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ABSTRACT

The response of tumour cells in vitro to ionising radiation can, to a certain extent, predict the response of tumours to various radiotherapy treatment modalities. This thesis considers some of the factors known to be involved in the radiation response of human tumour cells in vitro. These parameters include radiation-induced cell-cycle perturbations, apoptosis and DNA damage repair. A panel of eight human tumour cell lines with markedly differing radiosensitivities were assessed in order to determine the key factors governing their radiation response. A wide range of doses spanning both the low dose region (0.2 Gy and 0.5 Gy) and the clinically relevant region (1–4 Gy) were used to determine whether differences in responses could distinguish cells which were radiosensitive or resistant.

Ionising radiation produced a cell cycle delay in all cell lines in one or both of the cellular checkpoints. A G1/S delay was detected in those cell lines that expressed wild-type p53, and the duration of this delay appeared to be directly related to the level of constitutive protein. p53 protein stabilisation was observed after 4 h, even at doses of 0.2 Gy, although a G1/S delay was only detectable at higher doses. There was no direct relationship between p53 status and survival although wild-type p53 expression was more prevalent in the radiosensitive cell lines (3/4 sensitives are wild-type versus 2/4 resistsants). A G2/M delay could only be detected at doses of ≥1 Gy. This delay appeared to be dose independent in the resistant cell lines, suggesting a threshold dose of 1 Gy, above which no further effect is observed. A radiation-induced reduction of cyclin B1 protein was observed in all cell lines implicating this protein in the induction of a G2/M delay. The duration of G2/M delay was significantly longer in the radiosensitive cell lines at 4 Gy (7–20 h versus 4–6 h at 4 Gy). The proportion of cells that exited the G2/M block and re-entered G0/G1 phase was also significantly less in the radiosensitive cell lines at this dose and was directly related to the increased level of apoptosis observed in these cell lines. It is speculated that the induction of apoptosis occurs after cells have attempted to repair damage before the onset of mitosis, eliminating those cells with irreparable damage. Apoptosis at low doses were not significantly different to constitutive levels, suggesting a limited role of apoptosis in the low dose hypersensitivity response previously observed in some of the cell lines in this study. Radiosensitive cell lines also demonstrated reduced initial DNA damage, measured by CHEF, (1.3–1.7 versus 1.8–2.2 excluding mutant radiosensitive phenotypes) and DSB break repair (20–30% versus 15–20%). While the responses to radiation were expectedly varied it is speculated that radioresistance is associated with increased repair while radiosensitivity is associated with increased apoptosis.
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1.0 Introduction

1.1 Cancer growth and treatment

1.1.1 Cancer: The disease

In excess of eight million new cases of cancer are reported worldwide each year and is overwhelmingly a disease of older people with over 70% of cases occurring in people over sixty. Aging populations will undoubtedly lead to more cases if there are no reductions in cancer incidence rates. The incidence of cancer is reflected in the mechanisms which initiate the disease. Although cancer may originate from a mutation in a single cell, it must acquire other mutations or selective advantages over surrounding cells and the host, to progress and continue the malignant process. Continued proliferation of malignant cells results in the formation of a tumour and ultimately the invasion of adjacent normal tissue and metastasis. Failure to diagnose a tumour early in its lifetime and before metastasis occurs severely limits the success of curative treatment modalities.

Figure 1.1 summarises the life history of a tumour showing the relatively short window available for curative treatment after diagnosis. Reduction of the tumour population during the treatment period is achieved in about 60% of all cancers by local treatment, usually with surgery or radiotherapy alone or surgery in combination with radiotherapy or chemotherapy. In fact 50% of all cancers will receive radiotherapy with curative or palliative intent. One major shortcoming of radiotherapy is that ~40% of patients will recur locally after treatment. Evidence suggests that improvements in local control will lead to improved cure rates (Suit, 1986) and therefore research into improving radiotherapy regimes will benefit a large number of patients.

1.1.2 Radiotherapy: a brief review

Over a 100 years has passed since the discovery of X-rays by Röntgen, and one of their most important uses has been in the treatment of cancer cells. Radiotherapy is now considered one of the most important tools in clinical oncology. Radiation treatment results in the formation of clusters of highly reactive free radicals in the cell. The interaction of these free radicals with critical cellular components such as the DNA backbone can result in double and single strand lesions, leading to chromosome aberrations which can render the cell incapable of sustained proliferation and ultimately cell death (Radford, 1985). The chemistry of these free radical reactions is extremely complex (Gajewski et al. 1990; Teoule and Duplac, 1989). The extent of cellular death depends largely on the dose of ionising radiation applied (Puck and Marcus, 1956), the dose rate at which it is applied and the nature of the target cells. Major advances in the last twenty years in both the machinery available for the accurate delivery of ionising
Figure 1.1. Tumour development. The transformation of normal cells into cancerous cells on different tumour types is largely unknown. There is an heritable element in many tumours, and the prevention of tumour propagation can be aided by reduced exposure to certain risk factors, such as cigarette smoke inhalation. Once a cancer cell has escaped the body’s immune system, rapid proliferation results in the formation of a detectable tumour mass. Once detected, treatment can be initiated. The most important factors in patient survival after tumour treatment is the stage of tumour progression at the time of presentation, and early diagnosis is critical. The majority of cancers are treated using either surgery, radiotherapy or chemotherapy, or a combination of all three. Improvement of radiotherapy, either in the prediction of tumour response or in the delivery of the doses, therefore, would be hugely beneficial and is an area of active research.
radiation to the target and in laboratory research, has lead to the establishment of a number of clinical trials testing the efficacy of radiation at different fractionation schedules and doses (Schmitt and Wambersie, 1990; Saunders 1991) and in combination with various tumour modifying agents such as normobaric oxygen and carbogen (Rojas, 1992), hypoxic cell sensitisers (Dische, 1991) and cytotoxic drugs. On the whole these trials have been moderately successful though not to the degree predicted by *in vitro* models. The major problem lies in the difficulty in creating realistic tumour models *in vitro* that account for factors such as tumour doubling time, vasculature, blood flow, oxygenation and nutrient supply, all of which affect delivery.

In clinical radiotherapy there are four general strategies designed to improve the efficacy of treatment. These are to employ superior dose distributions e.g. conformal therapy, to increase the biological differential response between tumour and normal tissue, to expand knowledge of the natural history of tumours with particular reference to local and regional spread and to develop the capability of predicting the responses of tumour and normal tissue to standard radiation treatment and changes in response probability by modifying treatment strategy. It is the latter aspect of radiation therapy research, i.e. prediction of tumour response to radiation, which is the focus of this thesis.

Multiple biological factors can influence the outcome of radiotherapy but of prime importance are proliferation, hypoxia and intrinsic radiosensitivity. It has been suggested that, of these factors, intrinsic radiosensitivity may be the most important. Yet little is known about the mechanisms underlying the heterogeneity of the response of individual cells within the tumours or different tumours to the same dose of radiation. A variety of factors may be involved including DNA repair efficiency, cell cycle control mechanisms and fidelity of cell death pathways. It is hoped that accurate analysis of these parameters may help predict radiation response, helping to optimise individual patient radiotherapy treatments, with consideration for both the tumour and normal tissue. Many studies have shown the importance of intrinsic radiosensitivity as a factor in radiation therapy (Fertil and Malaise, 1981; West *et al.* 1993; Brock *et al.* 1989; Girinsky *et al.* 1993), as it correlates with survival and clinical responsiveness in various tumour cell types. In this thesis the factors affecting intrinsic radiosensitivity of several human tumour cell lines are analysed, with emphasis on the biological endpoints such as cell-cycle delay, programmed cell death and DNA repair and the proteins involved in the functional operation of these pathways. By analysing the radiation response of tumour cell lines with different primary histologies and intrinsic radiosensitivities, it was hoped to establish a molecular profile of each cell line which described the effects of DNA damage in terms of cell-cycle delay, DNA repair and cell death, both at low and clinically relevant doses. The possibility that the mechanisms underlying intrinsic radiosensitivity are common between all the cell lines was considered and it was hoped to determine how
useful the analysis of these biological endpoints was in the description of survival after radiation of these cell lines with a view to the prediction of radiosensitivity in other cell types.

1.1.3 Intrinsic radiosensitivity

The survival of cells after radiation treatment indicates the radiosensitivity of that cell population. Cells from different tissue origins display a broad range of radiosensitivities which is reflected in the clinic, where the observed local tumour control rates range from <5% to >95% for different tumour types (Tobias, 1992). For example, the dose to achieve a 90% local control rate (TCD 90) for testicular seminoma is 30 Gy whilst Hodgkin's disease requires 40-55 Gy and squamous cell cancer of the larynx would need 67 Gy. Yet 70 Gy applied to a glioma may correspond to a TCD 5 (reviewed by Hall, 1988). Hypersensitivity to ionising radiation has been demonstrated in both AT (ataxia telangiectasia) patients exposed to radiotherapy (Morgan et al. 1968) and cells in culture (Taylor et al. 1975; Chen et al. 1978; Arlett and Harcourt, 1980). The fact that radiation-sensitive tumours in vivo display a similar response in vitro has lead to the possibility that the response of tumour can be predicted from their survival characteristics in vitro.

One of the most widely used measurements of the radiosensitivity of cells involves the measurement of their colony-forming ability, which indicates cell survival. Classical in vitro radiosensitivity studies involve the construction of a survival curve which graphs the relationship between radiation dose and the proportion of cells that retain their reproductive integrity after radiation treatment. The standard method of radiosensitivity measurement was first described by Puck and Marcus (1956) and the technique continues to be used today. In this study they described the survival of the mammalian cell line HeLa. A known concentration of these cells was seeded into several growth dishes with supplemented medium and treated with doses up to 700 rads (7 Gy). Following incubation for 2 weeks under appropriate conditions, the dishes were stained and the number of resulting colonies, each assumed to be the progeny of a single cell, were counted. The surviving fraction was calculated from the ratio of the number of colonies to the number of cells originally plated, normalised by the surviving fraction of unirradiated controls. By plotting the surviving fraction with increasing dose, Puck and Marcus were able to determine the survival curve of HeLa. The fraction of surviving cells plotted on a logarithmic scale decreases with increasing dose, which is plotted on a linear scale, see Figure 1.2.

Cell kill is generally thought to be caused by the inactivation of primary sites of DNA within a cell, preventing stable replication. These sites can be thought of as targets and survival related to the number of target sites inactivated. This has led to a number of target theories to mathematically describe the shapes of the various survival curves.
Figure 1.2. The survival curve of the mammalian cell line HeLa (reprinted from Puck and Marcus, 1956).
obtained in vitro (reviewed by Steel, 1993). The simplest of these is the single-target single-hit inactivation theory which proposes that one hit of a sensitive target will inactivate the cell, and is represented by the following formula for each cell,

\[ p(\text{Survival}) = e^{-D/D_0} \]  

**Equation 1.1**

where \( D_0 \) is the dose that gives an average of one hit per target. When the dose applied (\( D \)) is equal to \( D_0 \), then the survival will be reduced to 37% of the initial population (i.e. \( e^{-D/D_0} = e^{-1} = 0.37 \)), where \( D/D_0 \) is the average number of hits per cell. The resulting semi-logarithmic plot of survival against dose is linear and is useful for describing the response of very radiosensitive human tumours, such as neuroblastomas.

A more general model is the multiple-site single-hit inactivation theory which proposes that the inactivation of each target site by one hit in a cell, having \( n \) sensitive sites, is required for cell death. This is described by the following equation

\[ p(\text{Survival}) = 1 - (1 - e^{-D/D_0})^n \]  

**Equation 1.2**

The cell survival curve described by this model has an initial shoulder, the size which can be predicted by the quasi-threshold dose (\( D_q \)) which is described as the dose below which there is no effect on survival (i.e. in the shoulder region of the curve). This model is useful for describing the survival of cells at higher doses, but often overestimates the survival at more clinically relevant and lower doses.

In order to describe with greater accuracy the survival of cells at more clinically relevant and lower doses, the multiple target theory can be modified by the addition of a single hit component, which takes into consideration that the response might not be flat for very low doses. The resultant two component model describes the radiation response of cells to a range of doses. This can be described by the following equation

\[ p(\text{Survival}) = e^{-D/D_1}(1 - (e^{-D(1/D_0^{-1}/D_1)})^n) \]  

**Equation 1.3**

where \( D_1 \) is the dose in the low dose region required to reduce the cell population to 37% of the starting population. Although this model does account for cell kill in the low dose region, it assumes that this is almost linear, implying no dose sparing below ~2 Gy, which is not experimentally true either in vitro or in vivo.

Therefore, perhaps the best description of radiation response is given by the Linear Quadratic model (LQ),

\[ p(\text{Survival}) = e(-\alpha D - \beta D^2) \]  

**Equation 1.4**
which describes the cell kill due to both the linear contribution ($\alpha$) and the quadratic contribution ($\beta$). Survival curves described by this model are continuously bending, the shape of which is determined by the $\alpha$ and $\beta$ factors. $\alpha/\beta$ represents the dose at which the linear contribution to cell death ($\alpha D$) is equal to the quadratic contribution ($\beta D^2$).

All survival curves constructed for mammalian cell lines treated with ionising radiation exhibit a similar shape to that described by Puck and Marcus (1956), the only major differences being in the width of the initial shoulder and the slope of the linear region. The $D_0$ for most mammalian cells lines in vitro was found to be between 1–2 Gy, except in AT cell lines which have a $D_0$ value of $\sim$0.5 Gy (a hypersensitivity that is observed in vivo in patients with this syndrome). However, the early use of these radiobiological factors for comparing intrinsic radiosensitivities revealed little differences between murine (Berry, 1973) and human tumour cell lines, which led to the initial lack of interest in the use of intrinsic radiosensitivity as a predictor of treatment outcome. An increasing number of studies, however, did find a difference in survival curves for different human tumour cell lines (Inada et al. 1976; Smith et al. 1976). It was subsequently shown that the culture conditions of different cell types can affect radiation response, Good et al. (1978). Utsumi and Elkind (1979) demonstrated altered levels of DNA repair and radiosensitivity in V79 cells cultured under different conditions (different media, gas concentrations etc.). To eliminate these variations, Fertil et al. (1981) collected the survival data for a range of tumour cell lines using identical culture conditions, at optimum plating efficiencies. Data from several independent experiments were fitted using the LQ model and results showed a significant correlation between the radiobiological parameter $SF_2$ (surviving fraction at 2 Gy $SF_2$ and 95% tumour control dose (TCD 95)) (Fertil and Malaise, 1981). Many studies have since shown a direct relationship between clinical outcome and $SF_2$ in whole range of tumour cell types (Deacon et al. 1984; Fertil and Malaise, 1985; Girinsky et al. 1993; West et al. 1993; Duschenne et al. 1997). The LQ model is also the most common model used to fit survival data.

The response of tumours depends on many factors of which the intrinsic radiosensitivity of the clonogenic cell population is only one and evaluation of this factor alone may not be adequate for final response determination. This is highlighted in several studies which have demonstrated no correlation between intrinsic radiosensitivity in vitro expressed as $SF_2$ with radioresponse in vivo (Brock et al. 1989; Ramsay et al. 1992; Taghian et al. 1993). These results, along with the fact that the evaluation of $SF_2$ of a cell line from a tumour biopsy takes two weeks, have prompted investigators to search for other radiobiological and molecular parameters which can be used to predict survival. As a result, considerable interest has been shown in the factors involved in the regulation of mammalian cell-cycle progression and the induction of cell death after
radiation. Correlation of the extent of cell-cycle delay has been related to radiosensitivity in many cell types (McKenna et al. 1991; Fitzgerald et al. 1990; Jung and Dritschilo, 1994; Nagasawa and Little, 1983; reviewed by Paulovich et al. 1997), as has the extent of radiation-induced apoptosis (Wheeler et al. 1995; Schwartz et al. 1995; Mitsuhashi et al. 1996; Levine et al. 1995, 1996; reviewed by Blank et al. 1997). These parameters were included in the comprehensive analysis of eight human tumour cell lines of different radiosensitivities in this thesis, in an attempt to identify whether the molecular decisions affecting survival in these cell lines can be identified and applied to other cell types to predict their response to a wide range of doses.

1.1.4 Low dose hypersensitivity

The existence of a low dose hypersensitive region in the survival curve of some cell types was first described by Eriksson in 1963 in which irradiated maize plants showed hypersensitivity to doses less than 1 Gy. Further work by Calkins (1967) suggested that protozoa show an increased sensitivity to radiation below a certain threshold dose. Koval (1984) described the low dose hypersensitivity of a lepidopteran insect cell line TN-368 irradiated in air or Nitrogen. The advent of more sensitive techniques to measure cell survival has facilitated the precise analysis growth characteristics of cells at very low doses. These include flow cytometry and cell sorting techniques, or a microscopic assay using a DMIPS machine (Dynamic Microscopic Image Processing Scanner; Palcic and Jaggi, 1986). The DMIPS, currently in use in the Gray Laboratory, allows the position of a number of cells to be memorised on computer and ‘revisited’ any number of days after the initial radiation treatment. Using the DMIPS to analyse survival to a wide range of doses, hypersensitivity was demonstrated in V79 cells (Marples and Joiner, 1993) and in four human tumour cell lines (Lambin et al. 1996). Figure 1.3 shows the survival curves for six of the cell lines analysed by Lambin et al. (1996), which were also included in the present study. At doses between 0.1–0.5 Gy, HT29, RT112, Be11 and MeWo cells demonstrate a significant increase in radiation sensitivity compared with that predicted by the LQ model (dotted line; see inset). In effect, X-ray doses in the low dose range exhibited a greater lethal effect per unit dose than higher doses of X-rays. Between doses of 0.5–1 Gy, the survival curve recovers to levels represented by the LQ model, in what appears to be an increase in resistance. This phenomenon has been termed ‘induced radioresistance' (IRR). The survival of the SW48 and HX142 cell lines are described well by the LQ model at all doses. It was subsequently shown that cell lines which demonstrated a low dose hyper-radiosensitivity (HRS) and IRR were more resistant to higher, more clinically relevant doses (as assayed by survival at 2 Gy). These improvements in the basic methodology of counting surviving colonies has lead to more accurate descriptions of the multiphase response of some cell lines to radiation doses, and the possibility that the low dose response may be
Figure 1.3. The survival curves of six human tumour cell lines. The survival curves have been fitted using the Linear Quadratic model (dotted line). This model can overestimate survival at very low doses (0-1–0.5 Gy), which is especially evident in the HT29 cells (see inset). The apparent plateau region at doses of around 0.5 Gy and the subsequent resistance to higher doses of radiation has been termed low dose hyper-radiation sensitivity (HRS) and induced-radioresistance (IRR) response, and was not evident in the radiosensitive SW48 and HX142 cell lines.
an important determinant of overall radiosensitivity.

The type of multiphasic response demonstrated by Marples and Joiner (1993) and Lambin et al. (1996) and others (reviewed by Joiner et al. 1996) cannot be due to two genetically distinct moieties (Bean, 1964) and led to the possibility that a sensitive sub-population, in a particular phase of the cell cycle may be responsible for the low dose effect. Although cell cycle position does affect radioresponse in asynchronous populations, since G2 phase cells are relatively more radiosensitive than S or G1 phase cells, it is clear these differences in radiosensitivity could not alone be responsible for the extreme low dose hyper-sensitivity observed in these cell lines. As an example, in HT29 cells 7% of the total cell population would need to have a mean activation dose $\bar{D} = 0.042$ Gy, thirteen times less than AT cells and eighty-one times less than the majority of the HT29 cell population (Lambin, 1994). The possibility that the HRS/IRR was caused by different phases of the cell cycle present in asynchronous populations was addressed by both Marples and Joiner (1993) and Wouters (1996), who showed that survival curves for partially synchronised populations still exhibit the HRS/IRR response. It is therefore evident that the IRR region represents an inducible radioresistance of individual cells which acts as an inherent protective mechanism in response to radiation. That cell lines demonstrating an HRS/IRR response appear to be more resistant at clinically relevant doses suggests that the elucidation of the mechanisms involved in the regulation of this response could contribute to the current understanding of the factors affecting intrinsic radiosensitivity. In the present study it was hoped to identify the mode of action of the low dose response. The degree of cell cycle perturbation and cell death at low doses was analysed and compared with the responses observed at subsequent doses in order to examine whether the HRS/IRR phenomenon could be coupled to an altered cell-cycle progression and/or cell death. The concept that radiation at lower doses in vitro may be more effective per Gray than at higher doses may have important implications for radiotherapy both in the prediction of response and in the improvement of the efficacy of existing treatment regimes.

1.1.5 Molecular mechanisms controlling radiosensitivity

As alluded to previously, differences in radiosensitivity between various tumour types can, in part, be attributed to a variety of key biological functions. In this study some of the biological pathways affected by ionising radiation such as the cell-cycle delay, DNA damage repair and cell death were examined to investigate the possibility that radiation response can be predicted directly from the effect on these pathways. An introduction to the regulation of these cellular processes and their role in radiation response is provided in the following sections.
1.2 Cell Proliferation

The sequence of events leading to the reproduction of a cell is referred to as the cell cycle and it has long been known that cultured cells respond to ionising radiation exposure by slowing or arresting their progression through the cell cycle (Okada, 1970). As cancer can be basically defined as uncontrolled proliferation of normal cells, information on how proliferation is regulated and consequently deregulated is of extreme importance in the understanding of the evolution of cancer. The rate of proliferation of tumour cells has important clinical implications as the pattern of proliferation appears to have some influence on the clinical outcome of some cancers. Fast proliferating head and neck tumours have significantly better local tumour control but worse survival than slow proliferating tumours, based on Tpot studies (Bennett et al. 1992). The cell cycle was first described in detail by Howard and Pelc (1951) using the roots of *Vicia faba*. Four distinct compartments were described as G1, S, G2 and M-phases, the nomenclature of the phases being based on the stage of cellular reproduction carried out in that phase (see Figure 1.4). G1 and G2 are the gap phases, S where DNA is replicated and M where mitosis occurs. How these phases are regulated is still very much under investigation although major advances in the understanding of the distinct phases and their interaction to complete one cell cycle have been made (reviewed by Nasmyth, 1996).

1.2.1 Cell-cycle progression

Entry into and progression through each phase in the mammalian cell cycle activated by mitotic stimuli is regulated by a family of proteins known as the cyclins. These proteins have come to be regarded as the workhorses of the cell cycle with seven different members identified, each functioning in different phases (see Table 1.1).

Cyclins interact with partner proteins known as cyclin dependant kinases to form active kinase complexes which are regulated through a series of phosphorylation and dephosphorylation reactions. Orderly progression through the cycle requires activation of several different cyclin-cdk complexes at specific stages in the cycle. Figure 1.4 outlines the sequence of complex assembly through the mammalian cell cycle. Their activation is regulated by feedback mechanisms preventing premature entry of cells into the next phase of the cycle prior to the completion of necessary events, such as completion of DNA synthesis in S-phase, before the entry of cells in G2 phase (Murray, 1992).

2.1.1.1 G1 phase

G1 was originally defined as a time interval, a gap between the readily observed events of mitosis and DNA synthesis. Recent advances in cell cycle studies have shown that the major responsibility of the G1 phase is to ensure the fidelity of cellular DNA
Cyclin Cdk Partner(s) Active Phase

<table>
<thead>
<tr>
<th>Cyclin</th>
<th>Cdk Partner(s)</th>
<th>Active Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclin D1,2,3</td>
<td>cdk 4, 5,6</td>
<td>G1, G1/S</td>
</tr>
<tr>
<td>cyclin E</td>
<td>cdk 2</td>
<td>G1/S</td>
</tr>
<tr>
<td>cyclin B1,2</td>
<td>cdk 1(cdc 2)</td>
<td>G2, G2/M</td>
</tr>
<tr>
<td>cyclin A</td>
<td>cdk 1, cdk 2</td>
<td>S/G2, G2</td>
</tr>
<tr>
<td>cyclin H</td>
<td>cdk 7(MO15)</td>
<td>G2/M</td>
</tr>
<tr>
<td>cyclin G1</td>
<td>cdk 1</td>
<td>early to late G1</td>
</tr>
<tr>
<td>cyclin G2</td>
<td>cdk 1</td>
<td>late S</td>
</tr>
</tbody>
</table>

Table 1.1 Mammalian cell cyclin/cdk complexes. Many cyclins and cdk partners have been identified and kinase activation trigger onset of the next cell cycle phase. Inactivation through phosphatase activity can also signal the end of a phase.

Figure 1.4. Cell-cycle progression in mammalian cells. Regulation is achieved by the activation of cyclin/cdk kinase complexes in a phase specific manner.

before it is replicated in the following S-phase (reviewed by Paulovich et al. 1997). Key regulators of G1 progression, in mammalian cells, include three D-type cyclins (D1, D2, D3), which assemble into holoenzymes with either cdk 4 or cdk 6, and cyclin E whose levels increase late in G1 phase to combine with cdk 2 (reviewed by Sherr, 1996). The targets, or pocket proteins of the active cyclin/cdk complexes are still very much under investigation. In G1 phase one of the major targets is the Rb gene product which is bound to the transcription factor E2F (Kato et al. 1993; Meyerson and Harlow, 1994). The phosphorylation of pRb occurring in mid-late G1, reverses its growth-inhibitory effect and enables cells to proceed from G1 to S phase. G1 cells can revert to a state of
quiescence (G0) under sub-optimal conditions (e.g. nutrient deprivation or serum starvation) or confluence in vitro but can re-enter the cell cycle in G1 phase when conditions are re-optimised. G0 and G1 cells both have the same DNA content, but differ in cells size (G0 cells are smaller due to the degradation of RNA and protein molecules), transmembrane transport (which is reduced in G0 cells), and G0 ribosomes are monosomal rather than polysomal (Pardee, 1989). The presence of G0 cells within a solid tumour population can affect treatment of the tumour due to increased resistance of G0 cells to most forms of treatment modalities, including radiation (Angioli et al. 1993).

Human cyclin G2 together with its closest homolog cyclin G1 defines a novel set of cyclins (Horne et al. 1996). Cyclin G1 is thought to function during G1 phase while G2 during late S, and while there is little information available on the precise action of these cyclins, there is evidence to suggest that both cyclins may be key negative regulators of cell cycle progression (Horne et al. 1997).

Before progressing from G1 into S phase, mammalian cells must first pass through a restriction point (R), late in G1 (originally identified in yeast and termed 'START'). This represents the point after which cells are irreversibly committed to proliferate and divide. Any alteration of the mitotic stimulation after this stage will have no affect on cell-cycle progression.

1.2.1.2 S phase

This is usually defined as the moment when DNA biosynthesis becomes detectable although the onset of S-phase is controlled by events occurring late in the previous phase. During S phase, the entire DNA content of the nucleus must be replicated before cells enter G2. This must be done precisely and completely. The length of this phase can vary between species and between individual developmental stages within a species (Edenberg and Huberman, 1975; Hand, 1978; Rizzino and Blumenthal, 1978).

Once the decision has been made to enter the proliferation cycle a whole sequence of events is initiated before replication begins. Expression of cyclin A occurs just before the onset of S, with the formation of cyclin A-cdk 2 and cyclin A-cdk 1(cdc 2) complexes (reviewed by Roberts, 1993 and Stillman, 1996). Some studies indicate that cyclin A is necessary for DNA synthesis as direct inhibition of its action by injection of antibodies or anti-sense oligonucleotides prevents the completion of S phase (Pagano et al. 1992; Girard et al. 1991). Cyclin A has been shown to co-localise with sites of replication in S-phase nuclei, indicating a role in the assembly or regulation of replication structures (Cardoso et al. 1993). Cyclin A has also been shown to complex with a sequence specific transcription factor DRTF1, in conjunction with pRb (Bandara et al. 1993) which suggests a role in transcription, perhaps of those proteins involved in replication. DNA replication can be broadly divided into two stages: a pre-synthetic,
initiation stage and a synthetic, fork elongation stage. Initiation events include the binding of a DNA recognition protein which triggers DNA unwinding and the assembly of a replication fork. Replication is carried out at multiple origins distributed along chromosomal DNA and activation of each origin of replication occurs only once ensuring that only two copies of each segment of DNA are produced. It is still uncertain whether all origins are activated at the same time i.e. when DNA replication is initiated, or sequentially.

Both the initiation and elongation stages of DNA replication are mediated by replication protein A (RPA) (Wold and Kelly, 1988; Wobbe et al. 1987). This is a three sub-unit protein complex where the p70 sub-unit encodes the single-stranded DNA (ssDNA) binding activity (Brill and Stillman, 1989; Kenny et al. 1990), while the function of the p11 and p34 sub-units remains unclear. Regulation of RPA through normal cell-cycle progression is maintained by phosphorylation of the p34 sub-unit at the G1/S boundary when the protein binds to ssDNA, formed as a consequence of the unwinding of the template (Fotedar and Roberts, 1992). The kinase that phosphorylates RPA is present in all phases of the cell cycle, which suggests that it is the binding of RPA to ssDNA that marks the time in the cell cycle when origin unwinding first occurs.

Proliferating cell nuclear antigen (PCNA) is also a key protein in the DNA synthesis machinery, identified as an auxiliary protein of DNA polymerase delta (Bravo et al. 1987; Prelich et al. 1987). Levels of PCNA increase at the onset of S-phase and remain relatively constant throughout S and G2/M phases, suggesting that synthesis is a prerequisite for initiation of DNA replication (Liu et al. 1989). It is interesting to note that PCNA has been found in complex with cyclin D (Matsushime et al. 1992) which links D-type cyclins to the replication process.

1.2.1.3 G2 phase

Before the onset of mitosis, cells progress through another gap phase in the cell cycle, G2. This phase is believed to serve as a final checkpoint of the integrity of cellular DNA before mitosis. B-type cyclins (cyclin B1 and B2) regulate progression through this phase in complex with its cdc2 (cdk 1) kinase partner. This kinase is a 34 KDa protein whose levels remain constant throughout the cell cycle but whose activity rises sharply at the G2/M boundary, in conjunction with its association with cyclin B. This complex is known as the maturation promotion factor (MPF) whose activation can lead to the onset and completion of mitosis (reviewed by Nasmyth, 1996).

In S. pombe, the cyclin B/cdc 2 complex is formed during S but is phosphorylated by the wee1 kinase at the Tyr 15 residue of the cyclin, and inactivated. At the end of DNA replication, at the S/G2 boundary, cdc25 dephosphorylates Tyr 15 and restores the activity of cdc2/cyclin B complex.
Similar control mechanisms have been identified in higher eukaryotes. Three cdc25 (cdc25 A, B, C) homologues have been identified in human cells (Datta et al. 1992). The kinase responsible for the activation of the MPF by phosphorylation of the Thr-161 residue of cyclin B is termed the cdc2-activating kinase (CAK, Solomon et al. 1993). Isolation of another cdk-related kinase, MO15 (Shuttleworth et al. 1993) revealed this protein to be the catalytic sub-unit of CAK (Fesquet et al. 1993; Poon et al. 1993). The relationship between cyclin/cdk binding and MO15 activity however remained unclear. While there was some evidence to suggest that CAK can phosphorylate the monomeric form of the CDK sub-unit (Poon et al. 1993), other studies demonstrated that CAK activity was dependent on cyclin binding (Solomon, 1993). Further studies found that isolation of CAK from mammalian cells revealed two major sub-units, MO15 and another 37 kDa protein, subsequently identified as cyclin H (Fisher and Morgan, 1994). Reconstitution of these sub-units \textit{in vitro} to form active CAK demonstrated that MO15 is a cyclin-dependant kinase (cdk7).

These findings indicate that cyclin/cdk complexes can function as regulators of other cyclin/cdk complexes and suggest that cyclin/cdk cascades may exist, regulating each other and the order of progression.

1.2.1.4 Mitosis

Mitosis is the process during which eukaryotic cells ensure equal division of their chromosomes before cell division. In mammalian cells, entry into M phase is triggered by the activation of the MPF factor, regulated by the levels of cyclin B/cdc 2 and cyclin A/cdc 2, which are targeted for ubiquitin degradation once the cell enters M-phase. Active MPF helps to initiate mitosis in part by breaking up the nuclear envelope. It does this by the addition of phosphate groups to proteins called lamins, which causes them to dissociate. Active MPF also controls many of the other processes key to mitosis completion such as the assembly of the mitotic spindle (reviewed by Murray and Kirschner, 1991). There are four major steps involved in the completion of M phase,

1. Prophase - where the chromosomes are compacted or condensed.
2. Prometaphase - where the condensed chromosomes are positioned, so that firstly, copy of each chromosome addresses one end of the cell and then to the cells' mid-plane.
3. Anaphase - where the chromosomes are separated into two identical parts.
4. Telophase - where the nucleus is reformed to re-establish the interphase condition in the two new daughter cells.

(reviewed by McIntosh and Koonce, 1989)
Mitosis ends when cyclin levels fall below a certain threshold, degraded by the ubiquitin pathway. Without the sufficient levels of cyclin A and B, the cdc 2 proteins are unable to remain active, resulting in an inactive MPF complex. In the absence of active MPF, no phosphorylation of nuclear lamins and further cascade type phosphorylation reactions can occur, which permits reformation of the nuclear envelope. Phosphatases also switch off the enzymes activated by MPF, but to ensure the continuation of the cell cycle, these enzymes also include the enzymes that degrade the cyclins. This leads to a gradual restoration of the cyclin levels and re-entry of the now divided cell into another round of replication.

1.2.2 Cell Cycle Control

Regulated progression through the cell cycle and faithful reproduction of cellular DNA before division is crucial to the sustained survival of the cell. Control is strictly enforced by a number of inducible and constitutive proteins in each phase of the cycle, resulting in the apparent delay in progression of cells through their cycle (see Figure 1.5).

Delay in progression across the G1/S and G2/M boundaries have been identified as the most common arrest points in the cell cycle, ensuring that progression into the DNA replication and division phases occurs only when cellular DNA is intact and without heritable mutations which may be disastrous to the progeny. Current understanding of the function of these cellular checkpoints is to allow time for the repair of any damage to DNA or induction of programmed cell death, as many cell types exhibit a phase delay in the presence of cytotoxic agents such as ionising radiation. Cancer can be described as unregulated cell growth, and extensive study into the control of the cell cycle has identified a number of key regulatory proteins.

1.2.2.1 cdk Inhibitors

Just as the binding of a cyclin to its cdk partner triggers the activation of this complex, recent studies have focused on the many additional regulatory proteins which bind to the active complex and inhibit its function. These proteins have become known collectively as cyclin dependant kinase inhibitors (CDIs). While many of these proteins act in response to external stimulus and others seem to be an intrinsic part of cell-cycle progression (reviewed by Elledge, 1996), all are now regarded as a crucial components of the cell-cycle machinery in specific cell types.

(i) p21

p21WAF1, Cip1, SDI1, PIC1 (el-Deiry et al. 1993; Harper et al. 1993; Johnson et al. 1994; Hunter, 1993) acts as a negative regulator of cell-cycle progression at the
Figure 1.5. Cell-cycle regulation in mammalian cells. Progression through the cell-cycle is maintained primarily through the regulation of cyclin/cdk complex activation. This is achieved through the action of inhibitory proteins which bind to cyclin/cdk complexes and physically prevent their activation by phosphorylation. Most of these proteins (termed cyclin-dependant kinase inhibitors, CDIs) have been isolated in the Gap-phases i.e. G1 and G2 of the cell-cycle.

G1/S boundary. p21 levels are elevated in the presence of DNA damaging agents such as ionising radiation but only in cells with functional p53 (el-Deiry et al. 1993). However, expression is also elevated in a p53 independent manner, in response to mitogenic stimuli such as platelet derived growth factor (Michieli et al. 1994). It can strongly inhibit the kinase activity of various cyclin/cdk kinases: cyclin A/cdk 2, cyclin E/cdk 2, cyclin D1/cdk 4, and cyclin B/cdc 2 to a lesser degree. One consequence of p21 binding to and inhibiting cdk 2 is to prevent cdk-dependant phosphorylation and subsequent inactivation of the Rb protein (Harper, 1993) which is essential for cell-cycle progression. This prevents cells from progressing through the G1/S boundary, resulting in a G1 delay. In normal cells, p21 may also block the onset of DNA replication by
blocking the ability of PCNA to activate DNA polymerase delta, the principal replicative DNA polymerase. It is thought that this inhibition is due to the physical interaction of p21 and PCNA, with allosteric modulation of the PCNA protein masking the DNA polymerase binding sites. p21 does not block PCNA-dependant nucleotide excision repair (Li et al. 1994) indicating a primary role for p21 in the arrest of cells in G1 rather than in repair. However, using a tetracycline inducible expression system, it has recently been reported that p21 enhanced the nucleotide excision repair capacity in colorectal carcinoma cells, in the absence of wild-type 53 (Sheikh et al. 1997). p21 has also been linked to p53 mediated radiation-induced apoptosis although its role in this pathway is less clear (el-Deiry et al. 1995). Duttaroy et al. (1997) found p21 upregulation in mouse 3T3 fibroblasts stimulated to die by serum depravation.

(ii) p27

The p27 product of the Kip1 gene is a inducible inhibitor of cyclin dependant kinase activity (Toyoshima and Hunter, 1994), isolated from TGFβ treated cells (Polyak et al. 1994). TGFβ (Transforming Growth Factor beta) is a growth inhibitory factor which blocks cells late in G1 (Moses et al. 1990), prior to phosphorylation of Rb by cyclin/cdk complexes (Laiho et al. 1990). Treatment with TGFβ inhibits cdk 4 expression while not affecting cyclin D levels (Ewen et al. 1993), thus affecting cyclin D/cdk 4 complex formation. Levels of cyclin E also increase without affecting cdk 2 expression (Geng and Weinberg, 1993), suggesting that p27^Kip1 is the mediator of G1/S delay by TGFβ treatment. Levels of p27 also increase in cyclic AMP treated macrophages, which also show a G1/S delay (Kato et al. 1994). Analysis of these cells for active CAK (cdk-activating kinase) do not show any decrease in activity of this kinase. Instead, the G1 delay is mediated through the binding of p27 to cyclin D/cdk 4 complexes, preventing access of the activating CAK.

(iii) INK4 Family

The search for other proteins that associate with human cdk 4 revealed a 16 KDa protein named p16^INK4a (inhibitor of cdk4, Serrano et al. 1994). p16 binds to cdk 4 and inhibits the catalytic activity of cdk 4/cyclin D holoenzymes. Activation of Rb through phosphorylation is the endpoint of this negative regulator of cell-cycle progression and p16 growth suppression has been demonstrated only in cells with a functional Rb protein (Medema et al. 1995). p16 has also been shown to bind to cdk 6, an alternative partner of the D type cyclins (Serrano et al. 1994). The p16 gene has been mapped to 9p21q (Quelle et al. 1995), a chromosome region frequently deleted in many tumour types, especially familial melanoma (reviewed by Marx, 1994). Other members of the INK4 family, which all share a 32-amino acid ankyrin motif, and selectively inhibit
activity of cdk 4 and cdk 6 but not other cdks include p15, p18 and p19 (Hannon and Beach, 1993; Hirai et al. 1995) all of which require functional Rb to cause a G1 delay.

1.2.2.2 p53

Originally identified in SV40-transformed cells, specifically bound to the large T-antigen (Lane and Crawford, 1979), p53 is now recognised as one of the most important proteins in tumourigenesis, with mutations in the gene found in 50% of all human cancers. It plays a major role in the control of cell division in response to DNA damaging agents. While endogenous p53 is not necessary for normal growth (p53 knock-out mice grow to maturity) the importance of p53 in cell growth regulation is apparent when it was shown that these mice regularly developed tumours.

p53 acts as a transcription factor, binding specifically to other proteins and controlling their expression (O'Rourke et al. 1990). The DNA binding site is sequence specific (Kern et al. 1991) and it is the DNA binding region which is frequently mutated in human cancer (Cho et al. 1994). In the cell cycle, the protein functions to halt cycle progression in response to DNA damaging agents, primarily by the transcription of the WAF1, Cip1, SDI1 gene. The p21 protein product inhibits the activation of cyclin/cdk complexes by binding directly to them. Presumably this gives time for the cell to repair DNA damage or commit itself to death before DNA replication is initiated. It is interesting to note that p53 has also been strongly linked to the regulation of the repair and apoptosis pathways in response to DNA damage by increased expression of GADD45 and bcl-2, key regulatory proteins in these pathways (Kastan et al. 1992; Miyashita et al. 1994).

MDM2, a 90 KDa protein, has been found in stable complex with p53 (Momand et al. 1992). p53 and MDM2 appear to operate in a negative feedback loop system whereby p53 induces the expression of MDM2 in response to DNA damage (Barak et al. 1993), whilst overexpression of MDM2 inhibits the ability of p53 to stimulate expression of target genes (Oliner et al. 1992), limiting the extent if G1 delay. Recently p90MDM2 protein was shown to form stable complexes with both E2F and Rb proteins. MDM2 stimulates the release of E2F from Rb and leads to the transcriptional activation of E2F target genes (Martin et al. 1995; Xiao et al. 1995), indicating a role for MDM2 in the release of the G1 delay mediated by p53. Either mutations in p53 or overexpression of MDM2 are found in almost 70% of human tumours (Leach et al. 1993).

Many studies have also reported the down regulation of various genes by p53 overexpression; Rb, IL-6, c-fos, PCNA (Shio et al. 1992; Santhanam et al. 1991; Kley et al. 1992; Mercer et al. 1991). Some of these reports are difficult to reconcile with what is known about the progression through G1. An example of this is the repression of the synthesis of Rb, whose own function is as a tumour suppressor which regulates the G1 checkpoint. It is clear however that p53 mediated G1 arrest in response to DNA
damaging agents such as ionising radiation is a universal checkpoint and vital to the initiation of faithful DNA replication. Recent evidence has also implicated p53 in the regulation of the G2/M checkpoint (Stewart et al. 1995; Guillouf et al. 1995; McKenna et al. 1996). It is evident that p53 plays a major role in the control of cell cycle progression in mammalian cells. Many studies have demonstrated a direct relationship between the status of this gene and radiosensitivity (reviewed by Bristow, 1996). The multitude of gene targets and biological pathways that appear to be regulated by p53 suggest however that the status of this gene alone would not universally describe the radiation response of mammalian cells. It is speculated that the biological function such as G1/S arrest in response to DNA damage might be activated by wild-type p53 but whether sufficient DNA repair occurs during this phase is further dependent on the expression of other genes such as the GADD family and the hMSH1 and hMSH2 genes (involved in mismatch repair).

1.2.2.3 Retinoblastoma (Rb)

Rb has a key role in the cell cycle, acting as a signal transducer connecting the cell cycle clock with the transcriptional machinery. First identified as being linked to the hereditary form of Retinoblastoma, a rare paediatric eye tumour (Knudson, 1971), it was subsequently demonstrated to be responsible for mediating reversible G1 growth arrest (reviewed by Weinberg, 1995). pRb mediates the control of the transcription of genes that promote advancement through G1/S, a critical stage in the decision to complete one cell cycle (Pardee, 1989). Inhibition of cell-cycle progression is due to unphosphorylated Rb inhibiting the activity of the E2F family of transcription factors (Nevins, 1992) through complex formation. Phosphorylation of the Rb protein under conditions that favour proliferation facilitates the release of the E2F transcription factors (Chellappan et al. 1991), which are now free to activate a host of genes responsible for G1 progression and initiation of DNA replication including c-myc, B-myb, cdc 2 and the E2F gene itself (reviewed by Nevins, 1992; Stein et al. 1996). The mechanisms controlling Rb phosphorylation are still very much under investigation, although accumulating information suggests that the components of the cell cycle mediate much and perhaps all of Rb phosphorylation. Evidence that Rb contains many phosphorylation sites suggest that it is the target of many kinases, although it is not known whether all the sites have to be activated in order to allow progression thorough to S.

The growth inhibitory signals that prevent Rb phosphorylation do not seem to impinge on Rb directly, operating instead by the modulation of the cdk5 responsible for Rb phosphorylation. Effectors of this inhibition in the presence of growth inhibitory factors such as cAMP and TGFβ include p27Kip1 and the INK4 family. Radiation and other DNA damaging agents also block Rb phosphorylation and G1 progression through the use of p21WAF1, Cip1, SDI1 (see 'cdk inhibitors'). The D-type cyclins are most
prominently implicated in the phosphorylation of Rb, and are unique in their ability to form physical complexes with the Rb protein (Corbrinik et al. 1992; Ewen et al. 1993). The INK4 family may function to disrupt this association.

1.2.3 Cell Cycle and radiosensitivity

The effect of radiation on the cell cycle is to delay its' progression through the induction of cell-cycle inhibitors in the gap phases, G1 and G2 and to a lesser extent S, phase. The accumulation of cells in G2 phase is the most commonly reported effect of DNA damaging agents on cell-cycle progression (Tobey, 1975; Rao, 1980; Weinert and Hartwell, 1989). Cells lacking this response (al-khodairy and Carr, 1992; Weinert and Hartwell, 1989) or cells treated with agents that abrogate this delay e.g. caffeine (Walters et al. 1974; Rowley et al. 1992; Tolmach et al. 1977) are more sensitive to the cytotoxic effects of ionising radiation (Busse et al. 1977). In this context, cells from patients with ataxia telangiectasia, known to be sensitive to radiation exposure, have been reported to show less radiation-induced delay in G2 phase than normal cells (Nagasawa and Little, 1983; Zampetti-Bossler and Scott, 1981). Conversely, an extended G2 delay has been associated with resistance of some cells to DNA-damaging agents (McKenna et al. 1991; Su and Little, 1993). This is in agreement with the theory that increased G2 delay after radiation increases the chances of survival by allowing more time for DNA damage repair. However, these studies were performed in oncogene-transfected cells which had a shorter doubling time than the non-transfected cells (Su and Little, 1993) which may have affected the length of radiation-induced delays observed. Under conditions of equi-lethal doses, Nagasawa et al. (1994) used a panel of seven cell types, both human and Chinese hamster cells with a wide range of radiosensitivity, and demonstrated a similar level of G2/M delay. They concluded that the length of G2 delay is closely related to radiosensitivity, and perhaps, to the level of unrepaired DNA damage in the cell as it approaches mitosis. Several studies have also shown a longer G2 delay in resistant cell lines than comparatively radiosensitive cell types (Ehmann et al. 1975; Bates and Lavin, 1989), whereas other reports show no differences in radiation-induced delays in human tumour cell lines (Smeets et al. 1994a). This has added confusion over the precise role of phase delays in radiation response and their use in predictive assays.

Radiation-induced delays in G1 are essentially related to the status of the p53 gene and cells lacking functional p53 protein generally lose the ability to arrest in G1 in response to radiation (Kastan et al. 1992; Kuerbitz et al. 1992; reviewed by Paulovich et al. 1997) leading to increased radioresistance (Lee and Bernstein, 1993; McIlwrath et al. 1994). Siles et al. (1996) have reported a close correlation between p53 status, G1 arrest and radiosensitivity in a panel of seven human tumour cell lines. The relationship between G1 arrest and radiosensitivity was further substantiated by the absence of a G1
arrest in radiosensitive fibroblast strains derived from patients with AT (Little and Nagasawa, 1985). This relationship is not universal however as some irradiated cells expressing wild type p53 do not arrest in G1. Nagasawa et al. (1995) compared three human cell lines of different radiosensitivities and p53 status and found no relationship with progression through G1 in the first post-irradiation cycle. It is argued that the cells have bypassed the usual mechanisms by which p53 controls their progression through G1. Li et al. (1995) also studied the level of radiation-induced G1 delay and p53 status in normal human fibroblasts and tumour cell lines, with both wild-type and mutant p53 status. No direct relationship was observed between G1 arrest and p53 status in the tumour cell lines. Only three out of the five cell lines with wild-type p53 exhibited a G1 block, involving 8-15% of the cell population. In contrast, all the wild-type p53 fibroblast strains showed an irreversible G1 block, involving 20-70% of the cell population. Again it is argued that the induction of a G1 arrest after radiation may be mediated by factors other than p53, resulting in a significantly reduced block. In a recent review Bristow et al. (1996), argues for the analysis of functional assays of wild-type p53 function in studies which attempt to correlate normal and tumour tissue radioreponse with p53 genotype. This was in light of several studies which demonstrated that although p53 status was determined, the presence or absence of a G1/S checkpoint did not affect survival after radiation.

Therefore, the role of cell-cycle delays in the prediction of radiation response is still very much under investigation. As many of the major tumour suppressor genes and oncogenes, identified in human cells, play a vital part in the progression and control of the cell cycle, it is reasonable to consider cell-cycle perturbations as a very important endpoint in radiation response. Study of the extent of these perturbations in the human tumour cell lines involved in this study, both at low and clinically relevant doses, may provide information on the observed differences in radiosensitivity.

1.2.4 Measurement of cell-cycle progression

Tumour cell kinetics in vitro has been studied extensively in order to determine the existence of positive correlation between the frequency of tumour cell DNA synthesis and treatment prognosis. A variety of techniques have been used, primarily based on the ability to distinguish DNA synthesising cells. Mitosis is the only phase that can be distinguished by light microscopy and the length of one cell cycle can be determined from the time required for mitosis to appear. In the fraction labelled mitosis technique (FLM) S-phase cells are labelled with tritiated thymidine \(^{3}\)H dThy. Autoradiographs from samples taken periodically show recognisable mitotic figures which are scored as either labelled or unlabelled. A plot of FLM with time shows a series of waves, each separated by one cell cycle time; the width of each wave is the DNA synthesis duration. In general, when the mitotic index is high the rate of proliferation is high.
Figure 1.6. Cell-cycle measurement with BrdUrd, PI and flow cytometry. In panel A the BrdUrd labelled population (R1), measured on the y-axis, are clearly distinguished from the unlabelled populations (R2). The different phases can also be defined by their differences in DNA content (x-axis) where G2/M cells have twice the DNA content of G0/G1 cells. S-phase cells have a range of DNA contents as synthesis is actively taking place. Analysis of the progression of cells into each phase was carried on the histogram profiles of each cell population. M1–M5 highlight the cells of interest on the total cell populations (panel B) and are overlaid onto histogram plots of the BrdUrd-labelled and unlabelled populations.
Studies of cell-cycle progression characteristics of drug or radiation-treated cells also require measurement of the amount of $[^3\text{H}]\text{dThy}$ by cells in S-phase. Such techniques are limited by their labour intensive nature and the subjectivity associated with distinguishing between unlabelled and weakly labelled populations. The amount of in vivo work is also limited to animal models due ethical constraints of administering radioactive DNA precursors to patients.

A non-radioactive DNA precursor for identifying DNA synthesising cells was used for cell-cycle progression measurement in this thesis. 5-Bromo-2-deoxyuridine (BrdUrd), a thymidine analogue is readily incorporated into DNA during the S-phase of the cell cycle, and can be detected immunochemically with monoclonal antibodies (Gratzner, 1982). Use of two-parameter flow cytometry to detect labelled cells in conjunction with cellular DNA labelled with propidium iodide (PI) (Dolbeare et al. 1983) provides a flexible method of analysis of the progression of each phase of the cell cycle. Discrimination between active (BrdUrd labelled) and inactive (BrdUrd unlabelled) S-phase cells can also be achieved (Wilson et al. 1985; Zolzer et al. 1993) and is applicable to experimental systems and human tumours in vivo. The procedure is based on the pulse labelling of exponentially growing cells with non-toxic doses of BrdUrd. Samples are harvested at regular time intervals after radiation treatment and stained with antibodies targeted to the DNA bound BrdUrd. The uptake of BrdUrd is confined to cells in S-phase actively synthesising DNA (Figure 1.6), and this is reflected in the fluorescence emission of the fluorescein isothiocyanate (FITC) conjugated antibody (y-axis) which binds to BrdUrd labelled cells (Figure 1.6 a). Analysis of cell-cycle progression is carried out on the PI histogram profiles of each population (i.e. BrdUrd labelled and unlabelled). Regions M1–M5 highlight the populations of interest (Figure 1.6 b) and progression of cells through each of these regions is readily followed with time. Analysis of the cell-cycle in this way provides a rapid, accurate test for cell-cycle delays and the potential to also analyse other parameters such as the temporal expression of proteins involved in cell cycle progression.

1.3. Programmed Cell Death

1.3.1 The Process

Programmed cell death, or apoptosis, is defined by morphologic changes resulting in non-pathologic cell loss. Apoptosis, so named by Kerr et al. (1972) results in cell death by a process clearly distinct from necrosis (Raff, 1993). It is necessary for homeostasis in the development of tissue mass and is induced after certain cytotoxic treatments. Apoptosis is a common feature of human tumours and has been shown to be
induced by radiation, chemotherapy and hormone therapy (Warters, 1992; Searle et al. 1975; Dyson et al. 1986; Szende et al. 1989).

The morphologic changes that occur in the process of apoptosis can be divided into five distinct steps, precondensation, condensation, fragmentation, phagocytosis and degradation (reviewed by D’Amico and McKenna, 1994), Figure 1.7. In this model, the precondensation phase represents the time when the cell has been exposed to the initial insult and has 'made the decision' to apoptose. This phase, which is indistinct histologically, allows time for the expression of various genes necessary for the completion of apoptosis. Endogenous protease activation results in cytoskeletal disruption, cell shrinkage and membrane blebbing, which can be distinguished using light microscopy. Some cell types show an increase in intra-cellular calcium which can activate cellular endonucleases (Kyprianou et al. 1988). These endonucleases initiate the condensation of the chromatin and the formation of apoptotic bodies, which contain intact cytoplasmic organelles. This is one of the major morphological features of apoptosis and the use of nuclear stains such as propidium iodide (PI) or Hoechst 33342, with fluorescent microscopy, are widely used for the determination of apoptosis in vitro. Once the membrane starts blebbing it becomes unstable, leading to the release of the apoptotic bodies and cell fragments into the surrounding area. In vivo, these bodies are phagocytosed by neighbouring endothelial cells and macrophages without the activation of an inflammatory response. In most tissues cell survival appears to be dependent on the continuous supply of positive signals from neighbouring cells (Ishizaki et al. 1995) and isolation of a cell from most organs will result in cell death apparently by apoptosis. This seems to suggest that apoptosis is a default pathway which is prevented from being switched on by the constant supply of survival signals. This is further substantiated by the fact that death in some cases does not require the expression of new proteins, suggesting that the regulatory proteins responsible for apoptosis are constitutively expressed (Raff, 1993; Jacobson et al. 1994). Dysfunction of the apoptotic pathway, has important implications for tumour progression as it might result in the continued proliferation of a population of cells carrying a heritable mutation. The ability of a cell to apoptose after radiation treatment, therefore is an important factor in the study of radiation sensitivity.

1.3.2 The apoptotic pathway

The majority of work into the genes involved in the apoptotic pathway has been carried out in the nematode C.elegans. This eukaryote only has over a thousand cells in total, of which one hundred and thirty one die by an intrinsic death program during normal development. Genetic analysis has revealed fourteen genes involved
Figure 1.7. The apoptotic process. This can be followed through a series of stages that are unique for apoptosis. After the initial insult, condensation of the chromatin leads to disruption of the cellular and nuclear membrane with the subsequent release and phagocytosis of the apoptotic bodies.

at different stages of this apoptotic pathway. These are termed the ced genes (reviewed by Hengartner and Horvitz, 1994) and isolation of families of highly conserved human homologues has lead to the partial elucidation of the implementation and regulation of the apoptotic pathway in human cells.

1.3.2.1 The bcl-2 family

Bcl-2 was originally cloned from the breakpoint of a t(14;18) translocation present in many human B cell lymphomas (Cleary et al. 1986) which resulted in the overexpression of the bcl-2 protein. It shows high sequence homology with the C. elegans ced-9 gene, which protects cells from programmed cell death (Hengartner and Horvitz, 1994). Bcl-2 protein has been localised to the inner mitochondrial membrane (Hockenbery et al. 1990) although some studies isolate bcl-2 to the endoplasmic reticulum and nuclear membranes (Jacobson et al. 1993). Bcl-2 suppresses cell death induced by a variety of cytotoxic agents including gamma radiation, hypoxia, growth factor withdrawal and chemotherapeutic agents (Sentman et al. 1991; Shimizu et al. 1995; Nuñez et al. 1990; Miyashita and Reed, 1993). Failure of bcl-2 to inhibit apoptosis by cytokine deprivation of IL-2 or IL-6 (Nuñez et al. 1990)
and the process of negative selection in thymocytes, (Strasser et al. 1991) suggest that multiple independent cellular mechanisms exist for the control of apoptosis.

Recently, it has been suggested that bcl-2 controls programmed cell death by suppressing the production of reactive oxygen species (ROS) (Hockenbery et al. 1993; Kane et al. 1993) and, therefore, functions as an antioxidant. However, the prevention of hypoxia-induced cell death by bcl-2 suggests that this oncogene can exert an anti-cell death function by a mechanism other than regulation of ROS activity (Shimizu et al. 1995).

Further analysis of this oncogene revealed it to be the founding member of a family of highly conserved homologues (reviewed by Rao and White, 1997). Members of this family fall into two functional groups, those that inhibit apoptosis and those that promote it. One such member of this family is the bax protein, which was found to heterodimerise with bcl-2 in vivo (Oltvai, et al. 1993) and accelerate cell death due to cytokine deprivation in an IL-3 dependent cell line. Other bcl-2 family members include bcl-xL and bcl-xS (Boise et al. 1993), bad (Yang et al. 1995), bak (Chittenden et al. 1995), Mcl-1 and A1 (reviewed by Blandino and Strano, 1997). Three highly conserved genetic regions within the bcl-2 proteins have been identified. These regions, BH1 and BH2 (Yin et al. 1994) and BH3 (Boyd et al. 1995) (bcl-2 homology 1, 2 and 3) are critical for dimerisation with partner proteins as mutation of these sites can completely abrogate the protein function. The protein-protein interactions of the bcl-2 family members can control the fate of the cell by promoting apoptosis if the ratio of repressor (e.g. bcl-2) is less than the inducer of apoptosis (e.g. bax), suggesting an antagonistic relationship. Certain complexes form more readily than others and Figure 1.8 outlines some of the combinations of hetero- and homodimers identified and their effect on apoptosis. It has been proposed that apoptosis in some cells is determined by the relative amount of bax homodimers (Korsmeyer, 1995). Therefore, while Bcl-2 and Bcl-xL bind with bax, preventing its homodimerisation and thus suppressing apoptosis, on the other hand bad binds to both bcl-2 and bcl-xL, freeing bax to homodimerise, thus promoting apoptosis.

1.3.2.2 The ICE family

From the work carried out in the C. elegans model, ced-3 was identified as a gene necessary for cell death. The ced-9 gene, whose human homologue is bcl-2, antagonises the function of ced-3 by protecting against cell death (Hengartner and Horvitz, 1994). Similar studies have identified the human homologue of ced-3 to be the cysteine protease interleukin-1 beta-converting enzyme (ICE), a protease important in the inflammatory response (Miura et al. 1993). Numerous cysteine proteases in mammals have been identified, the activity of one or more being increased during apoptosis (reviewed by Kumar, 1995). These include nedd-2/ich-1 (Kumar et al. 1994; Wang et
Figure 1.8. Dimérisation of the bcl-2 family and their effect on apoptosis. The protein-protein interactions of the bcl-2 family can promote or repress apoptosis induced by a variety of cytotoxic insults. The bcl-2/bcl-2 homodimer itself does not affect apoptosis; it needs to be complexed with bax or bad to exert its anti-apoptotic function. bax-bax homodimers are the most potent promoters of apoptosis and although it complexes with other promoters of apoptosis such as bcl-xL, bax shows higher affinity for the homodimer (bold arrow), which is more effective in apoptotic cell death promotion.

al. 1994), TX/Ich-2/ICErel-II (Faucheu et al. 1995; Kamens et al. 1995; Munday et al. 1995), MCH-2 (Nasir et al. 1997) and CPP32/YAMA (Alnemri-Fernandes et al. 1995). Overexpression of any of these five proteases in several cell types has been demonstrated to result in apoptosis, and indicate that proteolytic degradation is required for mammalian cell death. Identification of the downstream targets of ICE-like proteins is vital to understanding the exact role these regulators of the apoptotic pathway. There is evidence that ICE family members process themselves, and each other, proteolytically (Rao and White, 1997). Other substrates of these enzymes include poly-ADP ribose (PARP), DNA PK and lamins (Lazebnik et al. 1994, Casciola-Rosen et al. 1996; Lazebnik et al. 1995), suggesting that the ICE proteins regulate the physical processes, such as cellular shrinkage, that occur during apoptosis.

3.2.3 p53

In addition to playing a major role in the regulation of the mammalian cell cycle, p53 has been identified as key regulator of the apoptotic response to various cytotoxic agents in mammalian cells. Radiation-induced apoptosis in mouse thymocytes requires functional p53 (Lowe et al. 1993a). Treatment with chemotherapeutic compounds also
requires p53 for execution of apoptosis in mouse embryonic fibroblasts (Lowe et al. 1993b). The human lymphoblast cell lines TK6 and WTK1, which were derived from a single donor and have different p53 status, show differences in both the kinetics and the overall level of radiation-induced apoptosis (Xia et al. 1995). The introduction of wild-type p53 into human cancer cells with deleted p53 enhances apoptosis induced by chemotherapy (Fujiwara et al. 1994) and Hamada et al. (1996) have reported an increase of apoptotic cells in wild-type p53-expressing colorectal tumours compared with mutant p53 tumours.

The exact role of p53 and how it activates the apoptotic pathway is unclear as apoptosis can be induced via p53 independent pathways. Apoptosis is critical for the normal development and p53-knockout mice develop normally into adults (Donehower et al. 1992). However, apoptosis in response to DNA damaging stimuli such as radiation is, in general, p53-dependent. This is consistent with the findings that p53 levels are increased in response to DNA damaging agents (Lu and Lane, 1993). p53 is a well known transcription factor, and this raises the plausible possibility that p53 induces apoptosis via transcription of a 'death gene' in response to radiation. Studies into the effect of p21WAF1, Cip1 (which is transcriptionally activated by p53) on apoptosis have shown that increased levels of this protein has no effect on apoptosis (Kobayashi et al. 1996). However, Fan et al. (1994) have demonstrated increased levels of apoptosis in cells deficient in p21 when treated with nitrogen mustard. Treatment with other DNA damaging agents such as gamma irradiation, etopside and camptothecin produced similar effects. Analysis of cell-cycle progression revealed a G1/S block in the p21+/+ cells which was absent in p21-/- cells. Therefore, the role of p53 in the apoptotic response to DNA damage may be to transcribe the p21 gene, resulting in a G1 arrest. During this arrest the cell presumably attempts to repair the damage, which if it fails, undergoes apoptosis. This implies that the G1 arrest and apoptosis functions of p53, while being related, are two separate pathways. This is consistent with the findings that bcl-2 over-expression inhibits p53-triggered apoptosis but not G1 arrest (Wang et al. 1995) and that p21 knockout mice demonstrated apoptosis also with no G1 arrest (Deng et al. 1995). In predicting radiation response, p53 status has been shown to correlate significantly with survival in some tumour models, and its role in the apoptotic pathway may be partly responsible for this.

The molecular mechanisms involved in p53-dependent apoptosis are under investigation. It has been reported that p53 activates bax while reducing bcl-2 expression in M1 myeloid leukaemic cells transfected with a temperature sensitive p53 vector (Selvakumaran et al. 1994), and in ML-1 cells with endogenous p53 (Zhan et al. 1994). Therefore it would appear that p53 regulates apoptosis by regulation of the bcl-2/bax ratio.
1.3.3 Apoptosis and Radiosensitivity

The prediction of the radiation response of mammalian cells may be connected to the cells ability to undergo apoptosis, as alluded to above. Several workers have reported increased levels of apoptosis in radiosensitive cell lines when compared to more radioresistant cell lines. Russell et al. (1995) reported decreased apoptosis in a radioresistant human neuroblastoma cell line derived from a radiosensitive parental line. Similar responses in human lymphoblastoid and rat fibroblast cell lines (Schwartz et al. 1995; Aldridge et al. 1995) indicate that radiosensitivity may be due in part to an increased susceptibility to apoptosis. Stephens et al. (1991) showed that the extent of apoptosis varied between two murine tumours of differing radiosensitivity, and correlated with their ultimate response to local tumour irradiation. Analysis of the response of stage 1B cervical carcinoma by Wheeler et al. (1995) before and after radiation treatment showed that patients with a pretreatment level of apoptosis < 2% displayed a better overall survival than those with spontaneous levels of apoptosis >2% (p = 0.056). Radinsky et al. (1994) showed that malignant cells with lower levels of apoptosis demonstrated a higher pulmonary metastatic potential. However, not all tumour cell lines undergo radiation-induced apoptosis. Stapper et al. (1995) studied six human soft tissue sarcomas and fourteen glioma cell lines of different radiosensitivities and showed that ionising radiation-induced apoptosis was limited only to a sub-group of sarcomas.

The important question therefore is whether apoptosis is responsible for increased radiosensitivity. Although there are many studies to suggest that apoptosis does affect survival after radiation (reviewed by Blank et al. 1997), some studies have suggested that increased apoptosis in irradiated human cells does not affect the overall clonogenic survival (e.g. Aldridge et al. 1995). It must be remembered that apoptosis is not the only form of cell death affected by radiation as necrosis and mitotic cell death are also be induced in response to DNA damage (Nakano and Shinohara, 1994). In the present study, the extent of apoptosis induced by radiation during one cell cycle time was analysed in different human tumour cell types. It was hoped to determine whether apoptosis levels could be related to differences in the intrinsic radiosensitivity observed in these cell lines. There is sufficient evidence to imply a role of apoptosis levels before and after radiation in the survival of some tumour cells and therefore may prove and important tool in the prediction of radiation response.

1.4 DNA Damage and Repair

1.4.1 Radiation-induced DNA damage

Radiation affects many targets in the cell but DNA is the most sensitive site to damage in terms of its effect on cell survival. Exposure to ionising radiation produces a
number of chemical changes in the DNA of the exposed cell including single strand breaks (SSBs) and double strand breaks (DSBs), base damages and DNA protein-protein crosslinks (Ward, 1988), caused by multiple radical attack on local sites. Damage to an intracellular molecule can be traced through several distinct stages. These can be outlined as follows:

1. Physical deposition of energy
2. Production of primary radicals on the target molecule and in molecules surrounding the target.
3. Reaction of the radicals on the target and surrounding molecules
4. Reactions of unstable radicals leading to chemically stable damage.

(reviewed by Ward, 1990).

Since the vast majority of energy deposition events generally result in the production of more than one reactive species, it is probable that a significant number of sites on the DNA will contain more than one damaged moiety in a localised region (Goodhead, 1989). This type of damage, termed locally multiply damaged site (LMDS) (Ward, 1988) results from the combination of OH radical attack and direct ionisation of DNA molecules. Multiply damaged sites can occur in either strand or in both and it is these double strand breaks (DSBs) which seem to be most critical to the amount of cell kill and radiosensitivity. The presence of damage in one strand is not likely to be biologically significant as the unaffected strand can act as the template for the repair of the damaged strand. However, the presence of multiply damaged sites in opposite strands of DNA would be expected to be much more significant as there is a higher risk of loss of sequence information.

At biologically relevant doses (1–10 Gy) the yield of DNA damage is quite low. SSBs, which are the most commonly measured damage, are produced at a level of about 1000/Gy per cell. However it is the double strand break which is regarded as the lesion most likely to be the cause of the lethal effects of ionising radiation. Two main hypothesis have been proposed to explain differences in radiosensitivity between human cells in which it is proposed that cells may vary in both the amount of initial damage produced by radiation and the extent of repair. It is also proposed that cells vary in their tolerance to initial and residual DSBs and that this significantly affects survival after radiation.

1.4.2 Initial DSBs

The mechanisms underlying variations in initial DSB induction remain unclear but are likely to involve the presence of free radical scavengers and differences in chemical fixation of the damage. Several studies have analysed the relationship between initial DSBs and intrinsic radiosensitivity (reviewed by Nunez et al. 1996). The main objective of these studies has been to assess whether initial DNA damage levels can
predict radiosensitivity, which would represent a more rapid technique for survival
determination than the clonogenic assay. A direct relationship has been reported
between radiosensitivity and initial DNA damage in several cell types including breast
carcinoma (Villalobos et al. 1996), cervix carcinoma (Kelland et al. 1988),
neuroblastoma (McMillan et al. 1989), squamous carcinoma (Schwartz et al. 1988) and
CHO cells (Hu and Hill, 1996). However, the fact that several other studies have
revealed no correlation with radiosensitivity and initial DSBs (including Olive et al.
1994; Smeets et al. 1994b; Giaccia et al. 1992; Kysela et al. 1993; Schwartz et al. 1996)
suggests that downstream events initiated by DNA damage may be more indicative of
survival rather than the damage itself in some cell types.

In the present study, the level of initial DSBs was examined in eight human
tumour cell lines, using pulsed field gel electrophoresis (PFGE). This technique is based
on the principle that DNA fragments of different sizes can be separated in a pulsed field
gel. The relaxation and reorientation of the migrating DNA molecule during pulses
determines its position on the gel. It has the advantage over other techniques in that it is
sensitive to DSBs produced by ionising radiation at doses as low as 1 Gy. It was hoped
to determine whether differences in initial DSB levels could be detected between the cell
lines and whether they could be related to the extent of activation of other biological
pathways such as cell cycle-delay and apoptosis, and ultimately, radiosensitivity.

1.4.3 DSB Repair

The actual mechanisms of repair of DNA lesions caused by ionising radiation are
poorly understood. It is thought that the cellular checkpoints, G1 and G2, allow time for
the repair of potentially lethal lesions before the DNA replication and mitosis phases.
Jeggo et al. (1990) found that in xrs (X-ray sensitive) mutants, lack of DSB repair
ability was accompanied by an increase in radiosensitivity. Transfection of Ku80 cDNA
restored the DSB repair capacity and this resulted in increase in survival to the levels
observed in the parental cells. Other proteins identified in the repair processes include
PCNA and GADD45, whose activity is regulated at the cellular checkpoints G1 and G2.

DNA DSBs induced by ionising radiation usually have damaged termini and
many strand breaks are gaps rather than nicks. Two models for DNA repair have been
proposed, the single strand annealing model (Lin et al. 1984) and the double strand
break repair model (Szostal, 1983). The presence of two repair models may account for
the bi-phasic repair kinetics i.e. a slow and a fast component, observed in many DNA
repair studies. The fast component of repair is generally complete within 1–4 h while the
slow component can take up to 12 h. These two components of repair have been termed,
according to the type of lesion, type I and II respectively, with type III used to describe
residual DNA damage that has not been repaired during the incubation time (Dikomey
and Franzke, 1986). It is conceivable that the type I DSBs are repaired using constitutive
proteins (as this repair is quite rapid). Repair of type II lesions, would then involve genes in the repair process which have been induced in response to damage, while type III represents irreparable lesions.

The ability of cell to repair DSBs, therefore, might be expected to have a bearing on cellular survival after treatment with DNA damaging agents such as ionising radiation. Indeed, studies into the extent of repair, usually 4 hours and up to 24 hours post irradiation, have revealed a number of significant correlation to survival in various human tumour cell lines. Paterson et al. (1976) identified AT fibroblasts as being deficient in DNA excision repair of gamma radiation-damaged DNA and postulated that this might be the cause of the extreme radiosensitivity of these patients. Wlodek et al. (1988) reported a reduced capacity for repair in a radiosensitive mutant lymphoblastoid cell line, both in G1 and G2 phase cells. Kysela et al. (1993) has also reported a correlation with the levels of residual DNA damage and the radiosensitivity in Chinese hamster cells. Similar results have been observed in cells derived from squamous carcinoma (Giacca et al. 1992), colorectal adenocarcinoma (Lambin et al. 1992), cervix carcinoma (Kelland et al. 1988), ovarian and malignant melanoma cancers (Zaffroni et al. 1994). As demonstrated in studies analysing the initial levels of DSBs, a direct correlation between residual DSBs and radiosensitivity, however, was not observed in some studies (including Whitaker et al. 1995; Powell et al. 1992; Woudstra et al. 1996a, b and Smeets et al. 1994b). It is evident therefore that the exact nature of the relationship between DNA repair and radiosensitivity still needs to be established.

1.5 Aims of this thesis

As alluded to previously, the response of mammalian cells to radiation-induced DNA damage is probably mediated through several biological pathways.

Several biological endpoints were analysed in detail to determine whether variations in intrinsic radiosensitivity between human tumour cell lines, of different primary histologies, could be attributed to any specific parameter(s).

Cell-cycle delays at each phase of the cell-cycle, apoptosis and DNA repair were all analysed in response to increasing doses of ionising radiation. Some of the cell lines involved in the study were found to demonstrate low dose hypersensitivity and it was hoped to determine whether this phenomenon is mediated by altered regulation of these pathways at low doses compared to clinically relevant doses.

It was also hoped to assess the potential use of such multiparameter analysis in the establishment of a molecular profile for each cell line, that indicates radiation response, and whether it can be applied to other tumour cell types.
2.0 Materials and Methods

2.1 Introduction

This chapter describes the materials and methods used routinely throughout the course of this project. It includes descriptions of cell culture techniques, irradiations, cell cycle analysis, measurement of apoptosis and various protein levels. Any additional procedures or adaptations of existing protocols are described separately in the relevant chapters.

2.2 Cell Culture

2.2.1 Cell lines

Table 2.1 describes the primary histology, source and survival data of eight of the cell lines analysed in this study. The U1-S40b cells, which were kindly donated by Dr. Trevor McMillan, were isolated as a radiosensitive mutant of the MGH-U1 cell line (McMillan and Holmes, 1991).

HL60, a human leukaemia cell line was grown for use as a positive control for apoptosis and some primary skin fibroblasts were also cultured for use as controls in protein expression assays.

2.2.2 Media

The HT29, SW48, RT112, and Be11 cell lines were maintained routinely and experimentally in Earl's modified minimal essential medium (EMEM) with Earles salts supplemented with 10% foetal calf serum (FCS, Sigma), 20 μM L-glutamine (Sigma), 50 μg streptomycin (Sigma), 50 U/ml penicillin (Sigma), 1.5 g/L sodium bicarbonate (Sigma) and 10 ml/L of non-essential amino acids (10X stock, Sigma). All other cell lines were maintained in Ham's F-12 medium (Life Technologies) supplemented with 20% FCS, and additions as above.

Cells were maintained in exponential cultures with media changes every 2–5 days, depending on the growth rate, at 37°C and 5% CO₂/95% air. Cell cultures were changed every six months to avoid exhausting the cultures and were regularly tested for infections such as mycoplasma.

2.2.3 Cell line sub-culture

Cells were routinely passaged using 1–2 ml of a 0.5 g trypsin and 0.2 g ethylenediamine tetra-acetic acid (EDTA) litre⁻¹. Incubation was for 3 minutes or until the cells detached from the flask. The action of the trypsin/EDTA was stopped by the addition of a small amount culture medium containing serum and the cells were counted and plated as required.
Table 2.1. The eight human tumour cell lines involved in this study.

*American Tissue Culture Collection

2.2.4 Freezing cells for storage

Frozen stocks of cultures were prepared by harvesting cells and resuspending in culture medium to a final concentration of 2 - 4 x 10^6 cells/ml. The cell suspension was then mixed with dimethylsulfoxide (DMSO) to give a final concentration of 10% DMSO. Cells were frozen quickly to -70°C for twenty four hours to minimise cell loss, and stored in liquid nitrogen.

2.3 Irradiations

All irradiations were carried out using a Pantak unit operating at 240 kVp with 0.25 mm Cu + 1 mm Al filtration giving a half value layer (HVL) of 1.3 mm Cu. Dose rates varied according to the experimental jig used which is described in more detail in the relevant sections.

2.4 Cell-cycle analysis

The progression of cells through their cell cycle can be followed using a variety of techniques which, in general, rely on the cellular incorporation of a DNA precursor. In this study the thymidine analogue, 5'-bromo-deoxyuridine (BrdUrd) was used, which is incorporated into S-phase cells actively synthesising DNA detected with a monoclonal antibody (Gratzner, 1982). Only those cells initially in S-phase cell are identified using BrdUrd, while the other cell populations (G0/G1 and G2/M phases) are distinguished by
their DNA content using propidium iodide (PI). Using dual parameter flow cytometry, cells in all phases can be followed individually through their cell cycle. Any radiation induced delays in the cell cycle can be detected by comparison with an unirradiated control.

2.4.1 BrdUrd pulse labelling and irradiation

Cells in exponential phase were pulse labelled for 20 minutes with 20 μM BrdUrd (Sigma) / flask, which was first dissolved in warm PBS. All irradiations were carried out in a Stuart incubator at 37°C to minimise heat loss and possible cell cycle perturbations (see Chapter 4). Control sham irradiations were also carried out to determine the effect, if any, of heat loss during the transit of cells to and from the incubator on cell cycle progression. After irradiation, cells were washed twice in warm PBS (37°C), to remove excess BrdUrd, and incubated in warm fresh medium until harvesting.

2.4.2 Cell harvesting and fixing

Cells were harvested at regular times after radiation, usually every two hours up to 48 hours. The procedure was done on ice to prevent any movement through the cell cycle during harvest. Cells were detached using trypsin (as described previously), washed twice in cold PBS and centrifuged at 1000 rpm for 5 minutes. Cells were then resuspended in 200 μL of PBS and drawn slowly through a 25 G needle to obtain a single cell suspension. Cells were fixed in 10 ml of 70% ethanol and stored at 4°C until use.

2.4.3 Cellular staining for BrdUrd

2.4.3.1 Nuclei extraction and DNA denaturation

In order to detect incorporated BrdUrd immunologically, cellular DNA has to be partially denatured to allow access of the antibody. This was achieved using 2 M HCl in a process that is strictly controlled to allow detection of the low levels of BrdUrd, without disrupting the ability of PI to intercalate double stranded DNA. To optimise the amount of antibody accessing the DNA, the cellular membrane is digested with pepsin, as described by Schutte et al. (1987).

1. 1 x 10^6 cells were washed twice in PBS, centrifuged at 2000 rpm for 5 minutes and resuspended in 2.5 ml of 2M HCl containing 0.1 mg/ml pepsin* (Sigma).
2. This solution was gently vortexed and incubated at room temperature for 20 minutes. HCl denatures the DNA while the pepsin digests the outer membrane.
3. The resulting nuclei were carefully washed twice in PBS, to remove all the HCl / pepsin solution.

*The pepsin was first dissolved in PBS (~0.5 ml).
2.4.3.2 Antibody staining of BrdUrd

BrdUrd is incorporated into those cells actively synthesising DNA, i.e. S-phase cells, and can be readily detected using antibodies and flow cytometry.

1. Nuclei extracted as above were resuspended in 0.5 ml of PBS containing 0.5% Tween 20 (Sigma) and 0.5% normal goat serum (NGS, Sigma), a solution routinely known as PNT (PBS, NGS, Tween).
2. 25 μl of a rat anti-BrdUrd monoclonal antibody was added to the cell suspension, giving a final dilution of 1:200. The solution was incubated at room temperature for at least one hour, mixed gently and regularly.
3. Cells were washed with 5 ml PBS and resuspended in 500 μL PNT.
4. A secondary goat anti-rat IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugated antibody (Sigma) was added at a final concentration of 1:200. Incubation was also at room temperature for not more than one hour.
5. Cells were then washed once in 5 ml PBS and resuspended in 1 ml of PBS solution containing 1 mg/ml RNase (Sigma) and 20 μg/ml propidium iodide (PI, Sigma).

2.4.3.3 Detection of labelled nuclei

The BrdUrd labelled nuclei stained with a FITC conjugated antibody were detected and analysed on a FACScan flow cytometer (Becton Dickinson). The FL1 channel collected the green emission from FITC labelled cells which had incorporated BrdUrd while the FL3 channel collected the emission of propidium iodide. Doublet discrimination was used to eliminate debris, doublets, clumps etc. by gating on the FL3 area and width signals. The FL1 signal was collected on a log scale while the FL3 signal was collected on a linear scale to achieve optimum separation of the signals being analysed. In each experiment, 10 000 events were recorded.

Analysis of the sample was performed using the LYSIS II software package (Becton Dickinson) as described in Chapter 3.

2.5 Apoptosis

Apoptosis represents a form of cell death whereby cells undergo self destruction in response to various stimuli. The metabolic events leading to cellular disintegration involve the compaction of the nuclear chromatin into uniform dense masses and condensation of the cytoplasm, which can be distinguished by morphological analysis (reviewed by Kerr and Harmon, 1991). The procedure used in this study for morphological assessment of apoptotic cells was adapted from the routine test for mycoplasma (Chen, 1977) which involved the visualisation of cellular DNA by Hoechst 33342 incorporation. Another DNA stain, PI was also tested but was found to give decreased clarity and discrimination of apoptotic cells.
Biochemically, the characteristic event of apoptosis is the activation of an endonuclease which is responsible for internucleosomal degradation into fragments of approximately 185 bp multiples. Several techniques have relied on this characteristic to detect apoptotic cells. One of the most standard techniques involves the extraction of cellular DNA and its separation on an agarose gel. A 'ladder' of DNA observed in a lane demonstrates the fragmentation indicative of apoptosis. Several flow cytometry techniques have also been developed to detect apoptotic cells (Darzynkiewicz et al. 1992). Cell fixation in ethanol followed by rinsing in aqueous buffers such as PBS results in the partial extraction of the low molecular weight DNA fragments which show less PI staining than whole cell DNA, resulting in a sub-G1 peak (Afanasev et al. 1986). Other methods combine DNA fragmentation with membrane integrity using PI exclusion (Omerod et al. 1992). More recently, methods have been developed which can demonstrate an apoptotic cell by catalysing a readily identifiable marker on to the characteristic DNA 3' hydroxy fragment ends present in apoptotic cells. In this study, the in situ labelling is carried out using terminal deoxyribonucleotide transferase (TdT) which catalyses biotinylated dUTPs onto the fragment ends, as described by Schmitz et al. (1991) and adapted from the Apoptag™ kit (Boehringer Manheim). These end-labelled fragments were then visualised using an avidin-conjugated FITC secondary antibody. The intensity of the FITC signal was directly related to the level of apoptosis (see Chapter 5).

2.5.1 Terminal transferase and flow cytometry

2.5.1.1. Irradiation, harvesting and fixation

Cells were irradiated at 37°C to minimise any apoptosis induced by heat loss during irradiation. Cells were harvested at regular intervals after radiation, to obtain a time course for the level of induction of apoptosis over one cell cycle time (up to 36 h). Cells were detached by scraping directly into the medium in the flask. This cell suspension was placed on ice while any remaining cells in the flask were retrieved by washing with cold PBS. Cells were centrifuged for 5 minutes at 2000 rpm and the pellet resuspended in 200 µL of PBS. A single cell suspension was obtained by extrusion through a 25G needle and fixed in 10 ml 1% paraformaldehyde (Sigma), pH 7.4, for 15 minutes on ice. The solution was washed twice in cold PBS and resuspended in 70% ethanol. Samples were stored at -20°C and were stained within five days of fixation.

2.5.1.2 Preparation of positive controls

HL60, a human premyelocytic leukemic cell line, rapidly undergoes apoptosis after treatment with several cytotoxic agents (Del Bino and Darzynkiewicz, 1991). For use as a positive control, 2 x 10⁶ cells were incubated in 50 mM camptothecin (CAM, Sigma) for 3 hours. As these cells grow in suspension, harvesting involved
centrifugation at 1000 rpm, repeat washes with PBS and fixation as described above. A control level of between 10–20% apoptosis was used to ensure the sensitivity and reproducibility of the assay on a day to day basis.

2.5.1.3 Cell staining

Cellular staining for apoptosis by the TdT assay was adapted in the Gray Laboratory from the Apoptag™ kit (Boehringer Manheim), making the assay more economically viable for the large volume of samples for analysis.

1. $2 \times 10^6$ cells were washed twice in PBS at 2000 rpm for 5 minutes (as for all the washing steps).

2. Cells were resuspended in 100 μL sodium cacodylate buffer, pH 6.8 (see Appendix 1), vortexed for 5 seconds and centrifuged at 2000 rpm for 5 minutes.

3. The supernatant was removed by pipetting and the pellet was resuspended in 50 μL of sodium cacodylate buffer containing 5 units of TdT and 0.5 nmol biotin-deoxyuridine triphosphate (b-dUTP). Negative control samples were prepared by the omission of TdT in this step.

4. Incubation was at 37°C for 1 hour, mixing occasionally.

5. Cells were washed twice in PBS and resuspended in 100 μL of 4 X saline sodium citrate buffer (see Appendix 1) containing 5 μg/ml avidin FITC (Vector Laboratories), 0.1% Triton X-100 (Sigma) and 10% non-fat dry milk (Marvel). Incubation proceeded in darkness for 1 hour at room temperature.

5. The cells were washed twice in 0.1% Triton X-100/PBS, and resuspended in 1 ml of PBS solution containing 5 μg/ml of PI and 0.05 mg/ml RNAse (Sigma).

2.5.1.4. Detection of apoptotic cells using flow cytometry

Samples were analysed on a FACScan (Becton Dickinson). To ensure the assay had worked satisfactorily, the positive control samples i.e. the CAM treated HL60 cells were analysed first. The test samples were only analysed if the positivity of the HL60 cells was within the set limit (10–20% apoptotic cells). All samples were run on a predetermined FITC channel (FL1) of 600 on a linear scale, which was found to be adequate for all cell lines. Negative control samples were used to determine the lower limit of apoptosis detection by placing a computer generated region around these samples so that the amount of false positive cells in the defined region was <1%. This region was then overlaid onto the test samples and the actual number of positive cells determined (see Chapter 5)

2.5.2 Morphological analysis

2.5.2.1 Sample preparation

Samples were irradiated and harvested as described above.
1. Once a single cell suspension had been obtained, samples were fixed in a 3:1 methanol:acetic acid (BDH) solution, on ice for 15 minutes.
2. Cells were then centrifuged onto poly-L-lysine coated slides (BDH) at 500 rpm for 5 minutes (Shandon Southern Cytospins).
3. The slides were quickly dried with paper tissue and stained with Hoechst 33342 at a concentration of 0.05 μg/ml PBS, in darkness for 10 minutes.
4. Slides were washed thoroughly in distilled water and mounted in 50% glycerol/PBS solution.

2.5.2.2 Data collection

Hoechst stained cells were visualised using a Nikon UFX–II microscope under UV illumination. Four different fields on each slide were analysed with over 100 cells counted in each field, resulting in over 500 cells being scored in each experiment.

2.5.3 Apoptosis data analysis

The kinetics and extent of apoptosis induction varies in different tumour cell types. There are a number of possible time course of induction that each cell line might demonstrate. Induction may be rapid or delayed after the initial insult. With various doses, levels of apoptosis may also reach different peak heights or decrease at different rates. In consideration of this, it was reasoned that a single sample of apoptosis therefore could yield information that was not representative of the complete apoptotic system. In this study, levels of apoptosis were analysed at regular time intervals over a time course of one cell cycle, or 36 h. Both sets of data (apoptosis by flow cytometry and morphology), were analysed using a basic 'area under the curve' method in order to describe the results for the whole of the experiment. The data was analysed in time increments of 2 h and averaged over 36 h. This method allowed all cell lines to be analysed on an equal basis, whatever the kinetics of apoptosis induction, and gives an indication of the level of apoptosis not only at one time point but throughout the whole experiment. Analysis in this way also meant that apoptosis levels could be expressed in terms of one value (either cumulative or total apoptosis over the time course of the experiment) which facilitates a direct comparison both between cell lines and with other parameters such as intrinsic radiosensitivity.

2.6 Protein expression

Many oncogenes identified as possible prognostic markers of tumour susceptibility and/or treatment response have been strongly implicated in the biological pathways examined in this study. Important cell cycle regulators such as p53 and cyclin B1 (Bristow, 1996; Maity et al. 1994) have been positively correlated with radiation response in many tumour cell types. Bcl-2, a key protein in the regulation of radiation
induced apoptosis has also shown prognostic significance in some tumour types (Wilson et al. 1996). Therefore, it was decided to analyse the expression of these proteins both constitutively and after radiation in order to check for any correlation with survival, and to identify any disorders in these pathways which may be caused by a mutated gene or inactive protein.

Protein expression in all cell lines was analysed by flow cytometry and/or western blotting. Most of the staining protocols for each individual protein varied only in the concentration of the primary antibodies and the fixative used. For this reason, the general staining procedure will be described here and any variations noted in the relevant chapter.

2.6.1 Flow cytometry

2.6.1.1 Sample staining

1. 1 x 10^6 cells previously fixed in 70% ethanol, were washed twice in 5 ml PBS, at 2000 rpm for 5 minutes.
2. The supernatant was removed by aspiration and the pellet resuspended in 100 μL PNT containing a 1/25 dilution of the primary antibody, e.g. for p53, 4 μL of mouse anti-human p53 (DO-7, Dako) was added to 96 μL PNT.
3. Incubation was at room temperature for 1 hour.
4. Cells were washed in 5 ml PBS and the pellet was resuspended in 100 μl PNT containing a 1/25 dilution of a secondary goat anti-mouse FITC conjugated antibody (Sigma).
5. Incubation was at room temperature for 1 hour, in darkness.
6. Cells were washed in PBS and resuspended in 1 ml of PBS containing 1 mg/ml RNAse and 20 μg/ml PI.

Positive controls were prepared by the same protocol. Normal fibroblasts were used as standards for all the proteins analysed. Samples stained with an isotypic antibody instead of the primary antibody were used a negative controls. A sample known to be negative for the protein, e.g. HL60, which are null for p53 protein expression, were also used as negative controls where possible.

2.6.1.2 Sample collection and analysis

All samples were collected and analysed on a FACScan flow cytometer, using the LYSIS II analysis software (Becton Dickinson). Channel settings, FL1 (protein) and FL3 (DNA), were determined on the negative and positive control samples, with FL1 emission set on a linear scale. Samples were analysed using computer generated regions set on the negative control samples to contain <1 % of the total population. This accounts for any non-specific binding effects of the antibodies on the overall positivity.
This region was then overlaid onto the test samples and the positivity calculated by the number of cells in this region as fraction of the total.

2.6.2 Western blotting

2.6.2.1 Harvest and fixation

Samples were harvested by scraping and were washed twice with cold PBS. The pellet was resuspended in 1 ml PBS and transferred to an eppendorf tube. Samples were centrifuged for 5 minutes at 2000 rpm. The supernatant was aspirated and the pellet stored at -70 °C until use.

2.6.2.2 Sample preparation and loading

Cells were resuspended in 100 μL loading buffer (see Appendix 1), sonicated for 5–10 seconds at 30 mA (Ultrasonics Ltd.), and placed on ice. Samples were loaded on a 12% polyacrylamide gel (see Appendix 1) at a fixed cell number of 0.5 × 10^6. Rainbow (14.5–220 kDa) and ECL (Amersham International) protein markers were run at each end of the gel.

2.6.2.3 Running protocol and protein transfer

All samples were run first through a layer of 12% polyacrylamide stacking gel (see Appendix 1), to align the samples, and then through a 12% polyacrylamide running gel (see Appendix 1). Running conditions were 50 V and 150 mA, at room temperature for eight hours, in running buffer (Appendix 1). Samples were then transferred to a Hybond-ECL nitrocellulose membrane (Amersham International) in transfer buffer (see Appendix 1) overnight at 4 °C, with an applied voltage of 50 V and 200 mA.

2.6.2.4 Membrane staining

1. Visualisation of the proteins was achieved using a Ponceau stain (Sigma) for 10 minutes.
2. The membrane was then washed in 5% acetic acid (3 × 5 minutes), air dried and photocopied for a visual record of the gel.
3. Non-specific binding sites are blocked by incubation for 1 hour in blocking buffer (see Appendix 1) containing 5% non-fat dry milk (Marvel).
4. Primary antibody solutions were made up in blocking buffer containing 1% non-fat dry milk. Incubation was at room temperature for one hour, with slow agitation.
5. The membrane was washed for 3 × 10 minutes with wash buffer (see Appendix 1).
6. The secondary antibody was a goat anti–mouse horseradish peroxidase-conjugated antibody, which was diluted to a standard concentration of 1/10 000 in blocking buffer containing 1% non-fat dry milk.
7. A secondary antibody for the ECL protein marker was also added, at a concentration of 1/1500. Incubation was at room temperature for 45 minutes with slow agitation.
8. The membrane was then washed for 3 X 10 minutes in wash buffer (see Appendix 1).

2.6.2.5 Protein visualisation using enzyme chemiluminescence (ECL)
This was carried out as instructed in the ECL kit manual (Amersham International).
1. A solution consisting of equal volumes of detection reagents one and two was added to the protein side of the membrane. Incubation was for 1 minute without agitation.
2. The membrane was washed by holding vertically over tissue paper, gently touching the edge.
3. The membrane was then placed, protein side down on some cling film and covered.
4. Once wrapped the membrane was placed, protein side up in a film cassette, taking care to ensure that any air bubbles were removed.
5. The membrane was then exposed autoradiograph film, (Hyperfilm-ECL, Amersham International) in a dark room for ~15 seconds, depending on the intensity of the signal.
6. The autoradiograph was developed for 3 minutes and fixed.

2.7 DNA damage and repair
The initial and residual levels of DSBs after radiation are readily detected using pulsed field gel electrophoresis (PFGE) as described by Blocher et al. (1989). Damaged DNA is separated from total DNA and can be visualised by ethidium bromide (Sigma) and UV exposure. Pre-labelling with Carbon-14 (14C) facilitates the calculation of the fraction of DNA that has migrated through the gel, compared to that which has remained in the well, by monitoring the scintillation counts of each segment of the gel lanes.

2.7.1 Initial DNA damage
2.7.1.1 Labelling, harvest and irradiation of cells
1. Cells in exponential phase were incubated with 2-14C-thymidine (0-4 μM)(2-11 GBq/mMol)(Amersham International) to give a final concentration of 0-02 μCi ml⁻¹, for 48–72 hours or until cells were confluent.
2. Cells were washed in fresh medium for 15–18 hours prior to irradiation and harvested with 1 x trypsin/EDTA.
3. The cell pellet was resuspended in PBS and mixed with an equal volume of 1-6% agarose to give a final concentration of 2 x 10⁵ cells per 30 μL agarose (0-8%). Agarose/cell plugs were formed in plug molds (Biorad) and solidified at 4°C.
4. Plugs were irradiated in 25 cm² petri dishes containing 5 ml of cold media. Radiations using a dose rate of ~2 Gy min⁻¹ at doses 1–30 Gy were carried out on ice, to prevent repair. Control sham irradiations were carried out under the same conditions.
5. Plugs were incubated in 50 mM EDTA lysis buffer containing 1% α-laurylsarcosine (Sigma) and 1 mg/ml proteinase K (Sigma) for one hour at 4 °C, to allow permeation of the EDTA into the plug, while preventing repair. Incubation for a further 20 hours at 37 °C ensured complete lysis while the rapid action of the detergent prevented any repair at this temperature.

6. Cells were washed in 50 mM EDTA once every hour for three hours and stored for a maximum of two weeks at 4°C before being loaded on a gel. Longer storage times increase the background readings.

2.7.1.2 Sample loading and analysis
1. Plugs were loaded on a 2% agarose gel and run for 48 hours (CHEF DR III, Biorad) with two separate programs:

<table>
<thead>
<tr>
<th></th>
<th>One</th>
<th>Two</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial switch time (s)</td>
<td>1200</td>
<td>7</td>
</tr>
<tr>
<td>Final switch time (s)</td>
<td>2400</td>
<td>14</td>
</tr>
<tr>
<td>Run time (h)</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td>Volts/cm</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Included angle</td>
<td>106</td>
<td>120</td>
</tr>
<tr>
<td>Actual current (mA)</td>
<td>60–70</td>
<td>250–280</td>
</tr>
</tbody>
</table>

2. Unirradiated control samples were loaded on the same gel and run under the same conditions.

3. The gel was stained for 20 minutes with 0.5 g/ml ethidium bromide and visualised under 312 nm UV illumination, see Figure 2.1a.

4. The gel was cut into individual lanes, each divided into the well and two 4.5 cm segments of the lane. All three pieces were placed in individual scintillation vials (Biorad) with 200 μL of 1 M HCl.

5. Vials were then heated to 95°C for 1 hour to melt the agarose plugs and left to cool for 20 minutes. HCl prevents the gel re-solidifying on cooling. 10 ml of Beckman Ready Safe™ scintillation cocktail was added to each vial which were then counted using a Packard Tri-Carb 2000CA liquid scintillation analyser.

2.7.1.3 Data analysis
The fraction of DNA released from the well was determined from the total number of counts in the lane compared to the count for the plug. For each dose, the fraction of activity released (FAR) can be calculated using the following formula:
\[
\text{FAR} = \frac{\text{dpm in the lane}}{\text{dpm in the lane} + \text{dpm in the plug}} \quad \text{Equation 2.1}
\]

where dpm is the number of disintegrations per minute. An unirradiated control was used to determine the level of background using this technique, and this was subtracted from each sample. A plot of FAR versus dose is a straight line graph, see Figure 2.1b (Ward, 1990). The slope of the line relates to the amount of damage induced on average over the dose range and it is this value that is used to compare cell lines of different radiosensitivities in terms of their initial and residual DNA damage levels.

### 2.7.2 Residual DNA damage

#### 2.7.2.1 Sample labelling, harvest and irradiation

1. Exponential cells were labelled and washed as described for initial DNA damage measurements.
2. Cold medium was added to the flasks immediately prior to radiation, to prevent any repair, and warm medium immediately after.
3. Cells were irradiated on ice with doses in the range of 5–70 Gy with sham irradiations as controls.
4. Cells were incubated for 4 hours at 37°C to allow any repair to occur, after which they were washed twice with cold PBS and harvested with cold 1 x trypsin/EDTA.
5. Cells were centrifuged at 2000 rpm for 5 minutes, resuspended in PBS and mixed with an equal volume of 1.6% agarose so that the final concentration is 0.8% agarose containing 2 x 10^5 cells.
6. 30 μL of the suspension was placed into plug molds and left to set for 10 minutes at 4°C. The resulting plugs were placed in ice cold lysis buffer and incubated, washed and stored as above.

#### 2.7.2.2 Sample loading and analysis

Samples were run in a 2% agarose gel under the same conditions as for the initial DNA damage studies. The separation of the gel into lane segments and plugs and the melting conditions were also as for initial DNA damage.

### 2.7.3 Data analysis

The fraction of activity released (FAR) was calculated in the same way as for the initial DNA damage studies. The slope of the straight line plot of the FAR against dose gives a value for the amount of unrepaired damage, see Figure 2.1b. Residual DNA damage was analysed both as an individual measurement, or as a fraction of the initial
Figure 2.1A and B. DSB break analysis by PFGE. Damaged DNA was separated from intact DNA in a pulsed field gel (A). Damaged DNA migrates further into the gel due to smaller fragment size. The amount of damage increases with increasing dose (right to left, each dose in triplicate lanes) and all samples were compared to an unirradiated control (far left). Quantitation of the proportion of DNA in the lanes was estimated from the amount of scintillations of the $^{14}$C in the lanes compared to the well (b). The fraction of activity released (FAR) into the lane increases linearly with dose, the slope of which relates to the amount of damage.
DNA damage induced. This gave an indication of the amount of repair occurring in each cell line. Repair efficiencies can then be compared in different cell lines and correlated to radiosensitivity. Correlation with survival was analysed using the Mann-Whitney test for unpaired variables, with 95% confidence limits.
3.0 Cell-cycle characteristics

3.1 Introduction

Early kinetic studies on cell systems concentrated on the progression through mitosis. This phase is the only one visible under light microscopy and initially little information was available on what occurred in the interval between two mitoses. One of the most important contributions to the development of reproducible assays to study cell-cycle progression was the description of the phases between mitosis based on the nuclear incorporation of $^{32}$P into bean root cells (Howard and Pelc, 1953). They found that cells incorporate the radioactive label only during a certain well-defined fraction of the cell-cycle, a DNA synthesis-phase (S-phase). They also observed a gap between DNA synthesis and the beginning of mitosis. This gap was called G2 and by the subtraction of S+G2 and the mitotic duration from the intermitotic time, it was calculated that there was another gap between the end of mitosis and the start of DNA synthesis, termed G1. This view of the cell-cycle as a series of four stages still, in general, remains the current model.

The process of mammalian cell division occurs through a series of tightly regulated stages, collectively known as the cell-cycle (reviewed by Nasmyth, 1996). The four major phases of the cell-cycle are termed G1, S, G2 and M phases, where G1 and G2 are the gap phases preceding DNA synthesis (S) and mitosis (M) respectively, see Figure 3.1. Cells can also enter a quiescent G0 phase if growth conditions are not optimal. The main task of the cell-cycle is to ensure precise replication of cellular DNA in S-phase and to complete division of the cell during mitosis. Regulation of the cell-cycle in response to DNA damage, acquired either through routine replication or by the treatment with DNA damaging agents, is achieved primarily by the elongation of the G1 and/or G2 phases, which are regularly referred to as cellular checkpoints. Here DNA integrity is checked, and repaired where appropriate, before entry into replication and division phases. This inherent protection mechanism is vital to the survival of the cell as it prevents the carryover of DNA lesions which may be lethal to the cell genotype.

The mammalian cell-cycle control is strictly enforced, and entry into one phase is denied until the previous-phase is completed. This control is maintained through a complex network of phosphorylation reactions which activate phase-specific cdk5ks to bind to their cyclin partners (reviewed by King et al. 1996). Cdk/cyclin complexes can activate proteins necessary for completion of the phase (Figure 3.1) a process that is regulated by the binding of distinct cyclin dependant kinase inhibitors (CDIs).

De-regulated cell-cycle control is a characteristic of many tumour cells, and often results in altered proliferation rates compared to surrounding normal tissue. Studies into the possibility of assessing potential tumour response to treatments such as radiotherapy
<table>
<thead>
<tr>
<th>Cyclin–cdk complexes</th>
<th>Cell-cycle stage</th>
<th>Phase specific inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclin D/cdk 4</td>
<td>G1/G0</td>
<td>p14, p15, p16, p18, p19, p21, p27</td>
</tr>
<tr>
<td>cyclin D/cdk 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclin D/cdk 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclin E/cdk 2</td>
<td>G1/S</td>
<td></td>
</tr>
<tr>
<td>cyclin G1/cdk1</td>
<td>G1</td>
<td></td>
</tr>
<tr>
<td>cyclin A/cdk1</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>cyclin G1/cdk1</td>
<td>late S</td>
<td>p21</td>
</tr>
<tr>
<td>cyclin B1/cdk1</td>
<td>S/G2</td>
<td></td>
</tr>
<tr>
<td>cyclin B2/cdk1</td>
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<tr>
<td>cyclin H/cdk 7</td>
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</tr>
<tr>
<td>cyclin B1/cdk1</td>
<td>G2</td>
<td>p21, wee1</td>
</tr>
<tr>
<td>cyclin A/cdk1</td>
<td>M</td>
<td>p21</td>
</tr>
<tr>
<td>cyclin B1/cdk1</td>
<td></td>
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</tr>
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</table>

Figure 3.1: Mammalian cell-cycle regulation. The formation of each cyclin/cdk complex represented is necessary for progression through the relevant phase. These complexes are regulated by the binding of cyclin/cdk dependant inhibitors (CDIs) that modify their specificity or accessibility to regulators.

Using proliferation characteristics as a predictive assay could provide a way of optimising treatment strategies for individual patients.

Intrinsic radiosensitivity is now regarded as one of the most important factors influencing the efficacy of treatment regimes in local tumour control, and progression through the cell-cycle is implicated as a major determinant of radiation response. Intrinsic radiosensitivity is variable throughout the cell-cycle in human (Terasima and Tolmach 1963) and rodent cell lines (Sinclair, 1968; Dewey and Highfield, 1976). Cells irradiated in mid to late S are relatively radioresistant, compared to G2/M cells, while G1 cells are regarded as the most radiosensitive. In addition, human cells xenografted into mice and subsequently cultured, also show this order of radiosensitivity (West et al. 1988). Some human tumour cells however show no cell-cycle variation in radiosensitivity (Tang et al. 1994) while some appear to be more resistant in G1 and most sensitive in G2/M (van Oostram et al. 1990). In studies where cells are grown to plateau phase in culture, an increase in radiosensitivity has been observed compared to the corresponding exponential phase cultures (Wallen et al. 1985; West et al. 1988). As
cells in G1 were shown to be the most radiosensitive in the cell lines used in both these studies, and that growing cells to plateau phase increased the number of cells in G1/G0, this suggests that the proportion of cells in each phase may affect the survival determined for the total population. A higher proportion of radioresistant S-phase cells has also been correlated with increased radioresistance (Quiet et al. 1991). The chemosensitivity of a series of Lewis lung carcinoma lines was also influenced by both the proportion of cells in G1 and the length of this phase (Holdaway et al. 1992).

The diversity in tumour responses observed \textit{in vivo} and \textit{in vitro} suggests that the process underlying radiosensitivity is not a simple one. In order to identify, with some degree of accuracy, those patients who would benefit from radiotherapy over other forms of treatment, as much information as possible needs to be collected from each tumour sample. The variation in cell-cycle distributions have been shown to correlate with radiosensitivity in some tumour cells, although from the literature, the number of different tumour types analysed is relatively low. In the present study eight human tumour cells of different histologies, displaying a wide range in radiosensitivities were analysed in order to examine whether the cell-cycle characteristics of different tumour types and radiosensitivities could be related to survival. In addition, it was hoped to build a profile of the cell-cycle of each tumour cell line before radiation treatment, which would provide useful baseline information for subsequent analyses of the cell-cycle after radiation treatment.

\section*{3.2 Specific Materials and Methods}

\subsection*{3.2.1 Doubling time}

The doubling time of each cell line was calculated by monitoring cell growth over a period of seven days. 3 x 25 cm$^2$ flasks were harvested by trypsinisation and counted by haemocytometry each day. The rate of increase in cell number followed a sigmoidal shape, indicative of typical cellular growth pattern: i.e. lag phase followed by exponential growth and finally reaching stationary phase when cells were confluent. The linear portion of this response, indicative of exponential growth, was analysed using linear regression, the slope of which corresponded to the doubling time.

\subsection*{3.2.2 Flow cytometry data collection}

BrdUrd and PI staining were visualised using a FACScan flow cytometer equipped with an argon laser with an excitation wavelength of 488 nm (Becton Dickinson). The green FITC fluorescence emission signal ($515 < 1 < 545$ nm) related to BrdUrd incorporation and was collected on a log scale to discriminate the total labelled and unlabelled populations. The red PI emission signal (620 nm) was indicative of DNA content and was collected on a linear scale for best discrimination of each cell-cycle phase. Debris or clusters of cells were excluded from the sample population by the
application of a doublet discrimination module on the PI signal (Becton Dickinson). 1 X 10^4 cells from each sample were collected for analysis.

3.2.3 Flow cytometry data analysis

3.2.3.1 Proportion of cells in each phase and DNA index

Both of these parameters were calculated from DNA profiles of each cell line, analysed using the CellFit™ software (version 2.0.2, Becton Dickinson). Estimation of the proportion of cells in each phase was carried out using a ‘sum of broad rectangles’ model to fit each histogram, see Figure 3.2. Using this model, successive approximations to the actual histogram were calculated, fitting G0/G1 and G2/M populations with single Gaussian curves. Five Gaussian convolved rectangles were selected to fit S-phase in each sample.

The DNA index, which is an expression of the DNA content, was calculated relative to normal human fibroblasts for each cell line from the DNA histograms of PI incorporation. Analysis was carried out by the comparison of the mean G0/G1 peak channel number for the normal fibroblasts and each sample. A ratio of greater than one indicates DNA content greater than normal fibroblasts, or aneuploidy. Results are shown in Table 3.1. Three of the cell lines, HT29, Be11 and MeWo, have a DNA content (expressed as DNA index) greater than 1.0 and are thus considered aneuploid.

3.2.3.2 Length of each cell-cycle phase

Using a series of plots showing BrdUrd incorporation with DNA content as demonstrated in Figure 3.3a, BrdUrd labelled (R1) and unlabelled populations (R2) were distinguished by regions generated using the LYSIS II™ software (Becton Dickinson). At each time interval examined, cells in each of these regions were analysed separately for DNA content, Figure 3.3 b–d. The populations of interest, labelled M1–M4, were defined on a plot of total DNA content (b), and superimposed onto the histograms of the BrdUrd labelled (c) and unlabelled cells (d) only. These regions acted as analysis windows through which cell-cycle progression of each population was observed. This method was adapted from the cell-cycle analysis procedures reported by Karn et al. (1989) and Higashikubo et al. (1996).

i. Cell-cycle time

Cell-cycle time, T_c was calculated from the data generated by following the movement of cells through a narrow window, M3, located in mid-S (see Figure 3.3 b–d). The changing ratio of BrdUrd labelled to unlabelled cells in this region with time represented the movement of S-phase cells through the cell-cycle (see Figure 3.4). Re-
Figure 3.2. Cell-cycle phase distribution and DNA index. The propidium iodide (PI) signal is represented as arbitrary channel numbers on the x-axis (FL3-A). Cell number/channel is represented on the y-axis. G0/G1 and G2/M phases were manually identified by placing a marker at the peak height of each signal. The proportion of cells in each phase was calculated using CellFit™ software (Becton Dickinson), which applies a 'sum of broad rectangles' (SOBR) model to calculate the proportion of cells in S-phase. The DNA index of the sample population is calculated from a direct comparison with a standard population, such as normal human fibroblasts. A ratio of both G0/G1 phase DNA contents >1 indicates aneuploidy.
Figure 3.3a. Cell cycle analysis using BrdUrd and flow cytometry. Cells harvested immediately after incubation in BrdUrd label for 20 minutes. BrdUrd incorporation is represented by the FL1 emission signal on the y-axis. DNA content is measured using the PI emission, plotted on the x-axis. Regions outlining the populations of interest are generated using the LYSIS II software (Becton Dickinson). BrdUrd labelled cells are highlighted by R1 and unlabelled cells by R2.
Figure 3.3b–d. Cell cycle phase length analysis. Panel b shows the total cell population, and the markers (M1–M4) are initially defined on this plot. These are then overlaid onto individual plots of BrdUrd labelled (panel c) and unlabelled cell (panel d) only (from R1 and R2 respectively). markers key is as follows: M1 = G0/G1 cells, M2 = G2/M cells, M3 = mid S cells and M4 = G2/M and S phase cells (for relative movement analysis).
Figure 3.4 (a–d). The cell cycle time of human tumour cell lines using BrdUrd incorporation and flow cytometry. Data points have been joined using a simple smooth fit curve to highlight the shape of the graph. The distance between the two peak height maxim is $T_C$. Filled symbols represent the mean of at least three separate experiments (open symbols).
Figure 3.4. The cell cycle time of human tumour cell lines. Continued from previous page.
entry of labelled cells into this window (~24 h+, depending on the cell line) indicated the completion of one cell cycle. The initial ratio therefore was at a maximum value, close to 1, dropping to a minimum at a time equal 0.5 \( T_S \), and remained low until the BrdUrd population completed one cell-cycle and re-entered S-phase. Here the ratio reaches a second maximum, close to the initial value as the majority of cells divide and re-enter S-phase. The time interval between the two maxim is the cell-cycle time. Values for \( T_C \) can be estimated both by eye and by the application of a curve fit model (see Figure 3.5 for comparison of results for both analyses). Analysis by eye involved the visual analysis of the mid-S versus time curves generated for each cell line and estimation of the mid-point of the second peak height. The curve fit model used to fit the data, based on that reported by Watson and Taylor (1977) and adapted by M.C. Joiner (personal communication), employed normally distributed mean times for the intermitotic times. This model (see appendix 2 for formula) required the manual input of reasonable estimations of \( T_S \) and \( T_C \) in order to optimise the accuracy of fit for each cell line (see appendix 2 for computer generated curve fits).

### ii. S-phase

The length of S-phase, \( T_S \) was calculated using the relative movement technique as described by Begg et al. (1985), where the movement of S-phase cells relative to the positions of G1 and G2/M cells was calculated as follows,

\[
RM = \frac{F_L - F_{G1}}{F_{G2/M} - F_{G1}} \quad \text{Equation 3.1}
\]

where \( F_L \) is the mean red fluorescence of the BrdUrd labelled cells (M4), \( F_{G1} \) and \( F_{G2/M} \) are the mean red fluorescence for the total G1 (M1) and G2/M (M2) populations respectively (see Figure 3.3b–d). At time zero \( RM = -0.5 \) as labelled cells were uniformly distributed throughout S-phase, midway between the G1 and G2/M populations. This increased as labelled cells progressed towards G2, to unity, if all the cells originally in S-phase at time zero remain undivided in G2. Values for RM were calculated at regular time intervals and fitted using linear regression. \( T_S \) was estimated from the x–axis intercept of the extrapolated best fit line to \( RM = 1 \), see Figure 3.6.

### iii. G2/M phase

The duration of G2/M phase, \( T_{G2/M} \) was measured directly from the rate of entry of BrdUrd labelled cells into G1 phase (appearance in M1). At 0 h, cells in late S-phase are ready to enter G2/M. The appearance of these cells in G1 relates to the time taken to complete G2/M. All cells appearing in this window have completed mitosis, and divided
to form two daughter cells with G1 phase DNA content. This cell division was corrected for by application of the following formula for estimation of labelling index at time zero,

\[
\text{Corrected cells in G1} = \frac{0.5 \times G_1 \text{BrdUrd}}{\text{TotalBrdu} - 0.5 \times G_1 \text{BrdUrd}} \quad \text{Equation 3.2}
\]

where \( G_1 \text{BrdUrd} \) is the number of BrdUrd labelled cells in G1. The entry of cells into G1 with time, corrected for cell division, followed a sigmoidal trend and were fitted using a non-linear regression procedure (Johns and Joiner 1991) using a generalised logit equation as follows,

\[
Y = (U - L) \frac{\exp[(X - S)W]}{[1 + \exp[(X - S)W]]} + L \quad \text{Equation 3.3}
\]

where \( U \) and \( L \) are the upper and lower asymptotes respectively, \( S \) defines the inflection point and \( W \) is the slope of the line \( (dY/dX) \) at the inflection point \( (X = S) \). This fit was applied to the individual data points for each experiment, and the limits were optimally defined for each cell line. Extrapolation of the regression line in the linear region of the curve to the \( x \)-axis estimates the time taken for labelled cells to complete G2, i.e. \( T_g2 \), see Figure 3.7. This formula was also used to fit the response of radiation-treated cells in the calculation G2/M delay values (see chapter 4).

iv. G0/G1 phase

The duration of G0/G1, \( T_{G0/G1} \) cannot be calculated directly from the data collected using the BrdUrd method of cell-cycle analysis. However, an estimate can be obtained by subtraction of the sum of the calculated phase times from the cell-cycle time, i.e.

\[
T_{G0/G1} = T_c - (T_S + T_{G2/M}) \quad \text{Equation 3.4}
\]

3.2.4. Statistical analysis

All correlations with survival were tested for significance using the Mann–Whitney test for unpaired variables, with 95% confidence limits.
Figure 3.5. Comparison of the different techniques for cell-cycle parameter analysis. The calculation of cell cycle time by a curve fit model is compared with a 'by eye' estimation (panel A). S-phase length, calculated by the same curve fit model is compared with the relative movement technique (RM; panel B). The G1 phase length, which is estimated from the subtraction of G2/M and S-phase length from total cell cycle time, is also compared with both techniques (panel C). Values represent the mean of at least three separate experiments.
Figure 3.6. S-phase length of human tumour cell lines. The movement of S-phase cells relative to G2 and G1 cells (RM) increases linearly with time. $T_S$ can be calculated from extrapolation of RM values to 1, since when $RM = 1$, $t = T_S$. 

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Figure 3.6. S-phase length of human tumour cell lines. Continued from previous page.
Figure 3.7. G2/M phase length human tumour cell lines. \( T_{G2/M} \) is calculated from linear regression analysis of the entry of BrdUrd labelled cells into G1, extrapolated to the x-axis. Data represents the individual results for at least three separate experiments.
Figure 3.7. G2/M phase length of human tumour cell lines. Continued from previous page.
3.3 Results

3.3.1 Doubling time

The doubling time of each cell line was calculated over a period of seven days, after which all cell lines had progressed through exponential phase and reached stationary phase. All results are summarised in Table 3.2. These values do not take into account unproliferating cells or cell loss and consequently doubling times are generally higher than cell-cycle times. Most cell lines demonstrated a higher doubling time than cell-cycle time, $T_C$. Values ranged from 23h–44h. Correlation with radiosensitivity, SF$_2$, was not significant ($p = 0.243$, $r = 0.467$).

3.3.2 DNA index

The DNA content may be important in radiation response as it is conceivable that a cell line with an aneuploid DNA content will exhibit a higher probability of undergoing DNA damage after a single radiation dose than a cell line with diploid DNA. The results obtained for the cell lines in the present study are shown in Table 3.1. Only three of the cell lines tested demonstrated aneuploid DNA content; HT29, MeWo and Be11. Two of these cell lines are relatively radioresistant (HT29 and Be11). The effect of the aneuploid DNA content in these cell lines is as yet unclear, although it might be important when considering the amount of DNA damage induced by radiation in these cell lines. This is analysed in more detail in chapter 6.

3.3.3 Cell-cycle phase distribution

The distribution of cells throughout the cell-cycle is summarised in Table 3.1. Values did not vary significantly over the cell-cycle, which was analysed for at least 36 hours in each cell line. Data is expressed as the percentage of the total number of cells analysed in each experiment. In all cell lines the majority of cells were in GO/G1 phase, while the remaining proportion of cells were distributed (sometimes equally e.g. MGH-U1, SW48) between the S and G2/M phases. Distributions followed the general pattern of 60–80% cells in G0/G1, 13–20% in S and 10–25% in G2/M. The U1-S40b cell line however, clearly diverged from this pattern, with only 56% of the total population of cells in G0/G1 and 24% in G2/M phase respectively. These results were especially interesting when it is noted that this cell line was isolated as a radiosensitive clone of MGH-U1 cells, treated with ethyl methanesulfonate (McMillan and Holmes, 1991). The significance of the altered cell-cycle phase distribution of U1-S40b cells compared to the parental line in the decrease in radiosensitivity (SF$_2$ values change from 0.70 to 0.40) requires further investigation, namely into the specific radiosensitivity of each phase.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Primary histology</th>
<th>SF2</th>
<th>DNA Index</th>
<th>Cell-cycle Distribution (% cells in each phase ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>Colorectal adenocarcinoma</td>
<td>0.74</td>
<td>1.44</td>
<td>G0/G1: 72.2 ± 0.9, S: 18.0 ± 2.4, G2/M: 9.47 ± 1.2</td>
</tr>
<tr>
<td>SW48</td>
<td>Colorectal adenocarcinoma</td>
<td>0.18</td>
<td>1.03</td>
<td>G0/G1: 81.3 ± 3.4, S: 15.0 ± 2.1, G2/M: 10.4 ± 1.7</td>
</tr>
<tr>
<td>Be11</td>
<td>Malignant melanoma</td>
<td>0.70</td>
<td>1.69</td>
<td>G0/G1: 63.9 ± 5.3, S: 18.8 ± 4.1, G2/M: 13.4 ± 1.2</td>
</tr>
<tr>
<td>MeWo</td>
<td>Malignant melanoma</td>
<td>0.35</td>
<td>1.048</td>
<td>G0/G1: 64.6 ± 2.2, S: 18.5 ± 2.7, G2/M: 14.3 ± 0.48</td>
</tr>
<tr>
<td>MGH-U1</td>
<td>Bladder carcinoma</td>
<td>0.70</td>
<td>1.02</td>
<td>G0/G1: 74.6 ± 4.3, S: 12.3 ± 2.3, G2/M: 11.8 ± 1.0</td>
</tr>
<tr>
<td>RT112</td>
<td>Bladder carcinoma</td>
<td>0.62</td>
<td>1.01</td>
<td>G0/G1: 76.3 ± 2.6, S: 14.1 ± 2.0, G2/M: 9.33 ± 2.5</td>
</tr>
<tr>
<td>S40b-U1</td>
<td>Bladder carcinoma</td>
<td>0.40</td>
<td>0.99</td>
<td>G0/G1: 56.5 ± 1.7, S: 19.3 ± 1.2, G2/M: 24.1 ± 0.52</td>
</tr>
<tr>
<td>HX142</td>
<td>Neuroblastoma</td>
<td>0.03</td>
<td>1.05</td>
<td>G0/G1: 67.5 ± 3.1, S: 20.3 ± 2.1, G2/M: 12.2 ± 0.97</td>
</tr>
</tbody>
</table>

Table 3.1. Cell-cycle phase distribution of eight human tumour cell lines, arranged in order of primary histology and decreasing radiosensitivity, expressed as SF<sub>2</sub>. Data was collected using propidium iodide (PI) incorporation and flow cytometry and analysed using CellFit™ software (Becton Dickinson). Data is presented as the proportion of cells in each phase under exponential growth conditions expressed as the % total cells in the analysis (generally = 10 000). Values represent the mean of three separate readings throughout the cell cycle (±SEM).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Primary histology</th>
<th>SF2</th>
<th>Doubling time (h)</th>
<th>Cell-cycle time (h)</th>
<th>G1 phase (h)</th>
<th>S phase (h)</th>
<th>G2/M phase (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>Colorectal adenocarcinoma</td>
<td>0.74</td>
<td>44.0</td>
<td>40.8</td>
<td>22.1 (0.66)</td>
<td>17.5 (0.43)</td>
<td>1.2 (0.03)</td>
</tr>
<tr>
<td>SW48</td>
<td>Colorectal adenocarcinoma</td>
<td>0.18</td>
<td>35.2</td>
<td>31.2</td>
<td>11.7 (0.38)</td>
<td>16.3 (0.52)</td>
<td>3.2 (0.10)</td>
</tr>
<tr>
<td>Be11</td>
<td>Malignant melanoma</td>
<td>0.70</td>
<td>41.7</td>
<td>32.4</td>
<td>8.8 (0.27)</td>
<td>19.9 (0.61)</td>
<td>3.7 (0.12)</td>
</tr>
<tr>
<td>MeWo</td>
<td>Malignant melanoma</td>
<td>0.35</td>
<td>29.1</td>
<td>25.0</td>
<td>5.9 (0.24)</td>
<td>16.0 (0.64)</td>
<td>3.1 (0.12)</td>
</tr>
<tr>
<td>MGH-U1</td>
<td>Bladder carcinoma</td>
<td>0.70</td>
<td>23.4</td>
<td>22.7</td>
<td>6.5 (0.29)</td>
<td>13.7 (0.60)</td>
<td>2.5 (0.11)</td>
</tr>
<tr>
<td>RT112</td>
<td>Bladder carcinoma</td>
<td>0.62</td>
<td>38.7</td>
<td>37.1</td>
<td>16.6 (0.45)</td>
<td>17.7 (0.48)</td>
<td>2.9 (0.08)</td>
</tr>
<tr>
<td>U1-S40b</td>
<td>Bladder carcinoma</td>
<td>0.40</td>
<td>24.6</td>
<td>23.8</td>
<td>5.9 (0.25)</td>
<td>16.2 (0.68)</td>
<td>1.7 (0.07)</td>
</tr>
<tr>
<td>HX142</td>
<td>Neuroblastoma</td>
<td>0.03</td>
<td>34.0</td>
<td>34.8</td>
<td>14.8 (0.43)</td>
<td>15.7 (0.45)</td>
<td>3.5 (0.10)</td>
</tr>
</tbody>
</table>

Table 3.2. Cell-cycle characteristics of eight human tumour cell lines, arranged in order of primary histology. Survival data, presented as SF2 values are also shown as they are referred to in the text. Cell-cycle data were calculated using the BrdUrd and flow cytometry technique of cell-cycle analysis, and are presented as duration of each phase, in hours, and the fraction of total cell-cycle time occupied by each phase (in brackets). Results have been calculated from at least three experiments.
3.3.4 Cell-cycle time

The series of plots used to calculate cell-cycle time $T_C$, are shown in Figure 3.4 a–h. A simple smooth fit has been applied to highlight the pattern of change in the % labelled cells in mid-S with time. Cell-cycle time was analysed using both estimation by eye and application of the computer curve fit model and results are compared in Figure 3.5 a. Values obtained by each technique were statistically similar ($p = 0.03$, $r = 0.755$). It was decided, therefore to use the cell-cycle times calculated by the model for subsequent analyses, as it was reasoned that this technique would be less prone to interpersonal variation and therefore more accurate and robust. Table 3.2 summarises the cell-cycle times obtained using the curve fit model. Values ranged from 22.7–40.8 h and while this range was extensive, it did not correlate with the observed differences in radiosensitivity ($p = 0.819$, $r = 0.097$). Variations between cell lines from tumours of similar primary histology were also evident. As an example, the $T_C$ times of the two colorectal adenocarcinomas, HT29 and SW48, varied from 40.8 to 31.2 h respectively. An estimate of the fraction of the cell-cycle time occupied by each phase is also shown in Table 3.2.

3.3.5. S-phase

The length of this phase was calculated using the relative movement (RM) technique described by Begg et al. (1985). The data generated by the RM analysis were plotted against time and fitted using linear regression, not forced through the origin. Figure 3.6 a–h shows the curves generated using this type of analysis for each cell line (see appendix 2 for model fits).

S-phase length, $T_S$, was also calculated from the curve fit model used to analyse the cell-cycle time, as $T_S$ is approximately half the distance between the first and second peak heights of labelled cells in mid-S (Figure 3.4). When $T_S$ results obtained using both these techniques were compared (see Figure 3.5 b), values were found to be outside statistical significance ($p = 0.217$, $r = 0.49$). Most of the values were similar, with the exception of two notable differences, in the MeWo cell line ($T_S$ by RM $= 24.6$ h versus 16.0 h by model) and Be11 ($T_S$ by RM $= 23.6$ h versus 19.9 h by model). Values are summarised in Table 3.2.

It can be hypothesised that if a tumour cell population has a long S phase, then a higher proportion of cells would exist in this phase at any one time compared to cells with a shorter $T_S$. This may influence the radiation response of the cell population as a whole since S-phase cells, particularly those in late S, are generally more resistant than cells in any other phase of the cell-cycle (Sinclair, 1968). The $T_S$ values of the cell lines in this study calculated using the model fit ranged from 13.7–19.9 h. However, this range did not correspond with the observed differences in radiosensitivity ($p = 0.446$, $r = 0.315$), see Figure 3.8a. There did appear to be a trend towards longer $S$-
Figure 3.8. Relationship between cell cycle phase lengths and radiosensitivity. S-phase times (a) were calculated from curve fit model. All fractions: $F_S$ (b), $F_{G2/M}$ (d) and $F_{G0/G1}$ (f) were calculated from $T_C$ values estimated using the curve fit model. G2/M (c) and G0/G1 (e) phase lengths were calculated as described in the text.
phase length in the more resistant cell lines, with only the MGH-U1 cell line (T_5 = 13.65) deviating from what would otherwise have been a significant relationship (p = 0.02). The fraction of T_C occupied by S-phase, which will be referred to as F_S, was similar for each cell line and values ranged from 0.43 – 0.68. As a result, values did not correlate with radiosensitivity (p = 0.729, r = -0.464), Figure 3.8b.

3.3.6 G2/M phase

Figure 3.7 a–h shows the experimental data used to calculate T_{G2/M} for each cell line. Individual data points from at least three separate experiments were fitted with the logit equation described above (equation 3.3). The linear region of each curve was fitted using linear regression, not forced through the origin. This was extrapolated to intercept the x-axis, which provides an estimate of G2/M length. Mean values are presented in Table 3.2.

The G2 phase is generally regarded as the most radiosensitive phase of the cell-cycle (Sinclair, 1968) and it has been well documented in terms of its extension in length after radiation treatment, known as G2 delay. Lack of G2 delay has been associated with radioresistance in many tumour cell lines (reviewed by Paulovich et al. 1997) and it is conceivable that the length of G2 phase in untreated cells may have an affect on the length of radiation-induced delay i.e. a short G2 phase might be associated with a long G2 delay. In all cell lines, G2/M was the shortest phase with the Be11 cell line demonstrating the longest observed length for this phase, at 3.7 h. Correlation of T_{G2/M} values with radiosensitivity was not significant (p = 0.293, r = -0.426), see Figure 3.8c, although there was a slight trend towards increased G2/M length among the radiosensitive cell lines. The fraction of T_C occupied by cells in G2/M phase (F_{G2/M}) was modest, and values ranged from only 0.03 to 0.12 T_C. The small range of values observed for both the length of the phase and the fraction of T_C did not reflect the range of radiosensitivity in these cell lines, showing poor statistical correlation (p > 0.1, r = 0.289), see Figure 3.8d. Analysis of the changes in the G2/M phase length after radiation treatment might demonstrate a better correlation with radiosensitivity.

3.3.7 G0/G1 phase

T_{G1} was estimated indirectly, by the subtraction of T_S and T_{G2/M} from T_C. An individual estimation of T_{G1} was obtained for each cell line from at least three experiments and the mean values are presented in Table 3.2. There were significant differences in T_{G1}, and values ranged from 5.9 h – 27.1 h. Correlation of these values with radiosensitivity, expressed as SF_2 was not significant (p > 0.5, r = 0.074), see Figure 3.8e. The fraction of T_C occupied by the G0/G1 phase (F_{G0/G1}) varied between the cell lines, ranging from 0.25 – 0.66. These values did not correlate with radiosensitivity (p = 0.777, r = 0.120) was observed, see Figure 3.8f.
3.4 Discussion

The extent to which cell-cycle phase distribution and progression rates through the individual phases in mammalian cell influences responses to radiation is still unclear. Although variations in radiosensitivity throughout the mammalian cell-cycle have been well documented, the ability of kinetic and static cell-cycle parameters (phase lengths and distributions) to indicate the radiation response has not been equivocally established. In this study, the cell-cycle characteristics of a panel of human tumour cell lines was examined. It was shown that such analysis highlights basic cell-cycle differences between the cell lines, providing information useful for subsequent analyses of radiation-induced responses.

The cell-cycle times of the cell lines in this study are typical of those observed in human tumour cell lines cultured in vitro, where the division cycle is normally complete within 1–2 days. In clinical radiotherapy treatments, it is often the case that tumours from the same primary site respond very differently in terms of local tumour control. The cell lines analysed in this study demonstrated markedly differing radiation responses, in terms SF$_2$ values, and include cell lines that have been derived from tumours of similar primary histologies.

In the adenocarcinoma cell lines, $T_C$ varied from 31 h - 41 h. This difference was due to an extended G0/G1 phase in the HT29 cell line ($T_{G0/G1} = 22.1$ h compared to $11.7$ h in SW48 cells), as the S and G2/M phases were of similar lengths. The significance of this was unclear as it is generally accepted that cells in G1 are more radiosensitive than cells in other phases of the cycle (Sinclair 1968). More recently, this pattern was also observed in HT29 cells (Tang et al. 1994). The proportion of cells in G0/G1 was similar for both cell lines (72.2% and 81.3% for HT29 and SW48 respectively). The increased length of G1 phase in HT29 cells could be due to disruption of the regulatory factors involved in G1-S traversal.

Another potentially important cell-cycle variation between these two cell lines is the fraction of the cell-cycle time occupied by cells in G2/M phase, which was 0.1 $T_C$ in HT29 cells and 0.03 $T_C$ in SW48 cells. Although these values were low relative to those observed in the other cell-cycle phases, the potential effect on survival after radiation may be significant. In general, cells in G2/M are the most radiosensitive while those in S are radioresistant. Therefore, tumour cell populations with a greater proportion of cells in G2/M might display an increased radiosensitivity. A similar interpretation could be derived from the S-phase data as the resistant HT29 cells have a higher proportion of cells in S-phase than SW48 cells (18.0% versus 15.0% respectively). Such a correlation between pre-irradiation cell-cycle distribution and radiosensitivity was demonstrated by Quiet et al. (1991) who analysed the cell-cycle distributions of two squamous cell
carcinoma cell lines derived from head and neck tumours. The survival values in these
cell lines differed by a factor of almost two and the resistant cell line, JSQ-3B, had a
higher proportion of cells in S-phase, compared to the more radiosensitive SCC-61B
cell line (28% and 13.2% respectively). As both cell lines demonstrated increased
radioresistance in S-phase, as determined by elutriation studies, the author concluded
that the in vitro radiosensitivity of these cell lines may be related to the pre-irradiation
distribution of cells throughout the cell-cycle, in this case, to differences in the
proportion of cells in S-phase.

The two melanoma cell lines included in this study were characterised by a
significant difference in radiosensitivity (SF_2 = 0.70 and 0.35, for the Bell and MeWo
cell lines respectively). Their cell-cycle characteristics, however were very similar. Both
cell lines showed almost equal cell-cycle phase distributions. Cell-cycle times differed by
only 3 h (32 versus 29 h), which was due to a longer S-phase in Be11 cells. This was
reflected by a higher F_S value and may indicate a possible role of the S-phase distribution
in the different radiation responses of these cell lines.

The three bladder carcinoma cell lines involved in this study also displayed a
range of radiosensitivities. Ul-S40b cells were markedly more radiosensitive (SF_2 =
0.40) than the other two, whose SF_2 values were comparable (0.70 and 0.62 for MGH-
U1 and RT112 cells respectively). The Ul-S40b cell line is distinct to the others
involved in this study as it was been isolated as a radiosensitive clone of the MGH-U1
cell line (McMillan and Holmes, 1991). Characterisation of these cell lines revealed a
repair defect in Ul-S40b cells (Powell et al. 1992). However, no differences were
found in the extent of cell-cycle perturbations after radiation. This was in agreement with
other studies using mutant phenotypes showing altered radiation response (Schwartz et

In this study, analysis of the cell-cycle distribution of the three bladder cell lines
revealed differences both in phase lengths and phase distributions. Ul-S40b cell
demonstrated markedly different cell-cycle distributions, with only 56.5% of cells in
G0/G1. This was accompanied by a large proportion of cells in S (19.3%) and an even
greater proportion in G2/M (24.1%), relative to MGH-U1 cells (12.3% and 11.8% in S
and G2/M phases respectively). It could be argued therefore, that the isolation of the Ul-
S40b cells involved the disruption of the cell-cycle and that this might be responsible in
some way for the increased radiosensitivity observed in these cells. The number of S-
phase cells, generally regarded as radioresistant, was higher in Ul-S40b cells while the
proportion of G0/G1 cells was lower, compared to the parental MGH-U1 cells. This
would suggest an increased radioresistance in the overall population of Ul-S40b cells,
as more cells were in the resistant S-phase at the time of irradiation. This is clearly not
the case. If, however, maximum radioresistance was found to be in another phase of the
cell-cycle other than S, such as G0/G1 as demonstrated in a cell line derived from a
uterine cervix carcinoma (van Oostram et al. 1990), then the reduced proportion of cells in G0/G1 observed in U1-S40b cells (~56% compared to ~75% in the resistant parental line MGH-U1) may be significant in the radiation response observed for the total population.

U1-S40b cells also demonstrated a reduced G2/M phase length (1.7 h versus 2.5 h in MGH-U1 cells). The length of G0/G1 phase was similar for both MGH-U1 cells and U1-S40b cells (6.5 and 5.9 h respectively) but was significantly prolonged in RT112 cells (16.6 h). The HX142 was the only cell line in the study derived from a neuroblastoma. Despite its extreme radiosensitivity (SF2 = 0.03) it did not exhibit any unusual characteristics in the sense that its cell-cycle kinetic parameters were all within the ranges observed in this study. This suggests that while the process of radiosensitivity might be cell-cycle related, the effects might only be confined to certain cell types.

In conclusion, the results presented in this section demonstrate that cell-cycle phase lengths and distribution values, in a panel of human tumour cell lines, do not directly indicate radiosensitivity. While it is generally accepted that in most cell types the order of increasing cell-cycle phase radiosensitivity is S, G0/G1 and G2/M cells, this may not be the case in all cell lines. Characterisation of the differential radiosensitivity throughout the cell-cycle was outside the scope of this project, but such an analysis could provide information on the different sensitivities observed in the total populations when compared with the data presented above. This would facilitate a more detailed examination of the potential significance of observed differences in cell-cycle distributions and phase lengths. Changes in the cell-cycle after radiation treatment, such as an increase in G2 phase length, termed G2 delay, have been correlated to survival in many cell types (Wilson et al. 1994; McKenna et al. 1991; Nagasawa et al. 1994; Cheong et al. 1992) where the cell-cycle parameters before treatment were known to be similar. Analysis of radiation-induced cell-cycle delays may help clarify the significance of the role of the cell-cycle in the intrinsic radiosensitivity of this panel of human tumour cell lines.
4.0 Radiation effects on cell-cycle progression

4.1 Introduction

Mammalian cells replicate by a process known as cell division forming two daughter cells that each carry a chromosome compliment identical to the parental cell. Initially, when populations of cells were observed using conventional light microscopy the only event which was distinguishable was the actual process of cell division, or mitosis. Little was known about other the processes occurring between the time required for two visible mitoses of a cell, known as cell division time or mitotic cycle. Howard and Pelc (1953) were the first to identify other phases of this cycle, using autoradiography of bean root cells labelled with $^{32}$P. They found that cells incorporated the radioactive label only during a certain well-defined fraction of the cell-cycle, a DNA synthesis-phase (S-phase). They also observed a gap between DNA synthesis and the beginning of mitosis. This gap was called G2 and by the subtraction of S + G2 and the mitotic duration from the inter-mitotic time, it was calculated that there was another gap between the end of mitosis and the start of DNA synthesis, termed G1. This view of the cell-cycle as a series of four stages was also observed by several other groups using different mammalian cell systems including CHO (Ross and Sinclair, 1972), HeLa (Bedford and Mitchell, 1973) and mammary tumour in rat (Steel and Lamerton, 1966), and still remains the generally accepted view of the cell-cycle.

Following treatment with single 'acute' doses of radiation, a block in the progression of cells through the cycle was observed in many cell types including mouse L-cells (Whitmore et al. 1967), HeLa cells (Puck, 1964; Yamada and Puck, 1961), mouse intestinal cells (Elkind et al. 1963) and CHO cells (Dewey and Highfield, 1976). A block in G2 phase after radiation is the most common delay observed in mammalian cells (Sinclair, 1968; Leeper 1973; Bootsma, 1965; Gragg et al. 1978). Alterations in the cell-cycle progression however, have been found in all phases. Painter and Young (1959, 1975) demonstrated a slow transient reduction in the rate of DNA synthesis. A radiation-induced arrest of cells in G1 has been well documented (Kastan et al. 1992; O'Connor et al. 1993). The hypothesis of a surveillance mechanism that operates throughout the G2 and G1 phases of the cell-cycle to eliminate cells with altered or damaged DNA from the proliferating population of cells was first introduced by Tobey (1975). Progression through the mammalian cell-cycle is still currently regarded as a strictly regulated process and entry into one phase occurs only when the previous-phase is complete (reviewed by Elledge, 1996). Uncontrolled proliferation is the hallmark of cancer and many tumour cells have acquired mutations to genes directly associated with the cell-cycle machinery.

Faithful replication of cellular DNA is vital to cell survival and progression.
through the cycle can be arrested in the presence of DNA damage, primarily at the G1/S and G2/M transition points (Hartwell and Kastan, 1994) and in some cases during S (Hartwell and Kastan, 1994; Nagasawa et al. 1994; Schwartz et al. 1994; reviewed by Paulovich et al. 1997). These delays in cell-cycle progression allow time for repair of DNA damage and/or the induction of apoptosis (Sherr, 1996) thus reducing mutation frequencies and improving overall survival levels. The best evidence for such a role of cell-cycle checkpoints comes from studies of the G2 checkpoint in yeast cells lacking double strand break repair function (Weinert and Hartwell, 1988). In these cells the trigger for an increased G2 delay was shown to be unrejoined double strand breaks which prolonged G2 phase until the DNA break was repaired. Delay at the G1-S transition point has also been reported to be activated in response to DNA damage, mediated primarily through activation of the transcriptional functions of the tumour suppressor gene p53 (reviewed by Levine, 1997).

It has been reported in some cell types that the extent of cell-cycle delay is indicative of radiation response. Increased radioresistance has been observed in mammalian cells transfected with oncogenes such as H-ras and v-myc (McKenna et al. 1991) raf-1 (Fitzgerald et al. 1990) and SV40 virus sequences (Jung and Dritschilo, 1994), all of which show an increased G2/M delay. Cell lines derived from patients with AT (ataxia telangiectasia) are extremely radiosensitive and lack a G2 delay after radiation (Rudolph et al. 1989; Zampetti-Bosseler and Scott, 1981; Nagasawa and Little, 1983). Human tumour cell lines with a broad range of radiosensitivities exhibited similar levels of G2/M delay when treated with equi-lethal doses of radiation, indicating a parallel relationship between the length of G2/M delay and radiosensitivity (Nagasawa et al. 1994). Treatment of human tumour cells with agents that modify radiation response, such as caffeine, enhances radiosensitivity through the abrogation of radiation-induced G2 delay function (Busse et al. 1977). Interestingly, this radiosensitising effect was subsequently shown to be dependent on the status of the p53 gene and observed only in cells with mutant or absent p53 (Powell et al. 1995), and consequently has been shown to be absent in human normal cell lines (Jha and Bedford, 1996).

Few factors have been identified as being directly involved in the G2/M delay response. Entry into mitosis is generally thought to be regulated by the mitosis promoting factor (MPF), which consists of two proteins namely p34<sup>cdc2</sup> and cyclin B (Draetta and Beach, 1989; reviewed by Nasmyth, 1996). Accumulation of cyclin B in late S and G2 is followed by binding to p34<sup>cdc2</sup> and activation is initiated by tyrosine dephosphorylation of p34<sup>cdc2</sup> by the cdc25 gene product (Moreno et al. 1989). In human tumour cell lines radiation-induced G2 delay is accompanied by a decrease in the levels of cyclin B1 mRNA and protein (Muschel et al. 1993; Markiewicz et al. 1994; Smeets et al. 1994a).

Mammalian cells expressing wild-type p53 are generally proficient in arresting at
the G1/S transition point and p53 status has been shown to correlate with radiosensitivity in many tumour cell lines (reviewed by Bristow et al. 1996; reviewed by Levine, 1997). p53 mediates cell-cycle delay primarily through the transcription activation the WAF1 gene, which expresses the protein product p21 (El-Deiry et al. 1993). Increased levels of this protein result in the arrest of cells in G1 through the binding to, and consequent inhibition of, cyclin E and D/Cdk4 and 6 complexes present in this phase.

Since the work of Fertil and Malaise (1981), which demonstrated a good correlation between the in vitro radiosensitivity parameters and clinical response in human tumour cell lines, there has been considerable interest in the development of in vitro predictive assays for tumour survival. However, many studies investigating the potential use of radiation-induced cell-cycle delays in the prediction of radiation response have been tested on cells treated with relatively high radiation doses (4 Gy and over) (Schwartz et al. 1994, 1996b; Bernard et al. 1996; Nagasawa et al. 1994; Smeets et al. 1994a). Survival in vitro is determined using clonogenic survival curves fitted with the linear quadratic (LQ) model. This model has gained wide acceptance due to its reliability in describing the response of a broad range of mammalian cells to ionising radiation and survival curves are generally constructed using doses in the clinically relevant region of 1 – 4 Gy. Radiosensitivity is often expressed as the surviving fraction at 2 Gy (SF2), which is calculated from the LQ equation of best fit. It would be more informative, therefore to examine the degree of perturbation in the cell-cycle caused by doses in this range, such that realistic comparisons can be made with the cell survival data.

There is, however, much evidence to suggest that the LQ model can overestimate the survival of some mammalian cells to radiation doses less than 1 Gy (Marple and Joiner, 1993; Lambin et al. 1996; Wouters et al. 1996). In such cases cells demonstrate radiation hypersensitivity in the dose range 0.1 – 0.5 Gy followed by an apparent induced resistance to doses of 0.5 – 1 Gy. These linked phenomena have been termed hyper-radiosensitivity and induced radioresistance response (HRS/IRR). It has been hypothesised that cells which demonstrate this phenomenon are capable of inducing an adaptive response to higher doses of radiation. Thus, when the DNA damage levels are sufficient to trigger cellular responses such as cell-cycle delay, DNA repair and apoptosis, this mechanism will result in increased overall survival compared to cells lacking a HRS/IRR response.

In the present study, we examined the effect of ionising radiation, in the range 0.2 – 4 Gy on cell-cycle progression of eight human tumour cell lines. Six of these lines had previously been analysed for low dose hypersensitivity (Lambin et al. 1996) and it was found that the three resistant cell lines (SF2 > 0.5; HT29, Be11, RT112) and one radiosensitive cell line (SF2 = 0.25, MeWo) demonstrated HRS/IRR. This was in contrast to two radiosensitive cell lines (SW48 and HX142) which demonstrated no
HRS/IRR response. It was hoped to determine whether cell-cycle progression was altered differentially at low doses in those cells that did or did not show an adaptive response. The effects of radiation at doses above 1 Gy (up to 4 Gy), were also examined to determine the significance of cell-cycle perturbations at doses similar to those used in clinical radiotherapy.

Cell-cycle delays were measured using the bromodeoxyuridine (BrdUrd) pulse-labelling method and flow cytometry (as described in Chapter 2). BrdUrd is a thymidine analogue which is incorporated into cells actively synthesising DNA i.e. S-phase cells. This technique of cell-cycle measurement has several advantages over other methods commonly used. For example, it does not require synchronisation of the cell population which might affect normal cell progression. It also allows the analysis of several individual populations, which can be identified and followed through their cell-cycle through the examination of both their BrdUrd status and DNA content.

The gene products cyclin B1 and p53, which are known to be involved in the regulation of the cell-cycle, were examined in each cell line. The aim was to determine whether differences in cell-cycle responses to radiation could be affected by the relative expression levels of these proteins. The demonstration of such a relationship might have implications in the elucidation of the factors affecting the response of different tumour cell types to radiation and in the prediction of radiation response.

4.2 Specific materials and methods
4.2.1 Cell culture and irradiation
Cells were plated at a concentration of $2 \times 10^5$ per 25 cm$^2$ flask and left to attach and flatten for two days. Cells were then incubated in fresh medium and left to grow for a further three days. While still in exponential phase, cells were labelled with BrdUrd as described previously (see Chapter 2).

All irradiations were carried out using an experimental set-up which incorporated a Stuart warmbox set at 37°C (see Figure 4.1). Trays with polystyrene lids were used to transport culture flasks to and from the X-ray machine, ensuring minimum heat loss. These trays allowed the irradiation of 12 X 25 cm$^2$ flasks at any one time. The dose rate was $\sim 0.2$ Gy min$^{-1}$.

4.2.2 Sample collection and analysis using flow cytometry
Samples were collected using a FACScan flow cytometer (Becton Dickinson) and analysed using the Lysis II software package (Becton Dickinson). BrdUrd incorporation was detected using a monoclonal primary antibody to BrdUrd, and was revealed using a fluorescent (FITC) secondary antibody, which has a maximum emission spectrum of $515 < \lambda < 545$ nm. The green FITC fluorescence emission signal was directly related to BrdUrd incorporation, and was collected on a log scale. Parallel
incorporation of the DNA stain propidium iodide (PI) (maximum emission 590 <λ< 640 nm) allowed the simultaneous analysis of BrdUrd incorporation and DNA content. This type of staining protocol (Wilson et al. 1994; Nagasawa et al. 1994; Higashikubo et al. 1996) allowed the identification of the three main populations of interest: G0/G1, S and G2/M, whose movement could then be followed using a series of analysis windows generated in the software package (LYSIS II). Debris or clusters of cells were eliminated from analysis by the use of a doublet discrimination module on the PI signal.

The populations of interest used when analysing the cell-cycle data are shown in Figure 4.2. This shows the histogram profile of the total cell population, which contains both the BrdUrd-labelled and unlabelled cells. During analysis, these windows were overlaid onto plots of the individual populations, so the fraction of labelled and unlabelled cells can be calculated for each phase and analysed over time.

Figure 4.3 a–c shows examples of the plots generated during the dose response analysis of individual cell-cycle phase delays. A G2/M delay was observed in all cell lines. Estimation of the length of delay in the exit of cells from G2/M was performed by plotting the entry of BrdUrd-labelled cells, corrected for cell division (see Chapter 3), into G0/G1. The entry of cells into G1 with time followed a sigmoidal trend and was fitted using a non-linear regression procedure (Johns and Joiner, 1991) with a generalised logit equation as described in Chapter 3 (equation 3.3). This equation was fitted to the individual data points for each experiment, and the upper and lower limits were optimally defined for each cell line. The extent of G2/M delay of BrdUrd-labelled cells was determined from the shift in the inflection points of the best fit curves of unirradiated controls compared to and irradiated samples (Figure 4.3 a).

The G2/M delay of G2 phase cells was observed by following the movement of BrdUrd unlabelled G2 cells through this phase with time after radiation treatment. A delay in radiation treated cells was indicated by an increase in the time required for the fraction of cells in this phase to decrease with time, relative to unirradiated controls.

The extent of G1/S transition delay was analysed by plotting the entry of G1 cells into early S with time, see Figure 4.3 b. Differences between the half maximum peak height times were used to calculate the extent of delay at G1/S border. A similar analysis was performed for the determination of a delay in the progression of BrdUrd-labelled cells through S-phase, see Figure 4.3c.
Figure 4.1. Cell irradiation system for cell cycle perturbation experiments. The Stuart incubator in which the cells were irradiated was set at 37°C. Cells were transported to and from the X-ray machine to the long-term incubators using pre-warmed trays with polystyrene lids to minimise heat loss.
Figure 4.2. Cell cycle delay analysis of DNA histograms. Regions of interest were isolated using computer generated regions (LYSIS II software, Becton Dickinson). Each population is identified by differences in DNA content (x-axis). G2/M cells have twice the DNA content of G0/G1 cells, as they are preparing to divide. S-phase cells have a variable DNA content, as DNA synthesis is in various stages of completion. M1 shows cells which are in G0/G1 and this window was used to follow the entry of BrdUrd-labelled cells into this phase after progression through G2/M. This facilitates the analysis of a G2/M delay of S phase cells at the time of irradiation. M2 shows cells in early S and facilitates the analysis of G1/S delay. M3 shows cells in mid-S and facilitates the analysis of S-phase delay. M4 is placed around G2/M cells and allows the analysis of the progression of G2 cells at the time of irradiation through G2.
Figure 4.3. Radiation-induced cell-cycle delay. A: G2 delay dose response. Alterations in the rate of entry of SW48 cells into G1 with increasing dose indicate a delay in G2, and is quantified by the difference between curve inflection points. B: G1/S delay dose response. A delay in progression of BrdUrd-unlabelled cells through G1/S is estimated from differences in the time taken to reach half maximum peak height. C: S-phase delay. A delay in the movement of BrdUrd-labelled cells in mid-S through S phase is estimated from the time taken to reach half minimum peak height.
4.2.3 Western blot analysis

Cell lines were analysed for p53 and cyclin B1 expression using western blot analysis, as described in Chapter 2. p53 protein was detected using the mouse anti-human monoclonal DO-7 antibody (Dako), which recognises an epitope in the N-terminus of the human p53 protein. This epitope is known to reside between amino acids 19 and 26 and can therefore react with both wild-type and mutant protein. This antibody was diluted to a final concentration of 1/500. Cyclin B1 protein was detected using a goat anti-human polyclonal primary antibody to the human form of the protein (Santa Cruz) diluted to a final concentration of 1/200. Both proteins were revealed using a peroxidase conjugated secondary antibody, diluted to a final concentration of 1/5000, and visualised using ECL (chemiluminescence; Amersham International).

4.3 Results

4.3.1 Cell-cycle delay dose responses

4.3.1.1 S-phase delay

Delay in the progression of cells through S-phase was analysed by following the movement of BrdUrd-labelled cells through an analysis window placed in mid-S. Only cells progressing through their first cell-cycle after radiation treatment were analysed. The graphs generated by this type of analysis are shown in Figure 4.4 a–h. It was evident that progression of cells through S-phase in the majority of cell lines was not affected by radiation at any dose. This was characterised by the lack of alteration in the number of cells in mid-S after radiation compared to controls. Only the Bel 1 cell line demonstrated a delay in S-phase, which was observed only after treatment with 4 Gy. This delay was estimated at 4 h. All cell-cycle delay data are summarised in Tables 4.1 and 4.2.

4.3.1.2 G1/S delay

A delay in the transition of G1 cells into S-phase, termed G1/S delay, was analysed by following the movement of BrdUrd-unlabelled G1 cells through a small analysis window placed in early S-phase. All results are shown in Figure 4.5 a–h. Five out of eight cell lines demonstrated a slight delay in G1/S after irradiation, although in some cases this was only observed after 4 Gy (MGH-U1, Bel11 and U1-S40b). The HT29, RT112 and MeWo cell lines did not demonstrate any G1/S delay at any dose tested (panels a, d, and f). HX142 cells demonstrated a slight delay at both 2 Gy and 4 Gy, The SW48 cell line demonstrated the longest G1/S delay, estimated at 5 h, which appeared to be dose independent as this delay was observed to the same extent at 1 Gy, 2 Gy and 4 Gy. For simplicity in subsequent analyses, cell lines were described as either G1/S-delay positive or negative after individual radiation doses.
Figure 4.4. Radiation-induced S-phase delay in eight human tumour cell lines. The progression of BrdUrd-labelled cells through mid-S was followed with time. A delay is characterised by an increase in the time necessary for cells to exit this window.
Figure 4.4. Radiation induced S-phase delay in eight human tumour cell lines. Continued from the previous page.
Figure 4.5: G1/S delay in eight human tumour cell lines. The progression of unlabelled cells into early S is followed with time for increasing radiation doses. Errors represent the mean of at least 2 experiments (±SEM).
Figure 4.5: G1/S delay in eight human tumour cell lines. Continued from previous page
4.3.1.3 G2/M delay of S-phase cells

The entry of BrdUrd-labelled cells into the G1 phase indicates their completion of G2 and M phases and re-entry into G1 for another round of replication. A delay in the entry of these cells into G1 indicates a delay in their progression through G2 and M phases. Entry of cells into G0/G1 followed a sigmoidal trend, which was fitted using the general logit equation, 3.3. This allowed the calculation of the extent of this delay in each cell line in terms of hours per radiation dose. This type of G2/M delay analysis was less prone to interference by the entry of cells from other phases of the cell-cycle into the analysis window as only the BrdUrd cells (S-phase cells at the time of irradiation) were considered. All cell lines demonstrated a delay in the progression of BrdUrd cells through G2/M phase, see Figure 4.6 a–h. The extent of this delay, which was calculated by increased inflection points of the best curve fits of the irradiated samples compared to the controls, varied between cell lines (see Table 4.2). The dose response of G2/M delay with radiation is plotted in Figure 4.7 a–b. Low doses of radiation did not significantly alter progression of the more resistant cell lines through G2/M, compared to unirradiated controls (panel a). Indeed some of the delay values were calculated as being negative compared to the control, due to the small differences between the samples. However, the two most radiosensitive cell lines, SW48 and HX142, demonstrated positive delays of 1·0 h and 1·9 h at 0·2 Gy respectively (in panel b). These delays increased to 2·2 h and 2·1 h at 0·5 Gy respectively. At doses of 1 Gy and over, all cell lines demonstrated an appreciable delay in G2/M. There appeared to be a greater relationship between radiation dose and G2/M delay in the radiosensitive cell lines, as increasing radiation dose increased the length of the G2/M delay significantly in each cell line. In contrast, the resistant cell lines did not demonstrate significant alterations in G2/M delay with increasing doses of 1 Gy, 2 Gy and 4 Gy. As a result, the extent of delay after 4 Gy was markedly longer in all the radiosensitive cell lines. These values at each dose, expressed as the length of G2/M phase, were correlated with radiosensitivity expressed as SF2, in Figure 4.8 a–e. Only the G2/M phase lengths at 0·5 Gy and 4 Gy of ionising radiation were statistically significant with radiosensitivity (p = 0·032, r = -0·751, panel c; p = 0·004, r = 0·867; panel e respectively), although there was a trend towards increased G2 phase length with radiosensitivity at all doses tested. The survival and G2/M delay at individual radiation doses were also analysed, Figure 4.9 a–b. The overall shape of the curves appeared markedly different for the resistant cell lines (panel a) and the radiosensitive cell lines (panel b) primarily due to an extensive delay induced by 4 Gy in the sensitive cell lines. In the radioresistant cell lines the extent of delay at higher doses appeared to be dose (and therefore survival) independent. In the radiosensitive cells, the relationship between survival and G2/M delay at increasing doses of radiation appeared to be almost linear. This relationship deviated at higher doses (4 Gy), which may be related to the severity of this dose indicated by almost zero survival in these cells.
Figure 4.6. Radiation-induced G2/M delay in eight human tumour cell lines. The delay of BrdUrd-labelled cells in G2/M phase was estimated by analysis of the time to their appearance in G1 phase. The data were fitted using a generalised logit equation, on the raw data points. Data represent the mean of at least two experiments (± SEM).
Figure 4.6. Radiation-induced delay of BrdUrd-labelled cells in G2/M phase. Continued from the previous page.
Figure 4.7 G2/M delay of S-phase cells as a function of dose. The eight human tumour cell lines were divided into radioresistant (a) and radiosensitive cell lines (b). Cell lines were analysed over the dose range 0-2-4 Gy. Data points represent the mean of at least two experiments each dose (±SEM).
Figure 4.8. Radiation induced G2/M delay of BrdUrd-labelled cells and its relation to radiosensitivity. The length of G2/M was estimated by extrapolation of the linear portion of the sigmoidal curve showing the increase in labelled cells in G1 with time (see Chapter 3). Radiation-induced delays were estimated from the shift in the inflection point of the curve with increasing dose (see text). G2/M delay is represented in the above graphs as increasing G2/M length with radiation with radiosensitivity expressed as SF2.
Figure 4.9. G2/M delay and surviving fraction with increasing radiation dose. Surviving fraction was estimated from the Linear Quadratic (LQ) fit of survival data. The G2/M delay at each dose was calculated as described in the text. Radioresistant cell lines are grouped in panel a and radiosensitive cell lines in panel b.
especially SW48 and HX142.

It was evident from the analysis of the time course of progression of BrdUrd-labelled cells through G2/M that the fraction of cells which re-entered G0/G1 decreased with increasing dose. This effect (characterised by a reduction in the upper limit of the proportion of BrdUrd-labelled cells in G0/G1, Figure 4.6 a–h) was particularly evident in the more sensitive cell lines (compare panels a–d with panels e–h). It could be speculated that the cells which did not progress into G0/G1 were irreparably damaged and unable to overcome the radiation-induced delay. Also, the reduced survival observed in the radiosensitive cell lines might be directly related to this population of cells that do not progress into G0/G1. To examine this, the proportion of BrdUrd-labelled cells in G0/G1 was calculated from a ratio of the upper limit values (U) of the control with the irradiated sample populations shown in Figure 4.6. This value was termed the G2/M ratio (i.e. \( \frac{U_{\text{sample}}}{U_{\text{control}}} \) at each dose) as it represents the ratio of irradiated cells that have completed G2/M relative to the control. The results of this analysis are shown in Figure 4.10 a–d. At low doses (0.2 Gy and 0.5 Gy, panels a and b respectively) there appeared to be no relationship between G2/M ratio and survival. The statistical correlation was not significant (\( p = 0.965, \ r = -0.023; \ p = 0.932, \ r = -0.045 \) respectively). However, at higher doses where the effects of radiation were more obvious, there appeared to be a strong relationship towards cell lines with a high surviving fraction also demonstrating a high G2/M ratio, as both values for the 2 Gy and 4 Gy analyses were only just outside the range for significance (\( p = 0.078, \ r = 0.655; \ p = 0.055, \ r = 0.696 \) respectively). These results suggest that those cells unable to proceed through G2/M after radiation treatment (perhaps due to irreparable DNA damage) were subsequently eliminated from the population, through some form of cell death, thus reducing the overall survival of the population.

4.3.1.4 G2/M delay of G2 cells

The delay in the progression of G2 cells was analysed by following the movement of BrdUrd unlabelled cells in G2 through an analysis window generated around this population (see Figure 4.2). The proportion of BrdUrd-unlabelled cells decreases with time as cells exit this phase and a delay is represented by an increase in the time required for the cell number in this region to reach a minimum. The results of this analysis are shown in Figure 4.11 a–h. Cells were followed for at least 36 h but analysis was possible only up to a maximum of 24 h, or a time corresponding to S-phase length. This was due to the contamination of the analysis region by the entry of BrdUrd-unlabelled G1 phase cells into G2 phase (on their completion of S-phase) as two different but indistinguishable populations were present in the analysis window at this stage. This can be readily observed by the increase in the proportion of cells in G2 phase, which occurred after 10 h in all cell lines (Figure 4.11). The initial proportion of
Figure 4.10. Relation of surviving fraction of eight tumour cell lines to the fraction of cells which have progressed through G2/M, with increasing radiation dose. The fraction of cells which enter G1 represents those cells which have overcome the radiation induced-G2 delay and progress through mitosis. This value was calculated from the ratio of the upper asymptote of the plot showing the entry of BrdUrd-labelled control cells into G1, to irradiated cells. Surviving fraction at each dose was calculated from the LQ fit of the survival data for each cell line.
Figure 4.11. Radiation-induced G2/M delay of cells in G2 phase. BrdUrd-unlabelled G2 phase cells at the time of radiation were followed though G2 with time. A delay is represented by an increase in the time necessary for cells to exit this phase.
Figure 4.11. Radiation induced G2/M delay of cells in G2. Continued from the previous page.
cells in G2 varied between the cell lines with none of the values reaching zero at any time point tested. This could be indicative of either the constant progression of some unlabelled S-phase cells (perhaps apoptotic cells) through G2, or the persistence of cells destined to die prior to cell division. The HT29, MGH-U1 and Be11 cell lines did not demonstrate any detectable G2 delay of G2 cells (panels a, b and c). In the case of the HT29 cells, the initial proportion of cells in G2 did not vary much over the time course of the experiment, remaining at ~2.5% throughout, increasing only after 18 h when G1 cells enter the G2 phase analysis window having completed S-phase (HT29 S-phase length had been estimated previously at 17.5 h; see Chapter 3, Table 3.2). The remaining cell lines RT112, SW48, U1-S40b, MeWo and HX142 all demonstrated a G2 delay, but only at 2 Gy and 4 Gy, as values at these doses did not drop to the levels observed in the unirradiated samples. Instead, the proportion of G2 cells remained constant and increased only when G1 cells entered the phase. This was particularly evident in U1-S40b cells, where the values remained at ~20% of total unlabelled cells in samples irradiated with doses of 2 Gy and 4 Gy, while all others demonstrated a reduction to ~10%.

4.3.2 G2 phase length and G2/M delay

The delay of BrdUrd cells in G2/M was the only delay which was observed to some extent in all cell lines. In an attempt to identify some of the factors which might influence the extent of this response the relationship between the intrinsic G2/M phase length and delay was examined in each cell line. In Chapter 3, the cell-cycle distributions and phase lengths for each cell line were determined and discussed in terms of survival after radiation. It was speculated that the pre-irradiation characteristics of the cell lines might influence their response to radiation. The relationship between the G2 phase length and G2/M delay is shown in Figure 4.12 a – e. While none of the relationships reached statistical significance there appeared to be an inverse correlation between G2/M phase length and delay. This did not indicate radiosensitivity however, as the differences observed in the G2 phase lengths between each cell line were not related to radiation response.

4.3.3 G2 delay and G1 delay

Recent evidence has suggested that the G1/S and G2/M checkpoints are related. Russell et al. (1995) reported differential radiosensitisation of G1 checkpoint-deficient and competent cells by the abrogation of the G2 checkpoint, depending on the p53 status (Powell et al. 1995). Wild type p53, a major effector of radiation-induced G1 delay has been shown to positively modulate the exit of cells from the gamma radiation-induced G2 checkpoint in mammalian cells (Guillouf et al. 1995). The relationship between these phases in the eight tumour cell lines in the present study was examined (Figure 4.13). In
Figure 4.12. Relationship between G2 phase length and G2 delay with increasing radiation dose. G2 phase length was calculated on unirradiated controls (see Chapter 3). The radiation-induced delay in G2 phase is represented as an increase in G2/M phase length.
Figure 4.13. Radiation-induced G2/M and G1/S delay in eight human tumour cell lines. Each cell line was scored either positive (1) or negative (0) for G1/S delay at individual doses. This was related to the length of radiation induced G2/M delay at the corresponding dose.
this analysis, the presence of a G1/S delay was expressed as a function of G2/M phase length at different radiation doses. The existence of a G1 delay with one particular radiation dose in a cell line did not appear to affect the length of the G2 phase delay at that same dose, as both G1 delay positive and negative cells demonstrated a wide range of G2 delay lengths. In particular, the cell lines that demonstrated G1/S delay (primarily observed only at 2 Gy and 4 Gy) also demonstrated a range of G2/M lengths at these doses, suggesting that in these cell lines the extent of radiation-induced G2/M delay was not dependent on the presence of a G1/S delay.

4.3.4 p53 protein expression

The status of p53 protein expression was examined in each of the cell lines using Western blotting. Cells were tested for p53 expression 4 h following radiation doses of 0 Gy, 0.2 Gy and 2 Gy, and results are shown in Figure 4.14a–c. Three cell lines were identified as having mutant p53 expression; HT29, RT112 and MeWo, due to the relatively high constitutive levels of p53 protein and the lack of a radiation-induced increase in expression levels. This supports previous reports on the p53 status of these cell lines (Shao et al. 1996; Nagasawa et al. 1995; McIlwrath et al. 1994; Zolzer and Streffer, 1995). The remaining cell lines; MGH-U1, Be11, U1-S40b, SW48 and HX142, in contrast demonstrated low constitutive levels of p53 protein, with a clear post-irradiation increase, indicative of protein stabilisation, characteristic of wild-type p53 expression. SW48 demonstrated the highest level of protein, with lower levels detected in both the Be11 and HX142 cell lines. The level of protein in the MGH-U1 and U1-S40b cells was especially low and required much longer exposure times (30 minutes versus 2–5 min) during chemiluminescence development to detect the protein. When exposed for 30 minutes the induction of the protein in both cell lines can be detected (panel c). These results confirmed previous reports on the wild-type status of these cell lines (H. M. Warenius, personal communication; Zolzer and Streffer, 1995; T. J. McMillan, personal communication).

Of the five cell lines that demonstrated wild-type p53 expression, all exhibited a G1/S delay. Only the SW48 and HX142 demonstrated a G1/S delay at doses other than 4 Gy. None of the mutant p53 expressing cell lines delayed at G1/S. These results are consistent with the concept that in order for a cell line to demonstrate G1/S arrest their p53 expression must be wild-type.

4.3.5 Cyclin B1 protein expression

The progression of mammalian cells through G2 phase is mediated by the association of cyclin B1 with its cyclin dependant kinase partner, cdc2. Few factors have been identified in the initiation of G2/M delay, but it has been reported in some cell types
Figure 4.14. p53 expression in eight human tumour cell lines. Protein was detected using western blotting. 0.5 x 10^6 cells were loaded in each lane. Panel A represents the radioresistant cell lines while the radiosensitive cells are shown in panel B. Normal fibroblasts (Fibr) were run as positive controls. Wild-type p53 expression can be readily detected in the Be11, SW48 and HX142 cell lines, due to the characteristic increase in protein level after radiation. The level of protein in the MGH-U1 and U1-S40b cells was relatively low, but further examination (longer exposure times) demonstrated wild type-p53 expression in these cell lines also (panel C). Mutant-p53 expression was characterised by relatively high constitutive levels of protein with no increase after radiation. This was observed in HT29, RT112, and MeWo cells.
Figure 4.15. Cyclin B1 expression in eight human tumour cell lines. Protein was detected using western blotting (see Chapter 2). Each lane represents $0.5 \times 10^6$ cells. Panel A shows the resistant cell lines, treated with 0, 0.2, and 2 Gy of radiation. Panel B represents the radiosensitive cell lines. In both, normal human fibroblasts (fibr) and HeLa cells were run as positive controls.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>SF2</th>
<th>S delay (yes / no)</th>
<th>G1/S delay (yes / no)</th>
<th>G2/M delay of G2 cells (yes / no)</th>
<th>p53 status/expression (wt / mu)/++</th>
<th>cyclin B1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>0.74</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>mu&lt;sup&gt;a&lt;/sup&gt;, ++++</td>
<td>++</td>
</tr>
<tr>
<td>MGH-U1</td>
<td>0.70</td>
<td>no</td>
<td>yes (2 h at 4 Gy)</td>
<td>yes (at 2 and 4 Gy)</td>
<td>wt&lt;sup&gt;b&lt;/sup&gt;, +</td>
<td>++</td>
</tr>
<tr>
<td>Be11</td>
<td>0.68</td>
<td>yes (4 h at 4 Gy)</td>
<td>yes (5 h at 4 Gy)</td>
<td>no</td>
<td>wt. +++</td>
<td>++++</td>
</tr>
<tr>
<td>RT112</td>
<td>0.62</td>
<td>no</td>
<td>no</td>
<td>yes (at 2 and 4 Gy)</td>
<td>mu, ++++</td>
<td>++++</td>
</tr>
<tr>
<td>UI-S40b</td>
<td>0.40</td>
<td>no</td>
<td>yes (2 h at 4 Gy)</td>
<td>yes (at 2 and 4 Gy)</td>
<td>wt, +</td>
<td>++</td>
</tr>
<tr>
<td>MeWo</td>
<td>0.25</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>mu, ++++</td>
<td>++</td>
</tr>
<tr>
<td>SW48</td>
<td>0.18</td>
<td>no</td>
<td>yes (5 h at 0.5, 1, 2 and 4 Gy)</td>
<td>yes (at 1, 2 and 4 Gy)</td>
<td>wt, +++</td>
<td>++++</td>
</tr>
<tr>
<td>HX142</td>
<td>0.03</td>
<td>no</td>
<td>yes (1 h at 2 and 4 Gy)</td>
<td>yes (at 2 and 4 Gy)</td>
<td>wt, ++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 4.1. Cell cycle delay characteristics and protein expression in eight human tumour cell lines.

<sup>a</sup>: mutant
<sup>b</sup>: wild-type
<table>
<thead>
<tr>
<th>Cell line</th>
<th>SF2</th>
<th>G2 phase length (h)</th>
<th>G2/M delay of S phase cells (h)</th>
<th>Dose (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>HT29</td>
<td>0.74</td>
<td>1.24</td>
<td>0.11 ± 0.69</td>
<td>0.47 ± 0.87</td>
</tr>
<tr>
<td>MGH-U1</td>
<td>0.70</td>
<td>2.51</td>
<td>0.71 ± 1.52</td>
<td>-0.43 ± 0.75</td>
</tr>
<tr>
<td>Be11</td>
<td>0.68</td>
<td>3.71</td>
<td>0.19 ± 0.41</td>
<td>0.50 ± 1.64</td>
</tr>
<tr>
<td>RT112</td>
<td>0.62</td>
<td>2.88</td>
<td>-0.34 ± 1.06</td>
<td>-0.91 ± 0.90</td>
</tr>
<tr>
<td>U1-S40b</td>
<td>0.40</td>
<td>1.67</td>
<td>-0.47 ± 1.02</td>
<td>-0.13 ± 0.11</td>
</tr>
<tr>
<td>MeWo</td>
<td>0.25</td>
<td>3.13</td>
<td>-0.53 ± 1.14</td>
<td>0.87 ± 0.64</td>
</tr>
<tr>
<td>SW48</td>
<td>0.18</td>
<td>3.22</td>
<td>1.03 ± 0.77</td>
<td>2.17 ± 2.00</td>
</tr>
<tr>
<td>HX142</td>
<td>0.033</td>
<td>3.53</td>
<td>1.90 ± 1.16</td>
<td>2.14 ± 0.97</td>
</tr>
</tbody>
</table>

Table 4.2. Radiation induced G2/M delay of S-phase cells in eight human tumour cell lines.
that the alteration of cyclin B1 levels correlated with the time course of delay induction and release (Smeets et al. 1994a; Muschel et al. 1993; 1993; Bernhard et al. 1995; McKenna et al. 1996).

In the present study the level of cyclin B1 was examined in each cell line at 0 h and 4 h after irradiation by Western blotting and results are summarised in Figure 4.15 a–b. Normal human fibroblasts and HeLa cells were run as normal and positive controls respectively. Constitutive protein levels varied between cell lines, where Be11, RT112 and SW48 exhibited markedly higher constitutive levels of cyclin B1 compared to the other cell lines. In most cells the level of cyclin B1 was markedly decreased when exposed to 2 Gy. This might be indicative of cyclin B1 suppression during G2/M delay, similar to that reported by Muschel et al. (1993). Only the Be11 cell line did not appear to exhibit any significant change in cyclin B1 levels after radiation. This cell line also demonstrated the shortest G2/M delay at doses of 1 Gy and over. It could be speculated that this diminished delay was due to the lack of radiation-induced cyclin B1 suppression, resulting in only minor retardation of the progression of cells through G2/M, compared to unirradiated controls.

4.4 Discussion

Exposure of cells to ionising radiation results in alterations in their movement through the cell-cycle (Sinclair, 1968; Terasima and Tolmach, 1963; Bedford and Mitchell, 1973; Zampetti-Bosseler and Scott, 1981; Smith et al. 1985; McKenna et al. 1991; Su and Little, 1993; reviewed by Paulovich et al. 1997). Cell-cycle control is an important determinant of cell survival, although the factors involved in maintaining this control both under normal replication conditions and in the presence of DNA damage are yet to be fully understood.

In this present study, eight human tumour cell lines were examined for cell-cycle perturbations after treatment with increasing doses of radiation. It was hoped to determine whether differences in the extent of cell-cycle perturbation could be identified in cells of different tumour types and whether these could be related to differences in intrinsic radiosensitivity. The radiation doses of interest included those in the clinically relevant range, 2–4 Gy, and those in the low-dose region, <1 Gy.

Lambin et al. (1996) identified four human tumour cell lines with a pronounced low dose (0–0.5 Gy) hypersensitive region in their survival curves (HT29, Be11, RT112 and MeWo), which was not detected by the LQ model. This response was absent in two other human tumour cell lines (SW48 and HX142). The existence of this low dose hypersensitive region appeared to correlate with radioresistance to subsequent higher doses of radiation. The authors concluded that differences in the intrinsic radiosensitivity at clinically relevant doses might be due to an adaptive response
mechanism whereby resistant cells are hypersensitive to radiation at low doses because cell protection mechanisms, such as cell-cycle delays, are not triggered until higher doses. These linked phenomena have been defined as hyper-radiosensitivity and induced radioresistance (HRS/IRR). By examining the cell-cycle responses of these cell lines to radiation at low and clinically relevant doses of radiation it was hoped to determine whether the HRS/IRR response could indeed be linked to the differential response of the cell-cycle checkpoint pathways, which could then be used as a predictor of radiation response.

Cell-cycle progression was analysed using the simultaneous analysis of BrdUrd incorporation and PI staining. Radiation doses ranging from 0.2 Gy to 4 Gy were applied to exponentially growing populations of eight human tumour cell lines with a broad range of intrinsic radiosensitivities. Cell-cycle delays were analysed in G0/G1, S and G2/M phases at each dose and correlated to radiosensitivity.

The progression of cells through S-phase was not significantly affected by radiation in most cell lines. Only the Be11 cell line demonstrated a detectable delay in the progression of S-phase cells through S (see Figure 4.4, panel c), which was observed only at 4 Gy. The general absence of delay in this phase is concordant with several studies using mammalian cell lines (Higashikubo et al. 1996; Wilson et al. 1994). However, a transient S-phase arrest induced by radiation has been reported in a human melanoma cell line, M14 (Villa et al. 1996) and in primary rat fibroblasts transformed with the oncogenes H-ras and v-myc (Wang and Illiakis, 1992). Wyllie et al. (1996) also demonstrated an S-phase cell-cycle arrest following DNA damage in normal human diploid fibroblasts and found that, through manipulation of p53 function, this delay was independent of p53/p21 function. The reason why Be11 was the only cell line to undergo an S-phase delay is unclear. The fact that it was only observed at the highest dose tested (4 Gy) could indicate that this checkpoint is not activated until quite extensive DNA damage has been produced. It is generally accepted that cells in S-phase of the cell-cycle are the most resistant to radiation (Ross and Sinclair, 1972) and this could be the reason why no significant delay was observed in this phase in the majority of cell lines. It can be concluded therefore that S-phase cell-cycle arrest has a minimal influence on radiosensitivity of the cell lines in this study at these doses.

Radiation delay in the progression of cells through the G1/S transition point has been well documented (reviewed by Paulovich et al. 1997 and Nasmyth, 1996). The regulation of this checkpoint has been strongly linked to the p53 tumour suppressor gene and its transcriptional activation of the p21WAF1 protein, which has been shown to block the entry of cells into S-phase by inactivation of the cyclin D and E/cdk 4 and 6 complexes (reviewed by Levine, 1997). The persistence of cells in G1 phase after radiation treatment, in cell lines with wild-type p53 protein expression, has been shown to be a major determinant of intrinsic radiosensitivity. It is thought that activation of the
checkpoint facilitates optimal repair of DNA damage by delaying DNA replication (Kastan et al. 1992). In the present study, the extent of radiation-induced G1/S delay was examined by analysis of the movement of G0/G1 cells into early S-phase with time (Figure 4.5). Five cell lines demonstrated a detectable G1/S delay, but only two in response to doses lower than 4 Gy (SW48 and HX142). The delay observed in the other three cell lines was only detected after doses of 4 Gy (MGH-U1, Be11 and U1-S40b).

The response of the SW48 cell line was the most striking, where cells demonstrated a G1/S arrest at all doses except 0-2 Gy. The extent of this delay was estimated to be 5 h, irrespective of dose. This might indicate that, in this cell line, the G1/S delay checkpoint was activated in a dose independent manner and that DNA damage produced by 0-5 Gy of radiation was sufficient to activate the checkpoint pathway. The HX142 cell line did not demonstrate this sensitivity to G1/S delay activation after radiation. The proportion of HX142 cells which progressed into early S-phase over the time course of the experiment was less than in any other cell line, i.e. only a maximum of 6-5% of the total BrdUrd-unlabelled cell population was in this phase at any given time. This could be due to the relatively long duration of G1 phase (Tg1 = 15-7 h) resulting in slower progression of cells into early S. The low proportion of cells in early S made analysis of the G1/S delay in this cell line slightly more difficult. However, it was apparent that no G1/S arrest was induced at 0-2 Gy or 1 Gy and that a slight radiation-induced effect was observed at 2 Gy and 4 Gy. The existence of a G1/S delay in these cell lines therefore appeared to be independent of dose. This implies that the pathway was activated only when a certain threshold amount of damage was produced and increased DNA damage levels do not further increase the G1/S delay. Three of the four radiosensitive cell lines demonstrated a G1/S delay in response to at least one of the radiation doses tested: U1-S40b, SW48 and HX142. However, two radioresistant cell lines also demonstrated a G1/S delay: MGH-U1 and Be11. This suggests that the activation of the G1/S checkpoint does not provide a rational basis to explain the different radiosensitivities observed in these cell lines.

The status of the p53 gene in each cell line, which had previously been reported, was confirmed using western blot analysis of p53 expression after radiation (Figure 4.14). Five cell lines were found to demonstrate a wild-type p53 response (MGH-U1, Be11, U1-S40b, SW48 and HX142), characterised by the stabilisation of the protein after radiation, resulting in an apparent increase in protein level. Constitutive levels of p53 were characteristically high in the p53-mutant cell lines (HT29, RT112 and MeWo), while levels varied markedly between the wild-type cell lines (Table 4.1). Of the wild-type cell lines, SW48 cells exhibited the highest level of both constitutive and radiation-induced protein. The actual link between p53 status and cellular radiosensitivity is an area of ongoing investigation. Siles et al. (1996) analysed G1 arrest and p53 protein levels in a panel of eight tumour cell lines and reported a correlation between constitutive
p53 level and G1 arrest after radiation and radiosensitivity in some cell types. However, several other studies have demonstrated no relationship between p53 status and radiation response (reviewed by Bristow et al. 1996). In the present study, the SW48 cell line demonstrated the most obvious G1 arrest and the highest wild-type p53 protein level. It could be postulated therefore, that the activation of the G1/S pathway by p53 is effective only when a certain threshold level of functional protein exists. Cell lines which express wild-type p53 to a level below this may not demonstrate an observable G1/S delay. How G1/S delay is related to radiosensitivity is unclear, as the SW48 cell line is very radiosensitive ($S_{02} = 0.18$), and it is generally accepted that the function of the G1 arrest is to allow time to repair damaged DNA, thus increasing survival. It has been reported, however that SW48 cells are defective in mismatch repair (Branch et al. 1995), most likely due to a mutation in hMLH1 gene, which is involved in the initial stages of mismatch repair (Kane et al. 1997). A novel target gene of the p53 protein, hMSH2 has recently been identified (Scherer et al. 1996) whose product has been linked to the DNA binding stage of mismatch repair. It could be speculated therefore that, although SW48 cells delay in G1/S, the repair process is not functional during this delay due to the hMSH1 mutation. This would mean those cells with irreparable DNA damage either progress through the cell cycle, producing more resistant phenotypes or, and this is more likely, are eliminated from the population either at the G1/S stage or in the following G2/M phase. This is certainly consistent with the G2/M delay, apoptosis and DNA repair results (see following chapters) for this cell line. Interpretation of the G1/S delay data and p53 status and expression therefore, is speculative unless more details are known about the processes that occur during the block. It is plausible that what is more important to survival is the extent of repair, apoptosis or other processes that occur during the cell-cycle blocks rather than the extent of the block itself. This certainly could be applied to the SW48 cell line where, although wild-type p53 was present and a radiation-induced G1/S delay was observed at most doses tested (both of which intuitively suggest radioresistance), the cell line was still very radiosensitive, probably due to the lack of repair during the cell-cycle perturbation.

The role of the p53 protein in the promotion of radiation-induced apoptosis might also affect radiation response. Wild-type p53 has been reported to be involved in the induction of radiation-induced apoptosis (reviewed by Levine, 1997) and, in response to radiation-induced DNA damage, activates either a G1/S arrest to promote repair or apoptosis to eliminate cells which may have acquired irreparable lethal mutations. The majority of cell lines that demonstrate a G1/S arrest in response to radiation were found to be radiosensitive, suggesting that some form of cell death has occurred. As shown in the following chapter (Chapter 5), where the radiation-induced apoptosis levels have been analysed, SW48 cells demonstrate relatively high levels of apoptosis compared to the other cell lines in the study. This suggests that p53 might activate both pathways so...
that while a G1/S delay allows time for cells to repair damage, incomplete repair can result in apoptotic cell death. This is discussed further in the following chapters.

The significance of the G1/S arrest checkpoint and the role of p53 in cellular radiosensitivity is an area of ongoing investigation. A lack of radiation-induced G1/S arrest has been shown in several cell types expressing wild-type p53 (Nagasawa et al. 1995; Brachman et al. 1993), and the status of the p53 gene has failed to show correlation with radiosensitivity in some studies (Brachman et al. 1993; reviewed by Bristow et al. 1996). Considering the results obtained in the present study, it is difficult to visualise a significant role of the G1 arrest and p53 gene alone in the radiation response of the cell lines from the data available. Of the radioresistant cell lines, two demonstrate wild-type p53 expression, MGH-U1 and Be11, indicating that the status of the p53 gene does not indicate radioresponse. However, of the four radiosensitive cell lines, only one demonstrated mutant p53 expression (MeWo), which suggests that wild-type p53 is associated with increased radiosensitivity. The significance of the role of p53 and G1/S delay in the radioresponse of human tumour cells lines is probably dependent on the extent of cellular processes that actually occur during the G1/S arrest. In the following chapters, the level of radiation-induced apoptosis and DNA repair in each cell line has been analysed in an attempt to elucidate which pathways are activated in each cell line in response to radiation. It would be of interest to determine whether the p53-mediated G1/S arrests are accompanied by apoptosis or repair.

A radiation-induced G2/M delay of BrdUrd-labelled cells, i.e. S-phase cells, at the time of irradiation was detected in all cell lines (see Figure 4.6). Delays were observed by following the movement of BrdUrd-labelled cells into an analysis window placed around G0/G1 phase. Cells which appeared in this window represented those S-phase cells at the time of irradiation which have completed G2/M phase and re-entered the cell-cycle at G0/G1 for another round of replication. Cell numbers were corrected for cell division (as they have undergone mitosis) and the proportion of labelled cells which appear to be in G0/G1 at the start of the experiment, due to overlap of the cell populations (i.e. cells in early-S can appear to be in late G0/G1, as a result of the similarity in their DNA contents) were eliminated from the analysis where appropriate. The curves generated using this type of analysis were fitted using a generalised logit equation (Johns and Joiner, 1993) which quantitatively describes functional dose response data. This represented an effective way of describing the extent of delay obtained at each radiation dose in each cell line. The variables (upper and lower asymptotes and the inflection point) were estimated by eye and defined for each cell line in increments until the errors on the curve fit were minimised. Results are shown in Figure 4.6. These represent typical curve fits, but it must be pointed out that the actual values given in Table 4.2, and used in subsequent analyses were obtained using the raw data and not the mean data points which have been shown for clarity in Figure 4.6.
The dose response of each cell line plotted in Figure 4.7 demonstrates the differential effect of radiation on the G2/M delay in the eight human tumour cell lines tested. Cell lines have been grouped as either radioresistant (panel a) or radiosensitive (panel b). At the low doses (0.2 Gy and 0.5 Gy) no clear distinction could be made between these groups. The actual delay values (Table 4.2) range from -0.5 h–1.9 h in the 0.2 Gy treated samples to -0.9–2.17 h in the 0.5 Gy samples. While there was a slight trend towards increased delay at low doses in the radiosensitive cell lines, especially in the SW48 and HX142 cells, the large error bars show that the results were not significantly different from the controls. It is presumed therefore that the differences observed in G2/M phase length at low doses were due to random scatter of the data and can only be regarded as negligible compared to the controls. The extent of the delay at higher doses however was far more distinct and it was apparent that the radiosensitive cell lines demonstrated longer G2/M delay than the resistant cell lines. It was not possible therefore to conclude whether the cell-cycle perturbations at the G2/M checkpoint were involved in the HRS/IRR response (Lambin et al. 1996). None of the cell lines which demonstrated HRS/IRR exhibited significant differences in G2 phase length after irradiation from the control populations at low doses. Instead G2/M delay was only observed at higher doses of 1 Gy and over, suggesting that this was the minimum dose required to activate the factors involved in the induced radioresistance of these cell lines.

The extensive delay of the S-phase cells in G2/M, especially at doses over 1 Gy, was in agreement with reports showing that cells irradiated in S-phase are the most sensitive to G2/M delay (Terasima and Tolmach, 1963; Yu and Sinclair, 1967 and Tomasovic et al. 1980). It is speculated that this population would be most likely to demonstrate a detectable cell-cycle arrest at low doses. However, in each cell line the G2/M delay at low doses are too non-significant to speculate on whether cell cycle delays can be implicated in the HRS/IRR response. Analysis of alterations in cell-cycle progression at low doses is not without difficulty. The extent of radiation-induced perturbations at low doses was expected to be low due to the size of the dose, and were therefore limited by the sensitivity of the BrdUrd pulse-chase method to detect small differences in the movement of cells through their cell-cycle. The errors on the calculated values for G2/M delay at low doses were relatively high compared to the values obtained at higher doses. In some cases the errors (SEM) represented over 300% of the actual value (see B11 at 0.5 Gy, RT112 at 0.2 Gy; Table 4.2). Error margins were dramatically reduced at the higher doses tested. This shows that analysis of cell-cycle progression after low dose radiation treatment by this assay failed to distinguish between differences in cell cycle progression of cell populations treated with low doses of radiation over control samples and that in order to determine whether delays can be observed at low doses a greater number of repeat experiments need to be carried out.
The extent of G2/M delay observed at higher doses varied among the cell lines. The radioresistant cell lines all demonstrated comparable delays at 1 Gy, 2 Gy and 4 Gy. The extent of delay in each cell line at individual doses only increased by a maximum of 2-6 h (in the HT29 cell line) from 1 Gy to 4 Gy. This was in contrast with the radiosensitive cell lines which demonstrated an almost linear increase of G2/M delay with dose and a maximum increase of 16-6 h between the delay induced at 1 Gy and that at 4 Gy (in the SW48 cell line). When correlated to radiosensitivity, expressed as SF2, only the delays at 0.5 Gy and 4 Gy were significant (p = 0.032, r = -0.751; p = 0.004, r = -0.552 respectively, Figure 4.8). There appeared to be a trend towards increased G2 phase delay (G2 phase length at 0 Gy plus the length of radiation-induced delay) in the radiosensitive cell lines at all doses. The extensive G2/M delay observed in the radiosensitive cells relative to the resistant cells is in contrast with previous reports which suggest that an extensive G2/M delay is associated with radioresistance (McKenna et al. 1991; Russell et al. 1995; Warenius et al. 1996; Su and Little, 1993; Bernhard et al. 1996). However, there have also been reports which demonstrated no correlation between G2/M delay and intrinsic radiosensitivity (Smeets et al. 1994a; McIlwrath et al. 1994) and in some cases increased G2/M delay has been associated with increased radiosensitivity (Nagasawa et al. 1994; Meyn et al. 1993). Whether G2/M delay is a critical determinant of intrinsic radiosensitivity, therefore, is still an area of ongoing research. As alluded to previously, the actual processes that are presumed to occur during the delay, such as repair and apoptosis, are more likely to affect radiosensitivity. It remains to be determined whether the length of radiation-induced cell-cycle delays indicate the extent of the repair or apoptosis that has occurred during the delay, and whether this in turn indicates radiation response.

To analyse the relationship further, each cell line in the present study was analysed for G2/M delay at each dose expressed as a function of surviving fraction at that dose (Figure 4.9). At equal survival levels, 50% survival for example, both resistant (panel a) and sensitive cell lines (panel b) demonstrated a G2/M delay of between 4 - 6 h. At 4 Gy the surviving fraction of the radioresistant cell lines remained between 0.3 and 0.4. However, in the sensitive cell lines all survival values approach zero at 4 Gy and it is at this dose that a significantly longer G2/M delay was observed. It is possible, therefore, that if an extension of the radiation dose range to include higher doses (6, 8, 10 Gy) which would reduce the survival of resistant cells to zero, then similar delays to the radiosensitive cell lines would be observed. From the dose response curves it is evident that treatment of the different cell types with doses that produce similar survival levels also induce similar G2/M delay values. This suggests that analysis of the extent of radiation-induced G2/M delay alone does not indicate radiosensitivity.

It was evident that the fraction of BrdUrd-labelled cells that entered G0/G1, from which the delay values were calculated (Figure 4.6) was less with increasing dose
characterised by a decreased upper limit value). This was especially obvious in the sensitive cell lines (panels e–h). It was hypothesised that this reduction might be a reflection of the overall decrease in survival of each cell line with increasing dose, and that as this effect was more pronounced in the radiosensitive cell lines, could be indicative of radioresponse. To examine this the relationship between the proportion of cells in G0/G1 with radiosensitivity at each dose was examined in Figure 4.10. The ratio between the upper limit of cells in control samples relative to irradiated samples at each dose was calculated and termed the G2/M ratio. The higher the value for this ratio, the larger the proportion of cells that have progressed through G2/M and entered G0/G1. At 0.2 Gy and 0.5 Gy the relationship between the G2/M ratio and survival were highly non-significant ($p=0.965$, $r=-0.023$; $p=0.932$, $r=-0.045$ respectively). However, at higher doses, where the effects of radiation on cell cycle progression were more distinct, there appeared to be a definite trend towards increased G2/M ratio with a high surviving fraction (resistant cells). These relationships were only just outside the range of statistical significance ($p=0.078$ and $p=0.055$ for the 2 Gy and 4 Gy respectively). Therefore, at doses where a significant cell cycle response can be detected, the proportion of cells that progress into G0/G1 can be directly related to the overall survival of the cell population. These cells represent those that have survived the radiation treatment and would be expected to be indicative of radioresistance. It would appear, therefore, that the fraction of cells in G0/G1 gives better discrimination between radiosensitive and resistant cells than the length of time required for cells to reach this phase (G2/M delay) after radiation. Recent evidence has suggested a link between radiation-induced G2/M delay and apoptosis (Bernhard et al. 1996; McKenna et al. 1996). In both these studies, increased apoptosis in response to radiation was observed when G2/M delay was reduced or abrogated by caffeine treatment or transfection with c-myc and H-ras. The authors postulated that one intrinsic mechanism of enhancing cell killing by radiation was to trigger apoptosis by decreasing the G2 delay induced by radiation. This implies that cells which demonstrate extensive G2/M delays after radiation will exhibit relatively low levels of apoptosis. In the following chapter, analysis of the G2/M ratio with apoptosis was carried out as it was speculated that cells which are unable to exit G2/M delay have irreparable damage and are eliminated from the population by cell death, possibly apoptosis.

The length of the G2/M phase in untreated cells did not appear to influence the extent of delay in irradiated cells (see Figure 4.12). The relationship between G2/M length at 0 Gy and delay (expressed as the length of G2/M phase at 0 Gy plus the extent of radiation-induced delay at each dose) was significant in the 0.2 Gy and 0.5 Gy treated samples ($p=0.009$ and $p=0.022$ respectively) due to the short delay induced at these doses. At higher doses, however, when the induced delays were more extensive, there was no relationship between G2/M length and delay. The G2/M phase lengths of the
more resistant cell lines (HT29, BeII, MGH-U1 and RT112; black symbols) ranged from 1.5-3.5 h but varied in G2/M delay by only 0.84 h, 1.4 h and 1.4 h at 1 Gy, 2 Gy and 4 Gy respectively. Three of the more radiosensitive cell lines (SW48, MeWo and HX142; open symbols) all demonstrated G2/M phase lengths of over 3 h, and extensive G2/M delays at 4 Gy. It could be speculated that an extensive G2/M phase length would indicate a relatively shorter G2/M phase delay, and that different cell types might have similar combined times of G2/M phase length and delay at a given dose. Over the dose range tested, this did not appear to be the case. Instead there was a trend towards increased delay in the cell lines that demonstrated extensive G2/M phase length, especially at 4 Gy. This could indicate that, in addition to the factors involved in the control of normal proliferation, there are others which regulate the cell cycle both in response to external DNA damaging agents and the control of the cell cycle progression in the presence of DNA damage. Therefore, while the mechanism of cell cycle regulation under normal growth conditions and in the presence of DNA damage are probably the same, the processes are not interdependent, and cells with a long phase length can also exhibit a long G2/M delay.

Recent evidence has suggested that the G1/S and G2/M checkpoints might be related in some cell types. Perhaps the best evidence for this comes from studies using caffeine treatment, which abrogates radiation-induced G2/M delay (Musk and Steel, 1990). Russell et al. (1995) reported differential radiosensitisation of G1 checkpoint-deficient and G1 checkpoint-competent cells. They showed that human tumour cells which were radioreistant due to the loss of p53-mediated G1 checkpoint could be made more radiosensitive by abrogation of the G2 checkpoint. In this present study, G1/S delay and G2/M delay were examined at increasing radiation doses (see Figure 4.10). Most cell lines which arrested at the G1/S transition point did so only at higher doses (2 Gy, 4 Gy) and all demonstrated wild-type p53 expression. There did not appear to be a direct relationship between G1/S delay and G2/M delay. On the one hand SW48 cells demonstrated the longest G2/M delay and G1/S delay at all doses tested above 0.5 Gy yet U1-S40b cells, which also demonstrated an extensive G2/M delay, expressed a relatively small G1/S delay (2 h, only at 4 Gy ) whilst the BeII cells demonstrated a significant G1/S delay at 4 Gy (5 h) but exhibited the shortest G2/M delay at doses ≥ 1 Gy. These data make it difficult to formulate a coherent explanation for a link between G1/S and G2/M delay. Examination of the degree of apoptosis and DNA damage repair might provide more information on what is occurring during both checkpoints and might reveal how they are linked to radiosensitivity.

The molecular basis for radiation-induced G2/M delay are still poorly understood in human cells. Entry of cells into mitosis is regulated by the mitosis promoting factor (MPF), a complex of two proteins, p34cdc2 and cyclin B (reviewed by Nasmyth, 1996). In the present study the effect of different radiation doses on cyclin B1 expression was
analysed in asynchronous human tumour cell lines (Figure 4.15). Constitutive levels of protein varied between cell lines (Table 4.1). Be11, RT112 and SW48 demonstrated the highest level of cyclin B1 expression. The remaining cell lines in the study all exhibited similar, lower levels of protein, suggesting that initial levels of protein do not influence the extent of radiation-induced delay or radiosensitivity. There was a decrease in protein levels analysed at 4 h following 0.2 Gy and 2 Gy in all cell lines. This is consistent with a role of inhibition of cyclin B1 accumulation in the impairment of cell-cycle progression into M-phase. The level of reduction of cyclin B1 was similar in all cell lines suggesting that this reduction does not reflect inherent differences in radiosensitivity. Only the Be11 cell line did not demonstrate a measurable reduction in cyclin B1 level at either 0.2 Gy or 2 Gy. Interestingly, while this cell line demonstrated a G2/M delay, the extent of this delay was comparatively small (4.5 h at 4 Gy). It has not been shown whether the extent of cyclin B1 reduction can control the extent of radiation-induced delay. The general down-regulation of cyclin B1 observed in the other cell lines is in agreement with previous reports by Muschel et al. (1993) and Villa et al. (1996). In the latter study, a decrease in cyclin B1 (analysed by flow cytometry) was detected only after 10 Gy, while no differences were observed at 2 Gy or 5 Gy. In the present study a decrease in cyclin B1 was clearly observed at 2 Gy in all cell lines and at 0.2 Gy in some cells (HT29 and MeWo). This could reflect an enhanced response to radiation-induced alterations in cyclin B1 expression in this panel of cell lines as Smeets et al. (1994a) analysed cyclin B1 levels in two squamous carcinoma cell lines treated with 10 Gy radiation by western blot analysis and failed to observe any alteration in expression. It can be concluded that down-regulation of cyclin B1 is a general response to radiation in these cell lines but there did not appear to be a significant relationship between the extent cyclin B1 reduction and radiosensitivity.

In conclusion, the treatment of eight human tumour cell lines with increasing doses of radiation results in differential alterations in cell-cycle progression. All the cell lines that demonstrated wild-type p53 expression demonstrated a G1/S delay, primarily at higher doses and the level of p53 protein appeared to be indicative of the sensitivity to a radiation-induced G1/S delay. The extent of G2/M delay was the most extensive delay observed in all cell lines. This delay was probably mediated by a suppression in cyclin B1 protein, resulting in the inactivation of MPF. There appeared to be a trend toward increased G2/M delay in the radiosensitive cell lines at most doses tested, but the extent of delay was only significantly higher than the controls at doses ≥1 Gy. The extent of delay was similar in all cell lines at doses that produced equal survival levels indicating a limited role in of the extent of G2/M delay in intrinsic radiosensitivity. The fraction of cells that did not exit G2/M after radiation treatment however, varied between the cell lines, with a lower G2/M ratio in the radiosensitive compared to the resistant cell lines. This relationship was almost significant with survival at 2 Gy and 4 Gy suggesting that
increased cell death at the G2/M boundary might be related to radiosensitivity. It is not clear whether cell-cycle delays can be implicated in the HRS/IRR response observed in some of the cell lines in the study. G2/M delay values at low doses were not significantly different compared to the controls. However, it is evident that low dose irradiation induces a response in some cells as stabilisation of the p53 protein was observed at 0.2 Gy and a G1/S delay was observed in SW48 cells at 0.5 Gy.

Further analysis of the extent of processes thought to occur during the G1/S and G2/M checkpoints, namely apoptosis and DNA repair, might provide more information on the significance of the length of radiation-induced delays, and how they reflect radiation response.
5.0 Radiation-induced apoptosis

5.1 Introduction

The phenomenon of apoptosis, or programmed cell death, was first described by Kerr et al. (1972) as a process occurring naturally in the development of organs and tissues and after treatment with certain cytotoxic agents. Apoptosis of mammalian cells in vitro can be induced by a variety of treatments including serum or growth factor withdrawal (Evan et al. 1992), cytokines or glucocorticoids (Wyllie, 1980), chemotherapeutic drugs (Kaufmann, 1989) and ionising radiation (Hendry and Potten, 1982; Lowe et al. 1993). There is much evidence to suggest that the frequency of apoptosis is a factor in the intrinsic radiosensitivity of tumour cells, and has been shown to correlate directly with cell survival in many cases (reviewed by Blank et al. 1997). Such a link would have potential application in the treatment of human cancers both in the prediction of tumour radiosensitivity and in the improvement of existing radiotherapy treatments with promoters of apoptosis.

Ionising radiation produces 'classic' clonogenic cell kill by damaging DNA. This results predominantly in the production of single strand breaks (SSB) and double strand breaks (DSB), the DSB being most critical to cell survival. It is still unclear whether apoptosis occurs by a separate process distinguishable from clonogenic cell kill in response to radiation. Hendry and Potten (1982) first noted that levels of spontaneous apoptosis in normal human tissues were indicative of the amount of apoptosis induced by subsequent radiation treatments. Similar observations have been reported in murine tumours in vivo (Meyn et al. 1993). Stephens et al. (1993) reported higher levels of apoptosis in a moderately radiosensitive murine ovarian carcinoma than a radioresistant hepatocellular carcinoma. A direct correlation between radiosensitivity and apoptosis has been demonstrated in some human cell types in vitro (Schwartz et al. 1995; Mitsuhashi et al. 1996) and in vivo (Wheeler et al. 1995; Levine et al. 1995). However, there are several reports of experiments where apoptosis has been induced in mammalian cells with no effect on the overall clonogenic survival. McKenna et al. (1991, 1996) observed increased apoptosis levels in v-Myc transformed rat fibroblasts compared to primary rat fibroblasts yet the survival characteristics of both cell lines were virtually identical. Similar observations were reported by Aldridge et al. (1995).

In the low radiation dose range (<1 Gy) the clonogenic survival of some mammalian cell lines has been shown to diverge from the Linear Quadratic (LQ) model (Marples and Joiner, 1993; Wouters et al. 1996; Lambin et al. 1996). In all reported cases the survival was less than that predicted by the model, implying an enhanced effectiveness per unit dose of X-rays in the range of 0 Gy – 0.4 Gy...
compared to higher doses. Between doses of 0.5 Gy – 1 Gy the survival curve appears to recover to levels represented by the LQ model, i.e. an apparent increased resistance (IRR). This low dose hyper-radiosensitivity (HRS) has been extensively studied in an attempt to elucidate the mechanism of this biphasic response and it has been suggested that apoptosis may play a role. The elimination of cells with unreppaired DNA damage from a tumour cell population by apoptosis would prevent the continued proliferation of cells with genetic damage. Matthews (1997) hypothesised that at low doses, where relatively little damage is produced, cells with DNA damage apoptose rather than undergoing repair, thus sacrificing few cells without the expenditure of much energy. At higher doses, where more cells suffer DNA damage and the entire population is at risk, cells are arrested in their cycle and damage is repaired. Hyun et al. (1997) observed a reduction in apoptosis levels at 2Gy when a low priming dose (0.01 Gy) was applied to mouse lymphocytes compared to unprimed cells. This observation would be consistent with an involvement of apoptosis in the HRS/IRR response.

Differences in the rate of apoptosis induction have been reported in different tumour cell lines (Algan et al. 1996), which may have important implications in the analysis of apoptosis data. Several studies have suggested that radiation-induced apoptosis begins shortly after treatment (~0.5–1 h) with a peak in levels occurring approximately at 4 – 6 h (Stephens et al. 1993; Mirkovic et al. 1994; Meyn et al. 1993). However, there is an increasing number of studies which have reported radiation-induced apoptosis at much later times (in general 24–48 h), which might be indicative of a relationship between apoptosis induction with completion of individual phases of the cell-cycle.

The relationship between p53 and radiation-induced G1 delay and apoptosis has been well documented (reviewed by Bristow et al. 1996). Wild-type p53 expression is required for radiation-induced apoptosis in many cell types including thymocytes and crypt cells (Clarke et al. 1993; Lowe et al. 1993; Merritt et al. 1994). It has been suggested that p53 mediated G1 arrest and apoptosis are operated by separate pathways, controlled by p21 and bcl-2 respectively (Deng et al. 1995; Wang et al. 1995). Bcl-2 is a member of a highly conserved family of genes whose products control the induction of apoptosis with various stimuli through a series of dimerisation reactions. Apoptosis in many cell types is dependent on the balance of bcl-2 with its cellular partner bax. Bcl-2 is a negative regulator of apoptosis while bax is a positive regulator (reviewed by Blank et al. 1997). Levels of bcl-2 protein expression have been shown to correlate with outcome in a wide variety of human tumours including head and neck (Wilson et al. 1996), prostate (Colombel et al. 1993), lung (Pezella et al. 1993; Fontanini et al. 1993).
1995; Walker et al. 1995), renal (Lipponen et al. 1995), breast carcinomas (Silvestrini et al. 1994), and melanoma (Grover and Wilson, 1996).

While radiation-induced apoptosis has been observed in all phases of the cell-cycle it is thought that signalling occurs mainly in the G1 phase where p53 is active, although there is increasing evidence suggesting a role for p53 in G2/M phase. Treatment of HeLa cells with caffeine or staurosporine, which partially abrogates G2/M delay, increased the amount of apoptosis following radiation (Bernhard et al. 1996). McKenna et al. (1996) demonstrated increased apoptosis in rat embryo fibroblasts (REFs) transformed with both myc and ras compared to REFs transformed with myc alone. They found that the increased apoptosis was inversely proportional to the length of G2/M delay observed after radiation. Meyn et al. (1994), however reported that increased apoptosis in murine cell lines correlated with an extended G2 block. How cell-cycle delay and apoptosis pathways are related is therefore still unclear.

The measurement of apoptosis both in vitro and in vivo relies mainly on the detection of endonuclease DNA fragmentation, which is a classic characteristic of apoptosis. DNA fragments, consisting of ~185 bp, can be separated using gel electrophoresis resulting in a characteristic DNA ‘ladder’. However, this technique is not quantitative in terms of the number of apoptotic cells, or apoptotic index, within a test population. Flow cytometry is used widely for quantification of apoptotic indices, based on several parameters including cell size (Swat et al. 1991), ability to exclude propidium iodide in the presence of Hoechst 33342 (Ormerod et al. 1992), protein and RNA content and acridine orange assessment of DNA susceptibility to denaturation (Darzynkiewicz et al. 1992). More recent methods include in situ end labelling techniques which exploit the unique nature of the DNA fragment ends in apoptotic cells (Wyllie, 1993). Using this technique, apoptotic cells are detected using biotinylated nucleotides attached to the overhanging 5' ends by the action of DNA polymerase. These biotinylated fragments are then revealed using avidin conjugated fluorochnomes, usually fluorescein (FITC). Negative controls can be prepared from samples lacking DNA polymerase. However, morphological assessment for apoptosis is, in general, regarded as the most reliable method of measurement. Many different techniques are used to identify apoptotic cells (reviewed by Potten, 1996), all of which utilise the physical changes that occur to the membrane and nucleus during apoptosis. These include DNA stains such as propidium iodide (PI), Hoechst or DAPI, which allow visualisation of the nucleus, membrane stains such as acridine orange and eosin (with hematoxylin) which demonstrate membrane regularity, and electron microscopy which allows visualisation of the more subtle features of apoptotic cells such as the appearance of intact cellular organelles like the endoplasmic recticulum. The total number of cells
analysed by morphological assessment can be greatly increased by the use of automated systems (Matthews, 1997), improving the statistical significance of results.

In this study we report on the application of two techniques to determine apoptosis in eight human tumour cell lines. Morphological assessment was performed using Hoechst incorporation visualised with UV light, adapted from Chen (1977). Flow cytometry analysis was performed using the terminal transferase end labelling assay (TdT) as described by Gorczyca et al. (1993). Cells were analysed for apoptosis after treatment with doses of ionising radiation in the low dose range (0.2 Gy and 0.5 Gy) and the clinically relevant dose range (2 Gy and 4 Gy). These studies were undertaken to determine whether apoptosis could be identified as playing a role in the HRS/IRR response of some of the cell lines in this study and whether intrinsic radiosensitivity might be related to apoptosis at clinically relevant doses. Samples were analysed at regular intervals over a 36 hour interval, which represented at least one cell-cycle in most cell lines, in an attempt to identify both late and early apoptotic events within individual tumour populations. Bcl-2 levels were determined by flow cytometry and related to constitutive and radiation-induced levels of apoptosis. As shown in a previous section (see chapter 4), differences in cell-cycle progression were observed in the eight tumour cell lines which were related to radiosensitivity at clinically relevant doses of radiation. In this section, the occurrence of apoptosis was examined and compared with cell-cycle perturbations to examine the potential interdependence of both pathways in the radiation response of human tumour cell lines. The aim was to fully characterise the apoptotic response of each cell line in response to increasing doses of radiation, and to assess the role of this pathway in the overall radiosensitivity of each cell line.

5.2 Results

5.2.1 Time course of radiation-induced apoptosis

The levels of apoptosis in each cell line were analysed over a period of 36h after varying doses of radiation treatment. Figure 5.1a shows the nuclear staining of U1-S40b cells, treated with 4 Gy of ionising radiation and analysed 24 h later, using Hoechst 33342 visualised under UV illumination. Apoptotic cells were readily identified by chromatin condensation which is characteristic of apoptotic cells. In contrast, there was an obvious decrease of apoptosis in MGH-U1 cells treated with the same dose, represented in panel b. These differences were also observed in cells exposed to 4 Gy and analysed 24 h later using the TdT end-labelling assay (Figure 5.2a and b respectively). Positive apoptotic cells were identified by increased fluorescent intensity (cells in R1) over the non-apoptotic
Figure 5.1. Morphological detection of apoptosis in human tumour cell lines in vitro. Each cell line was stained with Hoechst 33342 and analysed under UV illumination. Apoptotic cells were clearly visible by the presence of discrete apoptotic bodies (see arrows). The frequency of apoptosis, which was higher in U1-S40b cells (A) than MGH-U1 cells (B), was determined from at least 400 cells per experiment.
Figure 5.2. Flow cytometric assessment of human tumour cells for apoptosis using biotinylated-UTPs and avidin-FITC. Apoptotic cells were identified by the level of green FITC fluorescence (R1) over viable cells (R2), plotted on the y-axis. The higher proportion of apoptotic cells in U1-S40b (A) was higher than MGH-U1 cells (B). Cell-cycle phase was also determined by the incorporation of propidium iodide which identified cells based on DNA content, plotted on the x-axis.
cells (R2). As described in materials and methods (see Chapter 2), the regions outlining the positive cells (R1) were defined using negative controls prepared from test samples stained in the same way but without the addition of the terminal transferase enzyme. This precludes cells displaying any spurious or background staining. Four of these controls were prepared in each experiment and the region R1 was set to contain < 1% cells. Using both morphology and end-labelling techniques a time course for apoptosis induction was established in all eight cell lines. Results are shown in Figures 5.3 a–h for the resistant cell lines and in Figure 5.4 a–h for the sensitive cell lines. Both sets of data for each cell line were plotted using the same axis limits. The length of one cell-cycle is highlighted on each graph.

The TdT assay (right-hand side panels in Figures 5.3 and 5.4) consistently identified more apoptotic cells than morphological assessment (left-hand side panels). The pattern of apoptosis induction was similar in those cell lines that showed a radiation-induced response i.e. a gradual increase in detectable apoptotic cells at around 10 h with greater separation of control and test samples at later times and higher doses. The resistant cell lines (Figure 5.3), exhibited lower levels of apoptosis (< 6% at all doses and time points) than the radiosensitive cell lines (Figure 5.4), with the exception of MGH-U1 cells (Figure 5.3, panels c and d). These demonstrated apoptosis levels between 10–15% at 36 h using the TdT assay (panel d), although this result was not concordant with the morphological assessment (< 5% apoptosis at 36 h; panel c). The radiosensitive MeWo cell line (Figure 5.4, panels c and d) also demonstrated relatively low levels of apoptosis at all time points (< 8%). Both techniques, however, indicated increased apoptosis in the radiosensitive cell lines.

5.2.2 Mean apoptosis over 36 h and one cell-cycle time course

The apoptosis measured over a 36 h time course gave an indication of the time frame for induction of apoptosis following radiation treatment to occur in each of the cell lines. In order to summarise the data the levels of apoptosis observed over the complete time course of the experiment were reduced to a single value in each cell line. This was achieved using a simple integration technique which facilitated both the direct intercomparison of apoptosis levels between cell lines and the correlation of apoptosis with the other cell survival parameters, such as radiosensitivity and cell-cycle delay length. A schematic of the approach used is shown in Figure 5.5. The levels of apoptosis were determined at equal time increments of 2 h and summed. The mean apoptosis over the time interval was determined by the division of the summed value by the number of time increments.
Figure 5.3 (a–d). Time course of apoptosis induction by ionising radiation in radioresistant human tumour cell lines. Apoptosis determined by morphology (using Hoechst 33342, adapted from Chen et al. 1977) is presented in the left panels (a and c). Over 100 cells were counted in four separate fields in each experiment. Error bars represent the mean of at least two experiments (±SEM). Apoptosis measured by TdT assay (using FITC labelling of biotinylated deoxyribonucleotides incorporated into the characteristic DNA fragment ends by terminal transferase, Gorczyca et al. 1992) is presented in the right panels (b and d). At least 5000 cells were counted in each experiment. Error bars represent the mean of at least two experiments (±SEM).
Figure 5.3 (e–h). Time course of apoptosis induction by ionising radiation in four radioresistant human tumour cell lines. Continued from previous page.
Figure 5.4 (a–d). Time course of apoptosis induction by ionising radiation in radisensitive human tumour cell lines. Morphology (left panels, a and c) and TdT assessment (right panels, b and d) of apoptosis as described previously (figure 5.3).
Figure 5.4 (e–h). Time course of apoptosis induction by ionising radiation in four radisensitive human tumour cell lines. Continued from previous page.
This analysis was done for both a 36 h time course (Figure 5.6a and b) and up to the length of one cell-cycle for each cell line (Figure 5.7a and b).

The mean level of apoptosis determined by morphological assessment was less than that detected using the TdT assay. However, there appeared to be a trend towards increased apoptosis in the radiosensitive cell lines over 36 h using both techniques. Analysis of apoptosis over one cell-cycle time in each cell line indicated a similar trend suggesting that 36 h could be used as a standard time course for analysis regardless of cell-cycle time. The constitutive levels of apoptosis over 36 h appeared to be higher in the radiosensitive cell lines when related to SF2 values (p = 0.297, r = -0.423; Figure 5.10, panel a). Only apoptosis levels in the HX142 cell line prevented this relationship from being significant (p = 0.042, r = -0.755 on remaining 7 cell lines). However, when apoptosis in the radiation treated samples were normalised for constitutive levels of apoptosis the differences between the radioresistant and radiosensitive cell lines was minimal (see Table 5.1). This indicates that although the levels of apoptosis in the radiosensitive cell lines appeared to be greater in both treated and untreated samples, the level of apoptosis induced by radiation does not appear to reflect the significant differences in survival observed in these cell lines.

5.2.3 Morphological analysis versus end-labelling (TdT) assay

It was clear that the results obtained using morphological assessment and end-labelling with flow cytometry were discordant, see Figures 5.3 and 5.4. These differences were also highlighted in Figures 5.6 and 5.7 where apoptosis levels were expressed as a mean of the values detected over both 36 h and one cell cycle time for each cell line. For example the S40b-U1 and SW48 cell lines demonstrated mean apoptosis levels of 10.5% and 13.1% respectively when analysed using the TdT assay. These values were significantly different to the 2.4% and 6.3% respectively when assessed using morphological analysis. This trend was observed in most of the cell lines analysed, although to a lesser extent. HT29 cells however, demonstrated apoptosis levels which were comparable. Moreover, MeWo cells exhibited lower levels of apoptosis when analysed using the TdT assay (1.3% versus 5.0%). It was speculated that an inherent difference between the cell lines might be responsible for the differences in response to one or other of the techniques. Differences in cell surface biotin levels might conceivably affect the level of 'positives' detected using the TdT assay. The process of harvesting the cells by scraping may also obscure the clarity between apoptotic and non-apoptotic cells analysed using morphological assessment.
Figure 5.5. Schematic for the estimation of mean apoptosis levels. The above represents a schematic of the procedure used to calculate mean apoptosis levels. The dose response curves, generated from actual data points (thick lines) were integrated over 36 h, at equal 2 hourly intervals (thin lines). The integrated figure was then divided by the total number of time intervals ($36+2 = 18$), to give a figure representative of the mean apoptosis observed over the time course of the experiment.
Figure 5.6. Mean apoptosis in eight human tumour cell lines over 36 h. Apoptosis was determined by a. morphology and b. end labelling (TdT). Mean apoptosis value were obtained by simple integration of the apoptotic time course over 36 h, with 2 h intervals. Graphs are plotted on equal y-axis scales to highlight the different results obtained using both techniques.
Figure 5.7. Mean apoptosis in eight human tumour cell lines over one cell cycle time. Apoptosis was determined by morphology (a) and end-labelling (TdT) assay (b). Mean values were calculated over the period of one cell cycle in cases where this was less than 36 h. Graphs are plotted on equal y-axis scales to highlight the different results obtained using both techniques.
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</table>

Table 5.1. Apoptosis values, by both morphological (left columns) and flow cytometric assessment (right columns), in eight human tumour cell lines. Values represent the mean apoptosis levels observed over 36h following radiation treatment (top panel) and are normalised for the constitutive levels in the lower panel.
To address these questions some additional experiments were performed. First, samples of unirradiated SW48, HT29 and MeWo cells, incubated for 24 h after mock irradiation were prepared for TdT analysis. In previous analyses apoptosis levels were determined from one set of controls (incubation of samples with b-UTPs but without the terminal transferase enzyme) and a test sample (b-UTPs with the terminal transferase enzyme). In addition to these, control samples incubated with the terminal transferase enzyme but without b-UTPs, and a sample with only the secondary antibody (avidin-FITC, i.e. no b-UTPs or terminal transferase) were also prepared. These were analysed as before and the results are shown in Figure 5.8 a–c. Using a control stained without b-UTPs but with the fluoresceinated secondary antibody it was hoped to detect some significant background staining over and above the levels observed using the original control sample (prepared with only the terminal transferase excluded). This would indicate spurious staining of cellular biotin. However, it was clear that this was not the case, especially in the SW48 cell line for which this test was particularly relevant as they showed the highest discrepancy between both assays.

The potential effects of scraping the cells during harvest on the level of apoptosis detected by both techniques was tested using HT29 cells, irradiated with 0.2 Gy and 4 Gy and harvested 4 h later either by scraping or trypsinisation. Samples were analysed by both morphological assessment and the TdT assay. Results are summarised in Figure 5.9 a and b. Only slight differences existed between the several combinations of harvest and detection technique. There was an indication, however that the trypsinisation of cells for TdT analysis showed a higher level of apoptosis than any other test, although these differences were not significant (p = 0.376).

The different protocols involved in each assay may have influenced the overall result. Both procedures required cellular fixation before staining. This could have selectively reduced the number of apoptotic cells in the original test population as cells characteristically undergo changes to the membrane during the apoptotic process. This could have resulted in increased fragility to treatments such as the paraformaldehyde/ethanol and methanol/acetic acid fixations that were used in the end-labelling and Hoechst staining protocols respectively. Both assays also involved a series of centrifugation steps that could have had the effect of eliminating apoptotic cells from the final analysis. This was especially relevant to the end-labelling assay which involved five centrifugation steps after fixation before final analysis. Morphological assessment on the other hand required only one cytospin step.

The lack of specificity of the end-labelling assays in the discrimination of strand breaks induced specifically by a particular treatment regimen, ionising
Figure 5.8. Comparison of cellular biotin detected by the TdT assay. The three cell lines represent the different types of response observed when comparing apoptosis by TdT and morphology where HT29 cells (A) showed similar apoptosis levels by both techniques, MeWo (B) showed more apoptosis when analysed using morphology while SW48 cells (C) showed more apoptosis by TdT. In panels (i), cells have been incubated with avidin-FITC only, (ii) with b-UTPs and avidin-FITC and (iii) with TdT and avidin-FITC. No significant difference was observed between the three control samples indicating that cellular biotin levels were not responsible for the differences between apoptosis level detected by morphology and TdT.
Figure 5.9. The effect of harvest protocol on apoptosis measurement. HT29 cells were harvested by either manual scraping (panels a) or trypsinisation (panels b). Aliquots of each sample were analysed for apoptosis at 0 h and 4 h using morphological assessment (top row) and end labelling (TdT) assay (bottom row).
radiation for example, over other strand fragments, such as those involved in the necrotic degradation of cells calls doubt to the specificity of these assays (Grasl-Krupp et al. 1995). And while this was not especially relevant to in vitro studies, evidence also exists that apoptosis can occur in the absence of any detectable DNA fragmentation (Yasuda et al. 1995). It has been suggested that assessment of DNA fragmentation by end-labelling and other techniques (such as the comet assay) should only be used as a supplement to morphological assessment of apoptosis (Russell et al. 1992; Potten, 1996).

The TdT assay, in general, detected more apoptosis than morphological assessment in the in vitro analysis of the eight human tumour cell lines in this study, although the trends of apoptosis induction were similar for both methods. Further analysis of apoptosis levels in relation to radiosensitivity and cell-cycle delay, therefore, was performed using the data generated by the morphological assessment, since this is generally the more accepted method of apoptosis determination (Yasuda et al. 1995; Collins et al. 1992; Potten, 1996).

5.2.4 Relationship between apoptosis and low dose hypersensitivity and intrinsic radiosensitivity

The relationship between the mean level of apoptosis observed at each dose point and intrinsic radiosensitivity expressed as \(S_F^2\) was examined in each cell line. Results are shown in Figure 5.10 a–f. Correlation with 0.2 Gy (b) and 0.5 Gy (c) radiation doses were not significant (\(p = 0.280\) and \(p = 0.139\) respectively). This suggests that the low dose hypersensitivity previously observed in the four most resistant cell lines (Lambin et al. 1996) was not due to increased apoptosis over 36 h. Indeed the resistant cell lines demonstrated relatively low apoptosis levels over the entire dose range. However, in general, apoptosis at doses in the clinically relevant range were more related to survival, almost reaching significance at 2 Gy (d) (\(p = 0.063\); \(p = 0.108\) at 4 Gy (e)). It must be noted that for almost all of the relationships between \(S_F^2\) and apoptosis presented in Figure 5.10, only the HX142 cell line prevents these from being statistically significant. This indicates that the addition of more cell lines to the sample population might statistically confirm the trend of increased apoptosis in the radiosensitive lines at all doses. Constitutive levels of apoptosis were not significant when related to \(S_F^2\) (a) (\(p = 0.296\)) but the relationship with apoptosis induction at 2 Gy was significant (f) (\(p = 0.008\)). This suggests that constitutive levels of apoptosis may be a determinant of the radiation-induced apoptotic response at clinically relevant doses.

The survival at each dose tested was also compared to apoptosis levels at the same dose, Figure 5.11a–d. At 0.2 Gy survival values were similar in all cell lines and correlation with apoptosis was not significant (\(p = 0.566, r = -0.298\)). At
Figure 5.10. The relationship between apoptosis at individual dose points and radiosensitivity expressed as SF$_2$. Mean apoptosis levels at each dose point tested are related to radiosensitivity expressed as SF$_2$ (panels a–e). Constitutive levels are also related to radiation induced apoptosis at 2 Gy (panel f).
Figure 5.11. Radiation-induced apoptosis and survival at each dose in eight human tumour cell lines. Mean apoptosis was determined by morphological analysis and the survival at each dose was calculated from the equation of the best LQ fit of the survival data.
higher doses (≥ 0.5 Gy) there appeared to an indirect relationship between the surviving fraction and apoptosis. The effect was more pronounced in the 2 Gy and 4 Gy samples where large differences in the surviving fraction were observed. Although the relationships at these doses were just outside the range of statistical significance (p = 0.060 and p = 0.061 in the 2 Gy and 4 Gy samples respectively), it was evident that a strong trend towards cell lines with the lowest surviving fraction after treatment with radiation showing the highest apoptosis. While the actual apoptosis values did not appear to reflect the large differences observed in the surviving fraction between the cell lines, it must be noted that the time of analysis of apoptosis was confined to one cell-cycle time whereas the clonogenic survival of each cell line was determined two weeks post-irradiation.

5.2.5 Apoptosis and bcl-2 expression

Bcl-2 protein levels were examined by flow cytometry and related to radiosensitivity and apoptosis in each cell line. It was hoped to determine whether cell lines vary in their apoptotic response in a manner dependent on bcl-2 levels. Results are summarised in Figure 5.12 (a–d). The relationship between radiosensitivity and bcl-2 was not significant, as bcl-2 levels were quite similar in all cell lines, with only one resistant (MGH-U1) and one sensitive cell line (MeWo) showing bcl-2 positivity greater than 10%. These values did not change significantly after radiation treatment (compare panel a (0 Gy) with panel b (4 Gy), as has been reported in many cell types (Wilson et al. 1996 and references therein).

When related to apoptosis levels (panels c and d), bcl-2 expression was not significantly different between those cell lines showing high and low levels of apoptosis, although some studies have reported an inverse correlation between apoptosis and bcl-2 levels (Sentman et al. 1991; Strasser et al. 1991; Hockenbery et al. 1993).

5.2.6 Relationship between apoptosis and cell-cycle delay

The extent of cell-cycle delay at the G1/S and G2/M checkpoints has been examined in a previous section (see Chapter 4). A G1/S delay was observed in three of the cell lines, but was not correlated to intrinsic radiosensitivity, expressed as SF2. A significant correlation was observed between the extent of G2/M delay after 4 Gy and intrinsic radiosensitivity. There also appeared to be a relationship between G2 delay at 2 Gy and intrinsic radiosensitivity.

In this section the relationship between the extent of radiation-induced apoptosis and cell-cycle perturbation in G2/M was examined in each cell line (Figure 5.13 a–e). At low doses (0.2 Gy and 0.5 Gy) the values of G2/M delay were not related to apoptosis. At the more clinically relevant doses of 2 Gy and 4 Gy a
Figure 5.12. Bcl-2 protein expression in eight human tumour cell lines. Bcl-2 positive cells (+) were determined by flow cytometry and are related to radiosensitivity expressed as SF$_2$ (a and b) and mean apoptosis levels (c and d), at 0 Gy and 4 Gy.
Figure 5.13. Apoptosis and G2 length with increasing dose. The length of G2 phase was calculated using BrdUrd incorporation and flow cytometry. Apoptosis was measured using Hoechst 33342 incorporation and UV illuminated microscopy. Apoptosis values represent the mean observed levels over 36h.
Figure 5.14. Radiation-induced apoptosis G2/M ratio in eight human tumour cell lines. Mean apoptosis was determined by morphological analysis. The G2/M delay ratio represents the proportion of cells that have progressed though G2 and M phases into G0/G1. The value was estimated from the upper asymptote of the plot showing the entry of cells into G0/G1 with time.
strong trend towards increased apoptosis in those cell lines that delayed longer in G2/M \((p = 0.037, r = 0.737; p = 0.15, r = 0.262\) respectively). At 4 Gy this relationship was significant when analysed in the absence of the radiosensitive mutant cell line U1-S40b \((p = 0.002, r = 0.966)\). This response was associated with radiosensitivity and suggests that cells, as opposed to either arresting in the cell-cycle or apoptosing, may in fact undergo both processes. This could mean that cells delay in G2/M phase before undergoing apoptosis perhaps due to irreparable damage.

From the analysis of the G2/M checkpoint it was apparent that the proportion of cells entering G0/G1 was less than the controls in some of the cell lines (Figure 4.6 a–h, chapter 4) and that this effect increased with dose. This implied that some cells did not progress through G2/M. It was speculated that this may be due to irreparably damaged cells being eliminated from the population through some form of cell death, perhaps apoptosis. To examine this an estimation of the proportion of cells in G0/G1 compared to unirradiated controls was determined by comparison of the upper limit of cells in this phase with time and related to apoptosis at each dose (Figure 5.14 a–d). At doses of 0.5 Gy and over there appeared to be an inverse relationship between G2/M ratio and apoptosis. Cell lines that exhibited a low proportion of cells in G0/G1 demonstrated high apoptosis at that dose. This relationship was statistically significant in the 2 Gy sample only \((p = 0.019)\) although there was an obvious trend in the 0.5 Gy sample \((p = 0.089)\). At 4 Gy the relationship was not significant \((p = 0.254)\), but elimination of the U1-S40b cell line from the analysis greatly improved the significance \((p = 0.049, r = -0.814)\).

Three cell lines in the study showed mutant p53 expression: HT29, RT112 and MeWo (see Chapter 4). These cell lines did not demonstrate a G1/S arrest at any dose tested nor did they demonstrate appreciable levels of apoptosis. This suggests that inactivation of p53 function has disrupted both the G1/S and the apoptotic response to DNA damage. The five wild type p53 expressing cell lines all exhibited a G1/S arrest, although some only at the highest dose tested (4 Gy). Three of these cell lines also demonstrated relatively high levels of apoptosis (U1-S40b, SW48 and HX142). The lack of radiation-induced apoptosis in Be11 and MGH-U1 cells suggest that a requirement for p53 in this pathway is not universal among all tumour types. However, it could also indicate bcl-2 mediated suppression of p53-dependent apoptosis (Chiou et al. 1994; Chiarugi et al. 1995, 1996), as MGH-U1 cells demonstrated high levels of bcl-2 and relatively low levels of p53 protein.

The major advantage of apoptosis measurement by the TdT assay is that it facilitates parallel determination of the cell-cycle phase of the apoptotic cells. Figure 5.15 shows the proportion of apoptotic cells in each phase of the cell-cycle. Only the U1-S40b cells demonstrated a high proportion of apoptotic cells in
Figure 5.15. Cell cycle phase-dependant apoptosis. The cell cycle phase in which apoptotic cells were detected was determined by dual parameter flow cytometry. Apoptosis was detected 36 h after 4 Gy by fluorescent intensity of the incorporated FITC fluorochrome. Cell cycle phase was determined by the parallel incorporation of propidium iodide, which allows each phase to be distinguished by DNA content.
Figure 5.16. Mean radiation-induced apoptosis in each cell cycle phase after one cell cycle. Apoptosis levels were determined using the end-labelling TdT assay. Parallel incorporation of propidium iodide facilitated the analysis of the cell cycle position of the apoptotic cells. Mean apoptosis values were calculated over a period of 36h following treatment with 4 Gy ionising radiation.
G0/G1 compared to the rest of the cell lines. However, the majority of apoptosis for all cell lines occurred in the S and G2/M phases of the cell-cycle. Figure 5.16 shows mean apoptosis in each phase of the cell-cycle of each cell line measured at 0 Gy and 4 Gy, over 36 h. The higher frequency of apoptotic cells detected in the S and G2/M phases might be related to the previous data which suggested that radiosensitivity might be related to the removal of cells unable to exit G2/M from the population by apoptosis (Figure 5.11).

5.3 Discussion

Radiation-induced apoptosis has been shown to occur in many human tumour cell lines but the relevance to clinical radiotherapy and therapeutic outcome is still very much under investigation. There have been conflicting reports over the nature of the relationship between apoptosis and clonogenic survival. Several reports have demonstrated increased apoptosis with radiation treatment without any affect on the overall survival of the cell population (Aldridge et al. 1995; Yanagihara et al. 1995). In contrast Hopcia et al. (1996) reported that although reduced clonogenic survival of HL60 cells irradiated with 12 Gy was about 0.002%, no apparent increase in apoptosis levels were observed. This suggests that, in these cell types, apoptosis is but one of a number of interchangeable mechanisms by which a cell undergoes death in response to radiation. On the other hand much evidence exists to support the hypothesis that apoptosis is directly related to radiosensitivity (Stephens et al. 1993; Hu and Hill, 1996; Lowe et al. 1993; Meyn et al. 1996; Levine et al. 1995). The identification of such a relationship in some cell types could have important implication in the identification of radiosensitive tumour types and the improvement of radiotherapy effects on tumour cells by the modification of the apoptotic response.

Whether apoptosis can be related to radiosensitivity relies very much on the method by which apoptosis in a population of cells is measured. The time course of apoptosis induction varies considerably between cell types from as early as 1 h (murine lymphoma cells; Mirkovic et al. 1994) to as late as 48 h (murine haemopoietic cells; Tauchi et al. 1994) after radiation treatment. Moreover, the total duration of the apoptotic process of most cell types is largely unknown. The continuous analysis of apoptosis over a period of 36 h after radiation treatment was an attempt to identify all possible early and late (after one cell-cycle) apoptotic events in each cell line in this study. The cell lines that demonstrated increased apoptosis did so 4–10 h following radiation treatment, which persisted at 36 h (Figures 5.3 and 5.4). Many studies using similar time courses have shown a peak time of apoptosis induction followed by a reduction to basal levels (Mirkovic et al. 1994; Fuks et al. 1994; Russell et al. 1995). The persistence of apoptotic cells in
this study up to 36h following radiation treatment might be indicative of a delayed apoptotic response, whereby apoptosis occurs after completion of a particular phase of the cell-cycle. Olive et al. (1996) reported a reduced rate of apoptosis induction (approximately three times less) in TK6 human lymphoblast cells irradiated in G1 than in any other phase of the cell-cycle. This suggests that the persistence of apoptosis at 36 h in the cell lines in this study may be related to differential perturbations in the cell-cycle.

The detection of apoptotic cells by morphological assessment and end-labelling techniques produced different results in terms of the number of apoptotic events detected. The trend of the time course of apoptotic induction was similar using both techniques and normalisation of the values at each dose point to the constitutive levels produced similar results (Table 5.1). The increased apoptosis observed in general using the TdT assay might be due to a lack of specificity of this technique to discriminate apoptotic cell DNA fragment ends over other fragment ends, such as those produced during DNA replication. Indeed, many of the apoptotic cells were detected in the S-phase of the cell-cycle (Figure 5.2, 5.15 and 5.16). It has also been reported that DNA fragmentation is not an absolute requirement for apoptosis and that the morphological changes may appear in the absence of DNA cleavage (Yasuda et al. 1995). It was evident that MeWo cells showed less apoptosis using the TdT assay than with morphological assessment. Analysis of each cell line for increased staining due to background biotin levels (Figure 5.8) and the effects of harvest technique (trypsinisation/scraping; Figure 5.9) failed to identify these as a means of explaining the differences between the cell lines (which could be accounted for in the final analysis). In a recent review, Potten (1996) expressed concern over the comparisons of apoptotic indices obtained using morphological and end-labelling assessment between different laboratories, and concluded that end-labelling assays should only be used as a reference technique, with morphological assessment used as the standard. In the present study all analyses of apoptotic data with other parameters such as cell-cycle delay, were performed using the results obtained by morphological assessment. End-labelling data was used to confirm these results and provide some information on the cell-cycle distribution of the apoptotic cells. That some discrepancies were observed using the two techniques indicates a need for further characterisation of the sensitivity of these assays.

In order to describe the cell lines in terms of apoptotic response expressed as a single value, the mean apoptosis value for each cell over 36 h was calculated using integration of the time course curve, with equal time intervals of 2 h. Increased levels of apoptosis were observed in the radiosensitive cell lines (Figure 5.6). These were related to the intrinsic radiosensitivity of each cell line, expressed
as SF$_2$, for each dose point tested (Figure 5.10). At all doses tested (panels a–f), including 0 Gy, there was a trend towards increased apoptosis in the radiosensitive cell lines, although none of the relationships reached statistical significance. Analysis of the apoptosis levels after one cell-cycle, which was less than 36 h in most cases, produced similar results to the 36 h analysis (Figure 5.7). This suggests that analysis of apoptosis over a given time course, such as 36 h, could be used as standard for different cell types, regardless of cell-cycle times.

Three resistant cell lines in the study (HT29, Be11 and RT112) and one radiosensitive cell line (MeWo) have been shown to demonstrate a marked low dose hypsersensitivity following doses of less than 0.4 Gy and an induced radioresistance between 0.5 Gy and 1 Gy. This has been termed the HRS/IRR response (Lambin et al. 1996). Included in the study were other radiosensitive cell lines (SW48 and HX142) which showed no HRS/IRR. It has been postulated that this phenomenon reflects an inducible repair process triggered by increasing doses of radiation and that this corresponds with intrinsic radiosensitivity at clinically relevant doses.

In this section it was examined whether apoptosis played a role in the HRS response of these six cell lines. If apoptosis was involved, similar levels of apoptosis would be expected in the radioresistant and radiosensitive cell lines at low doses and the relative amount of apoptosis would be greater in radiosensitive cell lines at subsequent higher doses. This was not the case as the mean values of apoptosis over 36 h varied between the cells lines (Figure 5.6, Table 5.1). At low doses (0.2 Gy and 0.5 Gy) the radiosensitive HX142 and U1-S40b cells and radioresistant HT29, MGH-U1, Be11 and RT112 all demonstrated similar apoptosis levels (~2%). At higher doses the resistant cell lines showed little evidence of a significant change in apoptosis levels at any dose tested, suggesting that radioresistance in these cell lines might be due to the inactivation of the apoptotic pathway. The radiation-induced apoptosis levels in the radiosensitive cell lines were higher than constitutive levels, especially in the U1-S40b and SW48 cell lines. The extreme radiosensitivity of the HX142 cell line (SF$_2$ = 0.03) was not reflected in radiation-induced apoptosis levels as, while values increased in a dose dependent manner, the overall levels indicate that apoptotic cell death was not the major cause of death in this cell line. However, the apoptosis response was analysed for only one cell-cycle duration (36 h) in comparison to the clonogenic survival which was analysed two weeks after radiation treatment. It has been suggested that the apoptotic response becomes more pronounced at longer times following treatment. Hopcia et al. (1996) reported that HL60 cells appear much more sensitive to radiation-induced apoptosis if assayed 2 – 4 days after treatment. It could be speculated, therefore, that differences between the apoptotic response of the cell lines in this study may be more pronounced if analysed at later times. This lack of
evidence to support the hypothesis that apoptosis plays a role in the HRS/IRR response could be affected by the limitations of the assay.

Great precision and sensitivity is required to detect the small differences in the radiation response of cells treated with 0 Gy compared to 0.2 Gy. Accurate determination of the low dose hypersensitive region present in some human cell types only became practical with the advent of modern techniques for precise cell counting such as the Dynamic Microscope Image Processing Scanner (DMIPS; Palcic and Jaggi, 1986; Spadinger and Palcic 1992, 1993). The techniques used in this study to harvest, fix, stain and score cells for apoptosis might not be sensitive enough to detect the subtle changes in cell numbers caused by low dose radiation treatment. As alluded to previously, the various fixative and centrifugation steps involved in both assays might also have eliminated some of the relatively fragile apoptotic cells out of the initial sample populations. Matthews (1997) reported the use of an automated microscopy system for the detection of apoptotic cells which operates on a similar principle to that of the DMIPS. This would allow more viable and accurate comparisons of low dose hypersensitivity apoptosis levels. In one such study, Matthews (1997) examined the low dose hypersensitivity of CHO cells with levels of apoptosis observed at low doses using an automated image cytometry system. No direct involvement of apoptosis in the HRS/IRR response was observed. However, increased apoptosis was observed after treatment with 0.25 Gy which the author speculates could indicate a higher propensity for apoptosis after this dose than treatment with either 0 Gy or 0.5 Gy. Therefore, while a role for apoptosis in this phenomenon cannot be ruled out, more evidence is still required to support the hypothesis.

The relationship between constitutive and radiation-induced apoptosis levels at 2 Gy was significant (p = 0.008; Figure 5.10: panel g). This could imply that the apoptotic response plays a minimal role in the radiosensitivity of the cell lines in this study, since constitutive and radiation-induced levels of apoptosis do not vary significantly and cannot be responsible for the large differences in radiosensitivity. However, it also suggests that the constitutive levels of apoptosis in these tumour cell types are indicative of the radiation-induced levels of apoptosis. This is in agreement with previous work showing a direct correlation between constitutive levels of apoptosis in pre-treatment biopsies and outcome to radiotherapy response (Wheeler et al. 1995).

The level of bcl-2 expression in each cell line was analysed using monoclonal antibody binding and flow cytometry. Bcl-2 is a negative regulator of apoptosis and when active, can inhibit the induction of apoptosis in response to a variety of stimuli including radiation (Reed et al. 1996). p53 is thought to down regulate bcl-2 in response to radiation to allow apoptosis to proceed (Chiou et al.
Bcl-2 levels have been shown to correlate with apoptosis levels in some tumour types (Wilson et al. 1996) and with therapeutic outcome (Grover et al. 1996). It was speculated that the differential levels of apoptosis and radiosensitivity observed in the panel of cell lines in this study might be reflected in their levels of bcl-2 protein before and after irradiation. There was no evidence of a direct correlation of bcl-2 and radiosensitivity expressed as SF$_2$, (Figure 5.12, a–b) either at 0 Gy or 4 Gy. MGH-U1 and MeWo cells demonstrated significantly higher levels of bcl-2 protein than any of the other cell lines, with only a slight decrease at 4 h following treatment with 4 Gy. Analysis of the remaining cell lines indicated a slight trend towards increased bcl-2 in the radiosensitive cell lines, with small decreases after radiation. No direct relationship existed between apoptosis levels and bcl-2 expression (Figure 5.12, c–d). The high level of protein expression in the MGH-U1 and MeWo cell lines was mirrored by a corresponding low level of radiation-induced apoptosis. Such a relationship was not observed in the HT29, Be11 and RT112 cell lines, where low bcl-2 levels exist in addition to low apoptosis levels. Since bcl-2 is only one member of an ever increasing family of highly conserved genes involved in the regulation of apoptosis, analysis of it alone may not provide sufficient information as to which mechanisms are controlling the apoptotic response. It has been shown that effects on apoptosis are more dependent on the balance between bcl-2 and its cellular partners like bax, than on bcl-2 quantity alone (Olive and Durand, 1997). Therefore, bcl-2 expression alone was not an indicator of apoptotic response and analysis of the ratio of bcl-2 with other family members, including bax and bad, might be more informative as to the role of bcl-2 in the apoptotic response of these cell lines.

Delay at the G1/S checkpoint and apoptosis in response to ionising radiation are thought to be mediated through p53. Cells either arrest or die at G1 through the action of proteins such as p21 and bcl-2, under p53-transcriptional control (reviewed by Levine, 1997). There is also increasing evidence to suggest that p53 exerts control over the exit of cells from G2/M (Guillouf et al. 1995) and work has been reported on delay and apoptosis at the G2/M checkpoint following radiation (reviewed by Blank et al. 1997). In the present study a prolonged G2/M delay induced by ionising radiation had previously been shown to indicate increased radiosensitivity (see Chapter 4), especially at higher doses (4 Gy). When analysed as a function of mean apoptosis at individual dose points (Figure 5.13), there appeared to be a trend towards increased G2/M delay in cell lines with the highest apoptosis level at 4 Gy. Analysis of apoptosis at 2 Gy with G2/M phase length (which is indicative of phase delay) was significant (p = 0.03). This is in contrast with the study by Bernhard et al. (1996) in which a reduced radiation-induced G2/M delay (caused by caffeine treatment) was associated with increased apoptosis, and
increased radiosensitivity. In the present study increased G2/M delay appears to be associated with increased apoptosis and radiosensitivity of these cell lines. The relationship measured at 4 Gy did not reach statistical significance but it is interesting to note that the elimination of the radiosensitive mutant U1-S40b cell line from the analysis increased the significance to p = 0.002 (from 0.145). U1-S40b cells were isolated from a radiosensitive clone of MGH-U1 cells (McMillan and Holmes, 1991). It was clear that these cells demonstrated significantly higher levels of apoptosis than the parental MGH-U1, a trend that has been observed in many mutant phenotypes. Mitsuhashi et al. (1996) reported reduced apoptosis in a radioresistant variant of a rat yolk sac tumour cell line, and similar results were reported in cells of a radioresistant variant of a human neuroblastoma (Russell et al. 1995) and lymphoblastoid cell lines derived from the same donor (Schwartz et al. 1995). In all cases no differences were observed in either cell-cycle progression or DNA repair capacity after radiation treatment and it is speculated that the apoptotic pathway is the major biological function affected during the isolation of mutant phenotypes with altered radiation responses.

When the fraction of cells that did not progress into G0/G1 after radiation treatment was analysed there appeared to be a direct relationship with apoptosis levels at each dose, which also corresponded with radiosensitivity (Figure 5.14). This was an interesting result as it could be speculated that the actual length of cell cycle delay induced in different cell types by radiation treatment is specific to each cell type and therefore not really relevant to survival. That is, if some cell types take longer to repair damaged DNA, thereby requiring an extended delay, as long as repair has been completed to the level necessary for progression through the checkpoint then survival levels should not be significantly different. What perhaps is more important to survival, therefore, is the number of cells that undergo the necessary repair compared to those that do not. An estimation of this number, by following the number of cells that appear in G0/G1 (G2/M ratio), provided a direct method of analysing cells lost from the population with, in this case, apoptosis. From the results (Figure 5.14) it was clear that in the more resistant cell lines there was a higher proportion of cells able to enter G0/G1 after undergoing a delay in G2/M, compared to the sensitive cell lines. When compared to apoptosis, the G2/M ratio was directly related, significantly so at 0.5 Gy and 2 Gy. Therefore, it could be speculated that the elimination of cells with irreparable damage occurs, at least to some extent, by apoptosis. Other studies have examined the relationship between G2/M delay length and apoptosis after radiation (Bernhard et al. 1996; Guo et al. 1997) and other agents such as tamoxifen (Ferlini et al. 1997) and staurosporine (Qiao et al. 1996). In general, it has been found that agents which induce a G2/M delay also induced apoptosis. In the present study it is implied that those cells which
undergo G2/M delay also apoptose, and that an estimation of the fraction of cells that do not complete G2/M delay may provide more information on the overall survival of the cell population than the length of G2/M delay.

No differences were observed in the p53 expression levels after radiation in both U1-S40b and MGH-U1 cells (see Chapter 4) but increased bcl-2 levels were detected in the MGH-U1 cells. This is consistent with the hypothesis that bcl-2 expression is inversely related to the level of apoptosis. Three cell lines demonstrated mutant p53 status (HT29, RT112, MeWo) and these showed very low levels of apoptosis. Of the cell lines that demonstrated wild-type p53 expression, only the Be11 cell line appeared to lack an apoptosis response. In a recent review (Bristow, 1996), the role of p53 in the radiation response of human tumour cell lines is questioned in light of conflicting reports over the induction of radiation-induced G1/S delay and apoptosis in cells with wild and mutant p53. In this review the author concluded that the role of p53 in the radiation response of human cells is unclear and that functional studies are required for the determination of wt p53 protein function in order to correlate normal and tumour cells radioresponse with p53 status and expression.

In the present study wild-type p53 expression appeared to be more associated with radiosensitivity than with radioresistance. Three of the four radiosensitive cell lines demonstrated wt p53 expression. All wild-type p53 expressing cell lines demonstrated a G1/S delay (Chapter 4) while only one wild-type p53 cell line did not demonstrate radiation induced apoptosis (Be11; Figure 5.6). The actual role of p53 in the regulation of the apoptotic response in each cell line is not fully understood. It has been suggested that p53 exerts an apoptotic response by lowering bcl-2 expression in response to radiation and has been implicated in the regulation of bcl-2 after radiation (Miyashita et al. 1994). However, no significant alterations were observed in bcl-2 levels which suggest that apoptosis may have occurred through a different p53-regulated pathway.

The relationship between G2/M delay and apoptosis at clinically relevant doses indicates that these pathways might be related and rather than one of the pathways being activated in response to damage i.e. cells either arrest to repair damage or apoptose, that both pathways are activated. The later appearance of apoptotic cells compared to G2/M arrest initiation (which can be observed as early as 1–3 h after radiation treatment (see Chapter 4) and the persistence of these apoptotic cells after 36 h suggests that cells delay in G2/M and then undergo apoptosis. This is in agreement with the G2/M ratio data (Figure 5.14) and implies that during the G2/M checkpoint, cells are arrested to repair damage and that apoptosis occurs in those cells that cannot sufficiently repair the damage, due to either the amount of damage or inactivation of repair mechanisms. The extent of
delay, therefore, does not necessarily indicate more repair and hence increased radioreistance. Meyn et al. (1993) reported increased growth delay in murine tumour cells prone to apoptosis. Similar results were reported in two CHO cell lines (Hu and Hill, 1996). In the study of Smeets et al. (1994a), the lack of any significant difference in the extent of G2/M delay in two human squamous carcinoma cell lines, despite differences in apoptosis levels, indicates the need for further characterisation of this response. Studies into the proportion of cells that did not progress through G2/M delay, as well as the extent of the delay of the cells that did in different cell types, might provide more information on what occurs during the G2/M phase checkpoint and how it affects survival in response to radiation.

The cell-cycle phase of apoptotic cells can be determined when using the end-labelling technique and flow cytometry by the parallel incorporation of propidium iodide into cellular DNA. Dual parameter analysis of green and red fluorescence facilitates the analysis of apoptosis together with DNA content, Figure 5.2, and this analysis represents a major advantage of the TdT assay. The proportion of apoptotic cells in each phase of the cell-cycle after 4 Gy are shown in Figure 5.15, and the mean levels in each phase over 36 h after 0 Gy and 4 Gy are shown in Figure 5.16. It was clear that the U1-S40b cells demonstrated the highest proportion of apoptotic cells, which were detected in every phase of the cell-cycle. In all cell lines the highest level of apoptotic cells were detected in S-phase and G2/M phases of the cell-cycle. The high proportion of apoptosis in U1-S40b in G0/G1 phase in the absence of any significant apoptosis in this phase in the other cell lines indicated an altered radiation response in this cell line which is absent or diminished in other cell lines. This might explain the increased mean apoptosis observed in this cell line, compared to its parental cell line MGH-U1 and the rest of the cell lines in the panel. Further investigation of this response could yield some insights into the molecular mechanisms involved in the generation of mutant phenotypes with altered radiation responses. That is, the process of isolation of mutant phenotypes may involve the disruption of the apoptotic pathway and it is this property which results in the altered radiation response. The majority of apoptosis appeared in the S and G2/M phases of the cell-cycle which is in agreement with Ling et al. (1995), who found an apoptotic fraction about twice as high for irradiation in S-phase and G2/M phase versus G1 phase. Cell-cycle phase independent apoptosis has also been reported in human lymphoblastoid cell lines (Olive and Durand, 1997). Knowledge of the cell-cycle dependent susceptibility of cells to apoptosis and length of cell-cycle phase and delays in response to radiation could have important implications in the planning and timing of potential radiotherapy treatment schedules.

In conclusion, it has been shown that in the cell lines in this study the levels of radiation-induced apoptosis indicates radiosensitivity but only at the
higher, clinically relevant doses of radiation. A role of apoptosis in the HRS/IRR response cannot be ruled out, but within the confines of the sensitivity of the techniques used for apoptosis assessment in this study, no evidence exists for low dose hypersensitivity due to apoptosis. The differences in apoptosis levels observed between the cell lines were not directly related to bcl-2 protein levels or p53 status which indicates that other members of the bcl-2 family need to be analysed. There is a direct correlation between the extent of G2/M delay and apoptosis at 2 Gy and 4 Gy and it is speculated that these pathways act together in deciding the fate of a cell after radiation treatment. The radiosensitive mutant cell line, U1-S40b, exhibits consistently higher levels of apoptosis than the parental MGH-U1, which may explain the altered radiation response observed in this cell line. It is speculated therefore, that the transformation of tumour cells into mutant phenotypes is commonly mediated by the alteration of the apoptotic response. G2/M and S phase cells in general demonstrate the highest proportion of apoptotic cells which suggests that the apoptotic response is activated before mitosis. Overall, the above results indicate that the radiation response of human tumour cell lines is likely to be affected by a number of key biological pathways and factors, and that extensive analysis of a number of these parameters together will be required in order to accurately predict radiosensitivity.
6.0 Radiation-induced DNA damage and repair

6.1 Introduction

The extent of local tumour control achieved in the clinic varies widely, and is dependent on a variety of factors such as oxygenation and cell proliferation. Intrinsic radiosensitivity however, is thought to be one of the most important determinants of radiation response in vivo (Fertil and Malaise, 1981). An understanding of the molecular mechanisms that control radiosensitivity could have important therapeutic benefits, both in the elucidation of novel treatment techniques and in the development of predictive assays.

Intrinsic radiosensitivity in vitro has been widely determined by clonogenic assay (Puck and Marcus, 1956; Deacon et al. 1984; West et al. 1993; Brock et al. 1989; Girinsky et al. 1993; Duschenne and Hutchinson, 1996; Lambin et al. 1996). In this procedure cell survival is measured as the ability of a cell to retain its reproductive integrity and form a visible colony after radiation treatment. The linear quadratic (LQ) model is widely used to fit clonogenic survival curves and predicts the decreasing surviving fraction (SF) with increasing dose (D). Differences in SF2 values (surviving fraction at 2 Gy) between tumour cells in vitro, calculated using this model, have been shown to correlate with clinical outcome (Fertil and Malaise, 1985; Deacon et al. 1984; reviewed by West et al. 1995) and SF2 values are now widely used as a measure of intrinsic radiosensitivity.

Radiation affects many targets in the cell with DNA being the most sensitive site to damage in terms of its' effect on cell survival. Two main hypotheses have been proposed to explain differences in intrinsic radiosensitivity between human cells: (1) cells may vary in the amount of DNA damage induced by a given dose of radiation and (2) cells may differ in their capacity to repair radiation induced DNA damage (Nunez et al. 1996). DNA damage in the form of double-strand breaks (DSBs) is regarded as most critical to cell survival (Frankenberg et al. 1981; Kemp et al. 1984; Radford, 1985; Ward, 1990). Several recent studies have examined the levels of initial and residual DNA DSBs in mammalian cells and related them to intrinsic radiosensitivity (SF2), using a variety of assays for DNA damage (see Table 6.1). The main objective of these studies has been to assess whether DNA DSB levels measured, either immediately following radiation or after a repair period (usually 2 – 4 h), could be used to predict radiation response. This would provide a more rapid non-clonogenic alternative for the determination of intrinsic radiosensitivity, with only 2 – 4 days required for a result as opposed to the two weeks necessary to complete a clonogenic assay. From Table 6.1 it was evident that analysis of both initial and residual DNA DSB does correlate with SF2.
<table>
<thead>
<tr>
<th>Initial</th>
<th>Residual</th>
<th>Cell types</th>
<th>Method of DSB detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>ND</td>
<td>Five human breast cancer and one human bladder carcinoma cell lines</td>
<td>PFGE</td>
<td>Ruiz de Almodovar et al. 1994a</td>
</tr>
<tr>
<td>Yes</td>
<td>ND</td>
<td>Three breast cancer cell lines</td>
<td>PFGE</td>
<td>Villalobos et al. 1996</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>Nine human tumour cell lines</td>
<td>PFGE</td>
<td>Whitaker et al. 1995</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>One human bladder, RT112 carcinoma and one human neuroblastoma cell line, HX142</td>
<td>Halo and modified PFGE</td>
<td>Woudstra et al. 1996b</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>Six human tumour cell lines</td>
<td>NFE and comet assay</td>
<td>Olive et al. 1994</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>Two human squamous cell carcinoma cell lines, SCC61 and SQ20B</td>
<td>FIGE</td>
<td>Smeets et al. 1994b</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>One human bladder carcinoma, RT112 and one human neuroblastoma cell line, HX142</td>
<td>Comet assay</td>
<td>Woudstra et al. 1996b</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>Two human bladder carcinoma, RT112 and MGH-U1 and one neuroblastoma cell line, HX142</td>
<td>PFGE and alkaline unwinding</td>
<td>Woudstra et al. 1996a</td>
</tr>
<tr>
<td>ND</td>
<td>No</td>
<td>One bladder carcinoma cell line, MGH–U1 and a radiosensitive clone, U1–S40b</td>
<td>NFE and PFGE</td>
<td>Powell et al. 1992</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>Five human squamous carcinoma cell lines</td>
<td>PFGE</td>
<td>Giaccia et al. 1992</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>V79 cells and a sensitive sub-line</td>
<td>PFGE</td>
<td>Kyseka et al., 1993</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>CHO and radiosensitive clone xrs–5</td>
<td>PFGE</td>
<td>Illiakis et al. 1992</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>Two human colorectal adenocarcinoma cell lines, HT29 and SW48</td>
<td>PFGE</td>
<td>Lambin et al. 1992</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>Twenty human tumour cell lines derived from ovarian and malignant melanoma cancers</td>
<td>PFGE</td>
<td>Zaffroni et al. 1994</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>25 human squamous cell carcinomas (SCC) and eight sarcoma (SAR) tumours</td>
<td>NFE</td>
<td>Schwartz et al. 1996</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Six human squamous cell carcinoma cell lines (including SCC61 and SQ20B)</td>
<td>NFE</td>
<td>Schwartz et al. 1988</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Five human cervix carcinoma cell lines</td>
<td>NFE</td>
<td>Kelland et al. 1988</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Two human neuroblastoma cell lines, HX142 and HX138</td>
<td>NFE</td>
<td>McMillan et al. 1989</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Two pairs of CHO cell lines (parental and mutant clones)</td>
<td>Neutral comet assay</td>
<td>Hu and Hill, 1996</td>
</tr>
</tbody>
</table>

Table 6.1. Correlation of the intrinsic radiosensitivity ($S_F^2$) of human cell lines with initial and residual DSBs.

a: Not determined  
b: Pulsed field gel electrophoresis  
c: Neutral filter elution  
d: Field inversion gel electrophoresis
in many cases, but it is not universal. This indicates that factors other than DNA damage and repair influence the intrinsic radiosensitivity of some cell types and that the exact nature of the link between DNA damage and cell lethality has yet to be established. It was also evident that different techniques produce different results on the same cell lines (for example compare results obtained by Woudstra et al. 1996b using both the halo and modified pulsed field gel electrophoresis (PFGE) assay with those obtained using the comet assay (Woudstra et al. 1996b), and Smeets et al. 1994b with Schwartz et al. 1988). The techniques indicated in Table 6.1 represent the majority of assays currently being used to measure DNA DSBs. Of these PFGE is regarded as the most sensitive to clinically relevant doses of radiation (Illiakis et al. 1991), although there are uncertainties regarding the cell cycle effects on mobility of DNA through the gel (Olive and Bannath, 1993; Mateos et al. 1996).

In this study we measured the level of initial and residual DSB levels using PFGE (Blocher et al. 1989; adapted by Elia and Nichols, 1993) in a panel of eight human tumour cell lines and correlated them to intrinsic radiosensitivity, expressed as SF$_2$. In addition, direct comparisons of SF$_2$ values with both initial and residual DSB levels with other biological pathways known to be affected by or initiated in response to radiation induced DNA damage, such as cell-cycle delay and apoptosis at various doses of radiation were also examined. These parameters have previously been measured and discussed (see Chapters 4 and 5).

6.2 Results

6.2.1 Cell line characteristics

The DNA content, relative to normal human fibroblasts, and cell-cycle distribution of each cell line at the time of irradiation were determined by propidium iodide incorporation (see Chapter 3). Analysis was performed using flow cytometry and quantified using the CellFit™ software program (Becton Dickinson). Results are shown in Table 6.2. Three of the cell lines, HT29, Be11 and MeWo, have a DNA content (expressed as DNA index) significantly greater than one and are thus considered aneuploid. The DNA index of each cell line may be important in the level of DNA damage induced as it is conceivable that a cell line with an aneuploid DNA content will have a higher probability of undergoing DNA damage after a single radiation dose than a cell line with diploid DNA (see section 6.2.2. and 6.2.3). It is possible also that aneuploid cell lines have a higher proportion of redundant DNA, which would lead to a reduced probability of cell kill when compared to diploid cell lines subjected to the same radiation dose.

The cell-cycle distributions of Be11, SW48 and HX142 cells at the time of radiation indicate a higher proportion in S-phase compared to the other cell lines.
Table 6.2. Cell cycle characteristics of human tumour cell lines at the time of experiment. Both the distribution and DNA content values were calculated using the CellFit analysis programme (Becton Dickinson) of DNA profiles generated using propidium iodide incorporation and flow cytometry. DNA indices were calculated by comparison with the DNA content of normal human fibroblasts.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>DNA Index (D1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>84.1</td>
<td>8.9</td>
<td>7.0</td>
<td>1.44</td>
</tr>
<tr>
<td>MGH-U1</td>
<td>91.0</td>
<td>6.6</td>
<td>2.4</td>
<td>1.03</td>
</tr>
<tr>
<td>Be11</td>
<td>68.3</td>
<td>14.6</td>
<td>17.1</td>
<td>1.69</td>
</tr>
<tr>
<td>RT112</td>
<td>92.4</td>
<td>4.4</td>
<td>3.3</td>
<td>1.01</td>
</tr>
<tr>
<td>U1–S40b</td>
<td>88.2</td>
<td>4.8</td>
<td>7.0</td>
<td>1.02</td>
</tr>
<tr>
<td>MeWo</td>
<td>82.6</td>
<td>8.2</td>
<td>9.2</td>
<td>1.48</td>
</tr>
<tr>
<td>SW48</td>
<td>76.5</td>
<td>14.9</td>
<td>8.7</td>
<td>0.99</td>
</tr>
<tr>
<td>HX142</td>
<td>70.3</td>
<td>19.5</td>
<td>10.3</td>
<td>1.05</td>
</tr>
</tbody>
</table>
These differences are most likely due to the fact that these particular cell lines could not be grown to plateau phase and instead detached upon contact. It has been suggested that S-phase cells have an altered migration rate through agarose gels (Stamato and Denko, 1990). The amount of DNA migration from individual cells irradiated in S-phase has been shown to be reduced by about 3 - 4 times compared to cells in G0/G1 or G2/M phase (Olive et al. 1991). This is probably due to a reduction in the electrophoretic mobility of DNA during replication (Illiakis et al. 1991, 1992). The differences in the proportion of S-phase cells observed in the cell lines in this study were considered as a factor possibly affecting the levels of initial radiation induced DSBs detected by PFGE (see section 6.2.2 and Figure 6.2b, c and f). Since residual DSB levels were measured 4 h after radiation treatment, the potential cell-cycle effects were largely unknown due to (1) possible redistribution of cells during this incubation period and (2) differential cell cycle delay responses in each cell line (see Chapter 4).

### 6.2.2 DNA DSB induction

The dose-response curves for DNA DSB induction for each cell line are shown in Figure 6.1. A number of protocols exist which estimate the level of DNA DSB using the PFGE assay (Ruiz de Almodovar et al. 1994b). In this study the most common of these was employed which measures total DSB induction (Blocher et al. 1989; see Chapter 2). Briefly, this involved the simple calculation of the amount of DNA which has been released from the wells into the pulsed-field gel. This was quantified using a radioactively labelled DNA probe, incorporated into proliferating cells over a period of at least 48 h prior to each experiment. This was then counted for relative activity in and out of the well. The fraction of DNA activity released into the gel (FAR) is the ratio of the isotope counts in each lane to the total counts (i.e. in the lane and the well, see equation 2.1). For the initial DNA damage studies FAR values were calculated over the dose range 0-30 Gy. Unirradiated samples were processed in the same manner to obtain relative background FAR values. These were subtracted from the FAR values obtained at each dose and the resultant dose–response curves fitted by linear regression through the origin. The slopes of these graphs relate to the amount of total damage over the whole dose range (Blocher et al. 1989). This was calculated for each cell line and all results are summarised in Table 6.3. Values range from 1.34 to 2.20% FAR/Gy, with the radioresistant cell line Be11 showing the highest level of initial DNA damage and radiosensitive SW48 cells the lowest. This reflected the overall trend observed when initial damage was related directly to the SF2 values of all eight cell lines (Figure 6.2a). Paradoxically, the resistant cell lines exhibited higher levels of initial damage compared to the radiosensitive cells and this comparison almost reaches statistical significance.
Figure 6.1. DNA DSBs induction in eight human tumour cell lines. The level of DSB induction is estimated from the levels of ¹⁴C activity released into the lanes of the pulsed field gel. Data points represent the mean of two experiments (±SEM), fitted to a straight line by linear regression, to allow inter comparison of slopes.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Initial Slope</th>
<th>Residual Slope</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>1.80 ± 0.12</td>
<td>0.31 ± 0.12</td>
<td>17.46 ± 3.0</td>
</tr>
<tr>
<td>MGH–U1</td>
<td>1.801 ± 0.14</td>
<td>0.36 ± 0.02</td>
<td>19.67 ± 2.86</td>
</tr>
<tr>
<td>Be11</td>
<td>2.210 ± 0.07</td>
<td>0.34 ± 0.04</td>
<td>15.46 ± 1.30</td>
</tr>
<tr>
<td>RT112</td>
<td>2.02 ± 0.12</td>
<td>0.34 ± 0.03</td>
<td>16.9 ± 2.13</td>
</tr>
<tr>
<td>U1–S40b</td>
<td>2.01 ± 0.24</td>
<td>0.17 ± 0.05</td>
<td>8.65 ± 2.12</td>
</tr>
<tr>
<td>MeWo</td>
<td>1.69 ± 0.16</td>
<td>0.34 ± 0.06</td>
<td>20.27 ± 3.4</td>
</tr>
<tr>
<td>SW48</td>
<td>1.34 ± 0.33</td>
<td>0.29 ± 0.10</td>
<td>21.95 ± 8.89</td>
</tr>
<tr>
<td>HX142</td>
<td>1.60 ± 0.24</td>
<td>0.49 ± 0.08</td>
<td>30.88 ± 7.79</td>
</tr>
</tbody>
</table>

Table 6.3. Slopes of initial and residual double break dose response curves constructed using PFGE. The ratio values represent the level of repair and were calculated as the ratio of residual double-strand breaks (DSBs) to the level of initial DSBs detected in each cell line. Errors represent the mean of two experiments (±SEM).
<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Initial DNA damage</th>
<th>Correlation with SF2 $p(r)$</th>
<th>Residual DNA damage</th>
<th>Ratio (residual/initial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.268 (0.446)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.601 (0.215)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.721 (0.151)</td>
<td>0.332 (-0.538)</td>
<td>0.271 (-0.491)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.025 (0.770)</td>
<td>0.170 (-0.538)</td>
<td>0.075 (-0.659)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.490 (0.287)</td>
<td>0.108 (-0.610)</td>
<td>0.113 (-0.604)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.822 (0.096)</td>
<td>0.131 (-0.581)</td>
<td>0.116 (-0.600)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>0.192 (-0.515)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>-</td>
<td>0.233 (-0.476)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.4. DNA damage correlated to survival at individual doses of radiation. Initial and residual levels of radiation induced DNA DSBs and the amount of repair (ratio) were analysed for significance with survival at the applicable individual radiation dose points used in the dose response curves (Figures 3.1 and 3.3). The data are expressed as significance values with the correlation coefficient in parenthesis. Statistical analysis was performed using the Mann-Whitney unpaired test for discrete groups, at 95% confidence level.
Figure 6.2 (a-f). Correlation of initial DNA damage levels with radiosensitivity in eight human tumour cell lines. DNA damage values were analysed with radiosensitivity (SF₂) as (a) values calculated directly from PFGE analysis, (b–c) in conjunction with S–phase fraction, (d–e) DNA index or (f) both. Legend is displayed in order of increasing radiosensitivity (left to right; top to bottom). Data points represent the mean of at least two experiments.
Analysis of the FAR (%) at individual doses also shows a higher proportion of damaged DNA in the radiosensitive cell lines at every dose point, see Table 6.4, reaching statistical significance at 10 Gy.

Further analysis of the total initial DSB levels and radiosensitivity was performed by attempting to equalise the differences observed in the S-phase fractions and DNA content of each cell line (Figure 6.2b–f). Cell lines with lower S-phase fractions show higher levels of initial DNA damage (Figure 6.2b) in agreement with the results observed by Olive et al. (1991). Subsequent division of the initial DSB values for each cell line by its respective S-phase fraction (Figure 6.2c) reduced the correlation with SF2 ($p = 0.274, r = 0.441$). The relationship between DNA index and initial slope (Figure 6.2d) was not expected to be significant as only three of the cell lines were aneuploid, one of which is radiosensitive (MeWo) while the other two were relatively radioresistant (HT29 and Be11). When the initial slope data was corrected for DNA content, Figure 6.2e, (by division of initial slope with DNA index values) the range slope was reduced to between 1.14–2.00 and the correlation with SF2 was also notably reduced ($p = 0.686, r = 0.171$). When the S-phase fraction and DNA content were both examined with initial slope values (Figure 6.2f) i.e. ((initial slope/S-phase fraction)/DNA index), the relationship with survival changed again ($p = 0.405, r = 0.344$). These results were in agreement with several findings that initial DNA DSB levels vary between cell lines, but in a manner independent of intrinsic radiosensitivity (see Table 6.1).

### 6.2.3 Residual DNA damage

The level of residual DSBs were measured 4 h after irradiation. Doses ranged from 0–70 Gy. Results are summarised in Table 6.3. Calculations were performed as for the initial DNA damage studies described previously (see Chapter 2 and section 6.2.2). For each cell line the slope of the FAR (%) versus dose plot was less than that observed for initial DNA damage, indicating that repair has occurred, see Figure 6.3. Unrejoined DNA DSB levels vary significantly between the HX142 and Ul-S40b cell lines. The fact that the Ul-S40b cell line was isolated from a radiosensitive mutant clone of the MGH-U1 cell line may be important. All other cell lines exhibited residual DSB levels ranging from 0.295–0.355. This implied that similar levels of unrejoined DSBs could be 'tolerated' in these cell lines despite their differences in radiosensitivity. Correlation with SF2 was not significant ($p = 0.50, r = -0.28$), Figure 6.4a. Damage levels at individual dose points were not significantly related to radiosensitivity (SF2) at any individual dose, see Table 6.4. When corrected for variations in DNA content between the cell lines, the range of unrejoined DSB levels was increased and the relationship with SF2 slightly enhanced ($p = 0.31, r = -0.42$), Figure 6.4b. Residual DNA damage data
Figure 6.3. Residual DNA DSBs in eight human tumour cell lines. The amount of DSBs remaining is determined after 4 h incubation at 37°C and is expressed as the fraction of $^{14}$C activity that has migrated into the lane of the pulsed field gel. Data points represent the mean of two experiments (±SEM).
Figure 6.4. Correlation of residual DSBs with radiosensitivity in eight human tumour cell lines. Damage was measured after 4h incubation at 37°C. Values were calculated directly from PFGE (a) and those corrected for DNA content (DNA index) (b) were correlated with radiosensitivity ($SF_2$). Data points represent the mean of at least two experiments.
was not corrected for any potential cell-cycle distribution differences as these had been measured prior to treatment without taking into account the changes induced by a 4 h repair period which would have resulted in redistribution of cells throughout the cell cycle (see Chapter 4).

It was evident from Figure 6.2 (a and f) that the initial levels of induced damage varied between cell lines. Analysis of residual DNA damage levels alone does not take into account these differences. To address this, the level of residual DNA damage was calculated as a fraction of the amount of initial damage observed in each cell line (ratio = residual DSBs / initial DSBs). These values represent the amount of original DNA damage that has not been repaired. The majority of the values fell between 15% – 23% of the initial damage (see Table 6.3). No statistical correlation was observed with SF\textsubscript{2} (p = 0.135, r = -0.576), see Figure 6.5a. There was a strong indication, however, that the resistant cell lines had less unrejoined breaks remaining than radiosensitive cell lines. It is interesting to note that the U1-S40b cell line (SF\textsubscript{2} = 0.40) showed a significantly lower level of residual DSBs (expressed either as residual DSBs alone or as a fraction of initial DSB levels) than its' radioresistant parental cell line MGH-U1 (SF\textsubscript{2} = 0.70). This may be due to a difference in the efficiency rather than the amount of repair as U1-S40b cells have been reported as having a lower fidelity of DSB rejoining (Powell \textit{et al.} 1992). When the repair values were 'corrected' for DNA content correlation with SF\textsubscript{2} was increased, Figure 6.5c, although it did not reach statistical significance (p = 0.109, r = -0.609). Previous analysis of the initial DNA DSB values involved compensation for differences in both the S-phase fraction and DNA content (see Figure 6.2f), as it was considered that these factors might have some influence on the number of DSBs detected by PFGE. Calculation of the residual DSBs as a function of this corrected value for initial DSBs did not improve the statistical relationship with SF\textsubscript{2} (p = 0.149, r = -0.56), Figure 6.5d.

6.2.4 DNA damage and cell-cycle delay

Radiation induced DNA damage activates many cellular pathways, in particular those leading to cell-cycle arrest (Hartwell and Kastan, 1994; Nagasawa \textit{et al.} 1994; reviewed by Paulovich \textit{et al.} 1997). Such delays presumably facilitate the repair of DNA damage (Sherr, 1996), and a prolonged G2/M and/or G0/G1 phase in response to radiation in mammalian cells has been shown to correlate with radiosensitivity (McKenna \textit{et al.} 1996; reviewed by Levine, 1997). In a previous section of this thesis, (see Chapter 4), the extent of radiation induced delays, both at the G2/M and G0/G1 checkpoints, was examined following increasing doses of radiation. A significant correlation was found between radiosensitivity (SF\textsubscript{2}) and the length of G2/M delay following 4 Gy (p = 0.005). A similar trend could be observed at 2 Gy. No correlation was observed between G0/G1 arrest and radiosensitivity.
Figure 6.5. Correlation of unrejoined DNA DSBs expressed as a ratio of initial DNA damage in eight human tumour cell lines. Values were correlated with radiosensitivity (a) and DNA index (b). Values were then corrected for differences in DNA content (c). Recalculation of the ratio values was performed using the initial DNA damage values corrected for differences in S phase fraction and DNA content (d), as in Figure 7.2d. Data points represent the mean of at least two experiments. Legend is arranged in order of increasing radiosensitivity (left to right; top to bottom).
Figure 6.6. DNA damage and G2/M delay in eight human tumour cell lines. G2 delay values (see Chapter 4) were correlated with initial DNA damage (a, b) residual DNA damage (c, d) and residual damage expressed as a fraction of initial damage (e, f). Data points represent the mean of at least two experiments. Legend is arranged in order of increasing radiosensitivity (left to right; top to bottom).
In this section the level of initial and residual DSBs were examined together with G2/M delay length, and results are shown in Figure 6.6 a–f. In panels a and b the original initial DNA damage slope value correlated significantly with the extent of G2/M delay at both 4 Gy and 2 Gy (p = 0.002 and 0.011 respectively). There was no significant relationship between G2/M delay and residual DSBs (c and d). However, the amount of unrejoined initial DSBs (ratio = residual damage / initial damage) may have some influence on the extent of G2/M delay (e and f) especially at 4 Gy (panel e; p = 0.136, r = 0.575). Correction of the DNA damage figures for S-phase fraction and/or DNA content differences (as performed in sections 6.2.2 and 6.2.3) did not improve any of the statistical correlations. These results suggest that in this panel of tumour cell lines, the more radiosensitive cell lines demonstrate less initial DNA damage, but a longer G2/M delay. The extent of repair, however, was greater in the resistant cell lines despite a shorter delay time.

6.2.5 DNA damage and apoptosis

Radiation induced apoptosis has been detected in many human tumour cell types and in some studies has been shown to correlate directly with radiosensitivity (reviewed by Blank et al. 1997). DNA damage is thought to be the primary effector of radiation induced apoptosis and of this damage, DSBs are believed to be the lesions most critical to cell survival after radiation. In this section, the level of DNA damage after radiation was compared with the apoptotic response of each cell line in order to examine more closely the relationship between radiation-induced DNA DSBs and apoptosis.

Radiation induced apoptosis was analysed by morphological assessment over 36 h after treatment in a previous section (see Chapter 5) and a close relationship was observed between intrinsic radiosensitivity (SF2) and mean apoptosis at 2 Gy (p = 0.063, r = -0.68). A similar trend was also observed at 4 Gy (p = 0.108, r = -0.658).

The relationship between mean apoptosis levels and (uncorrected) initial, residual and unrejoined initial DSB levels is shown Figure 6.7a–f. The level of apoptosis was indirectly related to the level of initial DNA damage (panels and b), and was almost significant at 2 Gy (panel b; p = 0.072, r = -0.666). The relationship between apoptosis at 4 Gy and initial DNA damage (panel a) was significant when the U1-S40b cells were eliminated from the analysis (p = 0.05, r = 0.835). This cell line was isolated as a radiosensitive clone of the MGH-U1 cell line and has been reported as having a reduced repair fidelity (Powell et al. 1992). These results were very similar to those observed during the G2/M delay and DNA damage analyses (Figure 6.5a and b) and suggest that, in these cell lines, the G2/M and apoptosis pathways might be directly related.

The level of residual and unrejoined initial DSBs remaining after 4 h did not demonstrate a correlation with apoptosis (panels c–d and e–f respectively). None of the above correlations were improved by the correction of the DNA damage values for S-
Figure 6.7. DNA damage and apoptosis in eight human tumour cell lines. Mean apoptosis levels, calculated over a period of 36 h after radiation of 4 Gy and 2 Gy (see chapter 5) were correlated to initial damage levels (a, b), residual damage (c, d) and residual damage expressed as a fraction of the initial damage (e, f). Data points represent the mean of at least two experiments. Legend is arranged in order of increasing radiosensitivity (left to right; top to bottom).
Phase and/or DNA content differences. These results suggest that radioresistance is associated with higher levels of initial DSBs followed by high DNA repair capacity, while radiosensitivity is characterised by lower levels of initial DSBs and repair capacity but increased levels of apoptosis.

6.3 Discussion

Intrinsic radiosensitivity is regarded as the most important determinant of radiation response in human tumour cells. Extensive research has been targeted towards the elucidation of the factors affecting intrinsic radiosensitivity with the primary aim of improving current radiotherapy cure rates. Several key biological pathways have been implicated in the response of human tumour cells to radiation. These include radiation-induced cell cycle arrest, DNA repair and apoptosis. Radiation is thought to affect these pathways primarily through the production of DNA damage. Many different types of damage to DNA occur in response to radiation, but it is the double-strand break (DSB) which is the most critical to cell survival (Radford, 1985). In this section, the level of radiation-induced DSBs were measured in eight human tumour cell lines by PFGE. These levels were analysed directly with intrinsic radiosensitivity, expressed as SF$_2$, and two other biological parameters; cell cycle delay and apoptosis. These were measured in two previous sections (see Chapters 4 and 5 respectively). It was reasoned that ionising radiation would have diverse effects on each pathway in different cell types, and that each pathway could be, and probably is, affected by another. It is generally accepted that radiation-induced cell-cycle delay, for example, is activated in response to DNA damage, and facilitates the repair of damage before the next stage of replication is initiated (reviewed by Paulovich et al. 1997). The results presented in this section indicate that high levels of initial DNA damage correspond to a prolonged G2/M phase and apoptosis, both of which indicate increased radiosensitivity. The extent of DNA damage repair however appears to indicate radioresistance in the cell lines tested.

Pulsed field gel electrophoresis (PFGE) enables fragments of high molecular weight to be separated in an agarose gel by alternating pulses of field direction or strength. The relaxation and reorientation of the migrating DNA molecule during pulses determines its position in the gel. It has the advantage over other techniques in that it is sensitive to DSBs produced by ionising radiation at doses as low as 1 Gy (Whitaker et al. 1995), while the majority of other techniques are limited to detection thresholds of about 10 Gy. Recent reports however, have suggested that sensitivity levels can be reduced to doses of around 0.1 Gy of radiation, using neutral filter elution (Kaur and Blazek, 1997) and the micronucleus assay (Vral et al. 1997). A comparative study of both these techniques with PFGE would be very useful. If the sensitivity of a technique to measure DNA damage can be reduced to detect damage induced by doses similar to
those used in clonogenic studies (generally up to 5 Gy), then direct correlation of both sets of data would be more meaningful.

The PFGE assay used in this study is based on the observation that the damage induced by ionising radiation, measured as the fraction of $^{14}$C activity released (FAR) into the pulsed field gel, is linear with dose. This was the case in all cell lines in the dose ranges tested for both initial and residual DNA damage assays (Figures 6.1 and 6.3). The slope of the FAR dose response represents DNA damage in each cell line. Initial DNA damage, measured immediately after radiation, varied between cell lines, in agreement with many other studies (for reviews see Olive et al. 1994; Nunez et al. 1996). Correlation with SF$_2$ was almost significant, but with radioresistance rather than radiosensitivity ($p = 0.07$, $r = 0.668$; Figure 6.2a). Analysis of initial DNA damage at individual dose points also indicated a relationship between radioresistance and DSBs, reaching significance at 10 Gy ($p = 0.025$; Table 6.4). A similar trend has been observed in some human tumour cell lines, using the same method of DSB analysis of the PFGE generated data (Whitaker et al. 1994; Ruiz de Almodovar et al. 1994a; Villalobos et al. 1996). Several other studies which have reported a relationship between initial DNA damage and radiosensitivity have shown that radiosensitive cell lines exhibit higher levels than radioresistant cell lines. This supports the hypothesis that radiosensitivity is due to an increased susceptibility to potentially lethal DNA damage. The results observed in the present study, which indicate that the level of initial damage is higher in the radioresistant rather than the sensitive cell lines, might reflect a higher tolerance to initial DNA damage levels in the radioresistant cell lines, and could be indicative of a higher capacity to repair damage. This is in agreement with the observation that resistant cell lines demonstrate increased repair (see Figure 6.5a). The correction of the initial DNA damage values for variations in both DNA content and cell-cycle distribution effectively randomises this relationship, and suggests that the role of initial damage in the radiation response is unclear and that the method of analysis of results should be carefully considered.

The experimental variations observed in the level of DNA DSB detection using different techniques, and between those observed using PFGE (Whitaker et al. 1994 and Woudstra et al. 1996b) suggest that the simple equation of DSB levels detected by PFGE with radiosensitivity must be interpreted with caution. Olive et al. (1992) demonstrated that the detection of DNA damage was influenced by the chromatin structure and DNA packaging at the time of assay, both in terms of the mobility of the DNA through the gel and the access of repair enzymes to the damaged sites. Consequently, differences in radiosensitivity have been related to differences in DNA structure rather than DSB levels. Woudstra et al. (1996b) observed equal DNA DSB levels in two cell lines, which differed in radiosensitivity (SF$_2$) by a factor of 20, using PFGE but showed higher damage in the radiosensitive cell line using the halo assay,
which allows the higher DNA structure to remain partially intact. The author concluded that these cells differed in their structural organisation of chromatin and hypothesised that, as a result, the same number of lesions have a different impact on cell survival. If the DNA structure affects the detection of DSBs, then the relationship of DSB levels with radiosensitivity cannot be treated as a simple one.

Giacca et al. (1992) argued that differences in initial DSB levels were artefacts from variations in cell cycle distribution as S-phase cells exhibit a lower mobility under PFGE, giving the appearance of less DSBs. To overcome this problem, DNA damage experiments by PFGE were performed on confluent cell populations. This reduced the amount of replicating cells to 9% or less of the total population. These conditions could not be produced during culture of the SW48, HX142 and Be11 lines, as these cells became detached before confluence was reached. This was reflected in their cell cycle distribution values at the time of experiment in which S-phase populations ranged from 14.6 to 19.5%. Olive et al. (1991) reported a reduced mobility of DNA from S-phase cells, in the order of about 3 – 4 times less than that of G1 or G2 cells. This difference is most likely the result of the formation of replicon clusters and could affect the level of DSBs detected by PFGE. The direct comparison of initial DNA damage levels appeared to be directly related to the S-phase fraction in most of the cell lines, Figure 6.2b. This was especially true in the case of two of the most radiosensitive cell lines, SW48 and HX142, which demonstrated the lowest level of initial DSBs and the highest S-phase fractions (14.9 and 19.5% respectively). The correction of each initial DSB value for differences in S-phase fraction resulted in the reduction of the range of damage values. This was reflected in the slight reduction in the significance of initial DSB with SF2. The exact nature of the influence of relatively small differences in S-phase fractions observed between the cell lines is unclear. Mateos et al. (1996) reported that S-phase fraction was only significant at values of ≥ 0.70 (70% of the total population) in the bladder carcinoma cell line MGH-U1. The values for S-phase fraction reported in the present study fall well below this level. However, it would require a similar study on each cell line to indicate whether the differences between the cell lines significantly affected the DSB levels detected.

The comparison of DSB levels to radiosensitivity could also be influenced by the cell-cycle as not only do plateau phase cells demonstrate relatively more radioresistance than exponentially growing cells (West et al. 1988) but that radiosensitivity varies throughout the cell cycle. Cells in late S-phase cells are generally regarded as the most radioresistant while those in G2, the most radiosensitive (Sinclair, 1967). In this thesis radiosensitivity studies were carried out on exponentially growing cells in clonogenic survival assays, while the DNA damage assays were carried out on plateau phase cells (where possible). Conclusions from a direct comparison of both results must, therefore, be interpreted with caution.
Measurement of the DSBs produced per DNA unit has been shown to improve the relationship between initial DNA damage and SF$_2$ (Ruiz de Almodovar et al. 1994a). The DNA index (DI) is defined as the ratio of the mean of the DNA/diploid G0/G1 value of the tumour sample and the internal control, such as normal human fibroblasts. This parameter has been shown to be prognostically significant in the prediction of survival some cancer patients (Kallioniemei et al. 1988; Friedlander et al. 1988; Klemi et al. 1990; Lage et al. 1992; Chen et al. 1994). Monasebian et al. (1996) demonstrated a correlation between DNA ploidy and radiosensitivity of hypopharyngeal squamous carcinoma which suggests a possible role of DNA content in the intrinsic radiosensitivity of some tumour cells. However, such a relationship was not evident in this study (see Chapter 3), in agreement with other in vitro analyses (McMillan et al. 1990; Zaffaroni et al. 1994). It was hypothesised that the level of DNA damage detected in each cell line might be influenced by DNA content. This was estimated for each cell line in this study relative to normal human fibroblasts using flow cytometry. Only three of the cell lines demonstrated aneuploid DNA content (DNA index >1), HT29, Be11 and MeWo and no direct correlation was observed with initial slope ($p = 0.329$; Figure 6.2d). Correction of the initial slope values for DNA content (by division of slope values by the respective DNA index values for each cell line) reduced the correlation between initial DNA damage with radioresistance ($p = 0.686$ versus $p = 0.07$; Figure 6.2e). Correction of initial slope values of each cell line for both differences in S-phase fraction as well as DNA content reduced the significance of the relationship between SF$_2$ (Figure 6.2f; $p = 0.405$ versus $p = 0.07$). While it could be argued that initial DNA damage could be directly related to DNA content (this was certainly true of the Be11 cell line, Figure 6.2d) due to an increased probability of striking a DNA target, this has not been substantiated in the literature. Indeed, in this study two of the three aneuploid cell lines (HT29, MeWo) demonstrated similar levels of initial DNA damage relative to the other (diploid) cell lines. An increased DNA content might also influence the probability of a radiation-induced break in a redundant part of the DNA. This would mean that although a break existed, the probability of cell kill due to this lesion would be reduced as it would not be critical to repair the lesion. While evidence for this was not clear from the results observed, it remains an interesting point for future studies. The interpretation of results obtained by the combination of cell-cycle distribution and DNA content with initial DSB levels, which did not result in any improvement of the statistical correlation with SF$_2$, must therefore be treated with caution until the precise effect of these factors are known.

The 'presentation' of DNA damage has been implicated in the intrinsic radiosensitivity response of mammalian cells and it has been postulated that cell lines differ in their response to radiation primarily by the mode with which they tolerate DNA damage. Many studies have implicated the repair capacity of human tumour cells as a major determinant of radiosensitivity (see Table 6.1). Residual DSB breaks were
analysed in the same way as the initial damage studies. The slopes of the FAR and dose curves relate to the amount of DNA damage (see Figure 6.3a and Table 6.3). It has been demonstrated in several studies that the majority of repair in mammalian tumour cells occurs in the first 30 minutes after radiation exposure and that repair is essentially complete in the period of 1–2 h following exposure (Schwartz et al. 1996a; Illiakis et al. 1992; Woudstra et al. 1996a; Whitaker et al. 1995; Zaffaroni et al. 1994; Powell et al. 1992). In this study residual DSB levels were analysed after a 4 h repair period, to ensure the majority of repair has occurred. Correlation of the level of residual DSBs was not significant with radiosensitivity expressed as SF$_2$ ($p = 0.499$, Figure 6.3a). Correction for differences in DNA index did not improve the relationship with SF$_2$ ($p = 0.305$, Figure 6.3b). All cell distributions were measured before treatment, and as a result the residual DNA damage values were not corrected for cell-cycle distributions due to the changes induced by the 4 h repair period which would have resulted in the redistribution of cells throughout the cell cycle (see Chapter 4).

Expression of residual damage as a fraction of the initial DSBs gives an indication of the extent of repair of initial lesions which has occurred in each cell line. This might be informative as many studies have reported a direct correlation between the extent of radiation-induced DNA DSB repair and radiosensitivity in human tumour cell lines (see Table 6.1). In this study there appeared to be a trend towards reduced residual DSBs in the more radioresistant cell lines (see Table 6.3) although this did not reach statistical significance with SF$_2$ ($p = 0.137$, $r = 0.574$; Figure 6.5a). While the DNA index values were not directly related to the ratio of damage (Figure 6.5b), correction of these values improved the overall correlation with SF$_2$ ($p = 0.109$, Figure 6.5c). As demonstrated previously the correction of initial DNA damage values for cell-cycle distribution and DNA content differences between the cell lines altered the overall relationship of these values with SF$_2$. The ratio value is expressed as a function of initial DSBs, and the significance of using the corrected initial DSB value in the calculation was also examined, Figure 6.5d. The effect on the statistical correlation between the ratio and SF$_2$ was minimal ($p = 0.1489$, $r = -0.56$). The results suggest that overall, resistant cell lines have an increased capacity to repair radiation-induced DNA DSBs.

In a recent review Nunez et al. (1996) attempted to summarise the information available on DNA DSB induction and repair and radiosensitivity in different experimental systems. These were grouped into four different categories and results summarised as data obtained from studies of

1) panels of human tumour cell lines
2) comparisons between parental cells and mutant phenotypes with altered radiation response
3) tumour cells irradiated in the presence or absence of radio-modifiers,
4) cells derived from human radiosensitive syndromes, such as AT
Seven of the eight cell lines analysed in this study were human tumour cell lines derived from various primary tumour sites, and only the U1-S40b cells were isolated from a radiosensitive clone of the MGH-U1 cell line (McMillan and Holmes, 1991). The categories outlined above would exclude this cell line from category one on the basis that it is a mutant phenotype (category two). Exclusion of this cell line from the analysis, so that only seven cell lines are considered, resulted in a statistically significant relationship between the amount of residual DSBs and SF$^2$ both in the raw and DNA index-corrected data respectively ($p = 0.019$, $r = 0.838$; $p = 0.045$, $r = 0.731$). This was in agreement with several reports which have suggested that the repair capacity of human tumour cell lines can be correlated directly to radiosensitivity in some cell types (see Table 6.1; reviewed by Nunez et al. 1996; Olive et al. 1994). Analysis of MGH-U1 cells directly with its' radiosensitive mutant phenotype U1-S40b indicate less residual DSBs remaining after 4 h in the U1-S40b cell line (8.7% versus 19.8%). Previous studies of these cell lines have attributed the increased radiosensitivity of the U1-S40b cell line to a reduced repair fidelity (Powell et al. 1992). This could conceivably affect the reorganisation of the U1-S40b DNA structure after repair and consequently its migration through the gel.

As alluded to previously, the impact of DNA DSB break lesions may vary in different cell types. Biological pathways implicated in the cellular response to radiation induced DNA damage and repair include cell-cycle arrest and apoptosis. The extent of cell-cycle delay at the G2/M checkpoint was analysed in a previous section (see Chapter 4). Re-analysis of these results as a function of initial and residual DNA damage levels indicated that the extent of G2/M delay, induced by both 2 Gy and 4 Gy, significantly correlated with the level of initial DSBs and that this was directly related to radiosensitivity (Figure 6.6a and b). This could be indicative of a greater impact of DNA DSB lesions in the radiosensitive cell lines, with relatively low levels of DSBs producing an extensive cell-cycle delay. The higher levels of initial DSBs and shorter delays in the resistant cell lines would conversely indicate a reduced impact of DSB, suggesting that radioresistance could be due to a higher tolerance to DNA damage. The levels of unrejoined breaks, measured independently (Figure 6.6c and d) or calculated as a fraction of the initial DSB levels (Figure 6.6e and f) did not appear to be related to G2/M delay either at 4 Gy or 2 Gy. It was speculated that the level of unrejoined DSBs after radiation might dictate to some extent the length of radiation-induced cell-cycle delay. This was not obviously the case, although there was a slight indication that G2 delay induced by 4 Gy of radiation was correlated to the ratio of unrejoined breaks (Figure 6.6e).

Similar trends were observed when the extent of radiation-induced apoptosis was analysed in conjunction with the extent of initial and residual DNA damage (Figure 6.7a–f). Mean apoptosis over 36 h induced by 2 Gy and 4 Gy of radiation was
determined in a previous section (see Chapter 5) and was shown to be related to radiosensitivity in these cell lines. The correlation of initial DNA damage with apoptosis levels in cell lines (see Figure 6.7a and b) revealed increased apoptosis in those cell lines that demonstrated the lowest levels of DNA damage and this appeared to be related to radiosensitivity. This relationship was significant when the U1-S40b cells were eliminated from the analysis of apoptosis at both 2 Gy (p = 0.017 versus 0.321) and 4 Gy (p = 0.05 versus 0.07). The levels of residual DSBs appeared unrelated to levels of apoptosis (Figure 6.7c–f). An important consideration here is that the detection of either initial or residual DSBs might be influenced by the levels of apoptosis in that the DNA fragmentation, characteristic of apoptosis, might be regarded as DSBs using PFGE. The levels of apoptosis observed in these cell lines (see Chapter 5) did not become significant until 10 h after radiation treatment. By analysis of residual DSB after 4 h, it was hoped to eliminate the possibility of apoptotic cell fragments being regarded as DSB. However, the time course of apoptosis may be significantly altered with higher doses, such as those used in the DNA damage assays. These effects would have to be investigated fully before any conclusions can be drawn from the data presented here. However, it could be speculated that radioresistance is characterised by a prolonged G2 phase followed by apoptosis and that DNA repair is a characteristic of resistant cell lines. Moreover, it could be speculated that the induction of a G2 delay allows time for the induction of repair and subsequently apoptosis, and is shorter in the resistant cell lines due to a lack of apoptosis induction.

The relationship between the level of radiation-induced DNA DSB induction and repair levels with both cell cycle delay and apoptosis suggests that in these cell lines, it is the initial damage levels which affects these biological pathways. The extent of repair, expressed as residual DSBs, was also inversely related to initial damage, although this did not reach statistical significance (p = 0.076, r = -0.658). The levels of residual DSBs remaining after repair did not vary significantly between the majority of cell lines. Indeed only two of the cell lines show residual DSB levels outside of a range of 15.5–22.0% of the initial damage. These included the U1-S40b cell line (8.8%), which has previously been shown to have repair fidelity defect (Powell et al. 1992), and HX142 cells, which appear to repair only 31% of the initial damage. It could speculated that DNA damage levels are reduced to a 'background' level independent of tumour cell type, either by repair of the damage or elimination of the cells with greater levels of damage by programmed cell death or other biological processes.

The majority of references in the literature regarding levels of DSBs induced in human tumour cell lines have analysed the relationship with intrinsic radiosensitivity, either as SF2 or other radiobiological parameters such as mean inactivation dose, D or the alpha component of the clonogenic survival curve. Hu and Hill (1996) analysed apoptosis levels in two pairs of CHO lines and postulated that the radiation-induced
apoptosis observed in these cell lines may be triggered by the residual DSBs remaining after 2 h. Schwartz et al. (1996b) demonstrated an inverse relationship between terminal deletions, the cytogenetic equivalent of unrejoined breaks and G2 delay and also suggests that the signal for cell cycle delay is an unrejoined break.

Before any conclusions can be drawn from a study which relates results from a DNA damage assay with those from a clonogenic study and a cell cycle study, the uncertainties of this procedure need to be considered. There are many differences between the assays used. DNA damage was measured after radiation treatment with doses up to 30 Gy in the initial and 70 Gy in the repair studies, while all other assays were performed with radiation doses in the dose range 0 – 5 Gy. Cells were irradiated on ice for both the initial and residual DNA damage experiments to prevent repair, while all other irradiations were performed at 37°C. The effects of these different conditions on the radiation response of the cell lines are unknown but could be important as Whitaker et al. (1995) has reported differences between the heat recovery of some tumour cell lines.

In conclusion, differences in the levels of initial and residual DSB do exist between the cell lines in this study. The validity of comparing results from assays which differ in irradiation conditions, which might affect radiation response, must be considered when interpreting all results. DNA damage levels were not significantly related to survival, but elimination of a radiosensitive mutant from the panel of cell lines resulted in a significant correlation between DNA repair and SF2. Factors that might influence the detection of DSB breaks with PFGE such as DNA content and S-phase did not greatly affect the results. Initial DNA damage levels correlate inversely with G2 delay and apoptosis, which can be directly related to radiosensitivity. It is hypothesised that in these cell lines, initial DNA damage activates the radiation response in the form of DNA repair, or cell-cycle delay and apoptosis. In these cell lines radiation sensitivity is linked to cell-cycle delay and apoptosis while resistance is characterised by an increased capacity to repair.
7.0 Final Discussion

In this thesis some of the molecular mechanisms linked directly to the intrinsic radiosensitivity of mammalian cells have been described for a panel of human tumour cell lines. These cell lines have been derived from various tumour types including colorectal adenocarcinoma, bladder carcinoma, malignant melanoma and neuroblastoma. In addition, this panel of cell lines displayed markedly differing radiosensitivities, with $SF_2$ values ranging from 0.033–0.74. In order to describe the molecular mechanisms involved in their individual radiation responses, each cell line was analysed for cell-cycle delay, DNA damage and repair and apoptosis in response to a range of radiation doses. Each of these parameters has been shown to individually track radiosensitivity in many tumour cell types in vitro. Comparable with many previous studies, each individual parameter analysed was compared directly with radiosensitivity. In addition, the possible inter-relationships between each of these pathways were considered for each cell line and, based on current knowledge of mammalian cell responses to DNA damage, the implications for survival were investigated. The hypothesis was that although the responses of different tumour types to radiation varies widely, radiosensitivity might be anticipated by analysis of such key pathways as interdependent functions, where the activation of one pathway such as apoptosis occurs only through the inactivation or failure of another, such as DNA repair. The results presented here demonstrate that radiosensitivity is affected by several parameters to different extents in diverse cell types. By analysing several key parameters as a series of inter-related events, however, a more accurate determination of the unique process of radiosensitivity in each cell line can be obtained, and indicates that radiation response may be anticipated from several in vitro parameters. This has important implications both in the prediction of tumour cell radiosensitivity and in understanding the complex series of processes involved in radiation response.

The principal effect of ionising radiation in mammalian cells is the production of DNA damage. The most critical lesion to survival is the DNA double-strand break (DSB). The levels of both initial and residual DSB breaks after radiation treatment have therefore been strongly coupled to the intrinsic radiosensitivity of human tumour cell lines. It is thought that cells can vary in both their tolerance of initial DNA damage and in their ability to repair DSBs. A summary of the most recent studies into the correlation of initial and residual DSBs with radiosensitivity, expressed as $SF_2$, is presented in Table 6.1 (Chapter 6). A large proportion of these studies (10/19) reported a correlation between either initial DSBs or residual DSBs and intrinsic radiosensitivity. A smaller proportion (4/19) demonstrated a correlation with both initial and residual DSBs while others (4/19) found no correlation with either initial or
residual DSBs. This indicates that the exact nature of the link between DNA damage and cell lethality has yet to be established and that factors in addition to raw DNA damage and its' repair determine the intrinsic radiosensitivity of some cell types. Woudstra et al. (1996a) suggested that the 'presentation', which is affected by chromatin structure, rather than the amount of DNA damage influences survival. This is thought to be the reason for conflicting results which have been obtained for the same cell lines obtained using different techniques for measuring DSBs (compare the different results obtained by Smeets et al. 1994b with Schwartz et al. 1988. While Smeets observed no correlation between the intrinsic radiosensitivity of two human squamous cell carcinoma cell lines, SCC61 and SQ20B, and their initial or residual DSB levels measured using field inversion gel electrophoresis (FIGE), Schwartz found a significant correlation between both the initial and residual DSBs, of these cell lines with radiosensitivity, measured using neutral filter elution (NFE)). What was evident from all these studies, however, is that different cell types vary in their levels of both initial and residual DSBs after radiation. In this thesis, a range of initial DNA damage values was also observed. It is speculated however, that it is the ability of a cell to process DNA damage that determines survival and that the analysis of several pathways downstream of the damage can describe the radiosensitivity in different tumour types.

The biological pathways known to be activated in response to initial DNA damage include cell-cycle delay, DNA repair and apoptosis. These have been analysed extensively in various tumour cell types in vitro and correlated with intrinsic radiosensitivity. The induction of a cell-cycle delay in response to DNA damage at the G1/S and G2/M checkpoints facilitates the repair of the damaged DNA before either DNA synthesis (S) or mitosis (M) occurs (reviewed by Nasmyth et al. 1996). An extensive delay in either phase, therefore, intuitively suggests radioresistance due to increased repair of damage. While such a relationship has been observed with regard to both the extent of G1/S and G2/M delays (McKenna et al. 1991; Warenius et al. 1996; reviewed by Paulovich et al. 1997), where increased delay has been correlated with radioresistance, there is now considerable evidence which suggests that the length of cell-cycle delays does not indicate survival in many cell types. Schwartz et al. (1996) observed a broad range of G2/M delay lengths in a panel of over 30 squamous carcinoma cell lines, which did not correlate with radiosensitivity, while several reports suggested that increased G2/M delay correlated with increased sensitivity to radiation in murine (Meyn et al. 1993) and human tumour cell lines (Nagasawa et al. 1994). This effect has also been observed in response to other cytotoxic agents such as cisplatin (Sorårenson et al. 1996), hydroxyurea (Kuo et al. 1997) and a panel of anticancer drugs (Li et al. 1997). It is speculated in this thesis that it is the extent of the processes thought to occur during radiation-induced cell-cycle delays, i.e. DNA repair
and apoptosis which determines survival to radiation and that the extent of G2 delay merely reflects these processes.

At present, the contribution of apoptosis to the radiosensitivity of tumour cells is unresolved. It has been demonstrated that, in general, radioresponsive tumours such as lymphomas undergo relatively higher levels of apoptosis than unresponsive tumours such as melanomas. Some studies have observed that spontaneous apoptosis levels correlate with radiation-induced apoptosis (Meyn et al. 1993; Hendry and Potten, 1982). Furthermore, radiation-induced apoptosis has been shown to correlate with radiosensitivity in tumour cells both in vitro and in vivo (Story et al. 1994, Russell et al. 1995, reviewed by Olive and Durand, 1997). These results would suggest that apoptosis was directly involved in the radiation response of mammalian cells. However, the lack of correlation between apoptosis and radiosensitivity demonstrated in several cell types (McKenna et al. 1991; Alderidge et al. 1995; reviewed by Blank et al. 1997) indicates that there might not be a simple relationship between apoptosis and intrinsic radiosensitivity. DNA damage and conflicting growth regulatory signals have been identified as the two main triggering events for apoptosis. However, membrane damage has also been shown to induce apoptosis through the hydrolysis of sphingomyelin to ceramide (Haimovitz-Friedman et al. 1994) and the observation that cells lacking nuclei (Jacobson et al. 1994b) are able to undergo apoptosis indicates that signals other than those originating in the nucleus, presumably by DNA damage, can trigger apoptosis.

Clearly, the ability of a cell to undergo apoptosis in response to a trigger signal depends on whether all the components necessary to implement the process are functional within the cell. A growing list of genes have been identified as key players in the apoptotic response, with p53 being identified as one of the most important. The wild-type status of the p53 gene is strongly implicated in both the activation of a radiation-induced G1/S checkpoint (Kuerbitz et al. 1992) and apoptosis (Lowe et al. 1993) in response to DNA damage. There is also evidence to suggest that p53 also plays a role in the regulation of the G2/M transition in some cell types (Argarwal et al. 1995; Guillouf et al. 1995; Powell et al. 1995; Jha and Bedford, 1996). It is generally accepted that p53 functions to arrest cells with damaged DNA to allow time for repair. If repair is not completed, either due to inactive repair processes or excessive damage levels, then the cell is signalled to apoptose. The inactivation of the p53 gene and protein function could lead, therefore, to increased resistance due to loss of p53-mediated G1/S arrest and apoptosis. However, the extensive literature available on the role of p53 in the radiosensitivity of mammalian cell lines indicates that this scenario is an over simplification, and that loss of p53 in many cell types does not affect radiosensitivity. Lack of apoptosis in cells expressing wild-type p53 (Merritt et al. 1997) indicates that downstream processes can disrupt the p53-mediated apoptosis
response. Bcl-2 over-expression has been shown to block p53-mediated apoptosis (Chiou et al. 1994). The observations that apoptosis can occur in the absence of wild-type p53 in tumour cells (Bracey et al. 1995) and that a G1/S arrest has been observed in cells with mutant p53 (Li et al. 1995) further indicate that the growth arrest and apoptosis functions of p53 may be cell-type specific. This has been demonstrated by the observations that the temperature inducible wild-type p53 expression in murine hematopoietic cells resulted in apoptosis (Yonisch-Rouach et al. 1991) but only growth arrest in other cell types such as fibroblasts (Michalovitz et al. 1990). Coupled with the requirement for all downstream targets of p53 to be functional for either growth arrest or apoptosis to occur it is clear that the status of the p53 gene alone provides limited information about the response of cells to radiation (as evidenced by conflicting reports on the correlation of p53 status with radiosensitivity, reviewed by Bristow et al. 1996) and that fuller analysis of the processes affected by p53 is necessary in order to adequately describe the radiation response of mammalian cells.

The aim of the present study was to re-examine the key molecular factors described above in different human tumour cells in order to elucidate how they interact in each cell line to govern radiation response. Eight human tumour cell lines, which were derived from tumours of different primary histologies, demonstrated markedly different radiosensitivities (see Chapter 2). Individual responses to radiation including cell-cycle characteristics (Chapter 3), the extent of radiation-induced delay in each phase (Chapter 4) and apoptosis (Chapter 5) were analysed in each cell line in response to a range of radiation doses, including low doses (0.2–0.5 Gy) and clinically relevant doses (1–4 Gy). DNA damage induction and repair was also analysed following doses of 0–70 Gy using PFGE (Chapter 6). Some of the key results identified from each of these analyses have been summarised in Table 7.1.

Differences in the pre-treatment characteristics between the cell lines were evident. Cell lines were analysed both as one panel and as groups based on primary histology. Neither the proportion of cells in each phase or the lengths of each phase correlated with radiosensitivity, as has been demonstrated in some cell types (Quiet et al. 1991; West et al. 1988). Examination of the DNA index of each cell line, a parameter which has been shown previously to correlate with radiosensitivity (Monasebian et al. 1996), identified three cell lines with aneuploid DNA content (HT29, Be11 and MeWo). No significant correlation was observed with radiosensitivity. These differences in DNA content were factored into the analysis of the extent of radiation-induced DNA damage as it was reasoned that the level of DNA damage might be greater in those cell lines that contained aneuploid DNA content. On the other hand, it was also considered that the relative amount of redundant DNA is likely to be greater in aneuploid cells, thus reducing the probability of a potentially critical DNA lesion. This would have implications in the survival of these cell lines...
after exposure to radiation. Correction of the initial DNA damage values for differences in DNA content eliminated the trend towards increased damage in the radiosensitive cell lines. Pretreatment cell-cycle distributions of each cell line was also accounted for while analysing DNA damage using PFGE. In order to reduce the proportion of S-phase cells which migrate differently through a pulsed field gel, cells are grown to confluence. Not all cell lines could be grown to this stage as they became detached before confluence was reached, resulting in altered cell-cycle distributions. Correction of the DNA damage values for differences in the proportion of cells in S-phase, however, did not significantly alter the results. Analysis of pretreatment apoptosis revealed higher levels in the radiosensitive cell lines. This suggests that the apoptotic pathway in these cell lines is operative and is likely to be further induced in response to radiation. This was supported by the observation in this thesis that apoptosis values induced by 2 Gy of radiation correlated significantly with the spontaneous levels (Figure 5.10) i.e. cells with low pretreatment apoptosis levels demonstrated corresponding low levels of apoptosis in response to radiation while cells with high apoptosis at 0 Gy demonstrated relatively higher levels of apoptosis after radiation.

The response of each cell line to low doses of radiation was analysed in order to examine the hypothesis that the low-dose hypersensitivity (HRS/IRR) observed in some of the resistant cell lines in this study (HT29, Be11, RT112; Lambin et al. 1996), might be cell cycle delay or apoptosis related. There is some evidence that the induced radioresistance response reflects the intrinsic radiosensitivity of the cell as it has only been observed in relatively radioresistant cell lines (Lambin et al. 1996; Wouters and Skarsgard, 1996). The biological mechanisms underlying this response are poorly understood. It has been speculated that sensitivity to low doses of radiation might reflect the elimination of cells with genetic mutations, possibly by apoptosis. At higher doses, cell defence mechanisms such as cell-cycle delay are triggered which facilitate the repair of damage resulting in relative resistance to these doses. Cells lacking in this inducible response are therefore relatively sensitive. In this thesis, the examination of apoptosis levels at low doses did not provide any evidence to support the hypothesis that this pathway might be involved in the HRS/IRR response. In addition, significant perturbations in the cell-cycle at low doses compared to the unirradiated controls could not be distinguished in the majority of cell lines. Only SW48 cells demonstrated a significant G1/S delay at 0.5 Gy, while all other cell-cycle delays in each cell line were significant only following doses of 1 Gy and above. The only other significant effect observed at low doses was the induction (stabilisation) of the p53 protein in all the wild-type p53 expressing cell lines after treatment with 0.2 Gy. Again, this was unrelated to the HRS/IRR response.

The detection of the HRS/IRR response in cells is facilitated by the use of
automated cell analysers which locate and memorise cell positions, which can be re-located and followed after radiation treatment. The advent of such equipment now allows the accurate analysis of cell behaviour after radiation, before which low-dose hypersensitivity was undetectable. By comparison, the techniques used to measure cell-cycle progression and apoptosis after radiation in this study are less sensitive, and it is possible that this is reflected in the lack of significant low-dose cell-cycle delay or apoptosis measured. Matthews (1997) described an automated technique whereby an extensive number of cells can be evaluated for apoptosis, reducing the statistical limitations of detecting slight changes in the apoptosis frequency expected at low doses. Using this more precise technique, again no significant alterations in apoptosis were observed in CHO cells treated with low doses of radiation although there appeared to be a trend towards increased apoptosis in cells treated with 0.25 Gy compared with 0.5 Gy. The author concluded that although his study did not demonstrate an unequivocal relationship of apoptosis with low dose hypersensitivity, a direct involvement of apoptosis in the HRS/IRR could not be ruled out and that the automation of apoptosis detection would aid further studies into this relationship.

Following treatment with clinically relevant (1–4 Gy) doses, significant cell-cycle perturbations over the controls were observed. A G2/M delay was detected in all cell lines and was more extensive in the radiosensitive cell lines (see Table 7.1). The relationship between G2/M delay and intrinsic radiosensitivity was only statistically significant at 4Gy (p = 0.004, see Figure 4.8) although there also appeared to be a trend towards increased delay in the radiosensitive cell lines at 1 and 2 Gy. All cell lines demonstrated a similar reduction of cyclin B1 protein after 2 Gy. This was consistent with the observations of Muschel et al. (1993), who demonstrated that cyclin B1 protein levels were downregulated during G2/M delay, and this suggests that cyclin B1-mediated radiation-induced G2/M cell-cycle delay is common to different tumour cell types. The increased delay in the radiosensitive cell lines is inconsistent with the hypothesis that the induction of a G2/M delay allows time for cells to repair damage before entry into mitosis, thereby implying radioresistance. However, the proportion of cells that did not exit G2/M during the time course of analysis was greater in the radiosensitive cell lines. Since some DNA repair has occurred in the radiosensitive cell lines, it is proposed that the extensive G2/M delay accommodates both time taken to carry out repair and, when failed, to eliminate those cells with irreparable damage from the population. A G1/S delay was observed in five cell lines, the most significant of which was of SW48 cells (a dose independent delay of 5 h detected at all doses from 0.5–4 Gy). All other cell lines demonstrated a G1/S delay only at 2 Gy or 4 Gy. All five of these cell lines exhibited wild-type p53 expression. None of the p53-mutant cell lines delayed at the G1/S border, which indicates that in these cell lines p53 is required for radiation induced G1/S arrest.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>HT29</th>
<th>MGH-U1</th>
<th>Be11</th>
<th>RT112</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Histology</td>
<td>Colorectal adenocarcinoma</td>
<td>Bladder carcinoma</td>
<td>Malignant melanoma</td>
<td>Bladder carcinoma</td>
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<td>SF$_2$</td>
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<td>0.70</td>
<td>0.68</td>
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<tr>
<td>G0/G1 phase length (h)</td>
<td>27.1</td>
<td>6.5</td>
<td>8.8</td>
<td>16.6</td>
</tr>
<tr>
<td>S phase length (h)</td>
<td>17.5</td>
<td>13.7</td>
<td>19.9</td>
<td>17.7</td>
</tr>
<tr>
<td>G2/M phase length (h)</td>
<td>1.2</td>
<td>2.5</td>
<td>3.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Max. G1/S delay (h)</td>
<td>none</td>
<td>2.0</td>
<td>5.0</td>
<td>none</td>
</tr>
<tr>
<td>Max. S delay (h)</td>
<td>none</td>
<td>none</td>
<td>4.0</td>
<td>none</td>
</tr>
<tr>
<td>Max. G2/M delay (h)$^d$</td>
<td>5.6</td>
<td>5.8</td>
<td>4.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Min. G2/M ratio</td>
<td>0.45</td>
<td>0.69</td>
<td>0.85</td>
<td>0.95</td>
</tr>
<tr>
<td>p53 status</td>
<td>mu$^c$</td>
<td>wt$^d$</td>
<td>wt</td>
<td>mu</td>
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<tr>
<td>p53 expression</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Cyclin B1 expression</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Constitutive apoptosis (%)$^b$</td>
<td>1.7</td>
<td>1.1</td>
<td>1.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Max. radiation-induced apoptosis (%)</td>
<td>2.3</td>
<td>2.4</td>
<td>2.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Constitutive bcl-2 expression (% of total cells)</td>
<td>1.5</td>
<td>14.7</td>
<td>0.82</td>
<td>0.98</td>
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<td>Max. post-radiation bcl-2 expression (% of total cells)</td>
<td>1.1</td>
<td>13.3</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Initial DNA damage</td>
<td>1.8</td>
<td>1.8</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Residual DNA damage</td>
<td>0.31</td>
<td>0.36</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>Repair (Residual/initial DNA damage)</td>
<td>17.5</td>
<td>19.7</td>
<td>15.5</td>
<td>16.9</td>
</tr>
</tbody>
</table>

Table 7.1. Radiobiological characteristics of eight human tumour cell lines. The four cell lines presented above represent those demonstrating relative radioresistance. All values have been presented in the individual Chapters elsewhere in this thesis.

a: Of BrdUrd labelled cells  
b: By morphological assessment over 36 h  
c: mutant  
d: wild-type
### Table 7.1. Radiobiological characteristics of eight human tumour cell lines. Continued from previous page.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>U1-S40b</th>
<th>MeWo</th>
<th>SW48</th>
<th>HX142</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Histology</strong></td>
<td>Bladder carcinoma</td>
<td>Malignant melanoma</td>
<td>Colorectal adenocarcinoma</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td><strong>SF₂</strong></td>
<td>0.40</td>
<td>0.25</td>
<td>0.18</td>
<td>0.033</td>
</tr>
<tr>
<td><strong>G0/G1 phase length (h)</strong></td>
<td>5.9</td>
<td>9.9</td>
<td>11.7</td>
<td>15.7</td>
</tr>
<tr>
<td><strong>S phase length (h)</strong></td>
<td>16.2</td>
<td>16.0</td>
<td>16.3</td>
<td>15.7</td>
</tr>
<tr>
<td><strong>G2/M phase length (h)</strong></td>
<td>1.7</td>
<td>3.1</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Max. G1/S delay (h)</strong></td>
<td>2.0</td>
<td>none</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Max. S delay (h)</strong></td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td><strong>Max. G2/M delay (h)</strong></td>
<td>7.4</td>
<td>12.6</td>
<td>20.7</td>
<td>14.5</td>
</tr>
<tr>
<td><strong>Min. G2/M ratio</strong></td>
<td>0.62</td>
<td>0.40</td>
<td>0.19</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>p53 status</strong></td>
<td>wt</td>
<td>mu</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td><strong>p53 expression</strong></td>
<td>+</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Cyclin B1 expression</strong></td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Constitutive apoptosis (%)</strong></td>
<td>2.4</td>
<td>5.0</td>
<td>6.3</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Max. radiation-induced apoptosis (%)</strong></td>
<td>7.7</td>
<td>4.9</td>
<td>9.1</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>Constitutive bcl-2 expression (% of total cells)</strong></td>
<td>3.8</td>
<td>26.6</td>
<td>8.7</td>
<td>8.1</td>
</tr>
<tr>
<td><strong>Max. post-radiation bcl-2 expression (% of total cells)</strong></td>
<td>1.1</td>
<td>21.3</td>
<td>5.6</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>Initial DNA damage</strong></td>
<td>2.0</td>
<td>1.7</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Residual DNA damage</strong></td>
<td>0.17</td>
<td>0.34</td>
<td>0.29</td>
<td>0.49</td>
</tr>
<tr>
<td><strong>Repair (Residual/initial DNA damage)</strong></td>
<td>8.7</td>
<td>20.3</td>
<td>22.0</td>
<td>30.1</td>
</tr>
</tbody>
</table>
There was a trend towards increased apoptosis, both constitutive and radiation-induced, in the radiosensitive cell lines. Radiosensitivity has previously been correlated with an ability to undergo apoptosis in many cell types (reviewed by Blank et al. 1997). The majority of apoptosis occurred in the S and G2/M phases of the cell cycle, with the exception of the U1-S40b cells, which also demonstrated apoptosis in G0/G1. Bcl-2, a known repressor of apoptosis, is significantly down-regulated in the radiosensitive cell lines.

The extent of radiation-induced initial DNA damage and repair were analysed using pulsed field gel electrophoresis (PFGE). Analysis of DNA damage is usually limited to doses above 10 Gy, but this assay was sensitive down to about 1 Gy. When each cell line was analysed there was a trend towards increased initial damage in the resistant cell lines. DNA repair, a crucial determinant of radiation response, was higher in resistant cell lines. This relationship was not significant (p = 0.135) due to the high level of repair observed in the radiosensitive U1-S40b. However, this cell line has been shown to display reduced repair fidelity (Powell et al. 1992) and elimination of this from the analysis increased the significance of the relationship between radiosensitivity and DNA repair to p = 0.019.

It is well documented that DNA repair, cell-cycle delay and apoptosis are all involved in the radiation response of mammalian cells. In the present study, the analysis of each parameter individually has identified some direct correlations with radiosensitivity. Due to the diverse range of cell types in this study, however, it was reasoned that the radiosensitivity of each cell line would be dictated by a unique sequence of events. It is speculated that these events would be inter-related such that the activation of one pathway is dependent on the extent of a proceeding pathway. For example, high levels of unrepaired DNA damage after radiation treatment in a particular cell line might signal apoptosis to eliminate cells with potentially lethal mutations from the overall population. However, if apoptosis in a particular cell type was p53-dependent, then the status of the p53 gene might be crucial to the survival. However, analysis of any of these individual parameters (repair, apoptosis or p53 status) alone would only provide a limited view of what would occur in response to radiation. Therefore, the possible interactions of each parameter analysed in response to radiation and the implications for survival were considered for each cell line. These results are presented in Figure 7.1 (see also appendix 3, Table A3) where the responses of each endpoint analysed following radiation have been grouped into strong, intermediate and weak in each cell line and integrated into a dynamic model of the current views on how mammalian cells respond to and treat DNA damage. The magnitude of each response has been colour coded for simplicity (refer to Table 7.1 for absolute values). This facilitates the analysis of each individual cell line, as well as groups, from which conclusions can be drawn regarding the specific processes
involved in the intrinsic radiosensitivity of each cell line.

Initial DNA damage levels varied between each cell line, with a slight trend towards increased damage in the radiosensitive cell lines. This indicates that the SW48 and HX142 cell lines were more sensitive to the effects of DNA damage and undergo more damage per Gy of radiation than other cell lines. Those cell lines with relatively low DNA damage (HT29, MGH-U1, Be11, RT112, MeWo) are radioresistant. The presence of DNA double-strand breaks (DSBs) in mammalian cells triggers a variety of responses that are all aimed at eliminating the damage. If a cell line is prone to more damage than another, then it could be speculated that the threat to survival is greater if the cell cannot cope with the damage. With regard to the initial damage data, it would appear that the SW48 and HX142 cells are at high 'risk' from the adverse effects of radiation compared to either the U1-S40b cells (which demonstrated an intermediary level of DNA damage) or the group of cell lines which demonstrated a low level of initial damage.

In response to DNA damage one of the most immediate effects is the stabilisation of the wt-p53 protein. Three cell lines did not demonstrate any stabilisation of protein levels after any radiation dose tested (HT29, RT112, MeWo). This was consistent with their mutant p53 status, and suggested that, as a result, no arrest at the G1/S border or p53-mediated apoptosis would occur in response to DNA damage. From the cell-cycle analysis, it was evident that these three cell lines did indeed lack a G1/S delay. However, DNA repair was high in HT29 and RT112 cells but relatively low in the MeWo cells. Inversely, the levels of apoptosis were higher in MeWo cells (despite relatively high bcl-2 protein levels) compared to HT29 and RT112. A similar trend was observed in the extent of G2/M delay whereby MeWo cells demonstrated a longer delay than either HT29 or RT112 cells. MeWo cells are more radiosensitive (SF$_2$ = 0.25) than either RT112 or HT29 (SF$_2$ = 0.62 and 0.74 respectively). Since some repair has occurred in the MeWo cell line, it is proposed that radiosensitivity is due to the elimination of cells with irreparable DNA damage by apoptosis, a process which does not occur in the radioresistant HT29 and RT112 cell lines. The significance of the p53 status on the radiation response in these cell lines is unclear as apoptosis in the MeWo cells obviously occurs independently of p53, as does the repair in the HT29 and RT112 cells. The increased radioresistance of the HT29 and RT112 cells could reflect the inactivation of the apoptotic response due to mutant p53. The level of repair in these cell lines however, might have been sufficient for survival thereby not requiring apoptosis to eliminate irreparable cells. It would be of interest to examine these responses at higher doses of radiation (where there is more likely to be cells with irreparable DNA damage) to determine whether apoptosis can be induced.
Figure 7.1. The route to radiosensitivity in human tumour cell lines. The extent of activation of each biological end-point analysed is represented in terms of a strong, intermediate and weak (see text).
Two cell lines, MGH-U1 and U1-S40b, demonstrated an intermediate level of p53 stabilisation. The U1-S40b cell line was isolated as a radiosensitive mutant of MGH-U1 cells (McMillan and Holmes, 1991). U1-S40b cells demonstrated more initial DNA damage compared to MGH-U1 cells, which might be indicative of their increased radiosensitivity. The similar low level of p53 stabilisation suggests that the extent of damage does not affect the degree of protein stabilisation. The wild-type status of the p53 gene also suggests that both cell lines would display a G1/S arrest and/or apoptosis in response to radiation. Further analysis revealed that these cell lines demonstrated a small delay (2 h) in G1/S after treatment with 4 Gy. In addition these cells display intermediate G2/M delay lengths (with similar cyclin B1 reduction) and persistence in G2. Consistent with these observations is the high level of repair observed in both cell lines. However, the U1-S40b cell line demonstrates significantly more apoptosis than MGH-U1 cells, which could be the major factor involved in the increased radiosensitivity observed in this particular cell line. It has been reported that the U1-S40b cell line has a reduced repair fidelity. It is possible therefore, that the process of isolation of this cell line has disrupted the repair process resulting in increased apoptosis and radiosensitivity. However, it is also possible that the high levels of bcl-2 observed in the MGH-U1 cell line (see Table 7.1) override p53-mediated apoptosis, an effect which has been demonstrated by Chiou et al. (1994). MeWo cells demonstrated a similar level of bcl-2 expression but higher levels of apoptosis. As MeWo cells demonstrated mu-p53 expression, it is hypothesised that bcl-2 only represses wt-p53 mediated apoptosis and that p53-independent apoptosis, which has been observed in other tumour cells (Bracey et al. 1995), also occurs independently of bcl-2, representing a distinct pathway of apoptosis. It has been proposed that the direct effects of X-rays on the nucleus initiate a cascade of signals in mammalian cells, resulting in the induction of apoptosis (Dewey et al. 1995; Radford and Murphy, 1994). Furthermore, it has recently been observed that the production of the lipid second messenger ceramide from sphingomyelin hydrolysis immediately following radiation contributes to the apoptotic response (Hamovitz-Friedman et al. 1994) and that loss of ceramide production confers resistance to radiation induced apoptosis (Chmura et al. 1997). It is possible therefore that apoptosis of the cell lines in the present study occurs via this p53-independent pathway and that the lack of apoptosis observed in some cell lines might be associated with a loss of ceramide production, not wild-type p53 status.

Three cell lines (Be11, SW48 and HX142) in the study demonstrated a strong induction of wild-type p53 protein after radiation. Of these only Be11 cells exhibited relatively low levels of DNA damage. In SW48 and HX142 cells, the relatively high level of initial damage indicates an increased sensitivity to DNA damage. This causes high levels of p53 stabilisation. Further analysis of the downstream events show that
all three cell lines demonstrated a G1/S delay, although this was more marked in the Be11 and SW48 cell lines. The G1/S delay of SW48 cells was of equal length (5 h) at all doses ≥ 0.5 Gy, which indicates that this response is dose independent (an effect which was also observed in the G2/M delay, especially in the resistant cell lines, see Chapter 4, Figure 4.7) and that a certain threshold dose needs to be applied before a delay is induced. The reduction of cyclin B1 in the Be11 cell line was lower than other cell lines, which may be related to the relatively low level of initial damage in this cell line. As the reduction of cyclin B protein level in response to radiation has been directly linked to the induction of a G2/M arrest Muschel et al. (1993), a short G2/M delay would be expected in the Be11 cell line. Further examination of this cell line showed that this was the case, as Be11 cells demonstrated the shortest G2/M delay at each dose (barely rising above 3 h after doses of 1 Gy, 2 Gy and 4 Gy). The level of repair in this cell line was high, suggesting that repair was rapid enough to be complete in the relatively short G1/S and G2/M total repair time. This could be related to the low level of initial damage in this cell line. Apoptosis levels in the Be11 cell line were also very low, consistent with the hypothesis that radiation response is determined by the ability of cells to repair DNA damage and that cells only apoptose if repair has failed. Therefore, as Be11 expresses wild-type p53, it is proposed that the resistance observed in this cell line is due to proficient DNA repair with minimal apoptosis. In the case of the SW48 and HX142 cell lines (the two most radiosensitive cell lines involved in the study), both demonstrate a detectable G1/S delay. The extent of G2/M delay was notably longer then any of the other cell lines, which suggests that some repair has occurred. Analysis of the DNA damage data however, revealed that these two cell lines demonstrated the lowest level of DNA repair of the cell lines in the study. Both cell lines also demonstrated a high proportion of cells which have remained in G2/M during the time course of the experiment (G2/M persistence). This indicates that a relatively high proportion of cells were unable to exit G2/M, presumably due to an inability to repair damage. SW48 cells also demonstrated high levels of radiation-induced apoptosis. It has previously been observed that SW48 cells are defective in mismatch repair (Branch et al. 1995) most likely due to a mutation in the hMLH1 gene, which is involved in the initial stages of mismatch repair (Kane et al. 1997). It is proposed therefore that in the SW48 cell line, the high radiosensitivity observed is due at least in part to the induction of apoptosis of cells unable to exit G2/M due to unrepaired DNA damage. In the HX142 cell line, the relative level of apoptosis was low and it seems likely that other types of cell death are involved in the extreme response of this cell line to radiation (SF2 = 0.033).

It is evident from the results presented in this thesis that the response of a diverse range of tumour cell lines to radiation is mediated by several different factors. However, amid this complex series of results, there does appear to be an emerging
trend. While the level of initial damage varies between the cell lines, in that the more sensitive cell lines accumulate more initial DNA damage, the downstream effects are more varied. A general consensus in radiobiology is that DNA repair is one of the most critical responses to radiation in terms of survival and that, ultimately, it is the repair of DNA damage that will lead to increased survival of the cell. If DNA repair fails, then cells must be eliminated from the population to prevent the fixation of DNA mutations. This process of elimination is thought to occur through the activation of cell suicide mechanisms, namely apoptosis. In agreement with this hypothesis, it has been demonstrated in this study that none of the cell lines which exhibited high levels of repair also showed high levels of apoptosis. This could be due to the redundancy of the ability to apoptose if DNA damage is repaired (at least for the one cell cycle time measured in this study). Conversely, in cells which demonstrated relatively high levels of apoptosis, this was accompanied by a relatively low level of repair. This suggests that apoptosis is inversely related to DNA repair and that radiosensitivity can be determined from the examination of whether a cell undergoes repair (and survival) or apoptosis in the presence of DNA damage. Therefore, analysis of the extent of repair should indicate the radiosensitivity of a cell line. However, it is clear that this simple relationship is not universal in light of the several studies which have found no correlation between the intrinsic radiosensitivity of human tumour cells and DNA damage (see Table 6.1, reviewed by Nunez et al. 1996). These results show that the exact nature of the link between DNA damage and cell survival has yet to be fully understood. A likely possibility to explain the lack of correlation between residual DNA damage and radiosensitivity is that some cell types vary in their tolerance to DNA damage. It has also been suggested that some cell types vary in their presentation of DNA damage, which might be related to their tolerance of it, and that differences in radiosensitivity are related to differences in DNA structure rather than DSB levels (Woudstra et al. 1996b; Olive et al. 1992). In a panel of different cell lines therefore, analysis of DNA damage alone would not necessarily indicate radiosensitivity, unless the DNA damage level for optimum cell kill was determined for each cell line and incorporated into the analysis. It is likely that cells of similar tumour types would display comparable levels of DNA damage tolerance, and in a recent study Schwartz et al. (1996) demonstrated a significant relationship between radiosensitivity and residual DNA damage in 25 squamous cell carcinoma cell lines. However, Smeets et al. (1994) did not observe such a correlation in two other squamous cell carcinoma cell lines. In this study, when cell lines derived from the same tumour types are compared (see Table 2.1 (colorectal adenocarcinoma: HT29 and SW48, malignant melanoma: Be11 and MeWo, bladder carcinoma: RT112, MGH-U1 and U1-S40b) there was a trend towards increased repair in the more radioresistant cell line in each group, which reflected the general overall trend between high repair
indicating radioresistance.

The induction of apoptosis appears to accompany low levels of DNA repair in the tumour cell lines in this study, indicating that apoptosis is induced in response to unrepaired DNA damage. While it is generally accepted that DNA damage can induce apoptosis in human tumour cells, providing the apoptotic pathways have not been inactivated during the process of tumourigenesis, it is not certain whether different types of damage preferentially induce apoptosis. It is also unclear if p53-mediated apoptosis is more sensitive to DNA damage than p53-independent forms of apoptosis, and whether the role of p53 in the apoptotic process is dependent on the type of damage produced in each cell type. Furthermore, the significance of apoptosis to clonogenic survival is questionable as it has been demonstrated that increased apoptosis in response to radiation is not accompanied by a reduction in the clonogenic survival in some cell types (Hopcia et al. 1997; reviewed by Blank et al. 1997). From the results obtained in this study, it is proposed that p53-dependent apoptosis occurs in response to unrepaired DNA damage, which correlates with radiosensitivity, and that apoptosis observed in the mutant-p53 cell lines is probably mediated by a membrane damage-dependent pathway. However, it is clear that the elucidation of the mechanisms by which cells signal that 1) damage exists, before and after repair has been attempted, and 2) whether it is critical to survival (thus eliciting a response such as apoptosis) will greatly advance our understanding of the mechanisms involved in radiation response.

Both apoptosis and repair occur during the cell-cycle checkpoints. G2/M delay was the only arrest observed in all cell lines, and there is increasing evidence that p53 is involved in the regulation of G2/M transition (Guillouf et al. 1995; Russell et al. 1995; Jha and Bedford, 1996). The persistence of cells in this phase (G2/M ratio) was greater in the radiosensitive cell lines and correlated significantly (p = 0.019) with apoptosis at 2 Gy (Figure 5.14). It is suggested that cells unable to repair damage in this phase are eliminated from the population through apoptosis. The increased length of G2/M delay in these cell lines also suggests that the induction of apoptosis occurs after repair has been attempted so that the length of the arrest is extended to accommodate initiation and implementation of both the repair and apoptosis processes. There is some evidence in the literature which supports this hypothesis. Orren et al. (1997) described an extensive G2/M delay followed by apoptosis in CHO cell lines deficient in repair, compared to those proficient in repair. Mitsuhasi et al. (1996) also demonstrated an increased G2/M delay and apoptosis in a radiosensitive rat yolk tumour cell line compared to a radioresistant variant. However, these results are in direct contrast with those published by McKenna et al. (1996) who reported that rat-embryo cells (RECs) transfected with H-ras and myc demonstrated increased G2/M delay and radioresistance compared to the wild-type cells. Abrogation of this delay by
caffeine reduced the survival of the transfected cells relative to the wild-type cells, accompanied by an increase in apoptosis. Bernhard et al. (1996) observed a similar effect of G2/M delay abrogation by caffeine on the apoptotic response. The authors concluded that one mechanism of enhanced cell killing by radiation is to trigger apoptosis by decreasing the G2 delay induced by irradiation. However, these results were obtained in only one cell type. In this thesis, it is proposed that the persistence of cells in G2/M is accompanied by apoptosis in those cell lines that cannot repair, and that this is indicative of radiosensitivity. However, less than half of the HT29 cells arrested in G2/M, following 4 Gy, exited G2/M. As this cell line is highly radioresistant (SF2 = 0.74) and demonstrates mutant p53 expression, it could be speculated that cells are permanently arrested in G2/M, without undergoing apoptosis. This result indicates the need for extensive analysis in order to elucidate each individual cells’ response to radiation. Similar measurements of the persistence of cells in G1/S was hindered by the entry of unlabelled G2 cells into the analysis window, but it was shown that some apoptotic U1-S40b cells were in G0/G1 phase (see Chapter 5, Figure 5.16).

It has been demonstrated in this study that the intrinsic radiosensitivity of human tumour cell lines is mediated by a variety of responses. The p53 gene is regarded as a major component of the response to radiation and activates several pathways dedicated to the processing of DNA damage. It would appear that the major cause of the different radiosensitivities observed in the cell lines in this study is whether cells are able to sufficiently repair DNA damage to ensure survival or whether they apoptose. Analysis, therefore of the level of both repair and apoptosis would appear to determine the ultimate radiosensitivity of the cell lines based on the results presented in this thesis. This proposed mechanism of radiosensitivity is outlined in Figure 7.2. Cells characterised by DNA repair only, with little apoptosis, are relatively radioresistant (HT29, Be11, RT112) while those that demonstrate some repair and apoptosis (characterised by an extensive G2/M delay) are relatively radiosensitive (U1-S40b, SW48, HX142). Although the MGH-U1 and MeWo both demonstrate these responses respectively (i.e. MGH-U1 cells have relatively high repair and low apoptosis, and are resistant) it is interesting to note that apoptosis in the MeWo cell line is p53-independent, and that inactivation of the apoptotic response in the MGH-U1 cells (which is present in its radiosensitive mutant) might be mediated by high bcl-2 levels. However, analysis of the other factors known to be involved in these processes, such as cell-cycle perturbation and protein expression provided more information, which could be assembled into a profile of each cell line, Figure 7.1.

The ultimate aim of this thesis was to examine the possibility of establishing a protocol to predict the radiosensitivity of human tumour cell lines. It has been demonstrated that while certain patterns exist that indicate radiosensitivity and
Figure 7.2. The key pathways involved in the radiation response of different human tumour cell lines. p53 is involved in the activation of both G1/S and G2/M delays. Extensive perturbation of either of these checkpoints indicates repair and apoptosis, which occurs in the radiosensitive cell lines. The high level of DNA repair and the lack of apoptosis is characteristic of radioresistance.

Radioresistance, it must be remembered that the mechanisms underlying intrinsic radiosensitivity are complex, involving many genes. However, in order to estimate the radioresponse of a tumour cell line for which no survival data is available (a situation similar to those encountered in the clinic from in vivo tumour biopsy samples) the following ‘checklist’, or priority tests, are proposed:

1. **Extent of DNA repair.** DSBs are regarded as the most crucial to survival so the level of their repair should have important implications for survival. Repair ideally should be estimated in response to radiation doses similar to those applied in the clinic, if the sensitivity of the assay permits this (PFGE is sensitive at doses of 5 Gy and lower). A high level of repair indicates radioresistance.

2. **Apoptosis.** Time course of assessment should extend to several days in order to assess the full response of the tumour population, including those cells which may have been permanently arrested in either G1/S or G2/M for more than one cell-cycle. Morphological assessment is the most accepted method of apoptosis determination, but parallel analysis using flow cytometry techniques provides
valuable cell-cycle information. Speculation on the significance of the proteins involved in apoptosis (bcl-2, p53, ceramide) can begin once the level of radiation-induced apoptosis has been assessed. High levels of apoptosis indicate radiosensitivity and coupled with low DNA repair suggest that residual DNA damage has triggered this suicide response.

3. Cell-cycle delays. Especially at the G2/M border, which is the most frequently observed delay in tumour cells. A G1/S arrest will generally indicate wt-p53 status. A long G2/M delay indicates radiosensitivity. Coupled with high apoptosis and low DNA repair, a long G2/M delay indicates that DNA damage has been detected (triggering the delay response). Failed DNA repair, perhaps due to extensive damage, triggers a second response (apoptosis), which also occurs during this phase. Exit from both G1 and G2 occurs only when both the repair and apoptosis signals are removed, hence and increased delay in radiosensitive cells.

4. Key proteins, such as p53, bcl-2, cyclin B. Analysis of these gene products in isolation provide little information on radiosensitivity if none of the above end-points have been analysed. Useful in the elucidation of the molecular processes involved in the implementation and regulation of repair, apoptosis and cell cycle delay.

In conclusion, the results demonstrate how varied the response to radiation is between human tumour cells of different types and the need to analyse several parameters thought to be involved in radiation response. Much of the work investigating the factors governing intrinsic radiosensitivity is open to speculation as to the significance of each end-point to survival. Extensive G2/M delay could imply increased DNA repair and radioresistance, but this clearly was not the case in this study. The signalling processes involved in deciding which pathway is activated in response to radiation would be the next area of investigation in this study. Questions that arise, such as how has apoptosis been activated in the radiosensitive cell lines (noting that MeWo demonstrated apoptosis while expressing mutant- p53 protein) and how repair has been activated in the resistant cell lines should provide answers that can explain the response to radiation on a molecular level. With such information it is hoped that a more distinct pattern can be identified which depicts how a tumour cell responds to radiation.
### 8.0 Appendices

#### Appendix 1: REAGENTS

**Apoptosis detection by terminal tranferase assay**

**Sodium Cacodylate buffer, pH6.8**
- 0.2 M sodium cacodylate (Sigma)
- 2.5 mM Tris HCl (Sigma)
- 2.5 mM cobalt chloride (Sigma)
- 0.025% bovine serum albumin (BSA, Sigma)
  in PBS
  pH with 1 M NaOH

**4X saline citrate buffer** (for 80ml of 20X stock)
- 17.53 g sodium chloride (Sigma)
- 8.82 g sodium citrate (Sigma)
- 80 ml distilled H2O
  pH with 10M sodium hydroxide

**Protein detection by Western blotting**

**Loading Buffer**
- 250 mM tris/HCL (pH 6-8)
- 4% sodium dodecylsulphate (SDS, BDH)
- 10% glycerol (Sigma)
- 2% mercaptoethanol (Sigma)
- 1% Bromophenol blue

**12% Polyacrylamide running gel (50ml)**
- 16.4 ml dH20
- 20 ml 30% acrylamide mix (Protogel)
- 1.5M tris (pH8.8)
- 10% SDS
- 10% ammonium persulphate (APS; BDH)
- 0.02 ml TEMED

**12% stacking gel (30ml)**
- 20.4 ml dH20
- 5.1 ml 30% acrylamide mix
- 3.75 M tris (pH6.8)
- 10% SDS
- 10% ammonium persulphate
- 0.02 ml TEMED

**Running buffer (2L)**
- 6 g Tris
- 28 g Glycine

**Transfer buffer (2L)**
- 6 g Tris
- 28 g Glycine
5 ml of 10% SDS
2 L in dH2O

500 ml methanol
5 ml of 10% SDS
2 L in dH2O

Block buffer
5% dry milk (Marvel)
10 mM Tris (pH 7.5)
100 mM sodium chloride
0.1% Tween 20

Wash buffer
0.1% Tween 20 (Sigma)
10 mM Tris
100 mM sodium chloride
Appendix 2: CELL CYCLE ANALYSIS USING MODEL

a. Formula

\[
\begin{align*}
\left( \text{normDist} \left( \frac{\text{Time (h) } + ts \cdot 0.5}{\sqrt{stc^2 \cdot 0.00001}} \right) - \text{normDist} \left( \frac{\text{Time (h) } - ts \cdot 0.5}{\sqrt{sts^2}} \right) \right) \\
+ \text{normDist} \left( \frac{\text{Time (h) } - tc + ts \cdot 0.5}{\sqrt{stc^2}} \right) - \text{normDist} \left( \frac{\text{Time (h) } - tc - ts \cdot 0.5}{\sqrt{sts^2 + stc^2}} \right) \\
+ \text{normDist} \left( \frac{\text{Time (h) } - tc \cdot 2 + ts \cdot 0.5}{\sqrt{stc^2 \cdot 2}} \right) - \text{normDist} \left( \frac{\text{Time (h) } - tc \cdot 2 - ts \cdot 0.5}{\sqrt{sts^2 + stc^2 \cdot 2}} \right) \\
+ \text{normDist} \left( \frac{\text{Time (h) } - tc \cdot 3 + ts \cdot 0.5}{\sqrt{stc^2 \cdot 3}} \right) - \text{normDist} \left( \frac{\text{Time (h) } - ts \cdot 0.5 - tc \cdot 3}{\sqrt{sts^2 + stc^2 \cdot 3}} \right) \right) + f
\end{align*}
\]
b. Curve fits

The curve fits obtained using the model described by Watson and Taylor, (1977) are shown below. The cell cycle time ($T_c$) and S-phase length ($T_s$) were calculated from the formula, and approximate standard errors are given for each.

A. HT29

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![Graph]

% BrdUrd-Labelled cells in mid-S

Time (h)
B. MGH–U1

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Time (h)
C. Bell

Nonlinear Fitting Control Panel

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% BrdUrd-Labeled cells in mid-S

Time (h)
D. RT112

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% BrdUrd-Labelled cells in mid-S

Time (h)
E. U1–S40b

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% BrdUrd-Labelled cells in mid-S

Time (h)
F. MeWo

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**Solution**

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</tbody>
</table>

**Graph**

- % BrdUrd-Labeled cells in mid-S
G. SW48

**Nonlinear Fitting Control Panel**

**Solution**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>ApproxStdErr</th>
<th>Lower CL</th>
<th>Upper CL</th>
</tr>
</thead>
<tbody>
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<tr>
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<tr>
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</table>

**Graph**

![Graph showing % BrdUrd-Labeled cells in mid-S vs. Time (h)](image)
### Nonlinear Fitting Control Panel

#### Solution

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>ApproxStdErr</th>
<th>Lower CL</th>
<th>Upper CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>tc</td>
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<td>stc</td>
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<td>ts</td>
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<td>f</td>
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</tbody>
</table>

#### Graph

- % BrdUrd-Labelled cells in mid-S
- Time (h)
### Appendix 3: Summary of radiation-induced cell responses

<table>
<thead>
<tr>
<th>Radiation-induced damage</th>
<th>p53 induction</th>
<th>cyclin B1 alteration</th>
<th>G1/S delay</th>
<th>G2/M delay</th>
<th>High G2/M ratio</th>
<th>DNA repair</th>
<th>Apoptosis</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HT29</strong></td>
<td>✓</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>0.74</td>
</tr>
<tr>
<td>Relatively low level of initial DNA damage, perhaps due to increased tolerance or constant rapid repair of damage</td>
<td>No p53 induction, high constitutive levels, due to mu p53 status, suggestive of altered/no cell-checkpoints and apoptosis responses</td>
<td>A reduction in cyclin B1 levels at 2Gy, indicative of a G2/M delay</td>
<td>No observable G1/S delay at any dose, due to mu p53?</td>
<td>A dose independent G2/M delay was observed at all doses above 1Gy</td>
<td>Only ~half of G2/M-delayed cells re-enter G0/G1, indicative of some cell death, through apoptosis?</td>
<td>Relatively high level of repair (only 17% of initial DSBs unrejoined), indicative of radioreistance</td>
<td>Relatively low constitutive apoptosis and little radiation-induced apoptosis and low bcl-2 levels. Due to mu p53 status?</td>
<td>Resistant, due to repair of DNA damage in G2/M phase and no apoptosis (either due to mu p53 or slow kinetics of apoptosis induction (&gt;36h))</td>
</tr>
<tr>
<td><strong>MGH-U1</strong></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>0.70</td>
</tr>
<tr>
<td>Low level of initial damage. Increased tolerance and/or rapid repair?</td>
<td>Low level of protein, with small increase after radiation, due to wild-type status. Suggestive of a G1/S delay and apoptosis</td>
<td>A reduction in cyclin B1 levels at 2Gy, indicative of a G2/M delay</td>
<td>Small delay at 4Gy (2h), related to low level of constitutive and radiation-induced p53 protein?</td>
<td>A dose independent G2/M delay was observed at all doses above 1Gy</td>
<td>70% of cells re-enter G0/G1, indicative of high repair, little cell death at this phase and radioreistance</td>
<td>80% of damage repaired (during G2/M block?), indicative of radioreistance</td>
<td>Little radiation-induced apoptosis (~2% over 36h). High bcl-2 (15% +ve), unchanged after radiation. High bcl-2 overriding p53 dependent apoptosis?</td>
<td>Resistant, due to high repair and low apoptosis levels, which may be due to high bcl-2 positivity overriding wt p53 mediated apoptosis.</td>
</tr>
<tr>
<td><strong>Be1</strong></td>
<td>✓</td>
<td>✓</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>0.68</td>
</tr>
<tr>
<td>Highest level of initial damage, slow repair?</td>
<td>Low level of protein but distinct induction after radiation. Suggestive of a G1/S delay and apoptosis</td>
<td>Low level of cyclin B1 reduction at 2Gy, indicative of a shorter (or no) G2/M delay?</td>
<td>Substantial delay, at 4Gy only (5h). Correlates with high induction of p53</td>
<td>Shortest delay observed at every dose. Correlates with cyclin B reduction. Indicative of reduced repair and/or increased cell death at this arrest point?</td>
<td>85% G2/M cells re-enter G0/G1. Indicative of high repair and survival</td>
<td>Low level of unrepaired damage (15%). Delay data suggests that repair occurs in G1/S and G2/M, and that repair is rapid in G2/M due short length of this delay</td>
<td>Very low apoptosis levels. Low bcl-2 levels Suggestive of the activation of repair not apoptosis p53 mediated?</td>
<td>Resistant, due to high repair and low apoptosis. May be due to repair activation rather than apoptosis in response to damage mediated by wt p53</td>
</tr>
</tbody>
</table>

Table A3. Factors affecting intrinsic radiosensitivity in eight human tumour cell lines. The processes known to be involved in the response of cells to radiation treatment and were analysed in this study are indicated above (see Figure 7.1). Activation of each response stage is indicated by (✓). No activation or response is denoted by (X).
<table>
<thead>
<tr>
<th>Radiation-induced damage</th>
<th>p53 induction</th>
<th>cyclin B1 alteration</th>
<th>G1/S delay</th>
<th>G2/M delay</th>
<th>High G2/M ratio</th>
<th>DNA repair</th>
<th>Apoptosis</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT112</td>
<td>√</td>
<td>X</td>
<td>√</td>
<td>X</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>0-62</td>
</tr>
<tr>
<td>High level of initial damage. Slow repair?</td>
<td>High constitutive levels of protein, and no induction, due to mu p53 status? Indicative of altered/no cell-checkpoints and apoptosis</td>
<td>Reduction of cyclin B1 at 2Gy, indicative of G2/M delay</td>
<td>No G1/S delay at any dose. Reduced repair levels?</td>
<td>A dose independent G2/M delay at every dose above 1Gy, correlates with cyclin B reduction</td>
<td>95% cells re-enter G0/G1 at 4Gy. Indicative of very high repair levels in this phase, and low apoptosis</td>
<td>Relatively high levels of apoptosis compared to other resistant cell lines (4% over 36h). Faster kinetics of apoptosis? Apoptosis in G1 or S phases?</td>
<td>Resistant, but the increased levels of apoptosis correlate with the reduced extent of SF2. The high level of repair and high G2/M ratio indicate resistance.</td>
<td></td>
</tr>
<tr>
<td>U1-S40b</td>
<td>√ √</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>0-40</td>
</tr>
<tr>
<td>High initial damage. Increased susceptibility to damage due to altered phenotype?</td>
<td>Low level of protein, with a small increase after radiation, due to wild-type status. Suggestive of a G1/S delay and apoptosis</td>
<td>A reduction in cyclin B1 levels at 2Gy, indicative of a G2/M delay</td>
<td>Small delay at 4Gy (2h), related to low level of constitutive and radiation-induced p53 protein?</td>
<td>Almost dose dependent increase in G2/M delay at doses above 1Gy. Correlates with cyclin B reduction</td>
<td>62% of cells re-enter G0/G1. Indicative of some repair and some apoptosis</td>
<td>Very high repair (91% DBSs repaired). Much higher than MGH-U1 parental line, but has low fidelity of repair. Indicative of increased apoptosis?</td>
<td>Relatively high apoptosis (8% over 36h). Possibly mediated by slight reduction in bcl-2 levels after radiation.</td>
<td></td>
</tr>
<tr>
<td>MeWo</td>
<td>√</td>
<td>X</td>
<td>√</td>
<td>X</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>0-25</td>
</tr>
<tr>
<td>Low level of initial damage, low tolerance or rapid repair?</td>
<td>Mu-p53 status, equally high constitutive and radiation-induced levels. Altered/no checkpoints and apoptosis?</td>
<td>Radiation-induced reduction of cyclin B1. Indicative of a G2/M delay</td>
<td>No G1/S delay, correlates with mu p53 status. Indicative of reduced repair? and/or increased cell death in this phase</td>
<td>Only 40% of G2 cells enter G0/G1. Indicative of reduced repair? High cell death, maybe apoptosis?</td>
<td>Relatively low repair levels (20% DSBs unrepaired). Correlates with low G2/M ratio.</td>
<td>Some radiation-induced apoptosis over 36h (5%). High bcl-2 levels (26%) with slight reduction after radiation (21%). p53-independent apoptosis?</td>
<td>Radiosensitive, due to apoptosis and reduced repair. Evidence for p53 independent-apoptosis?</td>
<td></td>
</tr>
</tbody>
</table>

Table A3. Factors affecting intrinsic radiosensitivity in eight human tumour cell lines. Continued from previous page.
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<table>
<thead>
<tr>
<th>Radiation induced damage</th>
<th>p53 induction</th>
<th>cyclin B1 alteration</th>
<th>G1/S delay</th>
<th>G2/M delay</th>
<th>High G2/M ratio</th>
<th>DNA repair</th>
<th>Apoptosis</th>
<th>Survival</th>
</tr>
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<tbody>
<tr>
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<td>✓ ✓ ✓</td>
<td>✓ ✓</td>
<td>✓ ✓</td>
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<tr>
<td></td>
<td>Lowest level of initial DNA damage.</td>
<td>High constitutive level of protein with large radiation-induced increase for wild-type status. Indicative of large cell-cycle delays and apoptosis?</td>
<td>Substantial G1/S delay at all doses above 0.5 Gy (5 h). Correlates with p53 expression. Indicative of either increased repair or apoptosis?</td>
<td>Extensive delay at all doses. Indicative of either high repair or apoptosis?</td>
<td>Very low G2/M ratio, only 19% enter G0/G1 at 4 Gy. Indicative of high apoptosis and low repair?</td>
<td>Relatively low repair (22% unrejoined). Correlates with low G2/M ratio</td>
<td>Highest apoptotic fraction (9% over 36 h). Correlates with low G2/M ratio. Slight radiation-induced change in bcl-2 (9%–6%). p53 mediated?</td>
<td></td>
</tr>
<tr>
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<td>✓ ✓ ✓</td>
<td>✓ ✓</td>
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<td>✓ ✓</td>
<td>✓ ✓ ✓</td>
<td>0.033</td>
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<tr>
<td></td>
<td>Low level of initial damage</td>
<td>Reducing in cyclin B1, indicative of a G2/M delay</td>
<td>Small G1/S delay at 2 Gy and 4 Gy. Correlates with low level of protein. Indicative of reduced repair?</td>
<td>High G2/M delay at all doses. Indicative of either high repair or apoptosis?</td>
<td>Only 29% of cells re-enter G0/G1 at 4 Gy. Indicative of reduced repair and high cell death at this point</td>
<td>Lowest level of repair (only 70% DSBs rejoined). Correlates with low G2/M ratio, indicative of high apoptosis?</td>
<td>Some radiation-induced apoptosis (5%), with reduction in bcl-2 positivity (8%–3%). p53 mediated?</td>
<td>Extremely radiosensitive, probably due to low repair and the presence of some apoptosis. Survival levels far too low to be explained by apoptosis alone.</td>
</tr>
</tbody>
</table>

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REFERENCES


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