An Investigation of Low Dose Hyper-radiosensitivity - Does it have a Role Clinically?

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ABSTRACT

Laboratory studies have demonstrated that some radioresistant tumours are hypersensitive to low doses of radiotherapy (<1Gy) and characteristically display features of radioresistance at more conventional radiation doses (2Gy per fraction), i.e. there is excess cell kill at doses <1Gy relative to that predicted by the linear quadratic (LQ) model. This phenomenon is called “low dose hyper-radiosensitivity” (LDHRS).

The aim of this thesis was to investigate if LDHRS could be demonstrated in tumours and in normal tissues and to assess whether there was a difference between them that could be exploited clinically. The epidermal basal cell layer of human skin was chosen as a model of normal tissue.

8 patients with metastatic tumour nodules to skin were recruited. The nodules were measured, their volume calculated and randomised to receive conventionally fractionated radiotherapy (1.5Gy/day) or ultrafractionated radiotherapy (0.5Gy TDS with a 4hr inter-fraction gap). Both groups were treated for 12 days. Measurements were taken on days 0, 5, 8, 12 & 26 and monthly until regrowth occurred. Time to regrowth to original volume was calculated and compared between groups using the Wilcoxon Signed Rank test. In addition skin biopsies were performed on days 0, 5, 8, 12 and 26; changes in BCD were compared using non-linear regression analysis. Proliferation was assessed using the proliferation markers Ki67 and Cyclin A.
Analysis of all re-growth data demonstrates greater tumour growth delay in the nodules treated with the "ultrafractionated" regime. This was most marked in tumours generally accepted as being radioresistant and known to show LDHRS in vitro, 2-tailed p-value 0.009. Analysis of the surrounding normal skin does not demonstrate any evidence of LDHRS The proliferative response was similar in both treatment groups.

In-vitro experiments carried out in parallel failed to demonstrate LDHRS following multiple low doses of radiation in Hs633T - a sarcoma cell line that has demonstrated LDHRS to single low doses.

It was concluded that there was a potential therapeutic window that could be exploited by using "ultrafractionated" radiotherapy for the treatment of "radioresistant" tumours ''. A feasibility study of "ultrafractionated" radiation in high-grade glioma (HGG) was initiated, with the ultimate aim of a larger phase II study. To date 2 patients have been recruited to the feasibility study.
ASSOCIATED PEER REVIEWED PAPERS AND PUBLISHED ABSTRACTS


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<td>DMIPS</td>
<td>Dynamic Microscopic Image Processing Scanner</td>
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<td>DNA</td>
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<td>DSB</td>
<td>Double strand break</td>
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<td>ER</td>
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<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<td>GMD</td>
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<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
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<td>HGG</td>
<td>High grade glioma</td>
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<td>LDHRS</td>
<td>Low dose hyper-radiosensitivity</td>
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<td>LD-RT</td>
<td>Low dose radiotherapy</td>
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<td>IR</td>
<td>Induced repair</td>
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<td>IRR</td>
<td>Induced radioresistance</td>
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<tr>
<td>LET</td>
<td>Linear Energy Transfer</td>
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<tr>
<td>LQ</td>
<td>Linear Quadratic</td>
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<td>Local Research Ethics Committee</td>
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<td>MRI</td>
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<td>OER</td>
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<td>Relative Biological Effect</td>
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OBJECTIVES OF THESIS

The aim of this thesis was to investigate whether or not LDHRS could be demonstrated in tumours and in normal tissues and to assess if there was a difference between them that could be exploited clinically in the treatment of tumours classically described as "radioresistant". This requires:

A model of normal tissue radiosensitivity. Human skin has been selected as it lies in the primary radiotherapy field more frequently than any other normal tissue. It is readily observable and the early and late reactions, at doses above 1 Gy/fraction following radiotherapy have been well documented. The choice of skin as the organ of interest for the assessment of normal tissue response allows correlation with historical data and forms the basis of analysis of other normal tissues for LDHRS.

Accessible tumours. Metastatic tumour nodules to skin were chosen; this enabled us to compare the effects of multiple low doses on the nodules and on the surrounding normal skin. It also allowed ease of dosimetry and tumour nodule measurement.

A suitable "radioresistant" primary tumour to assess the feasibility of using an "ultrafractionated" radiotherapy regime. High-grade glioma was chosen because as well as being an aggressive tumour with an appalling clinical outcome, LDHRS has been demonstrated in glioma cell lines and xenograft models.
Dedication

For Philip and Cate with love
CHAPTER 1: INTRODUCTION

1.1 Radiobiology applied to radiotherapy

1.1.1 Developments in radiotherapy

The use of ionising radiation for the treatment of malignant disease has undergone many changes over the course of the last century. The development, in the 1970’s, of Computerised Tomography Scanning (CT), and in the 1980’s of Magnetic Resonance Imaging (MRI) have revolutionised tumour diagnostics and the anatomic work-up for radiation planning. This phase matured in the 1990’s and has been amplified by an equally important development of radiotherapy equipment.

The past 30 years, therefore, have seen dramatic advances in the standard practice of radiation oncology; these have been mainly related to improved definition of the treatment volume with novel imaging techniques and the development of higher energy photon and electron sources with which these tumour volumes can be treated. As well as all this, major developments in radiotherapy fractionation have taken place developed largely from experimental and theoretical studies in radiation biology. In parallel with these advances, local control and 5-year survival rates have improved in a large number of tumours.

It does not seem unreasonable to expect a similar improvement in treatment outcome over the next number of years stemming from improved understanding of the mechanisms of action of both radiation damage and
treatment resistance and the application of therapies that target the specific characteristics of each tumour phenotype.

The biological effect of radiation on both tumours and normal tissues is dependent on a number of factors, namely total dose, dose per fraction, time between fractions and overall treatment time - the fractionation schedule used is as important as the dose delivered to the target tissues.

The introduction in the 1980's of the Linear-Quadratic (LQ) model allowed the most accurate estimation of dose-fractionation response over the dose range from large single doses down to doses per fraction of about 2Gy. The model describes the shape of the cell survival curve and the response of both tumour and normal tissues to changes in fraction size in terms of the same 2 parameters $\alpha$ and $\beta$ and their ratio $\alpha/\beta$. The clinical significance of this was recognised by Thames et al [1]. They realised that the differences in $\alpha/\beta$ ratios for early and late responding tissues could explain the differences in response of these organs to changes in fraction size and thus provided much of the impetus for the clinical interest in altered fractionation schedules.

At doses of more than 1Gy, early reacting tissues or tumours demonstrate predominantly a linear relationship ($\alpha$ component) between dose and effect. Late reacting tissues demonstrate a large part of their effect to the square of the dose prescribed ($\beta$ component). This is described by the equation:
\[ S = \exp (-\alpha d - \beta d^2) \]

Where \( S \) is the probability of survival, \( \alpha \) is thought to be related to linear cell kill due to single track events, and \( \beta \) is thought to be related to quadratic cell kill due to two track events. See Figure 1.1

Figure 1.1. Graphical representation of linear quadratic model. See text for explanation. (Steel 3rd Edition)

This is a continuously bending curve. It's shape is determined by the ratio \( \alpha/\beta \) which is the dose at which the linear contribution to damage (\( \alpha D \)) equals the quadratic contribution (\( \beta D^2 \)), a higher ratio of \( \alpha/\beta \) indicates a more linear survival curve and correspondingly less effect of fractionation. An \( \alpha/\beta \) ratio
of 3 is common for late responding normal tissues; this means that at 3Gy the linear cell kill is equal to the quadratic repairable cell kill of $D^2$ term. Tumours and acutely responding normal tissues have an $\alpha/\beta$ ratio of about 10 and therefore larger doses per fraction are better tolerated. The response of cells to densely ionising radiation e.g. neutrons is almost an exponential curve.

The biological basis of the LQ model is still debated, but it is widely used to model and predict the increase in total dose with decreasing dose per fraction needed for an iso-effective response to radiotherapy in normal tissues and tumours [1,2]. The model reflects the gradual decrease in radiation effectiveness with lowered doses due to these doses being further back on the shoulder of the underlying survival curve for the cells at risk. It has been successfully applied to a broad range of in-vivo and in-vitro experiments and is the currently accepted method of calculating isoeffect in radiotherapy [2].

1.1.2 Low Dose Hyper-radiosensitivity

Until recently laboratory confirmation of cell survival at doses less than 1Gy was limited due to the difficulty in defining cell populations and surviving colonies at such low doses. For many years the low dose region of the cell survival curve was predicted to follow the extrapolation of the LQ model, as accurate measurement in this region was not possible by conventional methods. These conventional “Puck and Marcus” [3] survival assays relied
on a certain number of cells being plated as a result of multiple dilutions. There are considerable errors inherent when using this method, including sampling errors, and the precise number of cells being plated may not be known.

Now however the availability of the Fluorescent-Activator-Cell-Sorter (FACS) and the Dynamic Imaging Processing Scanner (DMIPS) has enabled cell survival at low doses to be determined by defining cell number in irradiated populations more accurately [4].

The DMIPS is an automated microscope that scans a flask containing single cells, identifies the attached cells and stores their locations so that the exact cell positions can be revisited to manually score for colony formation. This allows a very accurate measurement of clonogenic survival on a cell by cell basis. The cell sorter uses the reflectance properties of cells when illuminated by laser light to sort individual cells on the basis of size and granularity without the use of a cell stain [5]. A precise number of cells can be plated before and after radiation, thereby omitting sampling error. These two methods have been used to measure the survival of tumour cells after very low doses of radiation.

Over the last decade work performed, mainly at the Gray Cancer Institute, has suggested that the LQ model does not apply at doses less than 1Gy and has identified a region of high sensitivity in the radiation survival response of mammalian cells at doses ~ 0.5Gy. This phenomenon is referred to as low dose hyper-radiosensitivity (LDHRS) and it precedes the
occurrence of an area of relative resistance to cell killing by radiation (~0.5-1Gy) – increased radioresistance (IRR). Above 1Gy the survival curve follows a smooth downward bending course in keeping with that predicted by the LQ model. LDHRS has been recorded in cell survival studies with yeast, bacteria, protozoa, algae, higher plant cells and insect cells [6] and there are now data available on over 50 human tumour cell lines of which >80% exhibit LDHRS [6-11]. Figure 1.2 shows a single dose cell survival curve using cell sort assay in T98G human glioma cells where the LDHRS/IRR at low doses is clearly evident [11].
Figure 1.2: Survival of asynchronous T98G glioma cells irradiated with 240kVp X-rays. The solid line represents the IR model and the dashed line represents the LQ model. At doses <1Gy the LQ model underestimates the effects of radiation. (Short 1999). The inset box is a close-up of the "low-dose" region of interest.
Other mammalian cell lines that have been tested for LDHRS/IRR to date include colorectal cancer, melanoma, prostate cancer, bladder cancer, lung adenocarcinoma, sarcoma, cervical squamous cell cancer, neuroblastoma, one non-malignant epithelial line and one primary fibroblast line. As a generalisation it may be stated that it is those cell lines that are most resistant to 2Gy doses that exhibit the most marked LDHRS/IRR and a number of the cell lines that do not exhibit a hypersensitive response are very radiosensitive at 2Gy [6].

A new radiobiological model termed the "induced repair model of cell survival" was developed to better describe the LDHRS/IRR effect [12,13].

1.1.3 The Induced Repair Model

Joiner has proposed a new radiobiological model, termed the "induced repair model of cell survival" [12,13]. It is a simple modification of the linear quadratic (LQ) equation allowing modelling of the dose response relationship below 1Gy. In this model, the alpha parameter (α) describing radiosensitivity is allowed to vary with dose, becoming larger at low doses; the degree of radioresistance is therefore dose dependent. The LQ model describes the dependence of cell survival (S) on dose (d) according to the equation:

\[ S = \exp (- \alpha d - \beta d^2) \]
In the Inducible Repair (IR) model, the basic LQ equation is extended (-αd is replaced) and becomes:

\[ S = \exp\left\{ -\alpha r \left( 1 + \left( \frac{\alpha_s}{\alpha_r} - 1 \right) e^{-d/d_c} \right) d - \beta d^2 \right\} \]

In this model, \( \alpha_r \) and \( \alpha_s \) describe sensitivity at high (conventional) doses and at very low doses respectively. \( \alpha_s \) is greater than \( \alpha_r \), representing increased sensitivity at very low doses. Thus at low doses, the IR equation becomes the LQ equation with \( \alpha = \alpha_s \) and at high doses the IR equation is an LQ equation with \( \alpha = \alpha_r \). The parameter \( d_c \) describes how rapidly this transition takes place with changing dose, so that when \( d = d_c \) the change from \( \alpha \) to \( \alpha_r \) is 63% complete. The \( \beta \) term from the LQ equation remains unmodified as this parameter only influences response at high doses.
Statistical fitting of this equation to the data from cell lines showing HRS has shown that this model is a good description of the observed outcome of low dose irradiation. Between different cell lines, $\alpha_s$ is similar whilst $\alpha_r$ varies considerably. Therefore since $\alpha_r$ largely determines response to conventionally sized doses, the parameter $\alpha_s/\alpha_r$ indicates how much more sensitive cells would be at low doses compared with high doses. $\alpha_s/\alpha_r$ has been found to correlate positively with the surviving fraction at 2Gy (SF2) and can reach values of 20 or more in those cell lines showing greatest high-dose resistance.

An alternative interpretation of this model would suggest that without "repair" mechanisms, all cells would follow the $\alpha_s$ survival curve (as seen with neutrons). But the interaction of photons with DNA at the molecular level is fundamentally different and allows for some recovery of cell damage, accounting for the $\alpha_r$ slope.

1.2 Possible mechanisms of low dose hyper-radiosensitivity (LDHRS)

Although now a well-established phenomena, the biological mechanisms underlying LDHRS/IRR are not fully understood. A number of hypotheses have been proposed but the precise explanation remains unclear. There follows a review of mechanisms that have been put forward.
1.2.1 Adaptive Response

It has been suggested that the phenomenon of LDHRS/IRR may be related to the "adaptive response" seen in some cell lines, where small initial doses of radiation induce resistance to a second higher dose [14-17]. This effect requires a time interval of several hours between the first and second radiation doses to occur, and like IRR, it is felt to be due to induction of repair mechanisms. If the adaptive response and LDHRS/IRR were consequences of the same underlying mechanisms then after a small initial conditioning dose there should be no LDHRS in response to a second dose. Marples et al [18] examined this using V79 hamster cells. They found that small priming doses of radiation induced resistance to a second given a few hours later. This effect was dependent on the initial dose given, with small initial doses (~20cGy) being more effective in abolishing second dose LDHRS than higher initial doses. They also noted that the induction of IRR in these cells was inhibited by cyclohexamide, which also inhibits the adaptive response in the same cell line. In these experiments 4-6hrs were needed for full induction of resistance with a return to the hypersensitive state occurring later again.

Although there appears to be a certain degree of homology between the adaptive response and LDHRS/IRR, a study by Wouters & Skarsgard [19] using HT29 cells suggests that they may be distinct phenomena. They found that priming doses prevent LDHRS but have no effect on the high dose
region of the cell survival curve. Further by Short [20] has suggested that in a number of human cell lines the time course for the effect to become apparent and the persistence of the effect is much greater in the adaptive response, mitigating against a mechanistic relationship.

1.2.2 Apoptosis

Apoptosis is programmed cell death and is a distinct entity from necrosis. Apoptosis is defined by morphological changes resulting in non-pathogenic cell loss. Evidence suggests that apoptosis is not involved in any way in LDHRS/IRR. An original hypothesis had suggested that small doses of radiation lead to cells undergoing apoptosis, as a means of removing genomically unstable cells from the whole population. This was investigated in the LDHRS positive T98G cell line. This particular cell line exhibits LDHRS at doses of 0.4Gy, but there was no evidence of an increase in apoptosis at this dose [11].

In the glioma cell lines tested for the presence LDHRS/IRR, almost 90% exhibit it, but apoptosis is not the primary mode of cell death in glioma cell lines [21]. In both U373MG (LDHRS negative) and T98G (LDHRS positive) cell lines, apoptosis does not occur due to an abnormally high activity of protein kinase C (PKC), which promotes the production of a protein that leads to inhibition of apoptosis – BCL-XL. However the inhibition of PKC induces apoptosis in these cell lines [22]. Power [23] investigated 2 cell
lines, HT29 and Be11, characterised by high levels of LDHRS/IRR. She found that they exhibit differing levels of apoptosis in both irradiated and unirradiated populations over a wide range of doses. It is therefore generally accepted that there is no correlation between apoptosis and LDHRS/IRR.

1.2.3 Cell Cycle Effect

The possibility has also been raised that the LDHRS/IRR phenomenon may be due to various sub-populations of cells demonstrating differing levels of radiosensitivity, for example cells at different stages of the cell cycle – the "two population" model. The argument being that at very low doses of radiation, cells in a particularly sensitive phase of the cell cycle would be eliminated and that this would influence the observed survival curve. Skarsgard [24] studied the low dose response of asynchronous and partially synchronised V79 cells and found such cell cycle effects did produce substructure in the asynchronous cells, but only at doses >1Gy.

Also, Short et al [11] examined T98G human glioblastoma cell lines and found that increased cell kill per gray is found at doses <0.5Gy in both asynchronously growing and G1 arrested cells. They concluded that LDHRS/IRR was a feature of the whole cell cycle and not due to a sensitive subgroup of cells. Mathematical modelling of the V79 data supported this supposition as 5% of cells would need to be ~50-60 times more sensitive than the remainder of the population in order to lead to the changes in seen in radiosensitivity over the dose range 0-1Gy [13].
Further work by Short et al [25] has demonstrated a variation in the magnitude of LDHRS throughout the cell cycle. Looking at T98G cell line, survival curves demonstrate the greatest LDHRS/IRR during the G2/M phase. The LDHRS/IRR effect is present during G1 to a lesser extent and is virtually absent during S phase. This increase in LDHRS/IRR parallels changes in high dose radiosensitivity during different phases of the cell cycle. Variations in DNA repair capacity during different phases of the cell cycle seem, therefore, to have an effect on clonogenic survival after high and low doses per fraction. These experiments suggest that LDHRS is not due to a subpopulation of sensitive cells, as all cells selected on the basis of a single cell-cycle phase for an experiment showed a similar non-linear radiosensitivity.

Papathanasiou et al [26] have demonstrated that radiation induces expression of genes involved in cell cycle arrest, but this was a higher doses than those at which IRR occurs. However, there are data to suggest that radiation, in the dose range corresponding to IRR, does cause induction of cyclins that are involved in cell cycle control [27].

Despite these data Marples et al in their 1997 paper postulated that it was unlikely that IRR was due solely to cell cycle effects [28]. The fact that LDHRS/IRR occurs in synchronised cell populations suggests that it is not due to a cell-phase specific checkpoint defect [8,28].
There is now evidence to suggest that although the HRS phenomenon occurs throughout all phases of the cycle, the magnitude of the effect varies with cell cycle phase [25]. It appears that LDHRS is primarily a G2 phase phenomenon and that the G2 response dominates that seen when asynchronous populations of cells are studied, although there is still an effect in G1.

This concept is given further weight by more recent work by Marples et al [29] who report that LDHRS may be explained by a substantial non-linear radiation dose survival relationship that is unique to cells in G2 and postulate that a recently identified G2/M checkpoint [30] may be implicated. This novel G2/M checkpoint occurs between 0 and 2 hours following radiation exposure, is dependent on ATM and is dose dependent over the dose range 0-10Gy. Most importantly it has been shown to be inactive at doses less than ~0.4Gy. The apparent function of this checkpoint is to arrest the progression of G2 phase damaged cells, thus preventing entry in mitosis. What this translates into is that G2 cells irradiated with doses less than ~0.4Gy would enter mitosis early, while harbouring unrepaired DNA damage, and would die: thus giving the appearance of heightened radiosensitivity. It may be that a radiation-induced arrest of cells that are in G2 at the time of irradiation represents the means by which PARP-mediated recognition of DNA damage is linked to repair of those lesions necessary for the development of IRR. Therefore LDHRS is simply a manifestation of the absence of this process [29]. This remains to be verified at a molecular level.
1.2.4 Alterations in DNA repair

Evidence has been accumulating that DNA repair is one of the main mechanisms underlying the LDHRS/IRR phenomenon. It was proposed that IRR results from activation of protective processes triggered by increasing levels of damage to the cell, while LDHRS is due to insufficient damage, that is not enough to trigger the mechanism [7,18,28]. Information gained from a number of studies using DNA repair modifiers and inhibitors of protein synthesis [18] point towards a need for the synthesis of new proteins involved in DNA repair for the development of IRR. This supports the theory that IRR reflects the induction of a radioprotective process. An alternative explanation may be that lower doses of radiation cause greater cell killing per unit dose because they cause little or no cell cycle arrest, during which time DNA repair would ordinarily take place. As stated previously [26] radiation, at higher doses than those at which IRR occurs can induce expression of genes involved in cell cycle arrest and there are data to suggest that radiation, in the dose range corresponding to IRR, does cause induction of cyclins that are involved in cell cycle control [27].

However, the fact that LDHRS/IRR occurs in synchronised cell populations suggests that it is not due to a cell-phase specific checkpoint defect [8,28]. Marples and Joiner [31] have studied the effects of radiation in the dose range 0-1Gy on clonogenic survival of V79-379A cells in the presence or
absence of three known modifiers of DNA repair, namely 3-aminobenzamide (3-AB), 9-D-arabino-furanosyladenine (ara-A) and novobiocin. The results gave a direct indication that specific DNA repair processes were probably involved in the mechanism of IRR. 3-AB inhibits the induction of IRR, so that the radiation response continues to follow the low dose hypersensitive pattern out to higher doses. This suggests that repair processes inhibited by 3-AB may be directly or indirectly involved. 3-aminobenzimide blocks the repair pathways associated with poly (ADP-ribose) polymerase (PARP). PARP has been implicated in DNA damage detection following radiation-induced damage [32], possibly by inhibiting DNA ligase activity [33]. The inhibition of IRR by 3-AB suggests that the formation of single (ssb) or double (dsb) are important for the development of radioresistance. As regards the other agents, neither ara-A, which inhibits double strand repair by inhibition of DNA polymerase [34], nor novobiocin, which inhibits topoisomerase II and therefore prevents relaxation of supercoiled DNA before excision repair [35], prevented the development of IRR. They concluded that the DNA repair pathways most likely involved in IRR were probably inhibited by 3-AB.

This concept of a causal relationship between LDHRS/IRR and DNA repair processes was given further credence in a paper by Vaganay-Juery et al [36], which implied that decreased DNA-PK repair complex activity was involved in the HRS/IRR phenomenon. This was, however, contradicted in a later paper by Marples et al examining DNA-PK activity directly [37].
1.3. The impact of Linear Energy Transfer (LET) and Hypoxia at low doses

The Linear Energy Transfer (LET) is defined as the mean energy given up to the medium by a particle travelling a distance of 1 μm. Differences in relative biological effectiveness (RBE) are dependent on the spacing of ionisation events in biological structures and high LET radiations, which are more densely ionising have a higher RBE. The presence of oxygen in the medium has an important influence on the outcome of radiation interaction.

In the presence of oxygen hydroxyls, hydroperoxides and peroxides are formed, which are more stable than the initial radical species and can be very damaging to DNA. Oxygen therefore produces 'fixation' of radiation damage. This results in increased radiosensitivity in the presence of oxygen, which is quantified as an oxygen enhancement ratio (OER) defined as the ratio of doses which produce equal levels of cell kill in anoxic and aerobic cells respectively.

LDHRS has predominantly been demonstrated in low dose radiation experiments that have used low LET radiation. High LET produces a monoexponential survival curve with no substructure [13]. This suggests cell kill in the low dose range is LET independent with induced radioresistance occurring at higher doses with low LET radiation only.

The theoretical effects of hypoxia appear complex at low doses of ionising radiation. In published experiments, low single doses of radiation tend to reduce the OER [38,39]. Marples has confirmed the presence of LDHRS
under hypoxic conditions [40]. The cell population studied was more resistant at high doses as expected but the resistant response was triggered at higher doses than in the oxygenated cells.

Denekamp and Dasu have attempted to model the interaction of cell survival, low dose radiation and hypoxia [41,42]. In contrast to the classical description of radioprotection in a hypoxic area their model suggests radiosensitization at low doses when LDHRS occurs in hypoxia. To achieve this effect it is assumed that in hypoxic cells the same level of free radical damage with induction of radioresistance occurs at a higher dose than in oxic cells. In the presence of LDHRS, the anoxic cells become more sensitive than oxic cells at around 1Gy. Laboratory experiments to substantiate this model are ongoing.

1.4. Evidence for the existence of LDHRS/IRR:

Evidence for the existence of LDHRS has been confirmed, excluded or disputed in cell line experiments, animal models and human normal tissue studies. Several laboratories using different techniques have confirmed the presence of LDHRS in a large number of cell lines.
1.41. Cell survival assays

(A) Single low dose radiation exposure

There are now data available on the response of over 50 different human cell lines to single low dose radiation exposures of which ~80% exhibit HRS [6-11]. Typically cells exhibit low dose hyper-radiosensitivity at doses < 0.6Gy above which radioresistance occurs which is maximal beyond 1Gy. The cell survival curve then follows the usual downward bending curve with increasing dose.

The cell lines investigated to date include colorectal carcinoma, malignant melanoma, bladder carcinoma, prostate adenocarcinoma, lung adenocarcinoma, gliomas, neuroblastoma and a number of normal tissue cell lines. Figure 1.3 summarises much of the data available to date and indicates by the wide diversity of cell lines investigated that LDHRS is widespread [6].
Figure 1.3: The ratio of the survival curve slope measured at very low doses (\( \alpha_s \)) to the slope extrapolated from the high dose response (\( \alpha_t \)) is plotted against high dose radioresistance as indicated by the surviving fraction measured at 2Gy. There is a trend for cell lines that are more high dose radioresistant to demonstrate the greatest gain in radiosensitivity as the dose is reduced to less than 10cGy, but this is not significant [6].
(B) Fractionated low dose radiation exposure

As well as data on the response of cell lines to single low dose exposures, there is a growing amount of data on the response of cell lines to fractionated low dose radiation. If LDHRS is to be of relevance clinically, there must be a hypersensitive response to each fraction of a multi-fraction treatment schedule, as, if the effects of low doses are indeed larger than predicted by the LQ model then the use of many low dose fractions will magnify this difference.

Smith et al [43] studied the effects of fractionated low dose γ-rays on both C3H 10T1/2 mouse embryo cells and V-79 Chinese hamster cells. The cells were exposed to 6Gy of γ-rays in fractions of 6Gy, 3Gy, 2Gy, 1Gy or 0.3Gy with time intervals of 3 hrs. The results from the two cell lines were virtually identical and were not inconsistent with some hypersensitivity at low doses, in that 20 fractions of 0.3Gy produced a slightly lower (though non-significant) surviving fraction compared with the same dose given in 2Gy fractions. However, the results of 0.3Gy x 20 exposures agreed well with the standard LQ model predictions based on higher dose per fraction. They concluded that there was no evidence of any effects at doses of <1Gy that are inconsistent with those predicted by the LQ formula.

This data must be interpreted in the light of a number of issues. Firstly, it should be noted that a 3-hour interfraction interval was used, which is shorter than that used in other experiments and may not be optimal. Secondly, the response of C3H 10T1/2 to single low dose exposures had not
previously been investigated—so the lack of LDHRS following fractionated
doses could be explained by the fact that LDHRS does not occur at all in
this cell line. As regards the V79 cell line; although it is known to exhibit
LDHRS to single low doses, the effect is much smaller than in other cell
lines.

More recently, Short et al [20] investigated the effects of fractionated low
dose radiation on four radioresistant glioma cell lines. Of these, three (T98G,
U87 and A7) had previously exhibited LDHRS to single low doses while the
fourth U373 had not. Not surprisingly, they found no evidence of LDHRS to
multiple low doses in the cell line that had not shown it with single low
doses. In the others they found a lower than predicted cell survival—
consistent with repeated LDHRS—when the doses are spaced by certain
intervals. In the T98G cell line a second hypersensitive response was only
seen when there was an interval of 2-6 hrs, in U87 cells it needed 1-5 hrs
and in A7 cells 2-8 hrs.

They concluded that a fractionated course of very low doses produces
increased cell kill when applied to radioresistant tumour cells in-vitro given
an appropriate inter-fraction interval and suggested that the same
phenomenon might occur in-vivo leading to better cell killing in radioresistant
tumours. See Figure 1.4.

In addition to this, Beauchesne et al [10] compared the effects of 0.8Gy TDS
(interfraction gap of 4 hrs) with the biologically equivalent dose in 2Gy
fractions OD on G5, CL35 and G152 glioma cell lines. They reported a
dramatic decrease in cell survival in G5 and G152 glioma cell lines with the
"ultrafractionated" regime – both of these cell lines had previously
demonstrated a hypersensitive response to single low dose fractions. There
was no difference between the 2 regimes in the CL35 cell line which was not
surprising as this cell line had not expressed the LDHRS phenomenon to
single low doses.
Figure 1.4: Asynchronous T98G cells following either 0.4Gy TDS or 1.2Gy OD for 5 days. Multiple low doses per day produced decreased clonogen survival compared with the same dose given as single daily fractions. Short [20].
The "Induced Repair" model predicts that this hypersensitivity to radiation at low doses would translate into a similar sensitivity when cells are exposed continuously to low-dose rate radiation. As with acute single low dose exposures, cells exposed to low dose rate radiation continuously will, in effect, be receiving very low doses of radiation per unit time. It is assumed, therefore, that insufficient damage will be caused to induce activation of repair within the irradiated cell. It may even be the case that low dose rate exposure is more effective than acute low dose exposure, as greater total doses may be given.

There has been a limited amount of investigation into the effects of continuous low dose rate exposures on cells. Previous studies have demonstrated an inverse dose rate effect at low dose rates on mutation induction in both Chinese Hamster V79 cells and on human lymphocytes i.e. increased mutagenesis at low dose rate [44,45,46,47]. In addition Colussi et al [48] have shown that continuous low dose rate exposure induces larger deletions at the human HPRT gene than high dose rate X-rays.

In a recent study Mitchell et al at The Gray Cancer Institute [49] studied and compared the effects of low-dose-rate $^{60}$Co gamma rays on a number of human tumour cell lines in-vitro. Three of these cell lines had previously been shown to demonstrate LDHRS (PC3 human prostate cancer & T98G & A7 human glioblastomas) and one had been shown to be LDHRS-negative (U373 glioblastoma). The LDHRS-positive cell lines demonstrated an
inverse dose-rate effect at dose rates <1Gy/h - that is, a decrease in dose rate led to an increase in effect per unit dose. This effect was not demonstrated by U373. It was therefore postulated that this inverse dose-rate effect might be related mechanistically to LDHRS i.e. a threshold effect on repair induction occurs at both acute low doses and low dose rate radiation.
1.4.2. LDHRS/IRR in vivo

(A) Animal normal tissue data

There is a body of data to suggest that the in-vitro phenomenon of LDHRS may translate directly into increased effectiveness of fractionated radiotherapy when given in very small doses. Joiner et al [12,50] investigated the effects of very low doses of radiation on mouse skin and kidney. Using a “top-up” experimental design they assessed the functional end-points of skin erythema and kidney function and demonstrated that when the dose per fraction is reduced to below 1Gy, the total dose needed to produce equivalent normal tissue damage decreased. Figure 1.5(a) [51] shows the total dose required needed to reach full acute skin tolerance in mouse skin as a function of the dose per fraction in the range 0.1-5Gy. Above 1Gy the data fit that predicted by the LQ model, however at doses per fraction of < 0.6Gy, the LQ model significantly underestimates the effect of the radiation. Figure 1.5(b) shows a similar response in mouse kidney. A similar experiment carried out by Parkins et al [52] demonstrated an equivalent effect in mouse lung.
Figure 1.5: Mouse skin erythema (a) and kidney function (b) at low doses per fraction. Relationship between total dose and dose per fraction for equal effect in mouse skin and kidney [51].
Further studies on mouse skin contradicted these results however. Vegesna et al [53] using wound tensile strength on day 14-post radiation as the end-point found no evidence of enhanced radiosensitivity to doses as low as 0.1Gy. This result appears to be supported in a study by Tzaphliidou et al [54]. Using electron microscopy they assessed the effects of doses of 0.5Gy, 1Gy and 2.5Gy on mouse skin collagen at 1, 4 and 8 weeks and noted no alterations in collagen. It may be that the doses used were not low enough - in previous positive studies LDHRS was only seen at doses < 0.5Gy.

Recent studies looking at the effect of fractionated low dose radiation on the skin of NMRI nude mice suggest the presence of LDHRS at very low doses. Using alterations in basal cell density (BCD) as the end-point Dorner et al initiated 2 separate studies [55]. The first compared 0.4Gy TDS (inter-fraction interval – 4 hours) x 6 weeks (7 days/week) with 1.68Gy OD x 6 weeks (5 days/week) - both regimes therefore received the same total dose (50.4Gy) in the same time period. Biopsies were taken on days 0, 4, 7, 14, 21 and 28 and assessed for changes in BCD. There was no difference between the 2 arms in this study. However, the second study compared 1.2Gy OD x 6/week with 0.2Gy x 6 per day (inter-fraction interval – 4 hours) to a total dose of 7.2Gy. Biopsies were taken on days 0, 2, 4 and 6 and once again assessed for changes in BCD. This showed a significant difference in basal cell density in favour of the low dose per fraction arm. They concluded that LDHRS could be demonstrated in the skin of nude mice following doses per fraction of 0.2Gy.
In any clinical trial studying altered fractionation regimens, the spinal cord is usually regarded as the critical organ with a tolerance of 48Gy when given in 2Gy fractions once daily. Obviously if LDHRS exists in normal spinal cord then using lower doses per fraction might result in a lowered tolerance dose. Ang et al [56] studied the effects of doses <1Gy on rat spinal cord and found that less sparing than predicted by the LQ model occurred when lower doses were given. Initially it was felt that this may be a manifestation of LDHRS, but it has since been interpreted as being due to the short (4hrs) interfraction interval used.

In another study involving rat spinal cord, Wong et al [57] concluded that the standard LQ model underestimated the sparing effect of low doses per fraction provided that sufficient time is left between fractions to allow for sublethal damage repair. They went so far as to suggest that cases of radiation myelopathy that occurred in patients undergoing altered fractionation regimes were due to the short interfraction times used. Therefore, from the above data it can be said that there appears to evidence to suggest the existence of LDHRS in animal skin, lung and kidney but not in neural tissue.
There have been a small number of studies looking at tumour responses to low doses of radiation in-vivo. Beck-Bornholdt et al [58] studied the effects of a number of different fractionation regimens on the R1H rat rhabdomyosarcoma xenograft model. Tumours were irradiated under ambient conditions to receive a total dose of 60Gy in 6 weeks. There were 5 different fractionation regimens studied – 10Gy once weekly (6 fractions), 2.85Gy three times a week (21 fractions), 2Gy five times a week (30 fractions), 1.42Gy per day (42 fractions) and 0.476Gy TDS (126 fractions). There was a substantial net growth delay in the 6 and 126 fraction arms, with the others being similar to one another but less effective. The net growth delay per gray was larger for the hyperfractionated, 126-fraction regimen, but there was no dependence on fraction number in the other regimens. This increased effect of multiple doses may be due to the induction of partial synchrony due to selective killing of cells in different cell cycle phases, a reduction in the effect of hypoxia because of the increasing number of fractions, or low-dose hypersensitivity.

Short [59] studied the effects of 2 radiation dose regimens on a T98G glioma tumour xenograft in nude mice. T98G is a cell line that has demonstrated LDHRS to both single fraction and multiple low dose fractions in cell survival assays. A total dose of 36Gy was given – 0.4Gy TDS for 30 days (interfraction interval of 6hrs) and 1.2Gy OD for 30 days – and tumour measurements were made twice weekly during treatment and for 60 days
after it. This time represents approximately 60 cell-cycle times, double that necessary to produce a 1g tumour (10^9 cells) assuming one surviving cell at the end of irradiation. The mean growth delay after 36 Gy given in 0.4 Gy fractions was 313.68 days, and after 1.2 Gy was 138.39 days. This growth delay did not reach statistical significance. Figure 1.6 demonstrates a trend for increased recurrence free survival for the low dose group but this also did not achieve statistical significance. This study was compromised because of a high infection rate, which meant that fewer animals than planned could be evaluated reducing statistical power.
Figure 1.6: Recurrence free survival for glioma xenografts (reproduced with permission, Short [59]. Refer to text for explanation.)
Beauchesne et al [10], in a follow-up experiment to that quoted previously, compared the effects of 0.8Gy TDS (interfraction gap of 4 hours) with the same biologically equivalent dose in 2Gy fractions OD on a G152 glioma xenograft. They found that tumour re-growth rate in the mice treated with the “ultrafractionated” regime was half that in the mice treated conventionally (p<0.05). They concluded that treatment of grafted glioma tumours with repeated low dose radiation results in a strong inhibition of tumour growth compared to a classical irradiation scheme and went so far as to suggest initiation of a clinical study using an “ultrafractionated” regime in the treatment of malignant gliomas.

Conversely, Krause et al [60] studied the effects of an “ultrafractionated” regime on an A7 glioma xenograft in nude mice. They compared 1.68Gy OD X 30 fractions with 0.4Gy TDS X 6 weeks. Both arms received the same total dose (50.4Gy) in the same treatment time (6 weeks). “Top-up” doses were given in both treatment arms at 2 weeks, 4 weeks and 6 weeks. The results, in both the “top-up” and non “top-up” experiments, demonstrated a significant improvement in local tumour control in the conventionally treated tumours (p<0.05). It should be noted, however, that although the A7 cell line has been shown to demonstrate LDHRS to single fraction low doses, the effect following multiple fractionated low dose exposures was much smaller than would be expected from extrapolation from the single fraction data. This experiment was carried out prior to information being available on the effect of cell cycle on LDHRS [S. Short, personal communication]. This experiment is currently being repeated using T98G glioma xenografts;
preliminary results appear to be similar to A7 study [M. Baumann, personal communication].

There is, therefore, some evidence to support the existence of LDHRS/IRR in vivo, and that the in-vitro hypersensitivity translates into an enhanced effectiveness of fractionated radiotherapy when it is given in very small fractions. This is exactly what would be expected from the cell line data and it also implies a rapid recovery from the adaptive resistance. This means that LDHRS may be exploitable clinically. If multiple small (~0.5Gy) doses were given, a regimen termed "ultrafractionation"; it may result in an enhanced therapeutic ratio. Obtaining an enhanced therapeutic ratio for ultrafractionation would be dependent on the tumour cells demonstrating more excess sensitivity than the surrounding critical normal tissues. This amount of increased sensitivity is dependent not only on the value \( \alpha_{SEN}/\alpha_{RES} \), but also on the rate at which \( \alpha_{RES} \) becomes \( \alpha_{SEN} \) with decreasing dose. Looking at the "worst case scenario" normal tissue investigated to date, mouse kidney (Joiner [51]), and comparing it with T98G glioma line (Short [59]) it can be calculated that 141 fractions of 0.5Gy to a total dose of 70.5Gy translates into 60Gy in 2Gy fractions to the normal tissue and 117Gy in 2Gy fractions to the tumour - a therapeutic gain of nearly 200%.

Clinically, if this effect were real and present in irradiated tissues, it may be of considerable significance as often critical normal tissues receive doses of~0.5Gy [61]. Additionally, if radioresistant tumours and normal tissues demonstrated differing level of LDHRS then this could be exploitable in the clinic
1.4.3. LDHRS/IRR in human normal tissues

Despite the evidence demonstrating LDHRS/IRR in vitro and in vivo there has been very little clinical data published. The erythematous response of normal human skin has in the past proven useful for testing the predictions of the LQ model. In a clinical study Hamilton et al [62] used reflectance spectrophotometry to assess skin erythema in response to exposure to radiation. They assessed the response to doses ranging from 0.4Gy to 5.2Gy per fraction and found that the non-visual erythematous reaction was greater than expected for the low doses per fraction compared with the higher doses. They concluded that either the standard LQ model does not apply for low doses per fraction in human skin or that erythema is not exclusively initiated by radiation damage to the basal cell layer.

In a clinical study by Turesson et al [6] the dose response in normal human skin to low doses per fraction was studied. They studied patients receiving standard radiotherapy for prostate carcinoma who consented to multiple skin biopsies during the course of the treatment, application of 5mm of bolus to the left lateral portal ensured that the dose per fraction was <1Gy. In the study, 3mm skin biopsies were taken before and regularly during a course of radiotherapy (70Gy in 35 fractions using 11 or 15 MV photons) from opposed lateral fields, with 5mm bolus on the left, and at 1.5 and 3 cm outside the fields. This allowed assessment of the effects of skin doses of 0.07, 0.2, 0.45 and 1.1Gy per fraction. The end points were epidermal basal cell density and Ki67 index. The mean dose responses for basal cell density
for doses of 0.45 and 1.1 Gy per fraction in 40 patients were compared and showed a significant reverse fractionation effect. See figure 1.7.

It was felt that the confounding effect of repopulation would lead to misinterpretation of these results, but in both arms the Ki67 index was effected in a similar fashion —there was depression during the first 3 weeks followed by a significant increase over the following 4 weeks. They suggested that repopulation might actually be obscuring the effect of LDHRS/IRR.
Figure 1.7: Dose response for basal cell density in skin, following daily fractions of 0.45 and 1.10 Gy. Mean values and 95% confidence intervals are shown for 40 patients. The difference in radiosensitivity, termed "Dose Modifying Factor" (DMF) is highly significant [6].
In a similar clinical study at Mount Vernon, Shah et al [63] investigated the response of human skin to low doses of radiation. 24 patients undergoing standard radiotherapy for prostate cancer were assessed. Without modification the dose to the skin on the lateral portals is in the range 0.3-0.5 Gy and application of 5 mm of bolus to one lateral portal increases the dose on that side to >1 Gy per fraction. Skin biopsies were taken prior to and during radiotherapy and assessed for changes in basal cell density.

A plot of BCD versus skin dose demonstrated a significant reduction in cell numbers for the low dose side, which was about twice that of the higher dose side. The results of this study were in complete accordance with those of Turesson et al and pointed towards the existence of LDHRS/IRR in normal skin. See figure 1.8
Figure 1.8: Dose response for basal cell density in skin following daily fractions of 0.45Gy and 1.1Gy. The right hip received ~1.1Gy per fraction and the left hip ~0.45Gy per fraction. An “enhancement ratio” of 2.03 was seen (95% CI 0.8-2.28) [63].
Clinical evidence of LDHRS/IRR has also been suggested in a study by Lambin et al [6]. 21 patients with a primary head and neck tumour treated with radiation therapy were assessed for alterations in salivary gland function in relation to radiation dose. The patients had CT and salivary gland scintigraphy (SGS) with free 99mTc pertechnetate performed prior to and 1 month following completion of radiotherapy. Six of the patients had a large gradient of doses in their parotid allowing assessment of doses per fraction of 0.57 Gy or less. Dose–response curves were constructed based on the dosimetric CT and the decrease of parotid function as assessed by scintigraphy before and after radiation. These dose–response curves demonstrated some evidence of hypersensitivity at low doses per fraction. See Figure 1.9.
Fig 1.9: Dose - response of parotid gland of one patient. The Y-axis shows the loss of function of the parotid after irradiation, computed as the difference between the salivary excretion fraction before and one-month after irradiation (Delta SEF). The X-axis shows the radiation dose; each slice of 5 mm of the parotid has been irradiated at various total doses in a fixed number of 25 fractions, therefore 10 Gy corresponds to a dose per fraction of 0.4 Gy. The fit to the data is linear except at the 2 lowest doses, which demonstrate increased radiosensitivity. Data points are mean ± SD [6].
1.5. Skin as a Model of Normal Tissue

Numerous studies have been carried out assessing the radiation response of normal skin. There are a number of reasons for this - skin lies in the primary radiotherapy field more frequently than any other normal tissue, it is readily observable and the early and late reactions, at doses above 1 Gy/fraction following radiotherapy have been well documented. Although it may not always represent the critical dose limiting normal tissue for radiotherapy, knowledge of its response is important for modelling other acute reacting tissues. Thus, the choice of skin as the organ of interest for the assessment of normal tissue response allows correlation with historical data and forms the basis of analysis of other normal tissues for LDHRS. The evaluation of epidermal basal cell density in the study of the biological effects of ionising radiation is well documented [64-67].

1.5.1 Structure of Human Epidermis

The skin is one of the major organs of the body constituting about 3% of the total body weight. One of its major functions is to provide a physical barrier to protect the body against the hazards of the environment. It controls liquid and electrolyte loss and also protects against infection. These functions have been shown to be of vital importance if the barrier is breached as a result of ionising radiation. The skin is also important in thermoregulation, detection of sensory stimuli, synthesis of vitamin D and is an integral part of the body's immune system.
The skin is composed of multiple layers, which can be grouped into two main structures: the outermost layers are referred to collectively as the epidermis and are derived from the embryonic ectoderm. The deeper layers, which are of mesenchymal origin, comprise the dermis. The dermis is infiltrated by specialised structures, the skin appendages, which are formed by an infolding of the epidermis. It is the epidermis that is of relevance to this study. See figure 1.10

The epidermis is a keratinised stratified squamous epithelium subdivided into several clearly defined layers; the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The cell kinetic organisation of the epidermis fits that of a steady state self-renewing tissue with hierarchical organisation (type H tissue) of stem cells, transit cells and functional end cells [68]. The outermost layer the stratum corneum is made up of flattened dead cells to form approximately 25% of the thickness of the epidermis [69,70]. The stratum granulosum lies beneath the non-viable stratum corneum [71]. Organelles are gradually lost from cells in this layer, the cells become flattened and eventually the nucleus degenerates. The main viable layers of the epidermis lie beneath the stratum granulosum. Their structural and proliferative organisation determine the response of the epidermis to radiation-induced injury. The upper layer of the viable epidermis, the stratum spinosum, is largely composed of post-mitotic cells. Cell division is to a large extent, but not exclusively, found in the basal layer of the epidermis. It is assumed that about 10% of the cells found in the basal layer
are stem cells. Other cell lines found at the basal layer include keratinocytes, Langerhans cells and melanocytes.

The sites of potential stem cells have been widely studied. Earlier studies using morphological criteria and radiolabelled thymidine uptake suggested a regular distribution of the stem cells along the basement membrane. Immunocytochemical studies with putative stem cell markers [72-75] demonstrate an irregular distribution of integrin positive cells along the tips of the dermal papillae and in the "bulb" region of the hair follicle. This has implications for studying the response of the epidermis to radiation from low energy X-rays, beta and alpha emitters in which there may be a considerable dose inhomogeneity complicating the assessment of specific cell types.
Figure 1.10: Skin at high power (X400). A= Stratum Corneum, B= Stratum lucidum, C= Stratum Granulosum, D= Stratum spinosum, E= Stratum Basale, F= Dermo-epidermal junction, G= papillary dermis, H= Reticular dermis.
1.5.2 Epidermal organisation and kinetics

The epidermis has been classically viewed as stratified squamous epithelium maintained by cell division within the basal cell layer, which is attached to the epidermal basement membrane.

Keratinocyte stem cells must give rise to all epidermal cell layers, but the majority of cells in epidermis are committed to terminal differentiation [76]. A transit amplifying population of epidermal keratinocytes can undergo a variable number of rounds of cell division before becoming postmitotic. Kinetic analysis of mouse epidermis using radioactive thymidine label retaining cells demonstrates that 10% of cells can form new foci of epidermis (stem cells) [77]. In vitro, there is heterogenous expression of β1 integrin for basal keratinocytes; basal cells expressing higher levels of β1 integrin show rapid adhesion to collagenous substrates, have a high colony forming efficiency and contain 45% of cells retaining tritiated thymidine, a potential stem cell marker. This suggests that β1-integrin bright cells contain the stem cell population. Recent work suggests these cells are located in the interfollicular epidermis and in a bulge region of the hair follicle [75].

Transit amplifying cells and postmitotic keratinocytes occur in the basal layer, but suprabasal keratinocytes can also undergo cell division, although committed to differentiation. It has been shown that 30% of cells undergoing DNA synthesis are above the basal cell layer [78]. Potten has suggested
that discrete "epidermal proliferative units" exist each with a stem cell surrounded by transit amplifying cells and postmitotic cells, and associated Langherhans and melanocytic cells. However, this organisation has been disputed in human skin studies as cited above.

Epidermal kinetics are complicated by the balance between growth, differentiation and cell death. The turnover time in skin is the time for the whole population to replace itself and depends on the cell cycle time and the growth fraction. The cell cycle or intermitotic time represents the interval between two successive mitoses. See figure 1.11. The growth fraction is the proportion of basal cells that are proliferative at any one time. The mitotic index of the basal layer is the fraction of basal cells that is in mitosis at any point, and the labelling index is the fraction of basal cells in DNA synthesis.
Figure 1.11: Diagrammatic representation of the cell cycle
These parameters have been summarised in the table below. Table 1.1. Perturbations from the resting state will result in significant changes in epidermal kinetics. This may be a physiological response e.g. to radiation or a pathological process e.g. psoriasis.

<table>
<thead>
<tr>
<th>Cell kinetic parameter</th>
<th>Human data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic index (%)</td>
<td>0.2-0.4</td>
</tr>
<tr>
<td>Labelling index (%)</td>
<td>4.3-8.1</td>
</tr>
<tr>
<td>Duration of mitosis, Tm (hours)</td>
<td>1.0-1.5</td>
</tr>
<tr>
<td>Duration of DNA synthesis, Ts (hours)</td>
<td>9.7-10.6</td>
</tr>
<tr>
<td>Cell production rate, k (cells/1000 cell)</td>
<td>5.1-8.8</td>
</tr>
<tr>
<td>Turnover time (hours)</td>
<td>120-184</td>
</tr>
</tbody>
</table>

Table 1.1: Epidermal kinetics in man [79].
1.5.3 The regulation of epidermopoiesis

The control mechanism for maintaining the constant epidermal cell production and cell loss consists of a balance of stimulatory and inhibitory signals. Stimulatory signals include the epidermal growth factor family of human epidermal growth factor, transforming growth factor alpha, amphiregulin and keratinocyte growth factor. Inhibitory signals include transforming growth factor beta, alpha and gamma interferon and TNF alpha.

A number of signal transduction pathways have been postulated to be important in the regulation of growth and differentiation in the skin, involving growth factors, hormones, cell surface receptors, second messengers and their control. These include the adenylate cyclase monophosphate pathway, protein kinase C, tyrosine kinase, p21-ras, NK kb transcription factor and nuclear receptors (steroid, thyroid, vitamin D and retinoic acid).

1.5.4 Terminal differentiation of the epidermis

As the epidermal keratinocytes move through the epidermis they undergo a complex process of terminal differentiation to produce the stratum corneum. The stratum corneum cells have lost their nuclei and other recognisable organelles and comprise 65% insoluble keratin proteins. There is synthesis of a highly insoluble cornified envelope derived from involucrin. In addition to the formation of keratin proteins and the cornified envelope, there are
changes in the expression of intracellular lipids, membrane glycoproteins, growth factor receptors, intercellular adhesion proteins, cell matrix adhesion proteins and blood group antigens [80].
1.6. The Response of Skin to Ionising Radiation

Knowledge of the dose tolerance and pathophysiology of irradiated skin is important.

1.6.1 Pathophysiology

The epidermis represents a constantly renewing, hierarchical type of tissue. Cell proliferation occurs in the basal and suprabasal layers [78], having a generation time of about 2.6 days. The minimum transit time for a labelled basal cell to pass from the basal layer, through the other layers and be shed from the corneal layer is about 26 days [81]. Following radiation there is a dose dependent reduction in stem cells, which results in a decrease in the influx of cells into the transit cell compartment. Pre-existing differentiated cells have an unaltered life span and in the absence of further proliferation, this would result in thinning of the epidermis with flattening of the papillae. If the entire differentiated population of epidermal cells is lost before a new transit cell compartment is re-established from a sufficient number of stem cells then loss of the skin surface will occur. Within a few days of irradiation there is a marked fall in the thymidine labelling index and the mitotic index of basal cells [82]. In pig skin [65], the rate of cell loss has been shown to be 2.6% per day after both single and fractionated irradiation. This rate of cell loss is independent of the final radiation dose [65], and also the time to the appearance of the early reaction and its rate of increase as they are related
to cell loss, which continues at the pre-irradiated rate. The peak reaction and degeneration are related to the dose - as they relate to the proportion of surviving stem cells. Following radiation repopulation of the basal layer occurs from proliferation of surviving stem cells within the irradiated volume, from cells at the periphery of the irradiated volume, and from the hair follicle regions [69].

A review by Archambeau et al [81] in 1995 summarised the effects of different radiation doses per fraction on skin as follows. Following a large single fraction of radiation, there is a linear loss of basal cells which reaches a nadir at ~21 days, this is followed by an exponential recovery which leads to control levels being reached at 28-32 days. The mitotic and labelling indices are increased during this period. Complete regeneration can be produced following all doses up to ~45Gy.

When assessing epidermal basal cell density following fractionated radiation exposure (2Gy/fraction), there is no alteration in BCD until doses in the range 20-25Gy have been given. This is followed by a period of cell loss which is maximal at ~50Gy, but which returns to control levels at ~60Gy due to cellular proliferation occurring during continuing irradiation.

These models suggest that the rate of reduction in basal cell density following exposure to radiation is independent of dose given, and cell loss occurs at a rate equivalent to it’s natural turnover time i.e. in the absence of proliferation, differentiation and cell loss will continue at the same rate.
1.6.2 Clinical Syndromes

Following radiation exposure, the changes in skin follow a reproducible pattern that is dose dependent.

**Early skin changes**

These occur during the first 70 days following radiation of up to 20Gy in a single exposure or up to 60Gy as a fractionated course. The initial feature is erythema, which is followed by pigmentation, epilation and desquamation. Moist desquamation may occur if the dose is large enough, which will either heal by 50 days or proceed to ulceration and necrosis.

**Late skin changes**

These tend to occur at >10 weeks following radiation and are characterised by atrophy leading to contraction of the irradiated area and the appearance of induration or "radiation fibrosis". The development of telangiectasia after 1 year demonstrates the progression of vascular injury to the dermis.

1.6.3 The Response of Skin to Low-Dose per Fraction Radiation

As previously stated, to date there has been a limited amount of investigation into the effects of low doses of radiation on normal human tissues. Three studies have focused on the effects of low doses per fraction on skin. In the first Hamilton et al [62] used human skin erythema as an
endpoint and concluded that the LQ model significantly underestimated peak erythema values at doses of <1.5Gy/fraction. In a later study Turesson et al [6] compared the effects of a range of doses per fraction on basal cell density (BCD) in human epidermis and demonstrated that when doses of 0.45Gy/fraction were compared with doses of 1.1Gy/fraction, there was a significant reverse fractionation effect in favour of the lower dose per fraction regime. Using a very similar technique Shah [63] compared doses of ~0.5Gy with ~1Gy and once again demonstrated greater cell kill per unit dose in the lower dose per fraction regime.

In summary, the patho-pysiological response of skin to ionising radiation has been extensively investigated. Descriptive studies focus on the development of skin reactions as a whole organ and are generally unable to distinguish specific epidermal and dermal contributions. Additional knowledge from the use of different doses and penetrative powers of ionising radiations combined with histological evaluation allows a fuller understanding of epidermal and dermal behaviour. This study assesses the response of the basal cell layer in isolation against the background knowledge of the complex cellular and functional interactions of skin subdivisions.
1.7. Conclusion

The LQ model adequately describes cellular response to ionising radiation at doses above ~1Gy. In the past it was difficult to accurately assess cell killing by ionising radiation below this dose. This has now been overcome by the development of the Fluorescent-Activator-Cell-Sorter (FACS) and the Dynamic Imaging Processing Scanner (DMIPS). Using this technology it has been shown that many cell types exhibit hypersensitivity to doses of less than 1Gy.

It has been proposed that this hypersensitivity might be exploitable clinically if it is practical to deliver radiotherapy as a very large number of very small (<0.5Gy). If some tumour types are found to exhibit enhanced radiosensitivity at low doses, the use of low doses to exploit hypersensitivity may be a way of improving local tumour control by overcoming radioresistance. The more effective application of low dose radiotherapy will depend eventually on uncovering the mechanisms of LDHRS, which may allow exploitation of the phenomenon to enhance tumour killing at all doses. An understanding of low-dose effects is also important to the understanding of the potentially harmful effects of non-therapeutic exposure to irradiation. Improved understanding of low-dose radiation effects might therefore influence both the treatment of radioresistant tumours and low-dose risk estimation.
CHAPTER 2: MATERIALS AND METHODS (CLINICAL STUDY)

The existence of LDHRS in tumours in vivo can only be exploited if the surrounding normal tissue exhibits lower levels of cell kill or improved recovery after fractionated low dose radiotherapy (increased therapeutic ratio). This study was designed to investigate the response of tumour and normal tissue (skin) to multiple low doses of radiation. Tumours metastatic to skin were chosen for a number of reasons, firstly they were easy to plan, treat and measure, and secondly direct comparison of the effects of low dose radiation could be made with the surrounding normal skin.

The term “ultrafractionation” has been used to define radiotherapy delivered at 0.5Gy per fraction in multiple fractions per day; in this study “ultrafractionated” radiotherapy was compared to once daily conventional radiotherapy.

Ethical approval was granted from Watford and Mount Vernon Hospital NHS Trust Ethical Review Committee.

2.1 Study Design

The design of the "ultrafractionated" regime was based on in-vitro data, which suggest that 0.4-0.5Gy at 2-6 hour intervals produces repeated LDHRS in a number of radioresistant cell lines [20]. The total dose and overall time were chosen to be comparable to a conventional palliative...
regime, to satisfy the requirements of research and to be acceptable to patients.

To make comparison with routine clinical practice we converted the dose fractionation schedules to single dose equivalent (gray) using the low-dose sensitivity parameter (αs), the α/β ratio and the SF2 for a number of tumour cell lines (including glioma, melanoma and bladder carcinoma) and for skin and kidney. For a total dose of 18Gy given as multiple low dose fractions, the single dose equivalent for radioresistant tumour cell lines lies between 7Gy and 13Gy, which is within the range commonly used for palliation in clinical practice. The primary endpoints of the study were threefold: tumour growth delay and, in the rim of normal skin included in all treatment fields, changes in BCD and the proliferation markers Cyclin A and Ki67.

2.2 Study endpoints

The endpoints of the study were as follows

- Time to re-growth of the tumour nodules in each treatment group.
- Changes in epidermal basal cell density (BCD) in the surrounding normal skin in each treatment group, assessed at regular intervals during and after radiation.
- Assessment of proliferation (using Ki67 & Cyclin A) in the surrounding normal skin, assessed at regular intervals during and after radiation.
2.3 Patient Selection

8 patients with multiple (>2) metastatic nodules to either skin or the subcutaneous tissues and for whom local palliative radiotherapy was appropriate were enrolled over a period of 2 years. Diagnoses were metastatic malignant melanoma (3 patients), metastatic leiomyosarcoma (2 patients), metastatic breast cancer (1 patient) and advanced Non-Hodgkin’s Lymphoma (NHL) (2 patients).

2.4 Irradiation Technique

Each patient entered into the study was offered accommodation within the Cancer Centre (CHART Lodge), with an accompanying person, for the duration of the treatment.

The patients were seen 1 week prior to commencement of treatment by the treating clinician, the physicist involved in the treatment planning and the radiographer involved in treatment set-up. At this time the lesions were measured in three dimensions using callipers with millimetre gradients (See Figure 2.1) and their volume calculated. They were then numbered consecutively according to volume and randomised in matched pairs to receive either an ultrafractionated regimen (0.5Gy TDS with a 4-hr gap, over 12 consecutive days) or a conventionally fractionated regimen (1.5Gy OD over 12 consecutive days). A total dose of 18Gy over 12 days was prescribed in both treatment schedules.
The treatment depth for each lesion was individually determined and each lesion was then planned and treated using appropriate electron energies to encompass the nodule entirely within the 95% isodose. Where necessary wax bolus was specifically designed and applied to ensure adequate coverage at depth but also ensure maximum dosage at the skin surface. (See figure 2.2)

The circumferential margin encompassing each nodule was chosen to allow for the electron isodose bowing effect at depth and also to allow for a normal skin biopsy to be performed – this margin was between 1-2cms in all patients (See Figure 2.3). The dose received at 0.1mm below the skin surface, the average depth for the basal cell layer of the epidermis, was verified by black carbon loaded thermoluminescent dosimeters (TLD).

2.5 Statistical Design

2.5.1 Tumour Nodules

A matched pair design was used to maximise the sensitivity of studying a relatively small number of lesions. Assuming a 50% difference in relative response rates between the two regimes, it was calculated that a total of 29 paired lesions would be needed to confirm a difference at the 0.05 significance level. The times to regrowth of individual nodules were compared using the Wilcoxon Signed Ranks Test.
For basal cell density comparisons, the analysis compared the ratio of the slopes of the log [basal cell density] versus time relationship for skin receiving 0.5Gy per fraction and 1.5Gy per fraction. This was termed the "Enhancement ratio (ER)." The null hypothesis was that the enhancement ratio was unity, i.e. that there was no difference in the slopes. An ER >1 implies a greater biological effect for skin exposed to 0.5Gy per fraction compared to skin exposed to 1.5Gy per fraction.

The method of calculating the ER was discussed between the prime investigator, Prof. Michael Joiner (Radiobiologist) and Prof. Soeren Bentzen (Biostatistician). In view of the baseline variability of normal skin BCD, the potential interaction of reduction of BCD following irradiation, response to dose and repopulation differences, a non-linear analysis of basal cell density response to radiation dose was chosen. Although this may not be the most ideal assessment, it was felt to be the most logical and practical approach as we were trying to determine whether there was a difference in BCD slopes between low and high radiation doses. The slope determined from non-linear regression analysis was treated as the best fit straight line. The comparison of these "best fit" lines then gave an ER for the doses studied.

The dose response relationship was assessed in each patient individually. Enhancement ratios were calculated by performing a non-linear regression
analysis on each set of data (low dose side and high dose side) then comparing the fit parameters. The basal cell density at origin (time 0) for each side was included as a dose dependent variable, without normalising the irradiated data to the original number of cells counted. ER's were calculated for each patient with 95% confidence intervals. A significant result was recorded if an ER>1 was associated with the lower confidence interval greater than unity or an ER<1 was associated with the upper confidence interval less than unity. In some patients, the software was unable to determine confidence intervals and ER was coded as indeterminate significance. The data were also analysed collectively to assess the overall estimated enhancement ratio for low doses per fraction.
2.6 Assessment of tumour growth delay

Tumour growth delay was assessed by tri-dimensional measurements of each nodule prior to treatment, during treatment on days 5, 8 & 12, on day 26 and then monthly until re-growth occurred. Measurement of nodules ceased when they reached, or exceeded their original volume.

The tumour volumes were derived from the formula -

\[
\text{Volume} = \text{length} \times \text{width} \times \text{depth} \times \pi/6
\]

Tumour volumes were then normalised to those on day 0 and plotted against time, in days, from the start of treatment. These data were then test-fitted to each of three different polynomial functions of degrees 1, 2 and 3 using least-squares non-linear regression (SAS JMP v4.0). The polynomial, which produced the best fit, i.e., the one with the smallest root mean square error (RMSE) was selected and/or which fitted the data best on visual inspection was selected. The equation of the fitted polynomial was then solved to give the number of days for the tumour to reach the starting volume. The mean number of data points used to fit the line was 9 (range 7-13).
2.7 Assessment of epidermal basal cell density (BCD)

Human skin biopsies for basal cell evaluation were obtained using a 2 or 3mm punch biopsy (Terumo - See Figure 2.4), similar to the Nyman technique [67]. Local anaesthetic (0.5-1ml lignocaine 1% ± 2% adrenaline) was infiltrated subcutaneously prior a single punch biopsy performed perpendicular to the skin surface. The skin defect was then covered with a standard medical plaster. Due to concerns regarding stimulation of proliferation by skin stripping [83], patients in the study had dry, non-adhesive gauze applied to the tissue defect thus eliminating any effect of skin stripping. The biopsy tissue was fixed in 4% formalin, embedded in paraffin, cut into 3um sections and stained in haematoxylin-eosin and PAS (periodic acid schiff). Basal cells were manually counted at high power (X400). Only the interfollicular epidermis was assessed and any melanocytes present were excluded from the count. The length of the basement membrane was determined on images captured using an Axioscope trans-illumination microscope (Zeiss) connected to a 3 CCD colour camera (JVC) connected to a 600MHz Pentium desktop PC with a Matrox Meteor frame grabber in a PCI bus. The length was measured using a calibrated line tool using standard image analysis routines (Visilog 5.02, Noesis Vision, Inc). The basal cell density was calculated as the number of basal cells divided by the length of basement membrane assessed (cells/mm). See Figure 2.5. The principle investigator (JH) performed manual counting of the specimens.
Number of images captured

For each punch skin biopsy, a minimum of 3 sections were captured at high power. Each section would give rise to eight to ten images from which the basal cell density was calculated. This results in twenty four to thirty high power images captured for each skin biopsy.

2.8 Immunohistochemistry and assessment of proliferative index

Immunohistochemical analysis of the skin was carried by staining for the proliferation markers Ki67 and Cyclin A.

2.81 Ki67

Ki67 is a mouse monoclonal antibody that identifies a nuclear antigen associated with the cell cycle [84]. Detailed cell cycle analyses has shown that the Ki67 antigen is tightly associated with proliferation, being expressed at all phases of the cell cycle except G0 [85]. It has been demonstrated by a number of authors that the amount of Ki67 antigen varies through the cell cycle, reaching a maximum during G2 and M phases [86,87].

The function of Ki67 is not completely understood, but it is generally accepted that it is an absolute requirement for the maintenance of the cell cycle and is essential for cellular proliferation [85].
Numerous studies have shown a good correlation between Ki67 immunoreactivity and other indices of cellular proliferation e.g. flow cytometry [88-90], thymidine labelling [88, 91-93] and bromodeoxyuridine incorporation [91,94,95].

Ki67 is a widely accepted measure of the growth fraction (GF) i.e. the actively proliferating cell pool [85,96]. Although some authors have stated that the fraction of cells showing Ki67 immunoreactivity may overestimate the growth fraction in tumours [97] it is accepted to be representative in normal tissues [96]

2.8.2 Cyclin A (CyA)

Cyclin A (CyA) is also a marker of proliferation, a member of the "mitotic" or "G2 Cyclin" family, and is involved in the control of G2/M and mitosis. It binds to and activates cyclin dependent kinase 2 (CDK-2), essential for initiation and progression through S phase. Cyclin A is responsible for the progression of cells through S-phase and the transition between G2 and M phases [98,99] and is expressed after the restriction point in G1, i.e. in S, G2/M and the early part of M [99].

2.8.3 Cyclin A: Ki67 Ratio

The Cyclin A: Ki-67 ratio was assessed at each time point as a surrogate for accelerated repopulation. It was taken as being representative of the number of cells occupying S and G2 phase in the growth fraction.
To assess each of these indices, de-paraffinised samples were put into a plastic rack and placed in a trough containing 250 mils of 10mM citric acid (pH adjusted to 6.0 using 2M sodium chloride). The lid was then put on and the trough put into a microwave at 750W where three, 4-minute pulses were given at high power. The trough was then left to stand at room temperature for 10 minutes.

The slides were then washed well in running tap water and rinsed in Tris buffer saline (TBS), drained and the section circled with a resin pen. DAKO Monoclonal Mouse Anti-Human Ki67 Antigen, used in a dilution range 1:75 – 1:150, and CASTRA Cyclin A mouse monoclonal antibody, diluted to 1:50 – 1:100, were then applied. The slides were then washed 3 times in TBS (3mins) and biotinylated anti-mouse antibody, diluted 1/4000, was applied for 1 hour. They were then washed a further 3 times in TBS (3mins) and AB Complex/Strept AB Complex was applied for a further hour. Following a further wash in TBS (3 x 3mins) Diaminobenzadine (DAB) solution was applied for 5mins. Then the sections were rinsed in distilled water, washed well under a running tap and the nuclei counterstained with Gills number 1 Haematoxylin for 60 seconds. They were then washed again, dehydrated and mounted in DPX.

For each punch skin biopsy, at least 3 sections were captured at high power for assessment of proliferative index. Each section would give five to eight images from which the Ki67 and Cyclin A indices were calculated. This
results in fifteen to twenty four high power images captured for each skin biopsy. The stained slides were analysed at high power with manual image counting. The total number of cells in the basal compartment were counted: this included both the basal layer and first supra-basal layer as at it is known that at least 30% of proliferation occurs in the suprabasal layers [78]. Then the number of basal cells that had clear-cut and strong nuclear staining for Ki67 and Cyclin A were counted. The indices of Ki67 and Cyclin A were calculated by dividing the number of stained cells by the total number of cells in the basal cell compartment. See Figure 2.6.
Figure 2.1: Metastatic skin nodules on the ankle of a patient with advanced malignant melanoma
Figure 2.2: Calliper measurement of metastatic nodule to skin in a patient with advanced NHL
Figure 2.3: Schematic representation of electron isodose curves encompassing a metastatic tumour in skin. Application of wax bolus ensures that the nodule is encompassed by the 95% isodose.
Figure 2.4: Metastatic skin nodules (sarcoma), treatment fields demonstrated by blue rings. Rim of normal skin included in treatment fields.
Figure 2.5: Metastatic skin nodules in a patient with advanced breast cancer. Blue rings demonstrate treatment fields. Rim of normal skin included in treatment fields.
Figure 2.6. Terumo 3-mm punch biopsy needle
Figure 2.7. High power view of skin biopsy.

Cell count is 39; length measurement is 390 micrometers. Basal Cell Density (BCD) is calculated as 100 cells/mm.
Figure 2.8: A. High power (X400) view of the proliferation marker Ki67.

B. High power (X400) view of the proliferation marker Cyclin A.

Cells with clear-cut and strong nuclear staining counted as positive. They are arrowed.
CHAPTER 3: RESULTS OF TUMOUR NODULE STUDY

3.1. Assessment of tumour nodule growth delay

8 patients were enrolled over a 2-year period (3M: 5F) with a median age of 71 years (range 64-81). Diagnoses included metastatic sarcoma (2 patients), metastatic breast cancer (1 patient), advanced NHL (2 patients) and advanced malignant melanoma (3 patients).

A total of 40 paired nodules with a median volume of 0.73cm$^3$ (range 0.007cm$^3$ to 114.0 cm$^3$) were analysed. 26 of these nodules were derived from tumours generally considered to be radioresistant and in which LDHRS has been demonstrated \textit{in vitro} (melanoma and sarcoma - patients 1,3,6,7 & 8), 10 were in tumours likely to be radiosensitive (NHL - patients 2 & 5) and 4 were in breast cancer (patient 4), a relatively radiosensitive solid tumour.

36 nodules have regrown after a median follow-up of 226.5 days (range 76 - 501 days). 4 nodules have not regrown after >500 days, these were all in a patient with NHL (patient 5). See Table 3. 41 & Figures 3.1-3.7.

Analysis of all regrowth data demonstrates a trend towards greater tumour growth delay in the nodules treated with the "ultrafractionated" regime (2-tailed p-value 0.14) as shown in Figure 3.8. If the data are re-analysed excluding the lymphoma nodules, that is leaving metastatic breast cancer nodules in addition to the melanoma and sarcoma nodules (30 nodules in total) the resultant difference in time to tumour regrowth is statistically significant in favour of the low dose per fraction group (P<0.05). Equally
when the radioresistant tumour nodules of types known to show LDHRS were analysed separately (melanoma and sarcoma, 26/26 regrown) a statistically significant tumour growth delay in favour of the "ultrafractionated" treatment group was demonstrated (2-tailed p-value 0.009), shown in Figure 3.9.

Within this group there is an apparent difference between sarcoma and melanoma nodules. Paired nodules 1-4 represent metastatic sarcoma nodules and the mean fractional difference in growth delay for the ultrafractionated group versus the conventionally treated group is 24% (range 11% to 45%). Melanoma nodules are represented by paired nodules 5-13 and in this group the mean fractional difference in growth delay is 11% (range -28% to +45%).

In 5 of the 18 treated paired nodules "ultrafractionation" decreased tumour growth delay. Three of these pairs were NHL; the remaining two pairs were breast cancer and melanoma. Figures 3.2, 3.4B & 3.7A.

There would appear to be a difference in volume between those nodules treated with "ultrafractionation" and those treated with conventional fractionation. If the nodules are ranked according to volume and the difference in volume analysed using the Paired Wilcoxon Ranks Test, the nodules treated with "ultrafractionation" are significantly greater in volume than those treated with conventional fractionation (p=0.002).
SPECIAL NOTICE

DAMAGED TEXT - INCOMPLETE IMAGE
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<tr>
<th>Nodule pair</th>
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<th>Pre-treatment volume (cm³)</th>
<th>Treatment (UF vs CF)</th>
<th>Time to regrowth (days)</th>
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Table 3.1. Details of treated metastatic tumour nodules and regrowth data.

UF = "ultrafractionation" and CF = conventional fractionation.
Figure 3.1 (A-C). Patient 1- metastatic sarcoma. Time to regrowth to original volume for 6 paired nodules. Ultrafractionation = dark bars. Conventional
Figure 3.2(A-C). Patient 2 - advanced NHL. Time to regrowth to original volume for 6 paired nodules. Ultrafractionation = dark bars. Conventional fractionation = light bars.
Figure 3.3. Patient 3 - metastatic sarcoma. Time to regrowth to original volume for 2 paired nodules. Ultrafractionation = dark bars. Conventional fractionation = light bars.
Figure 3.4 (A & B). Patient 4 - metastatic breast cancer. Time to regrowth to original volume for 4 paired nodules. Ultrafractionation = dark bars. Conventional fractionation = light bars.
Figure 3.5. Patient 6 - metastatic malignant melanoma. Time to regrowth to original volume for 2 paired nodules. Ultrafractionation = dark bars. Conventional fractionation = light bars.
Figure 3.6 (A-E) Patient 7 - metastatic malignant melanoma. Time to regrowth to original volume for 10 paired nodules. Ultrafractionation = dark bars. Conventional fractionation = light bars.
Figure 3.7 (A-C). Patient 8 - metastatic malignant melanoma. Time to regrowth to original volume for 6 paired nodules.

Ultrafractionation = dark bars. Conventional fractionation = light bars.
Figure 3.8. Analysis of all nodule data demonstrates a trend towards greater tumour control in the "ultrafractionated" group. $P = 0.14$. UF (dark bars): ultrafractionation. CF (light bars): conventional fractionation.
Figure 3.9. Analysis of sarcoma and melanoma tumour nodules demonstrates a statistically significant growth delay in those treated with the "ultrafractionated" regime. $P = 0.009$. UF (dark bars), ultrafractionation. CF (light bars) conventional fractionation.
3.2. Surrounding Normal Skin - Alterations in Basal Cell Density (BCD)

Basal cell density (BCD) measurements in skin surrounding metastatic tumour nodules irradiated with either 1.5Gy OD or 0.5Gy TDS for 12 consecutive days were available in 8 patients. This resulted in a total of ~2000 images being analysed.

There was a significant reverse fractionation effect, i.e. more loss of BCD, in the low dose arm in only 1 of 8 patients (patient 4). In this patient the BCD versus time relationship showed an enhancement ratio of 3.45 (95% CI indeterminate) and analysis of variance (ANOVA) demonstrated a significant reduction in BCD in favour of the low dose arm at day 28 (p<0.05). See Table 3.2 & Figure 3.13.

The remaining 7 patients showed no evidence of increased cell loss after multiple low doses compared to once daily doses. 1 of these patients demonstrated an enhancement ratio of 1.04 (95% CI 0.66-1.67) suggesting that the treatment regimes were equivalent, the remaining 6 patients demonstrated enhancement ratios of < 1 suggesting greater cell kill per unit dose in the higher dose arm, this reached statistical significance in 4 patients. See Table 3.2 & Figures 3.10 - 3.12 & 3.14 -3.17.

Analysis of variance of BCD at day 26 in each of these 7 patients showed a non-significant reduction in favour of the higher dose arm.

Analysis of the whole patient data set demonstrates an overall enhancement ratio of 0.75 (95% CI 0.61-0.89) See Figures 3.18 & 3.19.
<table>
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<td>8</td>
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Table 3.2: Patient characteristics, diagnoses and "enhancement ratios" from BCD measurements.

A statistically significant result is designated using bold type.
Figure 3.10. BCD data for patient 1. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.41 (95%CI 0.11-0.698).
Figure 3.11. BCD data for patient 2. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.619 (95%CI 0.277-1.019).
Figure 3.12. BCD data for patient 3. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 1.04 (95%CI 0.67-1.67).
Figure 3.13. BCD data for patient 4. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 3.45 (95% CI indeterminate).
Figure 3.14. BCD data for patient 5. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.49 (95%CI indeterminate).
Figure 3.15. BCD data for patient 6. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.526 (95%CI 0.315-0.753).
Figure 3.16. BCD data for patient 7. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.588 (95%CI 0.302-0.911).
Figure 3.17. BCD data for patient 8. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.477 (95%CI 0.391-0.565).
Figure 3.18. Non-normalised BCD data for all patients. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (△) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (○). Error bars denote 95% confidence limits. "ER" 0.75 (95%CI 0.61-0.89).
Figure 3.19. Normalised BCD data for all patients. Multiple low dose radiation exposures (0.5Gy TDS x 12 days) (▲) are compared to once daily conventional exposures (1.5Gy OD x 12 days) (●). Error bars denote 95% confidence limits. "ER" 0.75 (95%CI 0.61-0.89).
3.3. Surrounding Normal Skin - Alterations in proliferative indices (Ki67 & Cyclin A)

The mean pre-radiotherapy values of Ki-67 and Cyclin A labelling indices were $10.05 \pm 0.798$ (SE) and $4.81 \pm 0.696$ (SE) respectively. Repeated assessment of Ki-67 and Cyclin A following irradiation suggested similar responses in both treatment arms. In both schedules the Cyclin A and Ki-67 labelling indices (LI) decrease during the first 10 days and then increase over the next 2–3 weeks. This pattern of response occurred in all patients irrespective of treatment group. When the Ki-67 and Cyclin A were analysed collectively for all 8 patients, the decline in labelling Indices appeared to be slower in the "ultrafractionated" arm compared with the conventional arm and the depression persisted at day 12 whereas the conventionally treated skin had started to recover. See Figures 3.20-3.28.

The Cyclin A: Ki-67 ratio was also assessed at each time point. It was used as a surrogate for accelerated repopulation as it was taken as being representative of the number of cells occupying S and G2 phase in the growth fraction. In the untreated (pre-radiation) cells the Cyclin A positive fraction is expected to be 30 to 50% of the Ki-67 positive fraction. This value in Study 2 was $45.3 \pm 3.01$ (SE). This ratio increased rapidly in the conventionally treated arm, reaching 55% at day 5 and 70% at day 8. The increase in the ratio was not quite so marked in the "ultrafractionated" arm, but still reached ~65% at day 8. At day 26 the ratios were still elevated compared to the pre-radiation values in both treatment groups. See Figure 3.28.
Figure 3.20. Proliferation data for patient 1. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.21. Proliferation data for patient 2. Multiple low dose radiation exposures (△) are compared to once daily conventional exposures (●).
Figure 3.22. Proliferation data for patient 3. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.23. Proliferation data for patient 4. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.24. Proliferation data for patient 5. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.25. Proliferation data for patient 6. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.26. Proliferation data for patient 7. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.27. Proliferation data for patient 8. Multiple low dose radiation exposures (▲) are compared to once daily conventional exposures (●).
Figure 3.28. All patient proliferation data. Ki67, Cyclin A & Cyclin A: Ki67 ratio following exposure to "ultrafractionated" & conventionally fractionated radiation. Error bars denote 95% Confidence Interval.
CHAPTER 4: DISCUSSION OF TUMOUR NODULE STUDY

4.1 Tumour Nodules

"Ultrafractionated" radiotherapy using a regimen of 0.5Gy TDS (interfraction interval of 4hrs) significantly increased time to tumour regrowth in metastatic melanoma and sarcoma tumour nodules (P = 0.009). The fact that analysis of all of the nodules in the study did not yield a significant advantage in favour of "ultrafractionation" is unsurprising and explained by the fact that the group included a number of lymphoma nodules. It is generally accepted that lymphomas are exquisitely radiosensitive and therefore by comparison with the library of cell lines tested in-vitro, unlikely to show LDHRS.

When the data is analysed without the lymphoma nodules, thus including the breast cancer, sarcoma and melanoma nodules, the resultant difference in time to tumour regrowth is statistically significant (p<0.05).

Tumour growth delay is a commonly used end point in radiotherapy and chemotherapy protocols involving xenograft studies and in phase III randomised clinical trials. It is assumed that this is a valid surrogate for tumour cell kill, as a reduction in the clonogen population will lead to a delay in the tumour doubling time. However, this assumes the increase in size is directly related to tumour clonogens and it is known that a tumour mass also harbours several host cells (lymphocytes, macrophages, fibroblasts) also capable of proliferation and which may be killed by radiation. The tumour
regrowth pattern will also depend on the tumour microenvironment, which may be host, or tumour derived depending on the conditions of growth of the tumour; and tumour regrowth may be influenced by the microenvironment independent of which agent is studied. In vivo, a primary tumour and metastasis, from the same tumour, may behave differently to the same agent as different populations of tumour cells may coexist in different proportions at each site. Tumour regrowth, in this situation, may be a reflection of the response of a range of tumour cells, which are heterogenous. Overall, time to tumour regrowth was felt to be a valid endpoint in this study as the lesions studied were relatively small and therefore more homogenous, subcutaneous and all metastases. We felt this minimised the influence of the above factors and were satisfied that volume measurement was suitable in this study. It was also felt to be the most practical in this situation where palliative radiation would be standard treatment.

There are, however, a number of technical issues that should be addressed. The assessment of lesions was performed by tri-dimensional measurement using calibrated callipers. Artefactual measurements are known to occur in this setting with linear calliper measurements overestimating surface nodule size by a median of 23% [100]. In this study, a single investigator performed all the readings, which should eliminate interobserver variation. However, intraobserver variation could not be assessed as the nodules could not be measured blinded and frequent reassessment would have been impractical. In this respect, a 23% overestimation in each dimension would be likely to
have a significant impact when tri-dimensional measurement of surface nodules is used. Circumferential loop planimeter measurements may be more accurate with a median overestimation of 17% and better interobserver and intraobserver variation [100]. This instrument would merit further evaluation in the study of multiple cutaneous nodules treated with various fractionation schedules.

The patients were treated using electron energy with surface bolus to encompass the subcutaneous lesion to appropriate depths. Although the Biologically Effective Dose (BED) of electrons has been calculated at around 0.9, the 10% loss in biological effectiveness is unlikely to affect the outcome of the growth delay parameters as all the nodules were treated in a similar fashion. The treatment depth of the lesions was calculated to ensure full coverage with the 90% isodose contour, with a low probability of a geographical miss at depth. The use of surface bolus (wax) to increase the skin dose and in some cases, to reduce the depth dose, is unlikely to alter the growth behaviour of the nodules in favour of one regime over another.

Therefore, although this study demonstrates a significant increase in time to tumour regrowth it does not provide direct evidence of increased clonogenic cell killing by low dose fractions. We did not establish cell lines from these tumours and hence do not have any in-vitro data to directly support the clinical findings. However, the result strongly supports the existence of a true hypersensitive response to low doses per fraction in radioresistant tumour types clinically and is in accord with what would be expected from
extrapolation of existing in-vitro data in these tumour types. Nevertheless alternative explanations should be considered.

One possible alternative hypothesis is that the low dose radiation caused a change in the local immune response that differentially affected some metastatic tumours. It is well known that low-dose radiotherapy (LD-RT) has a significant anti-inflammatory effect and it has been used to treat a variety of inflammatory conditions such as rheumatoid arthritis, osteoarthritis, abscess and psoriasis [101]. This anti-inflammatory effect is in stark contrast to the pro-inflammatory effect seen following higher doses of radiation and is most marked in the dose range 0.3-0.6Gy/fraction.

Radiation treatment for benign disease became unpopular in many countries following the publication of data showing late harmful side effects [102] and, in particular, an epidemiological study of over fourteen thousand patients who had been treated for ankylosing spondylitis with radiation in the 1930s to 1950s which reported an increased incidence of leukaemia, aplastic anaemia and solid tumours in the irradiated organs [103]. However in many countries RT is still practised as the treatment of choice for such conditions as insertion tendinitis and osteoarthritis [101]. The underlying radiobiological mechanisms of this effect are not understood, but cell death and inhibition of proliferation as seen during treatments for malignant disease are not expected to be involved in the response to these low doses [104]
One postulate is that it may be partly due to a reduction in the production of nitrous oxide (NO) by macrophages [105]. It has been demonstrated that low radiation doses have quite different effects on the functional abilities of activated macrophages compared with higher doses with doses in the range 0.6-1.2Gy being most effective at suppressing NO [105]. A reduction in oxidative stress [106] and a reduction in adhesion of inflammatory cells to endothelial cells [104] have also been implicated.

Taken together, these effects on macrophages, lymphocytes and endothelial cells all oppose the inflammatory process and they are all observed in the low dose region, which correlates closely with clinical experience of anti-inflammatory effects. Schaue et al [106] have recently postulated that the variety of observed radiation effects at low doses may reflect different functional consequences that are specific for both cell type and cellular environment. What triggers the response is a matter of speculation: it may be an unspecific stress response, possibly with the induction of heat shock proteins or it may even relate to the effects of LDHRS on specific non-malignant cell populations, i.e. the involvement of differential induction of cellular repair mechanisms.

It is also postulated that LD-RT induced apoptosis in peripheral blood mononuclear cells (PBMC) [107,108], causes an increase in the secretion of anti-inflammatory cytokines such as IL-10 and a decrease in the secretion of pro-inflammatory cytokines, TNF-alpha, IL-1 and IL-12, from activated monocytes. Interleukin 10 (IL-10), in addition to having potent anti-
inflammatory and immunosuppressive activity, has demonstrated significant inhibitory effects on tumour growth, development of metastases and angiogenesis in a number of tumour types, including melanoma, in-vitro [109,110]. If the radioresistant tumours that we have treated were sensitive to such changes, this could explain the outcomes we observed in the low dose treatment regime.

Another possible explanation for these results that warrants exploration is the oxygenation status of the tumour nodules. Tumour oxygenation is recognised as an important determinant of the outcome of radiotherapy, it being generally accepted that hypoxia has a radio-protective effect, anoxia increases radioresistance by a factor of 2.5-3. Large numbers of clinical and experimental studies have demonstrated that tumour hypoxia has a direct effect on clinical outcome in many tumour types including sarcoma and melanoma [111-118].

There are a limited amount of data regarding the effects of oxygen status on the low dose response of cells, but assessment of the effects of hypoxia on LDHRS in vitro suggest that not only does it persist in hypoxic conditions, but that hypoxic tumours may actually be relatively more sensitive at very low doses than oxic tumours. In the only cell line where this has been studied in detail, Chinese hamster V 79 cells, the data indicate that hypoxic cells are also hypersensitive to low doses of radiation and exhibit an increased radioresistance response, albeit triggered at the slightly higher dose of ~0.7Gy (c.f. 0.5Gy) compared to oxic cells [40]. Mathematical modelling studies have taken these observations one step further. Dasu and Denekamp modelled four hypothetical cell lines using LQ/IR model and
demonstrated that at very low doses per fraction in a fractionated regime, hypoxic protection could be reduced, abolished or even reversed [119]. Further studies by the same group, using more cell lines, have gone so far as to suggest that 0.5Gy fractions may be more effective than oxygen mimetic chemical sensitisers [120]. They suggest that there is an optimum dose around 0.4-0.6Gy where hypoxic protection can be minimised if cells show inducible repair [42]. They concluded that the use of a standard low dose per fraction (of 0.5Gy) for all the cell lines came very close to minimising the hypoxic protection without the need to individualise the regime for each cell line, and that the potential benefit of ultrafractionation in minimising hypoxic resistance is greatest in those cells showing the highest intrinsic radioresistance.

These factors may be relevant to our observations if the melanoma and sarcoma nodules were hypoxic during treatment. Although nodules in the size range included in this study are unlikely to show a significant level of hypoxia, relative hypoxia compared to other tumour types could have increased the effect of low dose fractions compared to larger fractions specifically in these tumours. We have no histological material with which to confirm or refute this. Also, as there was a significant difference in volume between the two groups of nodules, those being treated with “ultrafractionation” being larger (p=0.002), it might be expected that this would effect the outcome, but if anything it would probably be in favour of the conventionally treated nodules as those treated with “ultrafractionation” are more likely to be hypoxic.
Whatever the mechanism, if the hypersensitive response in these radioresistant tumour nodules is real it has far reaching implications as it suggests that there may be a therapeutic advantage to using "ultrafractionated" radiotherapy regimens in the treatment of radioresistant tumour types. Such tumours would include melanoma and sarcoma, which were included in this study, but may also include other tumours that exhibit both clinical radioresistance and a high SF2 in vitro. One tumour type that warrants investigation is glioblastoma multiforme (GBM). It is designated "radioresistant" on the basis of in-vitro data (SF2) [121-124] and in-vivo data (TCD50) [125] and has an appalling clinical outcome with virtually no long-term survivors. "Ultrafractionated" radiotherapy may be applicable to these tumours, as LDHRS has been demonstrated in both glioma cell lines [9,10,11,28,126,127] and xenograft models [10,59].

In summary, in this study "ultrafractionated" radiotherapy appears to confer an advantage over conventionally fractionated radiotherapy in terms of tumour growth delay in metastases from "radioresistant" tumours. This is in accordance with a significant amount of laboratory data that demonstrates LDHRS in tumour cell lines and suggests that the same effect occurs in-vivo.

There are considerable inconsistencies in the literature between the results obtained in-vitro, in-vivo, in animals and clinically. Until now there have been no clinical data to support the hypothesis that radioresistant tumour types respond favourably to low dose per fraction radiation. This study, although small, adds to the body of evidence that point towards the existence of LDHRS in "radioresistant" tumours.
4.2. Normal Skin

4.2.1. Effect on Basal Cell Density

Historically speaking, the first evidence of an enhanced effect of low doses per fraction came from Joiner et al's 1986-study [50] looking at mouse skin. Using a "top-up" experimental design and assessing functional end-points, they demonstrated that at doses of less than 0.6Gy per fraction the LQ model under-predicted the effect of the radiation. This experiment suggested a maximal enhanced sensitivity factor of 2.88 for mouse skin, with maximum enhancement at doses of 0.1-0.2Gy per fraction. This study was the starting point for all subsequent investigation into LDHRS.

The evaluation of epidermal basal cell density in the study of the biological effects of ionising radiation is well documented [6,7,12,64]. Previous studies note a wide distribution of normal basal cell density in both human and animal skin specimens. The reasons quoted for this distribution include inherent biological variation and technical factors such as differences in the size of biopsy, the methods of tissue fixation, cell counting and measurement of the basement membrane length.

The effects of low dose per fraction radiation on normal skin demonstrated in this study need to be seen in the context of skin physiology and the normal response of skin to radiation. As stated earlier the epidermis is a constantly renewing, hierarchical type of tissue with cellular proliferation.
occurring in the basal and suprabasal layers [78,82] and having a
generation time of about 2.6 days. The minimum transit time for a labelled
basal cell to pass from the basal layer, through the other layers and be shed
from the corneal layer is about 26 days [81]. After exposure to radiation
there follows a dose dependent reduction in stem cells, resulting in a
decrease in the influx of cells into the transit cell compartment. Pre-existing
differentiated cells have an unaltered life span and in the absence of further
proliferation, this would result in thinning of the epidermis with flattening of
the papillae. If the entire differentiated population of epidermal cells is lost
before a new transit cell compartment is re-established from a sufficient
number of stem cells then loss of the skin surface will occur. After a number
days, following irradiation, there is a marked fall in the thymidine labelling
index and the mitotic index of basal cells [82]. The rate of cell loss in pig skin
has been shown to be 2.6% per day following single and fractionated
irradiation and is independent of the final radiation dose [65]. The time to the
appearance of the early skin reaction and its rate of increase are also
independent of the final dose, because they are related to cell loss and this
continues at the pre-irradiated rate. In contrast to this the peak reaction and
the extent of degeneration are related to the total dose: they relate to the
proportion of surviving stem cells.

These models suggest that the rate of reduction in basal cell density
following exposure to radiation is independent of dose given, and cell loss
occurs at a rate equivalent to its natural turnover time i.e. in the absence of
proliferation, differentiation and cell loss will continue at the same rate.
Two previous studies assessing changes in human epidermal basal cell density after different single doses of radiation, suggest a disproportionate decrease in basal cell density when doses of \( \sim 0.5 \text{Gy} \) per fraction are compared with doses of \( \sim 1 \text{Gy} \) per fraction or more [6,63]. The first of these studies by Turesson et al [6] compared the mean BCD response at different total doses given as 0.45 vs 1.1 Gy per fraction in 40 patients. The data suggested a significant reverse fractionation effect, meaning a greater effectiveness per unit dose of 0.45 Gy compared to 1.1 Gy on the cellular density of the basal cell layer. The ratio of the slopes of these dose relationships was termed the "Dose Modifying Factor" (DMF) and a value of 1.8 was quoted. The second of these studies used a very similar technique and compared doses of \( \sim 0.5 \) and \( >1 \text{Gy} \) per fraction [63]. The ratio of the slopes of these dose relationships was termed the "Enhancement Ratio" (ER) and here a value of 2.03 was quoted, equivalent to Turesson's 1.8 (DMF). These results may reflect the existence of LDHRS in vivo but this model has some limitations that are discussed below.

One hypothesis to explain the observed differences in BCD in both these studies is that the cells receiving a higher dose per fraction may initially sustain a higher cell kill in a shorter period, which stimulates early proliferation restoring the BCD to near original levels more rapidly in the higher dose per fraction group. The confounding effects of cellular repopulation could therefore mask a true biological effect on cell killing. However neither of these studies demonstrate a marked relative decrease in BCD for the higher dose fields at earlier time points, which would be expected if this hypothesis were correct. In other studies
[66,128], the onset of cell proliferation in skin has been observed from around day 21 regardless of the dose per fraction used. The majority of patients in the Shah study [63] completed treatment by 28 days with no suggestion of an overshoot in the BCD counts from the high dose side after day 21 i.e. no enhanced proliferation. Immunohistochemistry of proliferative markers, for example Ki-67, quantifying the proportions of cells in cycle would have been useful in corroborating the above data. In the Turesson study [6], the Ki-67 index was assessed - it was observed to be depressed below the value in unirradiated skin during the first 3 weeks of radiotherapy, followed by a significant increase during the next 4 weeks - equivalent in both treatment groups. For both high and low dose fields one conclusion could be that cellular repopulation may actually obscure the effect of the LDHRS phenomenon by rapidly replacing lost cells.

In addition to this, it could be argued that the accumulation of dose in unit time at the basal cell layer is less on the low dose side than on the high dose side. As each fraction was delivered with a 24-hour interfraction interval the resulting cumulative skin dose on the low dose side is always approximately 50% that of the high dose side. A standard plot of BCD versus skin dose for both of these studies demonstrates a reduction in BCD for the low dose side that is about twice that for the high dose side. See Figures 1.7 and 1.8 (Chapter 1)

If, however, the Shah data is re-analysed, plotting the BCD against time from the start of irradiation, this apparent effect is lost, figure 4.1, suggesting no significant sensitisation with low dose radiation.
Figure 4.1. Dose response for basal cell density in skin. BCD versus Time.
Right hip dose >1Gy/fraction, left hip dose ~0.5Gy/fraction. Error bars removed for clarity. “ER” = 0.718 (95%CI 0.659-0.781)
This BCD vs time result could, however, still be interpreted as being consistent with the presence of LDHRS, as reduction in BCD is equivalent at given times on both sides despite the fact that the low dose side has only received half the dose compared to the high dose side. However, the results could also be interpreted as a "non-specific" effect in which the rate of cell loss from the basal layer is independent of the dose given. This interpretation would be consistent with the model discussed earlier and put forward by Hopewell et al using data from the large white pig [129].

The study described in this thesis was initiated primarily to assess the difference between "ultrafractionated" and conventionally fractionated regimes on metastatic tumour nodules, but it also allowed comparison of the effects of regimes of equal dose intensity on normal skin. It was hoped that the information yielded by this study would help to distinguish between the two possible hypothetical models mentioned above, by eliminating time as a confounding factor and by simultaneously assessing proliferation in the basal cell compartment. The results lend support to the second interpretation, i.e. that a sequence of events is triggered following exposure to ionising radiation and that this sequence is independent of the dose given. The fact that the "ER" in this study (0.75) is very similar to that found when the data from the Shah study is re-analysed (0.718) would support this, as would the similar proliferative responses seen in both treatment groups in this study. In summary, therefore, the findings of Turesson [6] and Shah [63] suggest that LDHRS might exist in human skin, but each study was bound by the same limitations i.e. not taking into account the difference in dose intensity. It is interesting to note that in both
these studies, the enhancement ratio ("ER"), as quoted by Shah, or dose-modifying factor ("DMF"), as quoted by Turesson [6], approximate to 2 which is essentially the factor by which the time is different at any particular dose point. When the Shah data is re-analysed, taking the time factor into account, the apparent "reverse fractionation effect" is lost.

In our study we have taken this one step further, by ensuring equal dose intensity and demonstrating similar proliferative responses between the 2 arms, our data would appear to point to some sparing for the "ultrafractionated" arm. This would be in keeping with a normal fractionation effect, due to some repair occurring between fractions.

There are, however, a number of issues that need to be raised in regard to the interpretation of this study, firstly a direct comparison cannot be made with either of the previous studies [6,63] as the doses per fraction in the high dose arms are different (>1Gy & 1.1Gy vs 1.5Gy). This difference is important as 1.5Gy /fraction would be expected to produce a larger effect per unit dose than ~1Gy /fraction which could partially explain the "loss" of the LDHRS effect. Secondly, it may be that the 4-hour interfraction interval used in our study is too short to allow enough time for full restitution of the LDHRS effect between successive fractions or, thirdly, that the 0.5Gy per fraction dose was too great to cause a hypersensitive response, although the same remarks could be made about the other studies [6,63]. In the original work by Joiner et al [50] the effect was most marked at lower doses than those used in this study (0.1-0.2Gy per fraction) and larger gaps were left between treatments (8-hr inter-fraction intervals). More
recently and also using changes in BCD as the endpoint, Dorner et al [55] failed to demonstrate LDHRS in the skin of nude mice using 0.4Gy TDS (inter-fraction interval – 4 hours) x 6 weeks. However, in a follow-up study using 0.2Gy x 6 times per day (inter-fraction interval – 4 hours) to a total dose of 7.2Gy the phenomenon was evident.

Another, although less likely, explanation for this result is that the tumour nodules exert some sort of humoral effect on the surrounding skin causing it to become "stressed" and in doing so "lose" it's LDHRS.

It should be stressed at this stage that basal cell density is not a direct measure of epidermal cell clonogenic survival and therefore may not be the optimal endpoint with which to assess the radiation response of skin. A more appropriate method to determine whether or not LDHRS exists in normal human skin may be that described by Van der Aardweg et al which assesses clonogenic survival following radiation exposure directly [130,131]. They have demonstrated that the use of full thickness epidermal sheets, cultured in vitro, are suitable for the assessment of colony formation following exposure to ionising radiation and that epidermal radiosensitivity can be estimated from quantification of colony numbers.

In summary, data from previous studies supported the existence of LDHRS in normal skin using BCD measurement as the endpoint [6,63]. Our study, which excludes time as a confounding factor, contradicts this evidence, albeit in a limited number of patients. Our data also suggest that even if LDHRS does occur
in skin it is unlikely to be biologically relevant as proliferation is dose independent and easily overcomes any cell loss.
4.2.2 Effect on Proliferation

Human epidermis is a renewing cell population in which the rate of cell production equals the rate of physiological loss. Following an insult, i.e. radiation, the production rate tends to increase and remain high until the damage is repaired. How this process is controlled is not fully understood. To study the mechanism of this response, experimental work has been carried using the so-called "stripping" technique, whereby the stratum corneum layer is repeatedly removed by application of adhesive tape [83]. The period of post-injury recovery has been studied using a variety of techniques, including observation of mitotic figures [83,132,133], autoradiography [134] and flow cytometry [135]. Two possibilities have been proposed.

The first possibility is that, in human epidermis there is a resting G0 population of cells that has the potential to divide and that these resting cells are recruited into cycle from G0 following a certain stimulus [136,137]. Boezeman et al [139], using a mathematical analysis of DNA distribution at different times following injury, have suggested that a G0 population does indeed exist in resting epidermis in vivo and that following "stripping" with adhesive tape these cells are recruited into cycle.

Another possible explanation for the proliferative response is that there is a decrease in cell cycle time, which is brought about by a more rapid transition of cells from S into G2. In other words cells in cycle "speed up" in an effort to compensate for cell loss [139,140].
Ki67 antigen is expressed in late G1, S, G2 and M phase (but not G0) of the cell cycle and can be used as a marker for the growth fraction i.e. the actively proliferating cell pool [85,96]. Although some authors have stated that the fraction of cells showing Ki67 immunoreactivity may overestimate the growth fraction in tumours [97] it is accepted to be representative in normal tissues [96].

Cyclin A is responsible for the progression of cells through S-phase and the transition between G2 and M phases [98,99] and is expressed after the restriction point in G1, i.e. in S, G2/M and the early part of M [99]. Therefore, although also a marker of proliferation, its expression is short-lived in comparison to Ki-67; the results here reflect this, as the proliferative index assessed by Cyclin A was lower than that assessed by Ki-67.

We assessed the Cyclin A: Ki-67 ratio at each time point as a surrogate for accelerated repopulation as it was taken as being representative of the number of cells occupying S and G2 phase in the growth fraction. In the untreated (pre-radiation) cells the Cyclin A positive fraction is expected to be 30 to 50% of the Ki-67 positive fraction. This value in our study was 45.3 ± 3.01 (SE).

This pre-irradiation value of ~ 45 appears quite high when compared with results quoted from other studies, but it is important to note that the cell cycle kinetics of skin are by no means consistent in the literature. Castelijns et al [96] have calculated the S: GF ratio as being ~15%, but they only assessed S-phase cells, counted the cells in all layers of the epidermis and
counted per mm of the epidermis. Another study by the same group using flow cytometric methods calculated the percentage of basal cells occupying S and G2M as 5.5 [141]. A number of other flow cytometric studies quote values of 2.7 - 7.0 for cells in S and G2M [138,142,143,144]. These data are much more in keeping with our value of 4.81.

It is known that the percentage of cells occupying each phase of the cell cycle is effected by a number of external factors, namely age, time of year and part of the body [142]. On top of this, the proliferative response is a very complex issue; it is probably a mixture of perturbation due to cell cycle delays, alterations in cell cycle kinetics and recruitment of cells into cycle: neither Ki67 nor cyclin A may be the ideal markers with which to assess which of these is the main factor.

There is evidence in haematopoetic cells that Ki67 may not be expressed (or at least detectable) during the first transit of recruited cells through the G0 to G1 transition [145], therefore it may not be a sensitive marker of recruitment. It is not known if this is the case in epidermal cells. Also, as cyclin A is expressed in both S and G2 it will be effected by any radiation induced G2 delays and thus its level might not drop. However as Ki67 is not metabolised until mitosis, its expression would also be effected by a G2 delay. The fact that we are not seeing a significant fall in cyclin A relative to Ki67 suggests that higher levels of expression are due to more rapid cell cycling.
Although it is difficult to extrapolate information from static parameters into kinetic explanations, the changes in Cyclin A: Ki-67 ratio following radiation in this study are interesting. They would suggest that although the labelling indices (LI) are falling during the first 5-10 days in both treatment groups, the skin is already compensating, as the fall in Cyclin A LI is not as great as the fall in Ki-67 LI. This would be consistent with either speeding up of the cell cycle of the surviving cells or with cells being recruited into cycle to replace the damaged cells. The expression levels observed are more consistent with an increase in the rate of cell cycling during the initial period where both the Ki67 and the cyclin A fall as if the effect were purely due to cells being recruited into cycle the growth fraction would be increasing. The fact that the ratio is still higher than the untreated skin at day 26 would suggest that the epidermis has still not fully recovered i.e. there is still cell death triggering the process or the "healing" process is not complete.
4.3. Conclusion

In this study "ultrafractionated" radiotherapy appears to confer an advantage over conventionally fractionated radiotherapy in terms of tumour growth delay in radioresistant tumours. This is in accordance with a significant amount of laboratory data that demonstrates LDHRS in tumour cell lines and suggests that the same effect occurs in-vivo.

Until now there have been no clinical data to support the hypothesis that radioresistant tumour types respond favourably to low dose per fraction radiation. This study, while adding to the body of evidence that point towards the existence of LDHRS in "radioresistant" tumours, is small and does not answer the question definitively.

With regard to the effect of multiple low dose radiation exposures on normal skin, our study contradicts the previously available data, and suggests that LDHRS does not occur in skin. Also, given the almost identical proliferative responses seen following "ultrafractionated" radiation and the more conventionally fractionated radiation, even if it does occur it is unlikely to be biologically relevant as proliferation easily overcomes any cell loss.

The combination of these data (tumour nodules and normal skin) suggests a potential therapeutic window that could be exploited, by the use of "ultrafractionated" radiation as a treatment option for tumours traditionally described as radioresistant. Obviously further studies need to be performed
to resolve whether "ultrafractionation" consistently results in greater response in "radioresistant" tumours and to confirm the mechanisms underlying the phenomenon, which may provide a biological marker for tumours that show LDHRS and enable this effect to be fully exploited clinically.

It is also important to bear in mind, that the data presented here only reflect what happens when small volumes of normal skin are exposed to doses of 0.5Gy. Until we have a clearer understanding of the mechanisms that govern the response in this low dose region, these data cannot be used to predict the possible outcome if larger volumes of normal tissue were to be exposed to low doses of radiation (as in IMRT treatments), nor can they be used to predict risk of cancer induction following low dose radiation exposure.
CHAPTER 5: INVESTIGATION OF LDHRS IN Hs633T SARCOMA CELL LINE IN-VITRO

5.1. Introduction

The aim of this thesis is to ascertain whether LDHRS can be demonstrated in-vivo in normal tissues and in metastatic tumour nodules. One of the specific tumour types of interest is soft tissue sarcoma because it is radioresistant both in-vitro and clinically and a number of patients with metastatic sarcoma were included in the study (see Chapter 2). In vitro data using sarcoma cell lines is limited; we therefore carried out a series of parallel laboratory experiments to assess whether these tumours exhibit LDHRS in vitro following multiple low dose radiation exposures. We used a cell line that had previously been shown to exhibit LDHRS to single low doses. The aim was to compare survival after fractionated low dose exposures to equivalent single conventional doses. A similar dose schedule to that in the clinical study was used: 0.5Gy TDS X4 days vs 1.5Gy OD X4 days.
5.2. Materials and Methods

5.2.1 Cell line

The cell line used for the *in-vitro* experiment was **Hs633T**, a human fibrosarcoma cell line. It is a cell line that has previously been shown to exhibit LDHRS to single doses [pers. comm. S. Short]. See Figure 5.1. The cell line was supplied by The European Collection of Animal Cell Cultures (ECACC), ref. 89050201. It was originally established at The American Type Culture Collection (ATCC). This cell line is tumorigenic in nude mice.

5.2.2 Cell maintenance and sub-culture in vitro

Human tumour cell lines were grown as attached monolayers in Corning plastic tissue culture flasks (25, 75 or 150 cm\(^2\)) with canted necks and plug seal caps. Monolayers were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS). The culture flasks were maintained at 37°C in a CO\(_2\)-enriched atmosphere (nitrogen with 5% CO\(_2\) and 5% O\(_2\)) in Queue cell culture incubators with their caps loose. For all cell manipulations the flasks were transferred to a class II safety cabinet. Cells were routinely sub-cultured when they reached 50–70% confluence.
For sub-culture flasks were transferred from the incubator to a safety cabinet and the medium was poured off the cell monolayer into 2.5% hypochlorite solution. The monolayer was washed by adding 5-10 ml phosphate buffered saline (PBS) to the flask, re-capping it and gently rotating the fluid over the growth surface. The PBS was then poured off and 5-10 ml of pre-warmed calcium-free salt solution containing 0.1% trypsin and 0.04% EDTA was added. The flask was then re-capped and shaken gently, then the growth surface was inspected using a Nikon Diaphot inverted microscope under 40× magnification. When the cells could be seen floating in the trypsin solution an equivalent volume (5–10 ml) of pre-warmed EMEM was added to the flask to neutralise the trypsin. The time taken to remove cells from the growth surface varied between cell lines from approximately 0.5–5 minutes. The resulting cell suspension was then decanted into a labelled 20 ml plastic universal, which was then centrifuged at 1000 rpm for 5 minutes. The supernatant was then poured off leaving a cell plug, which was then whirl-mixed momentarily. The resulting cell suspension was then diluted, usually at 1:3 or 1:5, and aliquots were re-plated to plastic culture flasks. The re-plated flasks were then labelled and returned to the incubator.

5.2.3 Mycoplasma testing

All cell lines were tested for mycoplasma contamination before use and at six-month intervals thereafter. To test for mycoplasma cells were sub-
cultured into flasks containing antibiotic-free EMEM in which they were maintained for 3 days prior to testing. They were then removed from the growth surface using EDTA/trypsin and approximately $2 \times 10^2$ cells were inoculated to a covered slide ('Flaskette' chamber slide 177453 Nunc) with 5 ml of EMEM and placed in an incubator for 72 hours. The medium was then poured off and the cell layer was washed with fixative (3 washes with 3:1 methanol: acetic acid at 5 minute intervals) and left to air dry. The cells were then stained with a 0.05 μmml$^{-1}$ solution of Hoechst stain (Benzimidazole 33258 at 0.05 μgml$^{-1}$), which was left in contact for 10 minutes. The stain was washed off with double distilled water and the preparation was then inspected using a Nikon UFX–II microscope under UV illumination. The preparation was carefully inspected for bright cytoplasmic staining in discrete dots, which represents Mycoplasma contamination. An infection rate of 0.4% can be detected using this method. Lower rates can be detected if a larger number of cells are plated and stained [146].
5.2.4 Flow cytometry and Cell Sorting

(A) The Fluorescence Activated Cell Sorter (FACScan) is an automated cell analyser attached to a computer. Cell analysis is based on the principles of flow cytometry in which cells and cell sub populations are recognised based on their light scattering and reflecting properties when they are illuminated by laser light. Unstained cell populations can be recognised based on cell size (forward scatter of laser light) and granularity (side scatter). Cellular stains, which produce characteristic fluorescence, can also be used to differentiate sub-populations. The cells to be analysed are enclosed in a pressurised saline solution and passed through a flowcell where they are illuminated and generate up to 5 signal pulses simultaneously. These pulses are processed by an analogue-digital converter and stored and then further processed by the computer system for analysis.

In this experiment we used a Becton-Dickinson FACScan for the flow cytometry studies. See Figure 5.2. This FACScan is capable of measuring 5 optical parameters simultaneously; forward and side scatter and 3 different spectral regions of fluorescence. It has an air-cooled 15-milliwatt Argon-ion laser with an excitation wavelength of 488 nm. The sensors consist of 3 high performance photo multipliers with band pass filters of 530, 585 and >650 nm. The computer system displays the optical information as either a frequency histogram which shows the fluorescence intensity plotted against the number of events, or as a dual parameter correlated plot (dot plot) which
displays one parameter plotted against another. These computer-generated cytograms can be further analysed by setting polygons around the populations of interest for which the computer will generate statistical information.

(B) The Cell Sorter (CS) is a flow cytometer with an additional sort facility. In addition to the recognition of cell populations based on optical parameters, the cell sorter enables sub-populations of cells to be selected and individual cells from the selected population to be sorted and collected. It utilises a droplet sorting method in which the cells exit the flow chamber in a jet, which breaks up into regular droplets. The droplets contain the cells of choice, which have been selected using the computer system described above by gating a subset of cells on a cytogram. The cells of choice are charged and passed through a high voltage electrostatic field when they are deflected and so can be sorted as they emerge into a petri dish or flask. The cells are sorted individually and the exact number sorted can be displayed during the sorting process. A droplet sorting method is illustrated diagrammatically in Figure 5.3

In this experiment a Becton-Dickinson FACS Vantage cell sorter was used to sort and count unstained live cells. See Figure 5.4. A sterile cell suspension was introduced into the flow cell and single live cells were selected from the cell population by analysing the cytogram dot-plot of forward against side scatter. This displays cell doublets, debris and single cells as separate regions and the single live cells were selected for sorting.
using the computer system to generate a polygonal area around this population only. As these cells were sorted they were simultaneously counted and the count was displayed on the Vantage console. An exact predetermined number of live cells could therefore be sorted into a dish or flask and used for cell survival experiments. The method is a modification of that described by Durand [5] and used by Wouters and Skarsgard [24,147].
Figure 5.1. Survival of asynchronous Hs633T human sarcoma cells irradiated with 240kVp X-rays measured using the cell-sort protocol. Each data point represents 10-12 measurements. [S. Short, unpublished].
Figure 5.2. Becton-Dickinson FACScan
Figure 5.3. Diagrammatic representation of sorting method. The droplets containing selected cells are electrically charged as they pass through a high voltage electrostatic field and are deflected into a dish or flask.
Figure 5.4 Becton-Dickinson FACS Vantage cell sorter.
5.2.5 Measurement of cell survival after low-dose irradiation using the Cell Sorter

On the day before Cell Sort experiments twenty-four 25-cm² flasks were prepared. 5 ml fresh EMEM was added to each dish or flask and they were placed in an incubator at 37°C in a CO₂-enriched atmosphere (nitrogen with 5% CO₂ and 5% O₂) for 8–12 hours. Human tumour cells were harvested from monolayer growth in culture flasks using trypsin/EDTA, decanted into a plastic universal and centrifuged at 1000 rpm for 5 minutes to produce a cell pellet. The cell pellet was whirl mixed momentarily then 5 ml of fresh EMEM was added to the universal. The resulting cell suspension was passed through a 21-gauge needle attached to a 5 ml syringe 10–20 times to reduce cell clumps and whirl mixed again immediately before sorting. Aliquots of this cell suspension were decanted into 5 ml, round-bottomed polystyrene sample tubes (Becton Dickinson Labware 'Falcon 205L' tubes) which were positioned below the Vantage sampling port. 500–1500 cells were sorted directly into 25-cm² flasks. The number sorted depended on the radiation dose each one would receive. The exact number sorted in each flask was recorded on a standard result sheet and on each flask. Flasks were then placed in a cell culture incubator in a CO₂-enriched atmosphere (nitrogen with 5% CO₂ and 5% O₂) to allow the cells to attach. When all the flasks had been sorted and left for 30–60 minutes to allow cell attachment the flasks were irradiated (see next section for radiation protocol). Following irradiation on each day (days 1-4) triplicates were trypsinised and re-sorted from 2 flasks from each dose group. These flasks or dishes were returned to
the incubator and left for 10-14 days to allow for colony formation. They were then removed from the incubator and the medium was poured off. Approximately 2-ml crystal violet was then added to each flask or dish and left in contact for 20 minutes. This was then poured off and the flasks or dishes were rinsed twice in tap water. They were left to drain and dry at room temperature for 48 hours then the stained colonies could be counted manually using a colony counter. This is summarised in figure 5.5.

5.2.6 Irradiations

In the in vitro experiments irradiations were carried out using a Pantak X-ray unit at 240 kVp with 0.25 mm Cu and 1.2 mm Al filters and HVL 1.3 mm Cu to give a dose rate of 0.2 Gy min\(^{-1}\) for doses less than 1 Gy and 0.44 Gy min\(^{-1}\) for doses greater than 1 Gy. This change in dose rate at low doses was necessary because at doses below 1 Gy the time taken to give each dose at the higher dose rate is very short (less than 7 seconds for 0.05 Gy) and the time taken for the Pantak shutter to open is a significant proportion of this time. Reducing the dose rate reduced this 'shutter error'. In addition the flask(s) were arranged parallel to the shutter to reduce the effective opening time across the field defined by the flasks. No difference in cell survival has been found at these doses for changes in dose rate at low doses [13]. Flasks were irradiated in a Stuart perspex incubator heated to 37°C. This thermostatically controlled plastic housing can be kept at constant temperature whilst in position below the Pantak head. A plastic tray with cut-outs for 6 petri dishes or 6 X 25 cm\(^2\) culture flasks was placed inside the
incubator and dishes or flasks were positioned on this tray in the predetermined positions. If less than 6 flasks or dishes were used the remaining positions were filled with dummy flasks, containing 5 ml of medium. The temperature and atmospheric pressure were recorded at the start of the irradiations and regularly during irradiation and used to calculate the dosimeter divisions per gray for each dose separately. 0 Gy control flasks were positioned inside the incubator for 3 minutes without activating the Pantak unit. The time of starting and finishing each dose was recorded with the flask number(s), dose received and dosimeter divisions. A Pantak X-ray unit is shown in Figure 5.6.

In this experiment the effect of 2 radiation regimes on Hs633T cell line were compared. 0.5Gy TDS (4hr interfraction gap) X 4 days was compared with 1.5Gy OD X 4 days as follows. On day 1, 8 flasks were irradiated with 0Gy, 8 flasks were irradiated with 1.5Gy and 8 flasks were irradiated with 0.5Gy TDS. Four hours after the last irradiation, triplicates were sorted from 2 flasks from each dose group. On day 2, 6 flasks were irradiated with 0Gy, 6 flasks were irradiated with 1.5Gy and 6 flasks were irradiated with 0.5Gy TDS. Four hours after the last irradiation, triplicates were sorted from 2 flasks from each dose group. This protocol was repeated on day 3 with 4 flasks in each dose group and on day 4 with 2 flasks in each dose group. See figure 5.5.
Surviving fractions were calculated for each dose group on each day as follows:

\[
S.F = \frac{\text{P.E of treated observations}}{\text{P.E of untreated observations}}
\]

\[
P.E = \frac{\text{No. Cells observed}}{\text{No. Cells plated}}
\]

Where S.F is surviving fraction and P.E is plating efficiency.

Surviving fraction was then plotted against time for each dose group.
Figure 5.5. Hs633T Split Dose Experiment

Day 0
Hs633T cells grown to 60-70% confluency and plated to T25 flasks as follows
5E5 cells for day 2
2E5 cells for day 3
1E5 cells for day 4
5E4 cells for day 5 at 6 flasks per day
Warm up Pantak

Day 1
Irradiate 8 flasks @ 0Gy
Irradiate 8 flasks @ 1.5Gy
Irradiate 8 flasks @ 0.5Gy X 3 @ 6.00am, 10.00 am & 2.00 pm
4 hr after last treatment (6.00 pm) sort triplicates from 2 flasks from each
dose group and return to incubator X 10-14 days

Remaining flasks

Day 2
Irradiate remaining 6 flasks @ 0Gy
Irradiate remaining 6 flasks @ 1.5Gy
Irradiate remaining 6 flasks @ 0.5Gy X 3 @ 6.00am, 10.00 am & 2.00 pm
4 hr after last treatment (6.00 pm) sort triplicates from 2 flasks from each
dose group and return to incubator X 10-14 days

Remaining flasks

Day 3
Irradiate remaining 4 flasks @ 0Gy
Irradiate remaining 4 flasks @ 1.5Gy
Irradiate remaining 4 flasks @ 0.5Gy X 3 @ 6.00am, 10.00 am & 2.00 pm
4 hr after last treatment (6.00 pm) sort triplicates from 2 flasks from each
dose group and return to incubator X 10-14 days

Remaining flasks

Day 4
Irradiate remaining 2 flasks @ 0Gy
Irradiate remaining 2 flasks @ 1.5Gy
Irradiate remaining 2 flasks @ 0.5Gy X 3 @ 6.00am, 10.00 am & 2.00 pm
4 hr after last treatment (6.00 pm) sort triplicates from 2 flasks from each
dose group and return to incubator X 10-14 days
Figure 5.6. Pantak X-ray machine with a Stewart incubator used to keep the cells at 37°C during the irradiations.
5.3 Results

The results of the experiment comparing the effects of the two radiation regimens on asynchronously growing Hs633T human sarcoma cell line are shown in Table 5.1. The cell line received either 1.5Gy once daily for four days or 0.5Gy TDS (interfraction gap of 4 hours). These data demonstrate no enhanced killing from multiple small doses per day compared with the same nominal dose given as once daily fractions. These data are demonstrated in Figure 5.7. This result is in contrast to that obtained when the same cell line is exposed to single low dose exposures. See figure 5.1.

Of note, however, is that the surviving fractions on day 2 are significantly different, with greater cell kill in favour of the "ultrafractionated" treatment. By applying the single fraction data for Hs633T, we can calculate the predicted surviving fraction assuming LDHRS occurs following each successive fraction in this experiment. These data are plotted in Figure 5.9.
SPECIAL NOTICE

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Table 5.1. Asynchronous Hs633T human sarcoma cells. A result of irradiations over 4 days, given as either 0.5Gy doses at 4-h intervals or 1.5Gy single doses once daily. Surviving fraction = sf, plating efficiency = pe.
Figure 5.7. Survival of asynchronous Hs633T human sarcoma cells. A result of irradiations over 4 days, given as either 0.5Gy doses at 4-h intervals or 1.5Gy single doses once daily. Error bars are SEM. Overall multiple low doses per day did not demonstrate enhanced cell killing when compared with the same dose given as a single fraction. SF = Surviving Fraction.
Figure 5.8. Survival of asynchronous Hs633T human sarcoma cells. A result of irradiations over 4 days, given as either 0.5Gy doses at 4-h intervals or 1.5Gy single doses once daily. SF = Surviving Fraction. The red line demonstrates SF of Hs633T cells assuming LDHRS at each fraction and no proliferation between fractions.
5.4. Discussion and Conclusion

Although the phenomenon of LDHRS is well established there are limited data available on the effect of multiple consecutive low doses. The results obtained in this multifraction experiment are in contrast to predictions from the results of single fraction experiments, assuming a hypersensitive response to each fraction. See Figure 4.8. Hs633T human sarcoma cell line has clearly demonstrated LDHRS to single low dose exposures and the reasons that it has not demonstrated the phenomenon to multiple low dose fractions need to be explored.

The effect of the "adaptive" response on LDHRS and the relationship between it and LDHRS/IRR are uncertain. In classically described "adaptive" responses, a small "priming" dose produces enhanced resistance to a second "challenge" (usually higher) dose [14-17]. It is a distinct possibility that these two mechanisms share the same underlying process and that in some cell lines, in some circumstances an initial "priming" dose may initiate an "adaptive" response with increased radioresistance that abolishes LDHRS to subsequent low doses. In V79 hamster cells, which demonstrate LDHRS, small "priming" doses of radiation have been shown to induce resistance to "challenge" doses down to 0.05Gy delivered six hours later [18]. However this "adaptive" response appeared to be dose dependent, with doses in the range ~0.2Gy being more effective at abolishing "challenge" dose LDHRS than higher doses. In addition to this, cyclohexamide has been shown to inhibit the induction of induced
radioresistance (IRR) in the same cell line, thus demonstrating that protein synthesis is a requirement for the development of IRR. The "adaptive" response in this cell line is also inhibited by cyclohexamide [18]. This lends some support to the theory that these two phenomena are manifestations of the same underlying mechanism. As cyclohexamide inhibits protein synthesis, at least it suggests that both phenomena depend on de-novo protein synthesis, although not necessarily the same proteins.

However, this study [18] was limited to challenge doses of <1Gy and so an evaluation of the response at doses of >1Gy was not possible. Wouters and Skarsgard [7] studied the effects of consecutive low doses on HT29 cells, again a cell line that exhibits LDHRS to single low doses [9,148], and their results, while being consistent with those of Marples et al, offer a different explanation. Like the findings in the V79 cells, irradiation of HT29 cells 4hrs after a priming dose of 0.3Gy resulted in no LDHRS to the second dose. If, however, the response beyond 1Gy was studied it was seen to be, if anything, more sensitive than the unprimed population. Therefore there was no protection afforded by the 0.3Gy "priming" dose in these cells with respect to total doses of >1Gy. At total doses of 0.5-0.8Gy i.e. 0.3Gy "priming" dose followed by up to 0.5Gy "challenge" doses, they reported an increase in resistance relative to the single dose which may be attributed to an "adaptive" response.

Another possible explanation for the contradictory results between single and multiple low dose exposures in this cell line can be made on the basis of
cell-cycle effects. The efficiency and type of DNA repair and hence the radiosensitivity of cells is known to vary with position of cells within the cell-cycle in the high dose range. Short et al [11] initially suggested that similar variations may occur in the low dose range and then went on to study the effects of cell-cycle phase on the low dose response in two glioma cell lines [25]. This study suggested that the low dose response of both these cell lines alters when cells are irradiated during different phases of the cell cycle. In T98G, a cell line that demonstrates LDHRS/IRR, although the phenomenon was present in all phases, it was most marked in the G2 population of cells.

In the U373 cells, which had previously not demonstrated LDHRS/IRR in the asynchronous population, LDHRS/IRR was clearly demonstrated by those cells in G2. They concluded that LDHRS is primarily a response of G2 phase cells and that this response dominates that seen in asynchronous populations. This obviously has implications for fractionated low dose exposures, as the movement of cells into more or less sensitive phases of the cycle will impact on the magnitude of the hypersensitive response. Clearly the movement of cycling cells into more sensitive cell-cycle phases, such as G2, between successive low doses has the potential to markedly enhance the low dose response.

Another potential confounding factor may be the time interval between successive irradiations. In this experiment a 4-hr interval was used, this may be too short to allow restitution of the hypersensitive response, or too long
allowing significant cellular proliferation to occur. It has been known for some time that the phenomenon is dependent on the time interval between fractions. Short et al [20] studied the effects of multiple fractions of radiation on 4 different cell lines (T98G, U87, A7 and U373) and demonstrated that when consecutive low doses are delivered LDHRS only occurs, to each dose, when the doses are separated by certain intervals. In T98G, which demonstrates LDHRS to single irradiations, a second hypersensitive response was only seen when intervals of 2-6 hrs had elapsed, the same was true for A7 (2-8 hrs) and U87 (1-5 hrs), both of which show LDHRS to single low doses. However, in all four cell lines the surviving fraction increased with increasing time between fractions, this was felt to be due to cellular proliferation. In our experiment there was an overnight gap between successive irradiations (16-hrs) and some cell division may have occurred, but this should be equivalent between single and multi-fraction doses. Therefore it would cause a spurious increase to be observed in the surviving fraction, thus obscuring the LDHRS effect. In the same experiments, quoted previously, Short et al studied the effects of cellular proliferation between successive fractions. They demonstrated that qualitatively similar changes in surviving fraction with time between doses occurred in both asynchronous and G1 arrested cell populations. They postulated that although the extent of the LDHRS effect may be modulated by cell cycle position, cell cycle progression is not the underlying cause of the variation in surviving fraction seen following doses separated by differing times; it is more likely due to changes in underlying repair processes.
In another multi-fraction experiment Smith et al [43] studied the effects of fractionated low dose γ-rays on both C3H10T1/2 mouse embryo cells and V-79 Chinese hamster cells. The cells were exposed to 6Gy of γ-rays, given in fractions of 6Gy, 3Gy, 2Gy, 1Gy or 0.3Gy with time intervals of 3 hrs. They concluded that there was no evidence of any increased effect at doses of <1Gy inconsistent with those predicted by the LQ formula. These data must be interpreted in the light of a number of issues. Firstly, it should be noted that a 3-hour interfraction interval was used, which is shorter than that used in other experiments and may not be optimal. Secondly, the response of C3H10T1/2 to single low dose exposures had not previously been investigated—so the lack of HRS following fractionated doses could be explained by the fact that HRS does not occur at all in this cell line. As regards the V79 cell line, although it is known to exhibit HRS to single low doses, the effect is smaller than in other cell lines. Marples and Joiner [13] have shown that in the V79 cell line pre-treatment with x-rays can produce a priming effect that takes 6-24 hrs to diminish. It is therefore not surprising that there is no evidence of LDHRS in this cell line following exposure to multiple low dose fractions separated by only 3-hrs.

In many of the single fraction low-dose experiments that demonstrate LDHRS, doses of 0.4Gy per fraction were used. It may be that the dose of 0.5Gy used in this multiple fraction experiment is too high to elicit the LDHRS response.
Another possible explanation for the results of this experiment is that LDHRS may only occur over the first few fractions of the treatment regime with the effect then wearing off. This would be supported by the fact that at day 2 there is a significantly different surviving fraction for the multi-fraction regime compared with the single fraction regime, but as more time passes this effect disappears.

It is apparent from previous studies that the response to a second dose following an initial dose is dependent on a number of factors, namely dose, cell-line used and the time interval between successive fractions. This experiment merely highlights these issues and does not rule out the existence of LDHRS/IRR in this cell line. It does, however, raise similar questions as previous studies: if the LDHRS/IRR phenomenon is to exploited clinically then the time interval between fractions is as crucial as is the dose given.
6.1 Introduction

6.1.1 High Grade Gliomas (HGG)

Malignant gliomas are the commonest tumours of the CNS accounting for >50% of all such tumours in adults. The majority of these tumours are high-grade tumours (HGG), such as anaplastic astrocytoma and glioblastoma multiforme (GBM) - WHO grade III and IV respectively. These tumours are aggressive, highly invasive and neurologically destructive and are considered to be among the deadliest of human cancers.

Progress in the treatment of these tumours has been slow, in the most aggressive manifestation, glioblastoma multiforme (GBM); the standard of care has remained essentially unchanged for the last two decades: maximal safe surgical resection followed by postoperative radiation therapy ± chemotherapy. The prognosis of patients with GBM remains dismal despite improvements in neurosurgical and radiotherapeutic approaches [149], with the median survival being in the range of 9-12 months. Clearly there is a need for novel therapeutic approaches in order to improve survival rates in this patient population.
In the absence of a universally accepted staging system the prognosis of these tumours is based on a variety of clinico-pathological factors such as age, performance status and histology. By using these parameters these patients can be broadly classified into favourable and poor prognosis groups. Patients in the favourable prognosis subgroup are younger, have a good performance status and have a median survival of 12-24 months. Conversely in the poor prognosis group median survival is much reduced, being in the region of 6-9 mths.

6.1.2 Current Management Options

(A) Surgical resection

To date no prospective randomised controlled trials comparing surgical resection with no resection have been carried out. A number of investigators have retrospectively analysed the relationship between extent of surgical resection and outcome [150-152] and the consensus of opinion is in favour of more extensive resection where possible. Simpson et al [150] found median survival rates of 11.3 mths following complete resection, 10.4 mths following partial resection and 6.6 mths following biopsy alone. Other investigators [151,152] have reproduced these results. However, these studies are retrospective and as such are subject to selection bias in that the extent of the resection is influenced by the size of the tumour and the patient's general condition and performance status.
(B) Postoperative Radiation Therapy

Conventional Fractionation

Surgery alone achieves median survival rates of only ~14 weeks in GBM [149]. Two large studies have compared best supportive care with post-operative radiotherapy [153,154]. The BTSG 69-01[153] study showed an improvement in median survival from 14 weeks to 36 weeks in favour of radiotherapy while Kristiansen et al [154] found median survival in the patients who received radiotherapy to be 10.8 mths compared with 5.2 mths in those patients managed with best supportive care. Post-operative radiation treatment therefore remains standard therapy. Because of the fact that >90% of these tumours will relapse at or immediately adjacent to the primary site, a number of studies have been instituted to assess the need for whole brain radiotherapy (WBRT). These studies demonstrated no significant difference in survival rates for WBRT compared with local field treatment, which is now standard [155,156].

With regard to the issue of radiation dose, the evidence supports total doses of 50-60Gy using fractions of 1.8-2Gy. Of a number of studies assessing dose only the MRC (UK) study [157], which compared 60Gy in 30 fractions with 45Gy in 20 fractions, has shown a statistically significant benefit in favour of the higher dose. The joint ECOG/RTOG study [158] comparing 60Gy with 70Gy found no advantage to the increased dose, nor did the
addition of a brachytherapy boost to 50Gy in 25 fractions (ensuring a peripheral tumour dose of at least 60Gy) confer any advantage [155].

**Altered Fractionation**

The main aim of hypofractionation is to achieve equivalent tumour control with a shortened radiation regimen. The risk with this type of treatment is that there may be an increased incidence of late radiation morbidity. In the sole randomised study addressing this issue Glinski & colleagues [159] compared conventional RT with a hypofractionated regimen (3 courses of RT separated by a one-month intervals - the first 2 courses were 20 Gy in 5 fractions over 5 days to the whole brain, and the third a 10 Gy 'boost' over 5 days). They found at a preliminary analysis that the 2-year survival for GBM was significantly better in the hypofractionated arms (23% vs. 10%, p< 0.05).

Another option is to hyperfractionate i.e. to reduce the fraction size and/or to accelerate the treatment i.e. to give more than 1 treatment per day. A number of studies [160-173] have been carried out over the last decade comparing hyperfractionated and/or accelerated regimes with conventionally fractionated regimes in HGG and, in general the results have been disappointing. Only a single randomised trial has shown an improvement in median survival time in favour of the accelerated, hyperfractionated arm [168] and even then the gain in median survival was less than 3 months.
(c) Postoperative Radiation and Chemotherapy

Several studies have addressed the role of additional chemotherapy with postoperative radiotherapy with conflicting results. A meta-analysis of 16 randomised trials of radiotherapy +/- chemotherapy [174] suggested an improvement in median survival in favour of the radio-chemotherapy patients (12 months versus 9.4 months), but the prognostic factors were not comparable and there was a greater number of young, well patients in the combined treatment group. Brada et al [175] in the largest MRC trial to date found no conclusive evidence to support the addition of chemotherapy to radiotherapy in the treatment of HGG.
6.1.3 Poor Prognosis High Grade Gliomas (HGG)

As stated earlier, due to the lack of a universally accepted staging system the prognosis of these tumours is based on a variety of clinico-pathological factors including age, performance status and histology.

Most of the clinical trials studying high-grade gliomas have been carried out in young, good performance status patients, using standard management with surgery plus radiotherapy with the poor prognosis patients being excluded. However in the poor prognosis group of patients, who comprise 60% of patients with high-grade gliomas the same management may not be appropriate. These patients are usually older or have a poor performance status and the median survival is in the region of 6-9 mths – they correspond to the RTOG [176] recursive partitioning analysis group V and VI and have MST of 4.8 months.

The optimum treatment for these patients is not well defined – some practitioners treat with shortened courses of radiotherapy and others recommend symptom control with best supportive care only.

No randomised studies have specifically studied the effects of postoperative radiotherapy in poor prognosis high-grade glioma patients. In the studies quoted earlier [153,154] an accurate assessment of the effects of radiotherapy on these patients cannot be made because as the studies were not stratified according to prognostic factors.
A small number of retrospective studies have shown an improvement in median survival in patients with poor prognosis HGGs who received adjuvant radiotherapy when compared with those who received no radiotherapy (23-45 weeks vs. 6-9 weeks, p< 0.05) [177,178].

With regard to altered fractionation regimes, although there are no randomised trials comparing conventional radiotherapy with hypofractionated radiotherapy in these poor prognosis patients there is a significant amount of level III and IV evidence to suggest that hypofractionated radiotherapy may be equivalent [179-187]. There are no studies assessing the effects of hyperfractionated or accelerated regimens in the poor prognosis patient group. It is apparent, therefore, that there is not a clearly defined optimum treatment for poor prognosis high-grade glioma patients, in whom standard approaches yield generally poor results.
6.1.4 "Ultrafractionation" as a Feasible Treatment Option in High Grade Glioma (HGG)

It is widely accepted that the inherent radioresistance of some tumours is an important factor limiting the radiocurability. In vitro and clinical radioresistance in gliomas has been well-documented [123,124,188,190,191] and so any means of rendering gliomas more sensitive to radiation would be expected to improve local control. Ideally this would use a means that increased the effective dose to the tumour without increasing the toxic effects on the surrounding normal tissues.

By using an ultrafractionated radiotherapy regimen this may be possible, if LDHRS is a phenomenon specific to radioresistant tumour cells and does not occur in normal neural tissue.

(A) LDHRS in Glioma

There is a growing body of evidence to suggest that glioma may be a tumour that exhibits LDHRS and therefore may be suitable for treatment with an "ultrafractionated" radiation regime.

LDHRS has been demonstrated in significant numbers of glioma cell lines following single low dose exposures [6,7,11] and Short et al [20] have studied the effects of multiple low dose fractions in a number of radioresistant human glioma cell lines in vitro. They found LDHRS to be present in 5 of 6 cell lines tested; these 5 cell lines had previously exhibited LDHRS
to single low dose exposures. They suggested that LDHRS was a common, if not universal, finding in radioresistant human glioma cells lines and concluded that a fractionated course of very low doses produces increased cell kill when applied to radioresistant tumour cells *in-vitro* given an appropriate inter-fraction interval. They suggested that the same phenomenon might occur *in-vivo* leading to increased cell killing per gray in radioresistant tumours.

In addition to this, Beauchesne [10] studied the effects of an "ultrafractionated" regimen on 3 further glioma cell lines and reported a dramatic decrease in cell survival in 2 of these with the "ultrafractionated" regime – both of these cell lines had previously demonstrated a hypersensitive response to single low dose fractions.

The data for a hypersensitive response in glioma *in vivo* following low radiation doses are conflicting. Short [59] compared the effects of 0.4Gy TDS (interfraction interval of 6hrs) with 1.2Gy OD, each for 30 days, on a T98G glioma tumour xenograft in nude mice. T98G is a cell line that has demonstrated LDHRS to both single fraction and multiple low dose fractions in in-vitro cell survival assays. The results of in-vivo "ultrafractionation" demonstrated an increase in mean growth delay in favour of the "ultrafractionated" regime (313.68 days vs 138.39 days) but this did not reach statistical significance. Similarly, Beauchesne [10] compared the effects of 0.8Gy TDS (interfraction gap of 4 hours) with the same biologically equivalent dose in 2Gy fractions OD on a G152 glioma xenograft.
re-growth rate in the mice treated with the "ultrafractionated" regime was half that in the mice treated conventionally (p<0.05).

In contrast to these data, Krause et al [60] compared the effects of an "ultrafractionated" regime (0.4Gy TDS X 6 weeks) with a more conventionally fractionated one (1.68Gy OD X 30 fractions) using an A7 glioma xenograft in nude mice. They demonstrated a significant improvement in local tumour control in the conventionally treated tumours (p<0.05). Of note, although the A7 cell line exhibits LDHRS to single fraction low doses, the effect following multiple fractionated low dose exposures was much smaller than would be predicted from single dose studies [pers. comm. S Short]. This may explain the lack of effect in-vivo.

Therefore, the same group proceeded to repeat the in-vivo experiment using a T98G glioma xenograft, and the early data, also, appears not to demonstrate an advantage to the "ultrafractionated" regime [personal communication - M. Baumann].

(B) LDHRS in Normal Brain Tissue

Although HRS has not been investigated in brain tissue, studies examining rat spinal cord have shown no evidence of increased radiosensitivity with doses of 0.4Gy per fraction. In fact the reverse may be true, with more sparing than would be predicted by the LQ equation with low doses per fraction [57]. This would suggest that using "ultrafractionated" treatment
regimens to treat malignant brain tumours would allow large fields to be treated without increasing toxicity to normal brain. Using the experimentally derived induced repair parameters for cell lines that demonstrate LDHRS in vitro, it can be shown that an ultrafractionated regimen could be almost twice as effective as if the same total dose were given as 2Gy per fraction. For example, if T98G were the target tumour and kidney the critical normal tissue, 141 fractions of 0.5Gy giving a total dose of 70.5Gy would be equivalent to 117Gy in 2Gy fractions to the tumour and 60Gy to the normal tissue [6]. This increased effective tumour dose in the absence of increased toxicity to surrounding brain tissue would result in an increased therapeutic ratio.

In summary, these data are conflicting, but when added to the results of the tumour nodule study (chapter 3, [189]), which showed a statistically significant increase in tumour growth delay in "radioresistant" tumours following an "ultrafractionated" regime, they suggest that there may be a therapeutic advantage to using "ultrafractionation" in the treatment of HGG: a tumour that exhibits clinical, in-vitro and in-vivo radioresistance [123,124,125,190,191].

It was against this background, and given that there is no generally acceptable treatment for poor prognosis HGG that a feasibility study was initiated investigating the use of an "ultrafractionated" regime in this patient population. It was anticipated that this would lead to a larger Phase II study,
with the aim of ultimately performing a randomised study comparing an "ultrafractionated" regime with a standard palliative radiation regime.

### 6.1.5 Imaging Studies

Imaging studies provide an objective method of quantifying tumour response to treatment. As well as measuring tumour shrinkage (volume response to treatment), functional (physiological) parameters can also be assessed. Objective tumour shrinkage has long been widely adopted as a prospective end point for definitive clinical trials used to estimate the benefit of a treatment in a specific group of people, but a change in size may be delayed chronologically, often not becoming apparent until late in treatment or after completion of treatment. Reports from the functional imaging literature suggest that metabolic and physiological changes in tumour precede size changes. Therefore, functional imaging studies have the potential to provide early evidence of treatment effects and these may then be used as surrogate end points [192].

Commonly, tumours are characterised by neovascularisation and increased angiogenic activity. [193-195] As a consequence, tumours may have a high proportion of immature and therefore hyper-permeable blood vessels. It has been demonstrated in a number of animal models [196,197] that DCE-MRI can be used to quantify microvascular permeability in tumours.

Unlike conventional enhanced MRI, which simply provides a snapshot of enhancement at one point in time, DCE-MRI permits a fuller depiction of the
wash in and wash out contrast kinetics within tumours, and therefore provides insights into the nature of the bulk tissue properties and blood flow.

In most tissues except the brain, testes and retina, the contrast agent rapidly passes into the extravascular-extracellular space (EES, also called leakage space - \(v_e\)) at a rate determined by the permeability of the microvessels, their surface area and by blood flow. In tumours, typically 12-45% of the contrast media leaks into the EES during the first pass [198]. The transfer constant \((K^{\text{trans}})\) describes the transendothelial transport of low molecular weight contrast medium. Three factors determine the behaviour of low molecular weight contrast media in tissues during the first few minutes after injection; blood perfusion, transport of contrast agent across vessel walls and diffusion of contrast medium in the interstitial space. If the delivery of the contrast medium to a tissue is insufficient then blood perfusion will be the dominant factor determining contrast agent kinetics and \(K^{\text{trans}}\) approximates to tissue blood flow per unit volume [199]. This condition is commonly found in tumours. See Figure 6.1.

As low molecular weight contrast media do not cross cell membranes, the volume of distribution is effectively the EES \((v_e)\). Contrast medium also begins to diffuse into tissue compartments further removed from the vasculature. Over a period of several minutes to hours, the contrast agent diffuses back into the vasculature from where it is excreted (usually by the kidneys). When capillary permeability is very high, the return of contrast
medium is typically rapid resulting in faster washout as plasma contrast agent concentrations fall.

MRI sequences can be designed to be sensitive to the vascular phase of contrast medium delivery (so-called T2* methods which reflect on tissue perfusion and blood volume) [200,201]. T1-weighted sequences are sensitive to the presence of contrast medium in the EES and thus reflect microvessel perfusion, permeability and extracellular leakage space.

Susceptibility-weighted (T2*-weighted) spin-echo sequences are more sensitive to capillary blood flow compared with gradient-echo sequences, which incorporate signals from larger vessels [202]. The degree of signal intensity loss is dependent on the vascular concentration of the contrast agent, microvessel size [203] and density.

Tracer kinetic principles can be used to provide estimates of relative blood volume (rBV), relative blood flow (rBF) and mean transit time (MTT) derived from the first-pass of contrast agent through the microcirculation [200,201,204]. See Figure 6.5. MTT is the average time the contrast agent takes to pass through the tissue being studied. The most robust parameter that can be extracted reliably from first pass techniques is rBV, which is obtained from the integral of the data time series during the first pass of the contrast agent [205]. Absolute quantification of T2*W DCE parameters can be obtained by measuring the changing concentration of contrast agent in the feeding vessel, and in this way, quantified perfusion parameters in
normal brain and of low grade gliomas have been obtained [206,207]. From a practical perspective, it is not always necessary to quantify T2*-weighted DCE-MRI data to obtain insights of the spatial distribution of tissue perfusion. Simple subtraction images can demonstrate the maximal signal attenuation, which in turn has been strongly correlated with relative blood flow and volume in tumours (208,209). See Figure 6.6.

DCE-MRI was chosen as an appropriate investigative tool in this study as it was felt that it measured useful surrogate end-points for the evaluation of the efficacy and potential toxicity of "ultrafractionated" radiotherapy, as well as producing novel data on the radiation response of neural tissues.

6.2 Methods and Materials

This study was designed to investigate the feasibility of using an "ultrafractionated" treatment regime to treat high-grade gliomas (HGG). The term "ultrafractionation" has been used to define radiotherapy delivered at 0.5Gy per fraction in multiple fractions per day. In this study we used an "ultrafractionated" regimen with a 4-hour inter-fraction interval. Ethical approval was granted from Watford and Mount Vernon Hospital NHS Trust Ethical Review Committee. The patients receiving the "ultrafractionated" treatment were admitted to the hospital for the duration of their treatment.
6.2.1 Patient Selection

Patients with a histologically proven GBM, who were deemed poor prognosis on the basis of age alone (>60 years), or had KPS of >70 were deemed eligible for the study. Other eligibility criteria included

- A supratentorial tumour
- Able and willing to give informed consent.
- No previous brain radiation.
- No previous chemotherapy.
- RTOG Prognostic Class V or VI (Median Survival time-4.8 months). See Table I [176].

Patient numbers

It was hoped to recruit 3 patients into this feasibility study, before recruiting patients into a larger Phase II study. However, over a period of twelve months only 2 patients with HGG who fitted the inclusion criteria and in whom palliative radiotherapy was deemed the appropriate treatment option were enrolled. The Phase II study would be designed in such a manner as to detect a significant increase in MST.

6.2.2 Investigations

After bolus IV administration of contrast material, multiple images were obtained in rapid succession to evaluate the dynamics of tumour enhancement. The results were then analysed in a quantitative method to
quantify contrast uptake and, in turn, change in tumour perfusion as they were affected by the radiotherapy. In addition to T1 and T2 weighted images, other measures obtained included relative blood flow (rBF), relative blood volume (rBV), permeability surface area product (Ktrans), extravascular-extracellular leakage space (ve), mean transit time (MTT) and proton density (PD). Such measurements can be performed together with morphological information within 40 minutes. The scanning protocol is described below.

(A) Scanning protocol

- MR examinations were performed prior to and following "ultrafractionated" radiation treatment on a 1.5T Siemens Symphony scanner.
- Initial T1 and T2 weighted anatomical scans were performed to select an imaging plane through the centre of the tumour.
- Eight spoiled gradient-echo images of the central slice position were acquired with a range of different echo times (TE 5-75ms, TR 100ms, flip angle 40°, slice thickness 8mm) and the rate of signal decay R2* was calculated
- A proton density weighted (PD) image was acquired (TR 350 ms, TE 4.7 ms, flip angle 40°, slice thickness 8mm).
- Dynamic T1-weighted images were acquired at a time resolution of 12 seconds for 40 measurements (TR 11 ms, TE 4.7 ms flip angle 40°, total imaging time 8 minutes 5 seconds). Gadopentetate dimeglumine (Gd-DTPA)
was injected IV using a power injector (dose 0.1mmol/kg) at 4 ml/s during the fifth acquisition.

- Tumour regions of interest (ROIs), defined on the contrast-enhancing regions on early T1-weighted subtraction images (100 seconds), were drawn.
- Signal intensity was converted to T1 relaxation rate values using the PD image, in conjunction with a calibration experiment involving phantoms with known T1 relaxation rate values.

Gd-DTPA concentration was calculated for each pixel during the dynamic acquisition from the equation:

$$C_t(t) = \frac{1}{T_1(t)} - \frac{1}{T_1(0)} R_1$$

where $C_t(t)$ is the tissue Gd-DTPA concentration at time t after injection, $T_1(0)$ is the baseline tissue $T_1$, $T_1(t)$ is the $T_1$ at time t and $R_1$ is the longitudinal relaxivity of protons in vivo due to Gd-DTPA. The Gd-DTPA concentration-time curve was fitted to the Tofts and Kermode model of permeability$^{10}$ and values for $K_{\text{trans}}$ and $v_e$ were calculated.

- Dynamic $T_2^*$ images were acquired at a time resolution of 2 seconds for 60 measurements from a single slice position through the centre of the tumour (TR 30ms, TE 20ms, flip angle 40°, total imaging time 2 minutes). A second bolus of Gd-DTPA was injected IV using a power injector (dose 0.2mmol/kg) at 4 ml/s during the tenth acquisition. The relative maximum signal drop (rMSD), which has been strongly correlated with blood volume within tumours, was calculated from the signal intensity-time curve.
• A gamma-variate function can be fitted to the resultant signal intensity-time curve and maps of semi-quantitative relative blood volume, flow and mean transit time can also be derived.

(B) Scanning schedule

Scan 1+2: Pre-treatment (RT) - 2 scans within 5 days in the week leading up to the start of the radiotherapy to assess the reproducibility of kinetic parameters.

Scan 3: After completion of treatment (~4-6 weeks) to assess response (morphological and kinetic). Kinetic analysis to be done on an individual patient and group basis after calculating appropriate repeatability statistics from scans 1+2.

Scan 4: 12 week after completing treatment to assess further response (morphological study only)

The steroid dose was maintained during the period of the MRI studies (Scans 1-3) starting 1 week prior to the 1st DCE-MRI

6.2.3 Radiation Protocol

Dexamethasone 8mg per day was prescribed for all patients at initial assessment. This was because dexamethasone can alter the findings of the
DCE-MRI and this ensured that all patients could be assessed in the same fashion.

(A) Radiation Dose

Patients entered into the study received an "ultrafractionated" treatment. The standard palliative treatment protocol at this institution is 30Gy in 6 fractions given over 2 weeks. The "ultrafractionated" regimen was calculated to be biologically equivalent to the above "standard" protocol, from a tumour point of view, and is \(0.5\text{Gy TDS} \times 29\text{ days}\). See Appendix I. If LDHRS occurs in glioma cells in vivo to the same extent it does in vitro, then this is equivalent to 73.1Gy

(B) Radiation Treatment Plan

Patients were simulated in an immobilisation mask. The treatment was planned using conventional methods using CT and/or MRI scans to define planning target volumes. The patients were treated supine, using 2 parallel-opposed fields, ensuring appropriate margins around the tumour plus surrounding oedema (at least 3cm). The dose was prescribed to the mid-plane and single slice CT films were taken to ensure that the target volume was encompassed by the 95% isodose. The patients were treated using 6MV photons. See Figures 6.2 & 6.3.
6.2.5 End Points

- Feasibility of the "ultrafractionated" treatment.
- Feasibility of the scanning protocol.
- Overall survival (OS)
- Toxicity of "ultrafractionated" treatment
<table>
<thead>
<tr>
<th>Class</th>
<th>Age (yrs)</th>
<th>Histology</th>
<th>KPS (App V)</th>
<th>Mental status</th>
<th>Symptom Duration</th>
<th>Surgery Deficit</th>
</tr>
</thead>
<tbody>
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<td>AA</td>
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<td>AA</td>
<td>70-100</td>
<td>&gt;3/12</td>
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<td>&lt;50 &lt;50</td>
<td>AA GBM</td>
<td>90-100</td>
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<tr>
<td>IV</td>
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<td>GBM AA GBM</td>
<td>&lt;90 70-100</td>
<td>&gt;3/12 PR/CR</td>
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<tr>
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<td>&gt;50 &gt;50</td>
<td>GBM GBM</td>
<td>70-100 70-100</td>
<td>PR/CR</td>
<td>Yes Biopsy</td>
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<tr>
<td>VI</td>
<td>&gt;50 &gt;50</td>
<td>GBM GBM</td>
<td>70-100 &lt;70</td>
<td>Abnormal</td>
<td>Biopsy</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6.1. RTOG Prognostic Class** – based on RPA – RTOG [176]
Injection of IV contrast medium (e.g., Gd-DTPA)

Whole body interstitial space

Red Cell Fraction

Blood Plasma

$K_{trans} =$ Transfer constant

$K_{ep} =$ Rate constant

Tumor interstitial space ($v_e$)

Renal excretion

Figure 6.1. Body compartments accessed by low molecular weight, gadolinium containing contrast media inject intravenously
Figure 6.2. Plain X-ray (lateral) simulation film of patient 1. Parallel opposed lateral fields. The field margins are defined by most inferior light field and most anterior and posterior vertical light fields. Iso-centre is arrowed.
Figure 6.3. Patient 1 being placed into immobilisation device and ready for treatment
6.3. Results

Ultrafractionated radiotherapy was administered to 2 patients (1M: 1F) over a period of 12 months. Both of the patients had histologically proven grade IV glioma (GBM). The patient demographics are presented in table 6.2. Treatment was commenced at a mean time of 6.5 weeks from histological diagnosis (range 3-10 weeks) and 13.5 weeks from onset of first symptom (range 13-14 weeks). Both patients completed "ultrafractionated" radiation treatment within the specified treatment period, with no interruptions. Table 6.3

"Ultrafractionated" radiation therapy was generally well tolerated with no evidence of worsening cerebral oedema, as measured by steroid requirement. The only toxicity observed was alopecia: Patient I and Patient 2 developed grade II and III alopecia respectively during the course of their treatment. In addition Patient 2 developed steroid induced deterioration of his co-existent non-insulin dependent diabetes mellitus (NIDDM). This had previously been controlled with Metformin 500mg BD, but following initiation of Dexamethasone treatment necessitated the addition of Gliclazide 80mg mane and 120mg nocte in addition to Insulin given on a PRN basis.

Mean overall survival (OS), as measured from date of diagnosis, was 16.5 weeks (range 13-20 weeks).

Both patients tolerated the two pre-treatment MRI scans with no problems. Patient 1 was too unwell for the first post-treatment scan (at 4-weeks) and
died shortly after, patient 2 tolerated the first post-treatment scan but also
died shortly afterwards. The post treatment scan performed on patient 2
demonstrated significant disease progression with associated cerebral
oedema. In all the scans performed it was possible to obtain all of the
desired images. See figures 6.4 - 6.8.

With respect to the feasibility of using an "ultrafractionated" treatment
regimen in this patient population, all that we can state is that it is feasible in
our institution. It does however require the provision of 2-3 radiographers
three times/day including over the weekends, which obviously has cost
implications for the radiotherapy department.

The scanning protocol lasted 50-60 minutes in each case and, despite this
long duration, appears also to be feasible, although it is not possible to
extrapolate from these earlier scans just how well this patient population
would tolerate later scans of this duration.
<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
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<tr>
<td><strong>Patient demographics</strong></td>
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Table 6.2. Patient demographics
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</tr>
</thead>
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<tr>
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<td>Date RT start</td>
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<td>Date of death</td>
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<tr>
<td>Overall survival (OS)</td>
<td>20 weeks</td>
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</table>

Table 6.3. Scanning, planning and treatment dates.
Figure 6.4. Patient 1 pre-treatment scans. MRI (1).
30 ml of IV contrast Gd-DTPA was given after the 10th data point. First pass T2* susceptibility effects cause marked darkening of the tumour periphery. Darkening of the grey matter of the brain is greater than the less vascular white matter. The first pass and recirculation phases are indicated. Signal intensity changes for 4 regions of interest are shown in the insert (subtraction T2* image of the nadir point for the tumour ROI). An anatomic T2-weighted image at the same slice position is also shown for reference.

This is a typical T2*-weighted DCE-MRI image of a patient with a malignant glioma.
Figure 6.6. Patient 1. Model fitting of T2*-weighted data and parametric map formation.

T2* signal intensity data from figure 2 (tumour periphery) is converted into R2* (1/T2*) and then fitted with a gamma variate function. Parametric maps representing blood flow kinetics (relative blood flow (BF), relative blood volume (BV) and mean transit time (MTT)) are derived on a pixel-by-pixel basis. The computed values of rBV, rBF and MTT for this region of interest are 509, 21.3 arbitrary units and 24 seconds.
Figure 6.7. Patient 2 pre-treatment scans (MRI 1).
Figure 6.8. Patient 2, Treatment + 1 month scans (MRI 3).
6.4. Discussion and Conclusions

This study was initiated primarily to assess the feasibility of an "ultrafractionated" radiation regimen for the treatment of poor prognosis patients with high-grade glioma (HGG). It was planned to recruit patients who were designated poor prognosis purely on the basis of age, i.e. patients with biopsy proven glioblastoma multiforme (GBM) with excellent to good performance status (KPS > 70). It was planned to test feasibility in a small group of patients (i.e. 3) before designing a larger Phase II study. It was also hoped that valuable information on the effects of low dose per fraction radiation on HGG and on normal brain tissue could be evaluated using functional imaging.

Mount Vernon Hospital is a cancer centre with a strong tradition in research into altered fractionation radiation regimens. The CHART (Continuous Hyperfractionated Accelerated Radiation Treatment) and CHARTWEL (CHART Weekend Less) radiation regimes for head & neck cancer and non-small cell lung cancer (NSCLC) were developed and initiated at Mount Vernon. These regimes are used on a day to day basis at the centre and therefore it is acceptable and indeed standard practice for therapy radiographers to treat patients three times daily Mon-Fri and over weekends.

So, although we have demonstrated that "ultrafractionated" radiation treatment for HGG is feasible at Mount Vernon, a centre where thrice daily
and weekend treatment are common place, it is not possible to comment on
the feasibility of this treatment at other centres where this is not the case.

It is not possible to make any comment of the efficacy of the treatment in
this patient population based on the small number of patients treated. The
overall survival values of 13 and 20 weeks are in keeping with what would
be expected in this patient population following "conventional" palliative
radiation treatment. The lack of significant toxicity would suggest that the
treatment is safe, but again it is impossible to comment on any potential late
effects following this treatment.

With regard to the scanning protocol, it has been demonstrated that it is
feasible in these 2 patients, but unfortunately no information on treatment
effect could be ascertained. Patient 2, who had a post-treatment scan, had
unfortunately progressed following treatment so it was not possible to make
any comment on alterations in any of the measured parameters following
"ultrafractionated" radiotherapy.

In conclusion, this study set out purely to assess whether or not it was
feasible to treat patients with HGG using an "ultrafractionated" radiation
protocol. This study demonstrates that this treatment is feasible, albeit with
some limitations. There are significant implications as regards staffing,
hospital admission and cost, which it may not be possible to overcome in
centres other than this one.
In addition to this, to date only 2 patients have been assessed; it is impossible to extrapolate from these to other patients with HGG as these 2 patients may not be representative of the study population as a whole.

This study would suggest, given the bank of in-vitro and in-vivo data for LDHRS in glioma combined with the tumour nodule data and the successful application of "ultrafractionation" to these 2 patients, that one additional patient is assessed from a feasibility point of view. If the treatment proves feasible in this third patient we suggest that the initiation of a Phase II study is justified. Obviously, given the significant cost and resource implications of this treatment, any benefit from "ultrafractionated" radiotherapy would have to be significant to justify general use. This would need to be taken into account when designing the Phase II study, i.e. to set the power to be able to discern a significant increase in median survival in a small group of patients.
Appendix 1

Dose Calculations

- The standard protocol for poor prognosis HGG is
  \textbf{30Gy in 6 fractions over 2 weeks}

- BED was calculated for both tumour cells and normal brain tissue.
  $\alpha/\beta$ Ratio of 10 was assumed for tumour and an $\alpha/\beta$ ratio of 3 was assumed for normal brain tissue.

- The equation \( \text{BED} = D \left(1 + \frac{d}{\alpha/\beta}\right) \) was used, where
  \( D \) = total dose received and \( d \) = dose per fraction

- \( \text{BED (tumour)} = 30\left[1+\frac{5}{10}\right] \)
  \( = 45\text{Gy} \)
  \textbf{Equivalent to 37.5Gy in 2Gy fractions}

- \( \text{BED (normal tissue)} = 30\left[1+\frac{5}{3}\right] \)
  \( = 80\text{Gy} \)
  \textbf{Equivalent to 48Gy in 2Gy fractions}

\textbf{To give equivalent BED to tumour using 0.5Gy fractions}

Then \( 45 = N \cdot 0.5 \left[1+0.5/10\right] \) where \( N \) is the number fractions needed
\( N = 86 \) [29 treatment days]

\textbf{ULTRAFRACTIONATED REGIMEN = 0.5Gy TDS X 29 Days}
Assuming HRS exists in Gliomas

Using the IR model

\[ D = \frac{E}{\alpha_r \left( 1 + \left( \frac{\alpha_s}{\alpha_r} - 1 \right) e^{-\frac{d}{d_c}} \right) + \beta d} \]  

eq.1

Where E is effect, D is total dose and d is dose per fraction.

With \( d = 2\)Gy

\[ D_{2\text{Gy}} = \frac{E}{\alpha_r \left( 1 + \left( \frac{\alpha_s}{\alpha_r} - 1 \right) e^{-\frac{2}{d_c}} \right) + 2\beta} \]

Eq.2

From eq.1 and eq.2

\[ \frac{D_{2\text{Gy}}}{D} = \frac{\alpha_r \left( 1 + \left( \frac{\alpha_s}{\alpha_r} - 1 \right) e^{-\frac{d}{d_c}} \right) + \beta d}{\alpha_r \left( 1 + \left( \frac{\alpha_s}{\alpha_r} - 1 \right) e^{-\frac{2}{d_c}} \right) + 2\beta} \]

- \( \alpha_r = \alpha \) extrapolated from the high (conventional) dose-response, and \( \alpha_s \) is the actual \( \alpha \) derived from the response at very low doses.
- At very low doses, the IR equation therefore becomes a LQ equation with \( \alpha = \alpha_s \), while at high doses the IR equation becomes a LQ equation with \( \alpha = \alpha_r \).
- \( d_c \) is a parameter describing the range of doses over which the transition from LDHRS to IR occurs.
- Using the IR parameters from T98G glioblastoma cells [42] and substituting in the above equation with \( d = 0.5\)Gy.

\[ \frac{D_{2\text{Gy}}}{D} = 1.7 \]

Therefore, if we assume those high-grade gliomas have exactly the same hypersensitivity as glioma cell line then.

\[ D_{2\text{Gy}} = 1.7 \times 43 = 73.1\text{Gy} \]
CHAPTER 7: SUMMARY OF THE CONCLUSIONS OF THIS THESIS

The objectives of this thesis were threefold. Firstly to assess whether or not LDHRS could be demonstrated in normal tissues and malignant tumours and secondly to assess if there was a difference between normal tissues and tumours that could potentially be exploitable for the treatment of tumours classically described as being "radioresistant". If this was the case the third objective was to investigate the feasibility of an "ultrafractionated" radiotherapy regime in the treatment of a primary "radioresistant" tumour.

With regard to the first objective: does LDHRS exist in normal and malignant tissue? The answer appears to be that LDHRS is not exhibited by normal skin, but is exhibited by tumours classically described as being "radioresistant" such as melanoma and sarcoma, using the "ultrafractionated" schedule 0.5Gy TDS (4-hr inter-fraction interval). There are a number of possible explanations for this that have been discussed previously (see chapter 4). However, if this effect is real and consistent it suggests a potential therapeutic window that may be exploitable for the treatment of tumours such as melanoma and sarcoma and others such as glioma.

It was felt that these data coupled with the considerable bank of in-vitro and in-vivo data regarding LDHRS in glioma warranted the initiation of a feasibility study assessing "ultrafractionation" in patients with high-grade glioma. This feasibility study has demonstrated, albeit in a limited number of
patients, that the regime of 0.5Gy TDS x 29 days is feasible in this patient population. However, it must be said that there are significant manpower and cost implications associated with the delivery of this regime, even in a department with a long history of delivering multiple fractions per day radiotherapy. Any subsequent phase II study would need to demonstrate a significant benefit to patients to justify implementation of this regime into standard practice.

With regard to the parallel in-vitro study assessing LDHRS in Hs633T sarcoma cell line: the results are slightly disappointing given the fact that this cell line exhibits LDHRS to single low dose exposures and the clinical study suggests that there is a hypersensitive response to low doses of radiation in metastatic sarcoma nodules. Once again, there are a number of possible explanations for this that need to be considered namely cell cycle effects and interval between fractions. These have been discussed in detail in chapter 5.

This thesis demonstrates that the response of "radioresistant" tumours and normal tissues to fractionated low doses of radiation differs to an extent that may be exploitable clinically for the treatment of these tumours. It also demonstrates that an "ultrafractionated" radiotherapy regime for patients with high-grade glioma is feasible, albeit with certain limitations. In conclusion, therefore, the data generated by this thesis warrant continued investigation into the use of "ultrafractionation" for the treatment of high-
grade glioma through the continuation of the glioma feasibility and phase II studies.
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