THE EFFECTS OF NEOADJUVANT CHEMOTHERAPY
IN HUMAN OSTEOSARCOMA

by

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ABSTRACT

Samples of osteosarcoma examined upon resection or amputation following pre-operative (neoadjuvant) chemotherapy show areas which are "histologically altered", and are neither totally necrotic nor viable. Although the existence of these areas has been acknowledged by several workers, nothing has been published characterising these areas, in order to assess the prognostic indications of these "altered" areas.

The object of the present study was to characterise the nature of these "altered" regions in terms of morphology of chemotherapy-affected cells, differences in extracellular matrix molecules, metabolic status of affected cells, changes in expression of the multi-drug resistance molecule P-glycoprotein, and the recovery potential of affected cells.

Histological examination revealed a very heterogeneous morphology to osteosarcoma, with six distinct histological sub-classes being recognised. In the "altered" regions enlarged cells similar in morphology to "viable" tumour cells were evident in addition to enlarged cells with degenerative changes, and "viable" tumour cells.

Immunohistochemically, it was found that the expression of glycosaminoglycans epitopes was more pronounced in "altered" than in viable regions.

Histochemical assessment of cell death revealed that there were significantly more cells, including the enlarged ones, undergoing cell death in "altered" areas than in viable. Expression of proliferating cell nuclear antigen was also found to be greater in "altered" areas, but was only seen in the "viable" cells, and not the enlarged ones.

Immunohistochemical detection of P-glycoprotein revealed that there was a significant decrease in the expression of the epitope after surgery compared with at biopsy.

In assessing the in vitro recovery potential of "altered" cells in a drug-free environment, no differences were seen between outgrowth of cells from explants of "altered" tissue compared with viable.

In conclusion, "altered" areas do not show a good prognostic indication, since any "viable" cells present in these areas may still have the potential to propagate the tumour.
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# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Contents</td>
<td>4</td>
</tr>
<tr>
<td>List of Tables</td>
<td>7</td>
</tr>
<tr>
<td>List of Figures</td>
<td>8</td>
</tr>
</tbody>
</table>

## Chapter 1 - General Introduction

1.1 - Osteosarcoma                                                    | 10   |
1.2 - Treatment of osteosarcoma                                      | 11   |
1.3 - Evaluation of the effects of chemotherapy                      | 13   |
1.4 - Resistance to chemotherapy                                     | 16   |
1.5 - Cell proliferation                                             | 20   |
1.6 - Cell death                                                     | 24   |
1.7 - Aims                                                            | 27   |

## Chapter 2 - General Materials and Methods

2.1 - Tumour material                                                | 33   |
2.2 - Processing procedure for paraffin wax embedding                | 34   |
2.3 - Processing procedure and specimen manipulation for Spurr's resin embedding for ultrastructural studies | 36   |
2.4 - Processing procedure for hydroxyethyl methacrylate resin embedding for histochemical studies | 37   |
2.5 - Processing procedure for LR White acrylic resin for histochemical and immunohistochemical studies | 39   |
2.6 - Chrome alum-gelatin method for coating microscope slides       | 40   |
2.7 - Histochemical method for the detection of acid phosphatase     | 41   |
2.8 - Antibodies                                                     | 42   |
2.9 - Immunogold silver enhancement method                            | 43   |
2.10 - Tissue culture                                                | 44   |
2.11 - Histochemical assessment of alkaline phosphatase in explant cultures | 46   |
2.12 - Immunofluorescence detection method for explant cultures      | 47   |
LIST OF TABLES

Table 1.1 - summary of results for neoadjuvant chemotherapy for osteosarcoma 15
Table 2.1 - clinical data of patients used in this thesis 35
Table 4.1 - mean labelling profiles in osteoblastic viable areas 98
Table 4.2 - mean labelling profiles in osteoblastic "altered" areas 98
Table 4.3 - mean labelling profiles in fibroblastic viable areas 98
Table 4.4 - mean labelling profiles in fibroblastic "altered" areas 98
Table 4.5 - mean labelling profiles in chondroblastic "altered" areas 99
Table 4.6 - mean labelling profiles in undifferentiated viable areas 99
Table 4.7 - mean labelling profiles in undifferentiated "altered" areas 99
Table 4.8 - mean labelling profiles in epithelioid viable areas 99
Table 4.9 - mean labelling profiles in epithelioid "altered" areas 99
Table 6.1 - clinical data and chemotherapy regimens 143
Table 7.1 - the outgrowth rates and harvesting times of explant cultures from 8 osteosarcoma specimens 158
LIST OF FIGURES

Figure 1.1 - the pathways associated with P-glycoprotein 21
Figure 1.2 - differences in the morphological changes seen in necrosis and apoptosis 28
Figure 3.1a - age-sex distribution for patients 53
Figure 3.1b - site-sex distribution for patients 53
Figure 3.2 - histological sub-classification of osteosarcoma by evaluation of 1μm sections 55
Figure 3.3 - tumour imprints assessed histochemically for alkaline phosphatase 56-57
Figure 3.4 - osteoblastic viable tumour 62-65
Figure 3.5 - osteoblastic "altered" tumour 66-69
Figure 3.6 - fibroblastic viable tumour 70-71
Figure 3.7 - fibroblastic "altered" tumour 72-73
Figure 3.8 - chondroblastic viable tumour 74-75
Figure 3.9 - chondroblastic "altered" tumour 76-77
Figure 3.10 - epithelioid viable tumour 78-79
Figure 3.11 - epithelioid "altered" tumour 80-81
Figure 3.12 - undifferentiated viable tumour 82-83
Figure 3.13 - undifferentiated "altered" tumour 84-85
Figure 3.14 - telangiectatic "altered" tumour 86-87
Figure 4.1a - age-sex distribution for patients 94
Figure 4.1b - site-sex distribution for patients 94
Figure 4.2 - histological sub-classification of osteosarcoma by evaluation of 1μm sections 97
Figure 4.3 - immunolocalisation of GAGs in an "altered" osteoblastic area 102-103
Figure 4.4 - immunolocalisation of GAGs in an "altered" chondroblastic area 104-105
Figure 5.1a - age-sex distribution for patients (HEMA embedding) 112
Figure 5.1b - site-sex distribution for patients (HEMA embedding) 112
Figure 5.2a - age-sex distribution for patients (LR White embedding) 113
Figure 5.2b - site-sex distribution for patients (LR White embedding) 113
Figure 5.3 - PC10 immunolocalisation 118-119
Figure 5.4 - mitotic figures 120-121
Figure 5.5a - median-quartile plot for the percentage of PC10 positive cells 122
Figure 5.5b - median-quartile plot for the percentage of mitotic figures 122
Figure 5.6a - Mann-Whitney test for PC10 123
Figure 5.6b - Mann-Whitney test for mitotic figures 123
Figure 5.7 - cell death in HEMA-embedded material 125-126
Figure 5.8a - median-quartile plot for the percentage of cells undergoing programmed cell death for HEMA-embedded specimens 127
Figure 5.8b - median-quartile plot for the percentage of reactive cells for HEMA-embedded specimens 127
Figure 5.9a - Mann-Whitney test for cell death in HEMA-embedded specimens 128
Figure 5.9b - Mann-Whitney test for reactive cells in HEMA-embedded specimens 128
Figure 5.10 - cell death in LR White-embedded material 130-131
Figure 5.11a - median-quartile plot for the percentage of cells undergoing programmed cell death for LR White-embedded specimens 132
Figure 5.11b - median-quartile plot for the percentage of reactive cells for LR White-embedded specimens 132
Figure 5.12a - Mann-Whitney test for cell death in LR White-embedded specimens 133
Figure 5.12b - Mann-Whitney test for reactive cells in LR White-embedded specimens 133
Figure 6.1 - P-glycoprotein immunolocalisation using the C219 antibody 146-147
Figure 6.2 - the expression of C219 immunopositivity in biopsy and post-surgical sections of the 19 patients used 148
Figure 7.1a - age-sex distribution for patients 156
Figure 7.1b - site-sex distribution for patients 156
Figure 7.2 - explant cultures of osteosarcoma: cell morphology 159-160
Figure 7.3 - explant cultures of osteosarcoma: alkaline phosphatase positivity 161-162
Figure 7.4a - percentage of alkaline phosphatase positive cells in explant cultures from viable osteosarcoma tissue 163
Figure 7.4b - percentage of alkaline phosphatase positive cells in explant cultures from "altered" osteosarcoma tissue 163
Figure 7.5 - explant cultures of osteosarcoma: osteonectin and osteopontin immunopositivity 164-165
1 - GENERAL INTRODUCTION
1.1 - Osteosarcoma

Osteosarcomas (also sometimes referred to as osteogenic sarcomas) are the most common primary malignant tumour of bone (Malawer et al., 1985). Classical osteosarcoma is characterised histologically by proliferating tumour cells that, in most instances, produce osteoid or immature bone. Infrequently, such cells remain so undifferentiated that osteoid or bone is not elaborated, leading to difficulties in tumour classification (Resnick et al., 1985). Among the population as a whole, the incidence of osteosarcoma is low. A study over a 14 year period in the San Francisco Bay Area of all registered cases of osteosarcoma showed an incidence of 21 cases per 100 000 population per year (Katzman and Johnston, 1991). There is a slight male predominance. The Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute (NCI) conducted over the period 1973-1986 showed an incidence of osteosarcoma in males of 3.49 cases per million per year, compared with 2.89 cases in females (Homa et al., 1991). Osteosarcoma typically appears during childhood and adolescence, reaching its highest frequency between the ages of 15 and 19 (Kersjes et al., 1987). When it occurs in patients over the age of 40 it is usually associated with a pre-existing condition such as Paget’s disease or with bone irradiation due to the presence of bone metastases of another tumour (Malawer et al., 1985). Eighty to ninety percent of osteosarcomas occur in the metaphyses of long bones, with the distal femur being the most common site, followed by the proximal tibia and humerus (Dahlin and Coventry, 1967, Meister et al., 1979, Goorin et al., 1985, Picci et al., 1985). The axial skeleton is rarely affected (Uribe-Botero et al., 1977). The cause of osteosarcoma is unknown. The correlation of the age of most patients and the location of most of the tumours with the period of maximum bone growth indicates some relation to increased osteoblastic activity in terms of cell division and function (Goorin et al., 1985).

Diagnosis of osteosarcoma is based upon the direct formation of osseous intercellular substance by the tumour cells in the basic sarcomatous tissue (van der Huel, 1986). With the exception of serum alkaline phosphatase, which is increased in 45-50% of patients, routine laboratory findings are not usually helpful. However, increased alkaline phosphatase per se is not diagnostic because it is also found in association with other skeletal diseases, e.g. fibrous dysplasia and Paget’s disease. Also, alkaline phosphatase may not be detected in neoplastic foci which contain predominantly fibroblastic or cartilaginous tissue (Schajowicz, 1983). Leukocyte count and erythrocyte sedimentation ratio are normal, as are serum calcium and
phosphorous. Bone pain is the most common complaint. Physical examination demonstrates a firm, soft tissue mass fixed to the underlying bone with slight tenderness. There is no effusion into the adjacent joint and motion is normal. Incidence of pathologic fracture is less than 1%, and systemic symptoms are rare (Malawer et al., 1985).
1.2 - Treatment of Osteosarcoma

Before 1972, the prognosis for patients with osteosarcoma was poor and their quality of life compromised by amputation. Five-year survival figures after diagnosis were remarkably consistent - a mean of 19.7% (range 16-23%) in 1286 cases collected from world literature (Friedman and Carter, 1972). This figure of around 20% had remained largely unchanged for 30 years (Dahlin and Coventry, 1967). Historically, the control of primary tumour was achieved in most patients by amputation. However, around 80% of these patients must have had systemic micrometastases at time of surgery, since within 1 to 2 years of diagnosis of osteosarcoma, radiographically evident metastases appeared, usually pulmonary (Uribe-Botero et al., 1977).

Systemic chemotherapy has been shown to eradicate microscopic tumours in animal models. When applied to human patients with childhood mesenchymal tumours such as rhabdomyosarcoma and Ewing’s sarcoma, chemotherapy that produces tumour regression in patients with overt neoplasms is capable of eradicating micrometastatic disease and, therefore, increasing the rate of tumour-free survival (Farber, 1966). Therefore, as new forms of chemotherapy capable of producing tumour regression in patients with metastatic osteosarcoma were identified, they were used in an adjuvant setting (i.e. immediately after the removal of the primary tumour) in an attempt to decrease the relapse rate from micrometastatic disease (Goorin et al., 1985).

Many single agent and combined chemotherapy trials have demonstrated the effectiveness of methotrexate, doxorubicin, cisplatinum and cyclophosphamide in the treatment of osteosarcoma (Bramwell, 1987).

With the improvement in surgical reconstruction techniques (Mankin and Gebhardt, 1985) and endoprostheses (Kotz, 1983) whereby it is now possible to reconstruct a limb after large portions have been resected (thus partially restoring function of the affected extremity), neoadjuvant (pre-operative) chemotherapy was introduced in an attempt to decrease the primary tumour load (Rosen et al., 1976).

There are several theoretical arguments for the use of neoadjuvant chemotherapy (Goorin et al., 1985):
Chapter 1

1 - as stated above, neoadjuvant chemotherapy can produce regression in the primary tumour leading to more effective and less aggressive surgical treatment, i.e. en bloc limb salvage as opposed to amputation.

2 - the time from diagnosis to surgery is normally long enough for substantial enlargement in micrometastases to take place, with spontaneous mutations that produce drug-resistant progeny cells. Also, there is often a delay post-surgery before chemotherapy starts, which again adds to the problem of drug resistance in micrometastases. Thus neoadjuvant chemotherapy would limit the activity, and hopefully obliterate most of the micrometastases, before the susceptibility to chemotherapeutic agents was compromised.

3 - neoadjuvant therapy provides an opportunity to measure the effectiveness of a given treatment against the primary tumour in vivo. This permits the identification of different risk groups and allows alteration of the chemotherapy regimen for patients with the highest risk of relapse (i.e. those with poor responses).

4 - neoadjuvant therapy may decrease the chance that viable tumour will spread during the surgery to remove the primary lesion.

Groups that have included neoadjuvant chemotherapy in their treatment protocols have generally shown a substantial enhancement in the rate of disease-free survival (Table 1.1).
Table 1.1 - summary of results for neoadjuvant chemotherapy for osteosarcoma

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Drugs(^1)</th>
<th>%DFS(^2)</th>
</tr>
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<tr>
<td>Bacci et al., 1993</td>
<td>M,A,P</td>
<td>87</td>
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<tr>
<td>Bacci et al., 1994</td>
<td>M,A,P,B,C,D</td>
<td>82</td>
</tr>
<tr>
<td>Benjamin et al., 1992</td>
<td>A,P</td>
<td>61</td>
</tr>
<tr>
<td>Cassano et al., 1991</td>
<td>A,P,C,E</td>
<td>78</td>
</tr>
<tr>
<td>Link, 1993</td>
<td>M,A,P</td>
<td>63</td>
</tr>
<tr>
<td>Postma et al., 1993</td>
<td>M,V</td>
<td>63</td>
</tr>
<tr>
<td>Pratt et al., 1990</td>
<td>M,A,C</td>
<td>56</td>
</tr>
<tr>
<td>Rosen et al., 1979</td>
<td>M,A,C</td>
<td>75</td>
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<tr>
<td>Rosen et al., 1983</td>
<td>M,A,P,B,C,D</td>
<td>92</td>
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<tr>
<td>Solheim et al., 1992</td>
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<td>Winkler et al., 1993</td>
<td>M,A,P,B,C,D</td>
<td>69</td>
</tr>
</tbody>
</table>

**Key:**

1 - agents used for both pre- and post-operative chemotherapy: M = high dose methotrexate, A = adriamycin (doxorubicin), P = cisplatin, B = bleomycin, C = cyclophosphamide, D = dactinomycin, E = etoposide, V = vincristine.

2 - percentage of patients in study disease-free, with at least 3 years follow-up.
Chapter 1

1.3 - Evaluation of the Effects of Chemotherapy

With the advent of neoadjuvant chemotherapy came the opportunity to assess the response of the primary tumour to the therapy upon resection, and to evaluate the protocol for post-operative chemotherapy.

Various parameters have been looked at in order to make this assessment. Several non-invasive methods have been used to evaluate the response of the tumour during pre-operative chemotherapy including angiography (Bilbao et al., 1990), conventional radiography, computer-aided tomography (CAT) and radionucleotide scintigraphy (Sommer et al., 1987). The majority of the work on assessment of predictive factors has been done by analysing resected tumour specimens.

Several groups have assessed the degree of necrosis seen in resected specimens and based further post-operative therapy on the amount seen, with >90% necrosis being seen as a good prognosis (Rosen et al., 1979, Ayala et al., 1980, Rosen et al., 1982, Salzer-Kuntschik et al., 1983, Kempf et al., 1991, Petrilli et al., 1991).

Picci et al. (1985) observed the predominant sites of viable and non-viable tumour in primary lesions from 50 osteosarcoma patients after neoadjuvant chemotherapy. The study revealed several preferential sites where viable tumour was likely to persist which were: soft tissues, cortex, subcortex, ligaments, and areas in contact with the growth plate or articular cartilage. In contrast, necrosis appeared most concentrated in the central regions of the tumour. This study was carried out in order to assist surgical design of the operative procedure to remove the primary tumour, conserve useful muscle and tissue, and to safely decrease the size of the tumour-free margins on resection. This is of importance in view of a possible tendency to perform a less radical cancer operation under the assumption that chemotherapy has destroyed all or most of the tumour.

The effect of neoadjuvant chemotherapy has been evaluated and expressed as the degree of necrosis observed microscopically in large area sections from surgically removed tumour (Rosen et al., 1979, 1982, Salzer-Kuntschik et al. 1983). An individualised approach, based on the assessment of the degree of necrosis offered a possibility of tailoring the treatment. Thus, patients showing insufficient response to methotrexate were either given a higher dose of the same drug, or were allocated to another chemotherapeutic schedule of maintenance treatment, and this led to apparent success, at least in the short term (Rosen et al., 1982).
The amounts of necrotic/viable tumour tissue may also be prognostic indicators for post-surgical survival. An important question then arises: how much of the necrotic tumour tissue in patients who received neoadjuvant chemotherapy can be attributed to the therapy, and how much is spontaneous necrosis (Misdorp et al., 1988)?

Misdorp (1986) showed that the amount of necrotic tissue in patients with osteosarcoma who had not received neoadjuvant chemotherapy was considerable, but in the pre-treated patients, the amount of necrosis appeared to be significantly larger. He concluded that the mechanisms of necrosis in untreated cases differs from that in pre-treated cases. In the former, the necrosis is apparently associated with lack of blood supply, and thus lack of oxygen, whilst in the latter, the cytostatic action of the drug is responsible for cell death which can be expected to be maximal in parts of the tumour surrounding afferent blood vessels. Thus, in patients who received neoadjuvant therapy, chemotherapy induced necrosis was superimposed upon the spontaneous necrosis seen in untreated cases.

On the basis of these findings, a comprehensive comparative study of morphologic changes in pre-operative treated and untreated osteosarcoma patients was performed looking at 16 morphological tumour characteristics including tumour necrosis using qualitative, quantitative or semi-quantitative methods (Misdorp et al., 1988). Eight tumour characteristics were associated with treatment mode by using a step-wise logistic analysis: i) high grades of necrosis; ii) low amounts of viable tissue; iii) higher numbers of "bizarre cells"; iv) larger numbers of fully engorged blood vessels; v) increased angioblastic proliferation; vi) larger numbers of newly formed reactive fibroblasts; vii) increased reactive osteoblastic proliferation around necrotic tumour bone tissue, and viii) the increased presence of eosinophilic, poorly cellular fibrillar material. After regression analysis, only two of the variables proved to be of statistical significance: the amount of vital tumour tissue, and the amount of fibroblastic proliferation. The combined response to these two variables can be regarded as a very good response to chemotherapy, and this combination can thus help in selection of responsive patients in clinical trials.

A study in Florida (Springfield et al., 1991) conflicted with Misdorp in the extent of necrosis in pre-treated and untreated patient groups. Out of 76 patients who had not received therapy before surgery, 65 had less than 25% necrotic tissue within the area of the primary tumour, whilst out of 22 patients who had received neoadjuvant therapy, 12 had necrosis of over 80%. They thus concluded that the extent,
appearance and location of necrosis observed after the patient has received neoadjuvant chemotherapy is different from the necrosis seen in untreated patients, and therefore, that the presence of spontaneous necrosis should not effect the determination of the effects of chemotherapy.

Grundmann et al. (1983) examined details of cytological alterations of tumour cells after neoadjuvant therapy, reporting combined ultrastructural, histochemical and autoradiographic investigations. They noted the presence of many tumour cells with regressive changes often including degenerative alterations of the rough endoplasmic reticulum. However, they concluded that the ultrastructural picture of both regressive and necrotic changes appeared rather unspecific because similar alterations were commonly found in many necrobiotic cells.

Bacci et al. (1987) looked at a group of 85 patients who received neoadjuvant chemotherapy and examined several clinical parameters during therapy. They found that a decrease in serum alkaline phosphatase correlated well with a good response to therapy.

Delling et al. (1983) described the reaction of 45 primary osteosarcomas to the COSS-80 treatment protocol using several methods including routine histology, undecalcified bone sections, large area sections, histochemistry, X-ray of thick bone tumour sections, imprint cytology and electron image analysis of tumour cells and tumour compartments. They divided their patients into two groups: responders (\( \geq 90\% \) necrosis) and non-responders (\(<90\%\) necrosis). Using the various analyses they found many morphological differences between the two groups. Neoadjuvant chemotherapy was found to be effective in small osteosarcomas of osteoblastic differentiation with little or no production of cartilage and osteoid, and the presence of reactive macrophages. In contrast, non-responders were mainly large primary tumours of chondroblastic differentiation with large amounts of cartilage, osteoid or mineralised bone, with no macrophages.

Further studies by the COSS study groups (Apel et al., 1985) looked at nuclear polymorphism as a prognostic factor for the effect of neoadjuvant therapy. They retrospectively assessed nuclear size in biopsies of patients who had been subjected to the COSS-80 or COSS-82 treatment protocols using an image analysis system. They found that tumour cell nuclei of good responders to chemotherapy were significantly larger and showed a greater variance in size compared with cell nuclei from poor responders. Thus, they concluded that analysis of nuclear size at biopsy
Chapter 1

may be useful for predicting and assessing chemotherapy for osteosarcoma patients. It was also found by the COSS group that the extent of chondroid ground substance seen in biopsies has predictive importance for response to chemotherapy (Kersjes et al., 1987).
It can be seen from the previous sections that the advent of adjuvant chemotherapy has led to an increased success in the rate of disease-free survival of osteosarcoma patients. However, success is not 100%. Apart from the problems of severe toxic effects on normal tissues due to aggressive chemotherapy, for example with high dose methotrexate (Jürgens et al., 1983), failure to respond to, or relapse from, drug therapy are still common causes of death in osteosarcoma. While tumour unresponsiveness to drug therapy may be related to pharmacokinetic factors, tumour type and biology, host response, and drug sanctuaries, drug resistance may prove to be the most important obstacle to successful treatment (Chabner, 1986).

One of the most frequently described models of drug resistance is the P-glycoprotein-specific multidrug-resistant (MDR) phenotype (Chan et al., 1990). P-glycoprotein is a pleiotropic membrane transport protein found in many normal tissues and tumours. It differs from other membrane transporters in its ability to carry out active transport of an extraordinary variety of apparently unrelated organic compounds. Some of these compounds are cytotoxic drugs that play no role in normal physiologic processes. The physiologic substrates are not established (Weinstein et al., 1990).

The mechanism of action of P-glycoprotein is not fully understood (Hamada and Tsuruo, 1986). It is thought though that increased concentration of the transmembrane protein actively increases efflux of drug from the tumour cell by an ATP-dependent process, thus reducing the net intracellular accumulation of drug (Skovsgard, 1978). Figure 1.1 shows the pathways associated with P-glycoprotein (after Weinstein et al., 1990). Since a large number of chemically dissimilar chemotherapeutic agents and other compounds may bind to P-glycoprotein, it may increase efflux of many different compounds (Safa, 1988). Drugs involved in P-glycoprotein-mediated-resistance are some alkaloid compounds and the bacterial and fungal antibiotics and their derivatives, including vincristine, vinblastine, etoposide, adriamycin (doxorubicin), daunorubicin, and actinomycin D (Tsuruo et al., 1982). These compounds are frequently used in combination chemotherapy protocols for the treatment of many tumours including osteosarcoma.
Figure 1.1:- the pathways associated with P-glycoprotein (after Weinstein et al., 1990)

Pathways of selective lipophilic substance efflux in normal tissues and tumours. Lipophilic substances introduced into the extracellular compartment (LS-E), such as certain drugs, or lipophilic substances produced in the intracellular compartment (LS-I) are pumped out of the cell by the efflux transporter, Mdr1. The process is energised by the hydrolysis of adenosine triphosphate. (A) intracellular pathway, (B) extracellular pathway, (C) common pathway.
Since its discovery, there have been several attempts to establish a marker for P-glycoprotein-mediated-resistance, in order to assess the range of expression within normal and tumourous tissue. Methods were aimed at detecting the RNA for the MDR-1 gene which is assumed to encode for the P-glycoprotein, using gel-chromatography techniques (Fojo et al., 1987, Samuels et al., 1991), but as these are difficult and time-consuming methodologies in comparison with immunohistochemical techniques, monoclonal antibodies to epitopes on the P-glycoprotein molecule have been generated (Kartner et al., 1985, Hamada et al., 1986, Scheper et al., 1988). Using these antibodies, overexpression of P-glycoprotein has been extensively demonstrated in tumour cell lines selected in vitro for resistance, and also in clinical tumour samples, particularly from patients whose tumours have relapsed after chemotherapy (Dalton et al., 1989, van der Valk et al., 1990, Verrelle et al., 1991).

Some preliminary studies have demonstrated that the overexpression of P-glycoprotein leads to a bad prognosis for adjuvant chemotherapy in patients with osteosarcoma (Chan et al., 1991, Wunder et al., 1993).

While there is much evidence to support the model of MDR mediated by the drug efflux pump P-glycoprotein, several observations have suggested that this is not the only mechanism of resistance, although the actual mechanisms for other forms of resistance have yet to be elucidated (Morrow and Cowan, 1988, Garcia-Segura et al., 1990, Donenko et al., 1991).

When taking into account all the factors for resistance to therapy mentioned in this section, it is also found with osteosarcoma that an almost unique response is seen upon histological examination of tumour specimens on resection or amputation following neoadjuvant chemotherapy. With most tumours, there is either total response of areas to chemotherapy, with necrosis being the histologically visible sign, or there is resistance to therapy, with resistant cells in viable areas capable of maintaining tumour propagation. With osteosarcoma, and some other bone-related tumours, e.g. Wilm's tumour, an additional distinct area has been reported, which is difficult to interpret (Raymond and Ayala, 1988). Cells in these areas have cytological features that suggest significant "chemotherapy effect". Some of these cells contain nuclear and cytoplasmic vacuolisation as well as bizarre changes in size and morphology.
Chapter 1

Although these histologically "altered" areas have been reported, nothing is known about the "altered" cells in these areas in relation to the prognostic implications for patients who have remnants of tumour after surgery which have this "altered" area appearance. The metabolic status of the cells in these areas needs to be characterized, to see if they have proliferative potential, or are in the throes of death.
1.5 - Cell Proliferation

In order to maintain tumour propagation, there is a requirement for the cells of the tumour to be highly proliferative. The mechanisms which control the growth and division of a cell are encompassed within the cell cycle. The observation that animal cells duplicate their DNA during a discrete interval in interphase allows the cell cycle to be divided into four phases: G\textsubscript{1} phase (gap period between mitosis and the initiation of DNA synthesis), S phase (period of DNA synthesis), G\textsubscript{2} phase (gap period between S-phase and mitosis) and M phase (mitosis, entire cell-division phase) (Pardee et al., 1978). Cell cycle times vary from cell type to cell type, with some epithelial cells going through an entire cycle in as little as 8 hours, whilst neurons and skeletal-muscle cells may have cycling times of 100 days or more (Cheng and LeBlond, 1974, Potten et al., 1979). For most growing cell lines in tissue culture the interval between divisions is 10-30 hours (Pardee et al., 1978). Variation in cell cycle times is mainly due to variation in the length of G\textsubscript{1}, with the duration of S (6-8 hours), G\textsubscript{2} (2-6 hours) and mitosis (1 hour) being relatively constant.

In gaining an understanding of the proliferative status of a cell, one can use several different techniques with which firstly the viability of a cell or population of cells can be demonstrated, and secondly mark the cell at different stages of the cell cycle (usually S or M phase) to show that the cell is cycling.

Methods for assessing the viability of tumour cells include; the measurement of incorporation of radioactively labelled essential amino acids into cells (Freshney et al., 1975), trypan blue dye exclusion by viable cells (Finlay et al., 1984), neutral red dye uptake (Borenfreund and Puerner, 1984), fluorescein dye hydrolysis (Rotman and Depasquale, 1982), quantification of mitochondrial dehydrogenase activity using tetrazolium salts (Black and Speer, 1954, Mosmann, 1983), and protein staining using Sulphorhodamine B (Monks et al., 1983). These methodologies all only work on populations of viable cells in tissue culture, and are not suitable for retrospective assessment of viability on fixed, embedded tissue.

The different stages of the cell cycle were initially discovered by autoradiographic experiments by Howard and Pelc (1953), using incorporation of tritiated (\textsuperscript{3}H)-thymidine, a nucleoside that labels only the DNA molecule, into cells in S-phase. Incorporation is visualised as silver grains produced on the overlying emulsion. This method can be used on tissue preparations as well as cells by incubating the tissue in
medium containing $^3$H-thymidine, before fixation, processing and embedding (Lewis and Bowen, 1985). Other radioactive analogues of thymidine, such as io-dodeoxyuridine have also been used (Speth et al., 1989). However, in spite of its clinical usefulness, this method has not found widespread application in clinical practice due to the risk of radiotoxicity and the length of the procedure (at least 6 days for standard autoradiography).

A more practical approach for evaluating the percentage of proliferating cells relies on the use of immunocytochemical techniques, directing monoclonal antibodies against thymidine analogues, such as 5-bromo, 2'deoxyuridine (BrdU), after the incorporation of the non-radioactive analogue into the DNA of proliferating cells (Gratzner, 1982). BrdU incorporation can also be assessed in fixed and embedded tissue specimens (Veronese et al., 1989).

BrdU techniques still require pre-incubation of the tissue sample, which still provides problems if retrospective studies of proliferation in embedded tissue are to be done. Cellular proteins involved in cell proliferation have been identified, and monoclonal antibodies have been raised against these proteins. These include a growth related 145kD nucleolar protein (Orchs et al., 1985), Topoisomerase II (Heck and Earnshaw, 1986), and the most widely used, a proliferating cell nuclear antigen (PCNA) (Ogata et al., 1985, Waseem and Lane, 1990). Using monoclonal antibodies against cellular proteins negates the requirement for pre-treatment of tissue. Other advantages are maintenance of cellular and tissue architecture, the relative simplicity of the methodology and the rapidity of results (Hall and Levison, 1990).

PCNA, also known as cyclin, is a nonhistone nuclear polypeptide with a molecular weight of 36 kD, which is found in the nuclei of both normal and transformed proliferating cells, but not in resting cells, e.g. inactivated human peripheral blood lymphocytes (Takasaki et al., 1984). PCNA was first recognised when autoantibodies from patients with Systemic Lupus Erythaematosus, an autoimmune disease which affects connective tissue, were found to bind to nuclear antigen present in proliferating cells (Miyachi et al., 1978). The level of synthesis of PCNA has been found to correlate directly with rates of cellular proliferation and DNA synthesis (Celis et al., 1984). Elevated levels of PCNA appear in the nucleus during late G$_1$ phase immediately before the onset of DNA synthesis, become maximal during S phase, and decline again during G$_2$ and M phases (Takasaki et al., 1981).
Chapter 1

It has been hypothesized that PCNA is involved in the regulation of DNA synthesis and cell proliferation (Mathews et al., 1984).

Using immunohistochemical techniques, PCNA has been detected in proliferating cells of normal and tumourous tissues using cryosections and formalin-fixed, paraffin-embedded material (Robbins et al., 1987, Hall et al., 1990, Wilkins et al., 1992, Wolf and Dittrich, 1992).

Cells in the M phase of the cell cycle can easily be detected in routine histology sections as mitotic figures, with characteristic condensed chromosomes.
Chapter 1

1.6 - Cell Death

There are two major morphologically and biochemically distinct modes of death in nucleated eukaryotic cells: necrosis, and apoptosis (also known as programmed cell death) (Duvall and Wyllie, 1986).

Necrosis was the first to be recognized, and is a consequence of environmental insult. Causes of necrosis include complement attack, severe hypoxia, hyperthermia, lytic viral infection, or a variety of respiratory poisons or toxins such as agents used in cancer chemotherapy.

In contrast, apoptosis is thought to be under physiological and probably genetic control. Normal development of a multicellular organism is dependent upon programmed cell death, which is activated during specific periods during the life cycle (Bowen and Bowen, 1990). Examples of apoptosis in the regular life cycle include the production of the ovum, where for every ovum that is produced, three cells are extruded as polar bodies, and the removal of tissues between the fingers in the formation of the pentadactyl limb.

Figure 1.2 shows the differences in the morphological changes seen in necrosis and apoptosis (after Williams et al., 1992). The first step in necrosis is the increased permeability of the plasma membrane, either through alterations in its structure (complement, viral lysis), or by failure of the cationic ion membrane pumps (hypoxia, respiratory poisons), leading to an increased influx of water, and loss of calcium and sodium ion balance. Initially this produces a series of reversible changes in the injured cells. There is dilation of the endoplasmic reticulum, change in cell shape with "blebbing" of the membrane, increase in density of the mitochondria as the inner membrane shrinks away from the outer membrane, the nuclear chromatin flocculates, and protein synthesis declines. This reversible phase is followed, often with explosive rapidity, by irreversible changes. Mitochondria undergo "high amplitude swelling" with dilation of both inner and outer compartments, and the appearance of densely lipid rich aggregates within. There is increased activity of the Golgi apparatus, which is related to the rapid development of primary lysosomes containing degradation enzymes. With the osmotic shock caused by the ion imbalance, cell and organelle membranes are damaged leading to rupture and release of contents, autolysis, and loss of cellular compartmentalisation.
Figure 1.2: Differences in the morphological changes seen in necrosis and apoptosis (after Williams et al., 1992)
Chapter 1

There is increased clumping of nuclear chromatin, leading eventually to the characteristic nuclear pyknosis (Beaulaton and Lockshin, 1982, Duvall and Wyllie, 1986, Bowen and Bowen, 1990).

There are four cardinal elements involved in apoptosis (Wyllie, 1987):

First, there is a rapid volume reduction, with net intracellular fluid and ion loss, accompanied by a rise in cell density, convolution, and blebbing of the cell surface, compaction of cytoplasmic organelles, and dilation of the endoplasmic reticulum.

Secondly, there is chromatin condensation, first as a discontinuous band of densely granular material underlying the nuclear membrane, but then involving the whole nucleus. This morphological change is associated with activation of a 65kD nuclear endonuclease (Dykes et al., 1987) which cleaves chromatin at internucleosomal sites (Wyllie, 1980).

The third cardinal feature of apoptosis is an alteration in the cell membrane which permits recognition by phagocytic cells. It is thought that an endogenous, lectin-like molecule is involved, presumably recognising new glycan structures exposed on the surface of the apoptotic cell (Morris et al., 1984).

The fourth, and most characteristic, feature of apoptosis, is its dependence upon active protein synthesis. Although total RNA is eventually degraded, the abundance of mRNAs encoding several proteins is increased on apoptosis, although a specific requirement for any of the proteins produced has yet to be demonstrated (Williams et al., 1992). Some of these proteins are likely to be involved in the efficient disposal of the apoptotic cell, or control the crucial events leading to irreversible commitment to cell death.

Kinetic measurements of tumours indicate that cell loss is a prominent feature of tumour populations, but the precise mechanism and significance of cell death are complex (Sarraf and Bowen, 1986). Unlike necrosis, which normally involves groups of cells near the centres of tumour masses, apoptosis tends to affect scattered, individual cells throughout a tumour. It is, however, often particularly prominent near foci of necrosis (Walker et al., 1988). Apoptosis has been observed to occur spontaneously in untreated malignant tumours (Kerr et al., 1987, Sarraf and Bowen, 1986, 1988), and is enhanced in both neoplastic and normally proliferating cell populations by a variety of agents used in tumour therapy (Marks and Fox, 1991). Apoptosis is also induced in tumours by cell-mediated immune
Chapter 1

reactions (Ucker, 1987), and it is increased by necrosis factors in the same tissue (Walker et al., 1988).

The techniques for demonstrating cell death are many and varied. Some are based on detecting the cessation of the properties which are associated with life, for example respiration or reproduction. Others exploit the peculiar characteristics of dead and dying cells, for example nuclear pyknosis or the appearance of degradative products (Bowen and Bowen, 1990).

The simplest morphometric measurements may be helpful to illustrate the results of cell deletion and tissue regression. Measurements of wet and dry-weight, volume, area, length and width can provide such preliminary data (Bowen, 1984). Measurements of size may be of relevance at the cellular level, where changes in size due to the intervention of cell death may be analysed. Fine structural studies of using transmission electron microscopy, has added to a better comprehension morphologically of the changes taking place during cell death (Beaulaton and Lockshin, 1982, Allen, 1988).

There are many dyes which can be used in histological and cytochemical techniques, which can demonstrate areas of cell death. The nuclei of dead and dying cells are intensively chromophilic, and can be easily identified in iron-haematoxylin stained sections (Bowen, 1984). Vital dyes such as Nile Blue Sulphate (Saunders, 1966) are concentrated in dying cells, whilst another approach is to look at exclusion of dye, such as trypan blue (Drake et al., 1972), by viable cells. Another class of vital dyes includes reagents that demonstrate some vital cellular function, such as the tetrazolium salt, MTT (Mosmann, 1983) which stains mitochondria with active dehydrogenase activity.

There is extensive evidence of acid hydrolase involvement in cell death. This involvement is either secondary involvement with the process of digestion following phagocytosis, or primarily as an enhancement of free or non-sedimentable acid hydrolase activity as a prelude to death in the cell.

Phagocytosis is an important secondary response to cell death, with dead or dying cells and cell fragments engulfed by wandering macrophages and histiocytes, or in the case of apoptosis, activated neighbouring cells. Intra-vital dyes, such as Neutral Red selectively mark this process of secondary phagocytosis (Bowen and Bowen, 1990).
Chapter 1

The processes of autophagy (intracellular digestion within cell's own cytoplasm), heterophagy (phagocytosis by other cell), or free acid hydrolase activity, can readily be demonstrated by histochemical and cytochemical methods. Acid phosphatase is one of the most widely used hydrolytic markers. An azo-dye technique for acid phosphatase was introduced by Barka and Anderson (1962), which gives a fine homogenous red reaction product at the sites of enzymatic activity. Lysosomal and diffuse sources of acid phosphatase activity can be resolved. This technique can be combined with autoradiographical techniques mentioned in the previous section to give a method for the simultaneous estimation of cell proliferation and cell death (Lewis and Bowen, 1982, 1985, Sarraf and Bowen, 1986).
1.7 - Aims

As was stated previously, osteosarcoma has a unique response to chemotherapy, in that upon examination after resection or amputation of the tumour it is seen that as well as areas of total response or total resistance to therapy, one also sees areas of "histologically altered" tissue in which the cells present with changes in morphology and size (Raymond and Ayala, 1988). There are no reports in the literature of characterisation of the "altered" cells in these "histologically altered" areas, and yet it would be important to establish the exact status of these cells when planning post-operative therapy, since similar cells may still be present in secondary deposits in the patient’s body after removal of the primary tumour, and these cells may have the potential to further propagate the tumour.

Thus, the main aims of this thesis were to characterise at both light microscopy and ultrastructural levels the cells found in these "altered" areas in terms of morphology, and metabolic status.

Morphological studies were carried out on epoxy-resin embedded material, and the assessment of the metabolic status of cells in viable and "altered" areas of tumour, in terms of cell proliferation and cell death, was carried out using immunohistochemical and histochemical techniques in acrylic resin-embedded material. Further characterisation of the different areas of osteosarcoma, in terms of the distribution of glycosaminoglycans was also carried out using immunohistochemical techniques in the acrylic resin-embedded material. Also, by transferring the cells to cell culture, in a chemotherapeutic-agent-free environment, the recovery potential of cells once chemotherapy had been withdrawn was assessed.

Preliminary studies have demonstrated that the overexpression of P-glycoprotein leads to a bad prognosis for adjuvant chemotherapy in patients with osteosarcoma (Chan et al., 1991, Wunder et al., 1993). Neither of these studies have investigated the changes in P-glycoprotein expression before and after neoadjuvant therapy, which would give an indication of just how important P-glycoprotein expression is as a model for multidrug resistance in osteosarcoma. In this study, using immunohistochemical techniques on biopsy and post-surgical material, the expression of P-glycoprotein was assessed.
CHAPTER 2 - GENERAL MATERIALS AND METHODS
Chapter 2

SEE APPENDICES FOR COMPOSITION OF BUFFERS, FIXATIVES AND HISTOLOGICAL STAINS NOT COVERED IN THE TEXT OF THIS CHAPTER

FURTHER DETAILS FOR METHODOLOGIES CAN BE FOUND IN THE RELEVANT CHAPTERS

2.1 - Tumour material

Material was taken from patients diagnosed with high-grade malignant osteosarcoma upon resection or amputation of the primary tumour and within 2-3 hours of the surgical procedure. All patients had received pre-operative chemotherapy. Surgical procedures were carried out at the Royal National Orthopædic and Middlesex Hospitals, London. Table 2.1 presents the clinical data for all patients used in investigations within this thesis, along with details of the embedding procedures utilised.

For the patients used in the histological characterisation (Chapter 3), and multi-drug resistance studies (Chapter 6), routine paraffin-embedded biopsy material was also utilised.
## Table 2.1: Clinical data of patients used in this thesis

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**Key:**
- **SEX:** M = male, F = female
- **SITE:** fem = femur, tib = tibia, hum = humerus, pms = pulmonary metastases, ilm = ilium, scp = scapula, calc = calcaneus
- **EMBEDDING:** H = HEMA resin, S = Spurr's resin, L = LR White, P = Paraffin
2.2 - Processing procedure for paraffin wax embedding

Freshly excised tumour material was fixed overnight in 10% neutral buffered formalin and then decalcified in nitric acid. Tissue was then dehydrated through a series of alcohols as follows: 50% Industrial Methylated Spirits (IMS) for 1 hour, 70% IMS for 1 hour, 90% IMS for 1 hour, and 2 x 1 hour in absolute ethanol. Tissue was then cleared in xylene for 2 hours with a change after an hour. The tissue was then penetrated with molten paraffin wax at 56°C for 3 x 2 hours, and then the tissue was blocked out in clean wax into plastic moulds, and allowed to solidify by placing at 4°C overnight.
2.3 - Processing procedure and specimen manipulation for Spurr's resin embedding for ultrastructural studies

Freshly excised tumour material was placed immediately into cold (4°C) 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). The undecalcified tissue was then cut into 1mm³ pieces and placed into capped Wheaton histology vials (BDH, Poole), and left on a rotator overnight at 4°C to allow for penetration of the fixative. Subsequent processing was carried out at room temperature on a rotator in a fume cupboard.

After fixation, tissue was rinsed (x2, 5 minutes each) in 0.1M cacodylate buffer to remove excess fixative. The specimens were then post-fixed in 1% osmium tetroxide (Agar, Stansted) in cacodylate buffer for 1 hr. After 2 more 5 minute rinses in cacodylate buffer to remove excess osmium, samples were dehydrated through a series of alcohols as follows: 50% IMS for 10 minutes, 70% IMS for 10 minutes, 90% IMS for 20 minutes, and 3 x 20 minutes changes in absolute ethanol. Specimens were then cleared in 3 x 20 minutes changes of propylene oxide (PO) (BDH). During the last immersion in PO, the constituent chemicals (Agar) of the resin used for embedding, Spurr’s resin, were weighed out and mixed together in the following order:

- 20 g ERL 4206,
- 8 g DER 736,
- 52 g NSA,
- 0.8 g S-1.

The PO was then drained from the vials and a 1:1 mixture of 2 ml Spurr’s resin:2 ml PO was added. After two hours, the mixture volume was doubled by adding Spurr’s resin, and the vial cap was replaced. After mixing thoroughly, the vial caps were removed so that the PO could evaporate, and the sample vials were left on the rotator overnight to allow for penetration of the resin into the specimens. The following morning, the resin was drained by inverting the vials, and fresh resin added. After 2 hours the process was repeated. After a further 2 hours, each piece of tissue was carefully transferred to a separate pyramidal BEEM capsule containing fresh Spurr’s resin. The resin was polymerised by placing the capsules in an oven at 60°C for 16 hours. All sectioning of specimen blocks was carried out on LKB III or Reichert-Jung Ultracut microtomes. Initial trimming and 1 μm semi-thin survey sections were carried out using glass knives, whilst ultrathin sections were cut using a 45° Diatome diamond knife.
Chapter 2

In order to assess the suitability of tissue for ultrastructural examination, 1 μm semi-thin sections were cut dry and collected on drops of water on uncoated Superfrost glass microscope slides (BDH). After drying on a hotplate, the sections were stained with 1% Toluidine Blue/1% Borax, washed and mounted in DPX (BDH) mounting medium. These sections were surveyed and representative blocks were chosen for ultrastructural studies and trimmed to a trapezium, with a maximal block width of 3 mm.

Ultrathin sections (50-90nm, silver-gold refraction) were cut over water using a diamond knife, and mounted on bare copper grids. Post-embedding staining consisted of eight minute immersions in uranyl acetate and Reynold's lead citrate (in the presence of sodium hydroxide pellets to remove carbon dioxide) with thorough rinses in double-distilled water after each stain (See Appendices). Specimens were then examined in a Phillips 400 or CM 12 transmission electron microscope at an accelerating voltage of 80kV, and photographic records taken using 3 1/4 x 4" Kodak 4489 electron microscope film.

Further 1 μm semithin sections were taken adjacent to ultrathin sections and stained with toluidine blue as above, and photographs were taken on a Zeiss Photomicroscope III using PAN F film (Ilford) at 50 ASA.
2.4 - Processing procedure for hydroxyethyl methacrylate (HEMA) resin embedding for histochemical studies: (modified from Lewis and Bowen, 1985)

Undecalcified freshly excised tissue was fixed overnight in 10% neutral buffered formalin and was then cut into 5mm³ pieces and placed into Wheaton histology vials with caps. The pieces were washed with agitation in four 5 minute changes of acetone, and were then placed into HEMA monomer (all reagents from BDH) for 3 hours with agitation and 3 hourly changes. To minimise denaturation of the enzyme activity by exothermic heat during polymerisation, the monomer comprised:

- 40 ml 2-Hydroxyethyl methacrylate,
- 8 ml 2-Butoxyethanol,
- 0.135g Benzoyl peroxide.

Polymerisation by the addition of activator was performed in 12 x 18 mm plastic embedding moulds (Agar) covered by embedding stubs, and was carried out at room temperature under vacuum. Two drops of activator (15 ml polyethylene glycol 200 plus 1 ml N, N-dimethylaniline) (Aldrich, Gillingham) were added to 5 ml of monomer for polymerisation to occur. After allowing the blocks to harden in air for several days, 2-3μm thick sections were cut on a Reichert-Jung Supercut microtome using a tungsten-carbide coated knife and floated out on cold water before transferring to uncoated Superfrost glass microscope slides, where the sections were allowed to dry out at room temperature.
2.5 - Processing procedure for L R White (LRW) acrylic resin embedding for histochemical and immunohistochemical studies: (Newman and Hobot, 1987)

Undecalcified freshly excised tissue was placed immediately in 10% neutral buffered formalin and then cut up into 3-4mm³ pieces, and placed into Wheaton histology vials with caps. Further processing was carried out at 4°C using a tissue rotator. Tissue pieces were fixed in formalin for 16-48 hours, dependent upon when the specimen was received from the pathologist. After four 30 minute rinses in phosphate buffer (pH 7.2) to remove excess aldehydes, tissue was partially dehydrated as follows: 50% IMS for 15 minutes, 70% IMS for 15 minutes, and 70% absolute ethanol. After draining off as much ethanol as possible, a 2:1 mixture of LRW (Agar):70% ethanol (freshly made up from absolute anhydrous ethanol) was added for 30 minutes. After thorough draining, 4 x 20 minute changes of LRW were carried out. Polymerisation was executed by addition of accelerator (1.5 µl of accelerator per ml of resin). Pieces of tissue were placed in 00 gelatin capsules (Agar) containing the LRW/accelerator mixture, and the blocks were allowed to polymerise overnight at 4°C.

After removal of the gelatin capsule, 1 µm semi-thin sections were cut dry using a glass knife on a Reichert-Jung Ultracut microtome, and sections placed on a drop of filtered distilled water on Chrome-gel coated Superfrost glass microscope slides. Sections were allowed to dry in an oven at 45°C for 1 hour.
2.6 - Chrome alum-gelatin method for coating microscope slides: (modified from Pappas, 1971)

Subbed (coated) slides provide a hydrophobic surface on which a drop of water will sit when carefully applied. This technique is useful when working with small-area sections, such as those produced above in section 2.5, since when the water drop evaporates upon drying in an oven, the section is smoothed flat on the slide surface.

The chrome alum-gelatin solution (all reagents from BDH) was made by first completely dissolving 5 g of gelatin in 1 litre of distilled water warmed to 60°C, adding 0.5 g of chrome alum (chromium potassium sulphate), cooling the solution and then filtering through #1 filter paper (Whatman), before storing at 4°C for up to 48 hours.

Slides were briefly dipped in distilled water before a 2-3 second immersion in the chrome alum-gelatin solution. After dipping in the solution, the slides were dried vertically in a dust free atmosphere overnight at 37°C.
2.7 - Histochemical method for the detection of acid phosphatase

After air drying sections prepared in sections 2.4 or 2.5 above, they were then incubated in fresh incubation medium made up as follows (all reagents from Sigma, Poole):

- 2 mg naphthol AS-TR phosphate dissolved in 20 μl of DMSO,
- 10 ml acetate buffer pH 4.8,
- 0.2 ml of 10% manganous chloride,
- 6 mg Fast Red Violet LB salt

The incubations were carried out at 37°C in humid conditions to prevent evaporation of the incubation medium. The optimum incubation times were found to be 2 hours for HEMA processed tissue, and 5 hours for LRW processed tissue.

After incubation the medium was rinsed off thoroughly with distilled water, and the sections then counterstained with Harris' haematoxylin for 5 minutes, differentiated in running tap water for 2-3 minutes, and in the case of the HEMA sections, air dried and mounted in Gurr's Xam (BDH). The LRW sections were left in tap water and mounted in Aquamount (BDH).
Chapter 2

2.8 - Antibodies

All the primary antibodies used in this thesis were murine IgG monoclonals, and were stored in small aliquots at -80°C. These antibodies included:

KP1 - anti-human macrophage, CD68 (DAKO, High Wycombe)
PC10 - anti-human proliferating cell nuclear antigen (DAKO)
C219 - anti-human P-glycoprotein (CIS UK Ltd., Cambridge)
2B6 - anti-rat chondroitin-4-sulphate (*)
3B3 - anti-rat chondroitin-6-sulphate (*)
5D4 - anti-rat keratan sulphate (*)
7D4 - anti-chicken chondroitin sulphate (oversulphated epitopes) (*)
VD12 - anti-rat bone osteopontin (Developmental Studies Hybridoma Bank, Iowa)
AON-1 - anti-bovine bone osteonectin (Developmental Studies Hybridoma Bank)

(* - gifts from Prof. B. Caterson, University of North Carolina at Chapel Hill, USA)
2.9 - Immunogold silver enhancement method

This procedure was carried out at room temperature using humidified staining boxes, and at no time were the sections allowed to dry out. A 10X concentrated solution of 10mM phosphate buffered saline (PBS) at pH 7.3 was made by dissolving 10 Oxoid PBS tablets (Unipath, Basingstoke) in 100ml of distilled water. Dilutions of this stock solution were used in making up reagents and for washes in this procedure.

Tissue sections were encircled with a water-repellent wax pen (DAKO) to prevent dispersal of applied liquids from the section during the procedure and placed on the staining rack within the humidified chamber. Sections were covered with bovine serum albumin (BSA) in PBS (20 ml of 0.12 g BSA in PBS) for 5 minutes in order to block non-specific antibody binding sites. After blotting off most of the PBS/BSA with a tissue, a suitable volume (20-100 µl dependent upon section area) of the required primary antibody (see relevant chapters) diluted to the appropriate concentration with PBS/BSA was applied, and sections were incubated for 2 hours. Sections were then subjected to three 5 minute washes in staining troughs filled with 100 ml of PBS. Slides were then placed back into the staining box and the secondary antibody, either 5nm gold conjugated goat anti-mouse IgG (BioClin, Cardiff), or 5nm gold conjugated rabbit anti-mouse IgG (a gift from Dr. AC Hann, Dept. of Physiology, UWCC, Cardiff), applied for 2 hours. After 2 further washes in PBS, sections were covered with 1% glutaraldehyde in PBS for 5 minutes, in order to fix the gold particles in position over their binding sites, and then slides were given three 5 minute washes in AnalaR water (BDH) to remove excess fixative. Sections were then given 3 short washes with 0.75 M tris acetate at pH 7.5 to provide the right pH for the silver reagents.

The silver enhancement reagents (BioClin, Cardiff) were prepared immediately before use by adding 1 part Intensifier to 1 part Activator. After removing as much tris acetate as possible by blotting the sections with a tissue, the required amount of the silver mixture was applied to each section. Sections were then observed under an Olympus HO11 dissecting microscope for signs of development of the silver (brown/black granules). This reaction was normally visible after 12-20 minutes. The reaction was then stopped by gently washing the sections with distilled water. After two further 5 minute washes in troughs of distilled water, sections were counterstained with Harris' haematoxylin for 5 minutes, differentiated in running tap water for 3 minutes, and mounted in Aquamount (BDH).
Chapter 2

The system controls used with this technique were a primary antibody control, where murine non-immune IgGs were used at a similar protein concentration to the corresponding primary antibody, for the primary incubation step, and a silver control, where PBS/BSA was used for both primary and secondary antibody incubations.
Chapter 2

2.10 - Tissue culture

i - media
The culture media used were as follows:
a) Phosphate Buffered Saline (1 PBS tablet in 100 ml distilled water, sterilized by autoclaving at 121°C for 20 minutes)
b) Complete Dulbecco's Modified Eagles Medium (DMEM), (all reagents from GIBCO, Paisley) which comprised:

- 100 ml incomplete DMEM (without sodium pyruvate + 4500 mg/l glucose),
- 0.01 g ascorbic acid,
- 10 ml foetal calf serum (FCS),
- 0.8 ml of 200mM L-glutamine,
- 1 ml of 10 000 U/ml nystatin,
- 1 ml of antibiotic/antimycotic,
- 1 ml of 1 M Hepes buffer.

In order to sterilize medium b), it was passed through 0.2 μm pore disposable filters and collected in sterile universal tubes. Complete DMEM was stored at 4°C for no more than one week.

ii - osteosarcoma tissue explants
Freshly excised tissue was placed into a sterile universal tube containing sterile PBS. All further manipulation of tissue from this point was carried out in a Class II laminar flow tissue culture hood. The tissue was then transferred to a 90mm sterile Petri-dish (Falcon, Cowley) containing 10 ml sterile PBS, and the outermost layer of tissue which was most likely to come into contact with a septic environment removed and discarded. Using a fresh scalpel blade, the remaining tissue was cut up into 2-3 mm³ pieces. Three of these pieces were transferred to each of 10 tissue culture plastic 35 mm Petri-dishes containing 2 ml of complete DMEM. These dishes were then placed in an 37°C incubator under a 5% carbon dioxide, 95% air mixture and high humidity, and incubated for varying time periods, e.g. 3, 7, 10, 14, 17 and 21 days. The explants were maintained by carefully aspirating off old medium and adding fresh complete DMEM every 3 or 4 days.

At varying time intervals, once growth of cells out from the explants had been observed, dishes of cells were fixed by removing medium, washing 3 times with PBS, and fixing with 95% IMS for 5 minutes, before aspirating off the IMS and leaving the dishes to air dry for 20 minutes, and storing at -20°C before carrying out any further procedures.
2.11 - Histochemical assessment of alkaline phosphatase in explant cultures

The stored dishes were removed from the freezer and allowed to thaw. Fresh incubation medium was made up as follows:

- 10 ml of 0.2 M Tris buffer,
- 2 ml of 0.1 N hydrochloric acid (HCl),
- 28 ml of distilled water,

(the above reagents were mixed together and suitable 1M HCl or NaOH added until a pH of 9.0 was reached)

Add 10 mg Naphthol AS-BI (Sigma)

Mix 0.5 ml of 4% new fuchsin (Sigma) in 2N HCl and 0.5 ml of 4% sodium nitrite (Sigma) in distilled water, and add to above.

2 ml of the incubation medium was added to each dish, and the incubation was carried out in a humidified atmosphere at room temperature for 30 minutes. Medium was then thoroughly washed off with tap water, the dishes air dried, and the preparations mounted in glycerol (BDH).
2.12 - Immunofluorescence detection method for explant cultures

Cells were fixed, air dried and stored as in 2.10ii, and were then removed from the freezer and allowed to thaw. Areas of the dish containing cells were encircled with a water-repellent wax pen (DAKO) to prevent dispersal of applied liquids from the area during the procedure. Dishes were placed within a humidified and darkened staining chamber and all incubations were carried out at room temperature. PBS (see above) was placed on the dishes for 5 minutes and then aspirated off, and a blocking solution of Normal Rabbit Serum (NRS) (DAKO) diluted 1:20 in PBS added and the dishes incubated for 20 minutes. The NRS was then blotted off the dish, and a suitable volume (50-100\mu l) of the required primary antibody diluted to the desired concentration with 1:20 NRS was added, and the dishes incubated for 45 minutes. After three 5 minute washes in PBS, the secondary antibody, fluorescein-conjugated rabbit anti-mouse IgG (DAKO) at a concentration of 1:50 in 1:20 NRS was applied for 45 minutes. This was then aspirated off, and after three subsequent 5 minute washes in PBS, the cell areas were mounted in aqueous mounting medium containing the anti-fading agent 1,4-diazabicyclo[2.2.2]octane (DABCO) (Sigma).
CHAPTER 3 - HISTOLOGICAL CHARACTERISATION OF THE
DIFFERENT AREAS OF OSTEOSARCOMA AFTER NEOADJUVANT
CHEMOTHERAPY
Chapter 3

3.1 - INTRODUCTION

Osteosarcoma manifests itself as many different morphologies, even without exposure to modifying agents such as chemotherapy or radiotherapy. Clear variations of morphology are seen not only from case to case but even within single specimens. Due to this heterogeneous picture, there is an abundance of papers relating to attempt to characterise the morphology of untreated osteosarcoma.

Despite the differing histological appearances of osteosarcoma, initial studies of the malignancy recognised just one "basic tumour cell type", the malignant osteoblast (Ghadially and Mehta, 1970, Jenson et al., 1971).

Subsequently, further cell types were recognised, dependent upon the pattern of appearances for the tumour specimens under observation. Williams et al. (1976), and Ferguson and Yunis (1978), identified at least four tumour cell types: primitive undifferentiated mesenchymal cells, and cells with the morphological features of osteoblasts, chondroblasts and fibroblasts. Additional cell types to those above have also been described upon ultrastructural examination. Myofibroblasts and osteocytes were reported by Reddick et al. (1980). Grundmann et al. (1981) defined 7 cell categories: anaplastic (undifferentiated); osteoblastic; osteocyte-like cells surrounded by mineralised matrix; fibroblastic; myofibroblastic; chondroblastic, and angioblastic (telangiectatic). Yoshida et al. (1988) also recognised telangiectatic areas. Similar findings to Grundmann et al. were reported by Aho and Aho (1982) who recognised 6 cell types: unclassified; osteoblastic; chondroblastic; fibroblastic; myofibroblastic, and histiocytic.

Other workers classified osteosarcoma by the histological type, with the predominant cell type giving the sub-classification of the area. Meister et al. (1979) subclassified osteosarcoma into osteoblastic, chondroblastic and fibroblastic types, and found no correlation between the histological typing and patient survival. More detailed ultrastructural studies of the cells found in each of osteoblastic, chondroblastic and fibroblastic areas was carried out by a group in Sweden (Stark et al., 1983, 1984a, 1984b). They ascertained that the osteoblastic, chondroblastic and fibroblastic cells found in their respective areas could be further sub-categorised on the basis of their maturation and differentiation.

With the advent of pre-operative chemotherapy, pathologists could assess the effect of therapy by assessing histological changes in osteosarcoma specimens at resection.
or amputation. Classification systems for histologically assessing the effects of neoadjuvant chemotherapy based on amount of necrosis observed have been utilised (Rosen et al., 1979, Ayala et al., 1980, Picci et al., 1985, Malcolm et al., 1988, Bramwell et al., 1992). Although these systems have differences in the number of gradations of tumour response, they all work on the same idea of an increase in necrosis denoting an increase in response.

Very little has so far been written on the assessment of chemotherapy-affected tissue. Huvos et al. (1977) examined surgical specimens in 20 patients who had received pre-operative chemotherapy and found that unlike tumours in other tissues where necrotic and viable areas are clearly differentiated, variable degrees of tumour destruction were apparent.

A more detailed study of the cytological alterations found in tumour cells under combined cytostatic therapy, including ultrastructural findings was carried out using material from 12 patients undergoing the COSS-80 protocol (Grundmann et al., 1983). They termed the areas of tumour which were neither completely viable nor necrotic, "necrobiotic" areas. Ultrastructurally, these areas revealed a striking cellular heteromorphism. As well as viable highly malignant tumour cells, with their characteristic ultrastructural appearance, they also found cells that had clear regressive alterations, a large number of macrophages, and giant cells similar to those found in granulomas.

Raymond and Ayala (1988) also observed that as well as areas of necrosis, there is an additional population of cells which have cytological features that suggest significant "chemotherapy effect".

None of the above studies assess alterations made to tumour cells within the different histological sub-classification areas due to the effect of chemotherapy, and only the paper of Grundmann et al. (1983) includes some description at the ultrastructural level of such effects.

In this study, a characterisation of the various cell types and morphologies found in different histological sub-classification areas of osteosarcoma after preoperative chemotherapy for thirty-one patients was carried out by histological observations at the light and electron microscopic levels. Uniquely, enlarged, irregular "altered" cells seen at the light microscope level were further characterised ultrastructurally.
3.2 - MATERIALS AND METHODS

Tissue samples for this study were taken upon resection or amputation from 31 patients with clinically diagnosed high grade malignant osteosarcoma treated at the Royal National Orthopaedic and Middlesex Hospitals. Their clinical data are shown in figure 3.1.

Before processing, imprints were made of the tumour material on to microscope slides and allowed to air-dry. Imprints were then stained with alkaline phosphatase as described in Chapter 2 (section 2.10).

Tissue samples were either fixed and processed for paraffin embedding as described in Chapter 2 (section 2.2.), or for Spurr’s resin embedding as described in Chapter 2 (section 2.3). Eight micron thick sections of paraffin-embedded material were taken and routinely stained with haematoxylin and eosin (see Appendices). For the Spurr’s resin embedded material, one micron semi-thin sections were taken adjacent to ultrathin sections and stained with toluidine blue. These semi-thin sections were assessed by the pathologist for histological classification of the area (osteoblastic, chondroblastic, etc.) and the response of the area to chemotherapy. Photographs of imprints, paraffin-embedded and resin-embedded material were taken on either a Zeiss Photomicroscope III or Leitz DM/RB microscope using 35mm PAN F 50 ASA film (Ilford) at 100 ASA (to enhance contrast), or Kodak Gold 100 colour 35mm negative film used at 100 ASA.

Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Phillips 400 or CM 12 transmission electron microscope at an accelerating voltage of 80kV, and photographic records taken using 3¼ x 4” Kodak 4489 electron microscope film.
Figure 3.1a:

Age-sex distribution for patients in this study

Figure 3.1b:

Site-sex distribution for patients in this study

Key: fem = femur, tib = tibia, hum = humerus, ilm = ilium, pms = pulmonary metastases, scp = scapula, calc = calcaneus
3.3 - RESULTS

Sixty-two areas from the 31 specimens were examined at the light microscope (LM) and ultrastructural levels. On scrutinising the semi-thin sections, and by observing the major connective tissue and cell types present, 6 different histological areas were classified: osteoblastic, chondroblastic, fibroblastic, undifferentiated, epithelioid and telangiectatic. In assessing chemotherapy effect, a classification system based upon Bramwell et al. (1992) was used as follows:

\begin{itemize}
\item \( V \) = viable, undamaged tumour
\item \( D_3 \) = scattered pleomorphic and bizarre ("altered") cells that show nuclear and cytoplasmic changes more in keeping with damaged tumour cells than cells found in untreated osteosarcoma - less than 10\% necrosis
\item \( D_2 \) = acellular, fibrotic tissue with moderately large numbers of "altered" cells - 10-90\% necrosis
\item \( D_1 \) = relatively acellular hyalinised tissue that contained widely scattered pleomorphic/pyknotic cells - greater than 90 \% necrosis
\item \( N \) = no tumour, totally necrotic tumour, or non-neoplastic reparative tissue.
\end{itemize}

Figure 3.2 shows the frequency distributions of the histological areas and therapeutic responses seen for the assessed sections. Histologically, enlarged, "altered" cells were seen in most areas classified as \( D_2 \), and sometimes the odd "altered" cell was seen in \( D_1 \) and \( D_3 \) areas.

When imprints of tumour assessed histochemically for alkaline phosphatase activity (the pathologist's method for ascertaining if the presented tumour is an osteosarcoma) were examined, it was seen that as well as regular tumour cells, the enlarged, "altered" cells demonstrated positivity for the enzyme (figure 3.3a and b). Osteoclasts were negative for alkaline phosphatase (figure 3.3c).

For the histological classification and assessment of chemotherapy effect, the photomicrographs for the "viable" areas (figures 3.4a, 3.6a, 3.8a, 3.12a) were taken of \( V \) class areas (with the exception of figure 3.10a which was of a \( D_3 \) area), and the "altered" areas (figures 3.5a, 3.7a, 3.9a, 3.11a, 3.13a, 3.14a) were of \( D_2 \) areas.
Figure 3.2:--

Histological sub-classification of osteosarcoma by evaluation of 1μm sections

Key: osteo = osteoblastic, fibro = fibroblastic, chond = chondroblastic, telan = telangiectatic, epith = epithelioid, undif = undifferentiated
Chapter 3

**Figure 3.3 - tumour imprints assessed histochemically for alkaline phosphatase**

Figure 3.3a - photomicrograph of an area of a tumour imprint which has been stained for alkaline phosphatase activity. Positivity is seen as red granularity in the cell membrane of "regular" tumour cells (arrows). Magnification x 440.

Figure 3.3b - photomicrograph of an area of a tumour imprint which has been stained for alkaline phosphatase activity. Positivity is seen as red granularity in the cell membrane of enlarged, bizarre tumour cells (arrows). Magnification x 440.

Figure 3.3c - photomicrograph of an area of a tumour imprint which has been stained for alkaline phosphatase activity. A multinucleate osteoclast is negative for the reaction (arrow), whilst an adjacent tumour cell shows intense red positivity. Magnification x 440.
3.3.1 - Characterisation of osteoblastic areas (figures 3.4a-f and 3.5a-f)

In viable tumour, in which there was no evidence of chemotherapy effect, these areas were characterised by the presence of varying amounts of fine trabecular strands of osteoid or bone which were lined by cells, and enclosed cells (figure 3.4a). This tumour osteoid or bone could be clearly differentiated from remaining normal or reactive bone (figure 3.4b) which was a lot thicker.

The lining cells were mainly polygonal with single irregular-shaped nuclei, and 1-2 large nucleoli (cell diameter approximately 10µm). Ultrastructural examination of these cells (figure 3.4c) revealed a condensed peripheral chromatin pattern to the nucleus, and that the cytoplasm was comprised mainly of anastamosing profiles of dilated rough endoplasmic reticulum (RER).

Also associated with the trabeculae were giant cells (figure 3.4d) with a typical osteoclast-like appearance (multiple profiles of ovoid nuclei) (diameter approximately 25-45µm). Ultrastructural examination of these giant cells revealed a homogeneous distribution of condensed chromatin in the nuclear profiles, which had distinct nuclear membranes. The cytoplasm of these cells appeared denser than for the osteoblast-like lining cells. Vast expanses of cytoplasm were packed with mitochondria. Golgi apparatus, RER and primary lysosomes were also evident in some cells. Cell membranes appeared to possess microvilli-like structures in places, giving a "ruffled" border effect.

The enclosed cells (figure 3.4e) were round or polygonal in shape (diameter approximately 10µm), and appeared to have shrunk away from the surrounding matrix. Ultrastructural examination revealed condensed peripheral chromatin, and cytoplasm with few features, mainly undilated RER and a few mitochondria.

The areas surrounding the trabeculae were of loose connective tissue with polygonal-shaped cells and ovoid cells with a similar nuclear appearance to the above lining cells. At the ultrastructural level, these cells were seen to be similar in appearance to the lining cells (figure 3.4f).

With an increase in the effects of chemotherapy, there was a decrease in the density of cells associated with the trabeculae and in the surrounding loose connective tissue (figure 3.5a and b). Also observed with an increase in chemotherapy effects were histologically "altered" cells which were large and mainly round (diameter approximately 25-45µm) with high cytoplasm(C):nuclear(N) ratio (C > N). These
cells were mainly mononuclear, and the nuclear appearance varied considerably from regular ovoid to irregular. Vacuolation of the cytoplasm was sometimes evident. Ultrastructural examination of these enlarged cells also gave a diverse picture. Some of the enlarged cells (figure 3.5c) had the same characteristics as the osteoblast-like lining cells described above. Many other cells had peripheral nuclear inclusions, and there was considerable variation in condensed chromatin distribution patterns between cells, and even between nuclear profiles in the same cell. The distribution, quantity and condition of mitochondria, RER and other cytoplasmic organelles also varied greatly. In some cells (figure 3.5e), bundles of microfilaments (diameter approximately 6nm) in the perinuclear region and throughout the cytoplasm were evident. As necrosis became more apparent (figure 3.5f), there was an increase in the occurrence of large, clear vacuoles, which appeared to be membrane-bound. There was also a marked increase in the degeneration of structure seen in mitochondria. Apoptotic bodies were occasionally seen (figure 3.5d).

3.3.2 - Characterisation of fibroblastic areas (figures 3.6a-d and 3.7a-d)
In viable tissue, areas were characterised by a collagenous matrix, with a high density of spindle cells containing a single regular spindle-shaped nucleus with usually a single prominent nucleolus (N>C) (cell length approximately 10-20μm) (figure 3.6a). Ultrastructural examination of these cells (figure 3.6b-d) revealed slight condensation of chromatin around the periphery of the nucleus. The main organelles evident in the cytoplasm were dilated RER, and dense elongated mitochondria.

As the effects of chemotherapy became more apparent (figure 3.7a), a decrease in the density of tumour cells was evident, along with an influx of small characterless cells (diameter approximately 7-10μm). At the ultrastructural level (figure 3.7b), these cells had darker nuclei compared to tumour cells, due to a greater amount of condensed chromatin. The main cytoplasmic feature of these cells was dense, occasionally membrane-bound bodies of varying shapes and sizes.

Also apparent as the effects of chemotherapy became more visible were histologically "altered", large, irregular cells. Ultrastructurally (figure 3.7c and d), as for "altered" cells in osteoblastic areas, these cells exhibited a diversity of morphologies, with both round and spindular morphologies observed.
3.3.3 - Characterisation of chondroblastic areas (figures 3.8a-d and 3.9a-d)
In viable tissue, areas showed a characteristic "cartilaginous" appearance (figure 3.8a). Single rounded cells (diameter approximately 10\(\mu\)m) in lacunae with either one or two regular nuclei surrounded by a thin rim of cytoplasm were seen. Each cell appeared to be surrounded by 2-4 distinct rings of densely staining basophilic matrix (indicative of high glycosaminoglycans content) at the LM level. Ultrastructural examination of these cells (figure 3.8b-d) revealed a homogeneous distribution of condensed chromatin within the nuclei. Mitochondria were the main organelles seen, and there were pseudopod-like projections of the cytoplasm.

"Altered" cells (figure 3.9a) were characterised by a large increase in lacunal size, with incorporation of large vacuoles in the cytoplasm (diameter approximately 25-45\(\mu\)m). At the ultrastructural level, whilst some of these large cells were morphologically similar to the viable chondroblast-like cells, the nuclei of many of these cells appeared to be without any condensed chromatin (figure 3.9c). With increasing necrosis, there was an increased vacuolation of cells and incorporation of lipid (figure 3.9b). Also evident in some cells were peri-membraneous and perinuclear microfilaments (diameter approximately 6nm) (figure 3.9d).

3.3.4 - Characterisation of epithelioid areas (figures 3.10a-d and 3.11a-d)
In the most viable areas, there was a dense packing of cells with little connective tissue (figure 3.10a). Cells were large, regular and polygonal (C > N) (diameter approximately 15-25\(\mu\)m). The cytoplasm showed little vacuolation, and the nuclei were irregular in shape with 1 or 2 nucleoli. Ultrastructural examination of these cells (figure 3.10b) revealed homogeneous clumping of chromatin. Round mitochondria and dilated and undilated RER were the most frequently observed organelles. Also evident within the cytoplasm of most cells examined were distinct desmosome-like structures comprising thick and thin dense and light lines (figure 3.10c). In areas of adjacent cells, densities suggesting simple tight cell junctions were evident (figure 3.10d).

With "altered" cells (figure 3.11a), there was an increase in cell size (diameter approximately 25-40\(\mu\)m) and vacuolation of the cytoplasm. There was also an increase in the frequency of reactive cells. Ultrastructurally (figure 3.11b-d), apart from the cytoplasmic characteristics described for the epithelioid cells above, the irregular cells showed an increased vacuolation and inclusion of lipid.
3.3.5 - Characterisation of undifferentiated areas (figures 3.12a-d and 3.13a-d)

In the viable tissue, areas were characterised by a dense population of cells with very little detectable connective tissue (figure 3.12a). Cells were small and rounded (diameter approximately 7-10\(\mu\)m) or spindle-shaped (N > C) (length approximately 10\(\mu\)m). Cells were normally mononuclear, and the nuclei contained small nucleoli. Ultrastructural examination (figure 3.12b) revealed a thin rim of condensed peripheral chromatin. The main features of the cytoplasm were mitochondria and perimembraneous microfilaments (diameter approximately 6nm) (figure 3.12c). Where cells were adjacent, simple tight cell junctions were apparent (figure 3.12d).

With an increase in chemotherapy effect, there was a decrease in cellularity (figure 3.13a), with greatly enlarged cells of differing morphologies in evidence. Some of the larger cells had a similar appearance to the viable cells, and ultrastructurally showed similar characteristics to the viable ones. Others had a bizarre appearance, and demonstrated vacuolation, lipid infiltration and mitochondrial degeneration (figure 3.13c and d). Reactive cells packed with a dense pigment which was probably haemosiderin were also seen (figure 3.13b).

3.3.6 - Characterisation of telangiectatic areas (figure 3.14a-d)

No viable areas of telangiectatic appearance were detected. The chemotherapy-effected telangiectatic areas were characterised by large amounts of haemorrhage, with many erythrocytes, and the presence of numerous vascular elements (figure 3.14a). Ultrastructural examination of cells in these vascular elements (figure 3.14b) revealed that the nuclei had condensed peripheral chromatin. Mitochondria and undilated and dilated RER were present in the cytoplasm. Primitive tight cell junctions were apparent between adjacent cells. Interspersed among the erythrocytes were "altered" cells in clusters or solitary cells. These cells (figure 3.13c and d) were greatly enlarged and had varying morphologies (diameter approximately 25-40\(\mu\)m). The N:C ratio varied between cells, and in many cells the cytoplasm appeared greatly vacuolated. Ultrastructural examination also revealed a diversity in the profiles of these cells. Nuclei varied from having no evident chromatin condensation, to large clumps of condensed chromatin covering most of the nucleus. As well as discernible mitochondria and RER dilated to varying degrees, many of the cells examined had cytoplasm that invaginated into villous-like projections. Most of the cells had vacuoles of varying size and density, some obviously membrane-bound, and many had lipid droplets and/or dense lysosomes.
Chapter 3

Figure 3.4 - osteoblastic viable tumour

Figure 3.4a - photomicrograph of a viable area of osteoblastic tumour showing polygonal cells (arrows) adjacent to fine trabeculae of osteoid. Haematoxylin and eosin. Magnification x 350.

Figure 3.4b - photomicrograph showing osteoblasts lining normal bone (arrowhead), osteocytes enclosed by bone (arrow), and large multinucleate osteoclasts (O). Haematoxylin and eosin. Magnification x 350.
Figure 3.4 (continued)

Figure 3.4c - electronphotomicrograph of polygonal osteoblast-like cell adjacent to osteoid. Evident in the cytoplasm are profiles of dilated RER (arrowhead), and mitochondria (m). A prominent nucleolus (n) is seen in the nucleus. Magnification x 4,155.

Figure 3.4d - electronphotomicrograph of osteoclast-like cell adjacent to bone. Evident are two nuclear profiles (N). The cytoplasm is packed with mitochondria (arrowheads), and a Golgi apparatus (G) is seen near to one of the nuclei. A ruffled border effect is seen in the cell membrane which borders onto the bone (arrowheads). Magnification x 4,155.

Figure 3.4e - electronphotomicrograph of enclosed cells. Cytoplasm contains dilated (arrowhead) and undilated (arrows) profiles of RER. Magnification x 4,155.

Figure 3.4f - electronphotomicrograph of cell in loose connective tissue. Nucleus and cytoplasm are of similar appearance to that of the bone lining cells. Magnification x 7,420.
Figure 3.5 - osteoblastic "altered" tumour

Figure 3.5a - photomicrograph of "altered" osteoblastic tumour area (D2 classification) showing less cellularity than comparable viable area in Figure 3.4a. Enlarged, irregular cells are evident (arrowhead) as well as "viable" tumour cells (arrow). Haematoxylin and eosin. Magnification x 350.

Figure 3.5b - photomicrograph of severely "altered" area (D1 classification) showing less cellularity than in Figure 3.5a. The area is almost totally acellular with remnant tumour osteoid. Haematoxylin and eosin. Magnification x 350.
Figure 3.5 (continued)

Figure 3.5c - electronphotomicrograph of enlarged cells of similar appearance to "regular" osteoblast-like cells. Cytoplasm is packed with dilated RER (arrowheads). Magnification x 4,155.

Figure 3.5d - electronphotomicrograph showing two osteoclast-like cells (O), and a shrunken, apoptotic body (A). Magnification x 4,155.

Figure 3.5e - electronphotomicrograph showing perinuclear and cytoplasmic microfilaments (arrows) in an "altered" tumour cell. Magnification x 19,292.

Figure 3.5f - electronphotomicrograph of a severely "altered" cell showing large amounts of vacuolation (V), and shrunken dense nuclear profiles (N). Magnification x 5,654.
Figure 3.6 - fibroblastic viable tumour

Figure 3.6a - photomicrograph of viable area showing spindle-shaped tumour cells (arrows) surrounded by a highly fibrous matrix. Haematoxylin and eosin. Magnification x 350.

Figure 3.6b - electronphotomicrograph of spindle cells. Nuclei contain a large nucleolus (n). Cytoplasm is mainly full of RER (arrows). Prominent collagen fibres (arrowheads) are evident in the extracellular matrix. Magnification x 4,155.

Figures 3.6c + d - electronphotomicrographs of less-spindular cells seen in viable fibroblastic areas of tumour, which have similar cytoplasmic features to the spindle cells. The cytoplasm is mainly full of RER (arrows) and mitochondria are also evident (arrowheads). Magnification x 5,654.
Figure 3.7 - fibroblastic "altered" tumour

Figure 3.7a - photomicrograph of "altered" area showing less cellularity than comparable viable area in Figure 3.6a. Enlarged, irregular cells are evident (arrowhead). Haematoxylin and eosin. Magnification x 350.

Figure 3.7b - electronphotomicrograph showing spindle cell with vacuolated cytoplasm (V), and a round cell with cytoplasm packed with small very dense lysosome-like bodies (arrowheads). Magnification x 4,155.

Figure 3.7c - electronphotomicrograph of enlarged irregular spindle cell with highly vacuolated cytoplasm (V). Most of the vacuoles appear to be enclosed by a distinct membrane (arrow). Magnification x 5,654.

Figure 3.7d - electronphotomicrograph of "altered" spindle cell. Cytoplasm contains few organelles compared with viable spindle cells (figure 3.6b), the most prominent being 2 dense lysosomes (arrowheads). Magnification x 5,654.
**Figure 3.8 - chondroblastic viable tumour**

Figure 3.8a - photomicrograph of a viable chondroblastic area of tumour. Rounded cells are surrounded by rings of densely staining basophilic matrix (arrows). Haematoxylin and eosin. Magnification x 350.

Figure 3.8b,c + d - electronphotomicrographs of chondroblastic cells. The most prominent organelles seen are mitochondria (m). Pseudopod-like projections of cytoplasm are evident (arrowheads). Magnification x 5,654.
Figure 3.9 - chondroblastic "altered" tumour

Figure 3.9a - photomicrograph of "altered" chondroblastic area of tumour. Cells are greatly enlarged and vacuolated (V) compared with the chondroblastic cells in Figure 3.8a. Haematoxylin and eosin. Magnification x 350.

Figure 3.9b - electronphotomicrograph of severely damaged tumour cells. Cells are greatly shrunken, and the cytoplasm is packed with lipid vacuoles (arrows). Also evident are large "lakes" of dilated RER (arrowheads). Magnification x 800.

Figure 3.9c - electronphotomicrograph of "altered" tumour cell. Cytoplasm contains few organelles compared with viable chondroblastic cells (figure 3.8b-d). The nucleus also lacks the peripheral condensed chromatin seen in the viable cells. Magnification x 5,654.

Figure 3.9d - enlargement of figure 3.9c showing perinuclear microfilaments (arrows). Magnification x 19,292.
Figure 3.10 - epithelioid viable tumour

Figure 3.10a - photomicrograph of viable epithelioid area. Cells are large and have differing morphologies and a variety of nuclear profiles are evident. Toluidine blue stained resin semi-thin section. Magnification x 350.

Figure 3.10b - electronphotomicrograph of epithelioid cells. Cytoplasm contains dilated RER (arrowheads) and small mitochondria (m). Some vacuolation is evident (V). A distinct desmosome-like structure (arrow) is seen in the cell on the left. Magnification x 4,155.

Figure 3.10c - enlargement of figure 3.10b showing desmosome-like structure (arrow). Magnification x 19,292.

Figure 3.10d - electronphotomicrograph of adjacent cells showing simple tight cell junction (arrows). Magnification x 4,155.
Figure 3.11 - epithelioid "altered" tumour

Figure 3.11a - photomicrograph of "altered" area with some cells greatly enlarged than in the comparable viable area in figure 3.10a. Cells appear more vacuolated and damaged (arrows). Toluidine blue stained resin semi-thin section. Magnification x 350.

Figure 3.11b - electronphotomicrograph of an "altered" tumour cell showing slight vacuolation of the cytoplasm (arrowhead) and electron-dense mitochondria (arrows). Magnification x 4,155.

Figure 3.11c - electronphotomicrograph of "altered" tumour cells demonstrating greater degeneration than in figure 3.11b. A simple cell junction (arrows) is evident between the 2 large cells, whose cytoplasm is electron lucent and contains small dense lysosome-like bodies (arrowheads) and degenerate mitochondria (m). Lipid is also evident (l). Magnification x 1,484.

Figure 3.11d - electronphotomicrograph of binucleated "altered" tumour cell showing lipid accumulation (arrowheads). Magnification x 5,654.
Figure 3.12 - undifferentiated viable tumour

Figure 3.12a - photomicrograph of undifferentiated cells. Tumour cells are mono- or multinucleate, with distinct nuclei with little evident condensed chromatin. Haematoxylin and eosin. Magnification x 350.

Figure 3.12b - electronphotomicrograph of multinucleate cells showing large N:C ratio. Mitochondria (arrows) are the most prominent organelles. Magnification x 5,654.

Figure 3.12c - electronphotomicrograph showing perimembraneous microfilaments (arrows). Magnification x 19,292.

Figure 3.12d - electronphotomicrograph of mononuclear cells. Simple tight cell junctions are evident (arrows) between the cells. Magnification x 4,155.
**Figure 3.13 - undifferentiated "altered" tumour**

Figure 3.13a - photomicrograph of "altered" area showing less cellularity than the comparable viable area in figure 3.12a. Greatly enlarged cells are evident (arrows). Haematoxylin and eosin. Magnification x 350.

Figure 3.13b - electronphotomicrograph of reactive cell. Cytoplasm contains mitochondria (m), and irregular and regular membrane-bound lysosome-like bodies of varying size and densities (arrows). Magnification x 4,452.

Figure 3.13c - electronphotomicrograph of "altered" tumour cells (A) with sparse cytoplasm. Magnification x 1,929.

Figure 3.13d - electronphotomicrograph of more severely damaged cells than in figure 3.13c showing vacuolation (V), and condensation of nuclear chromatin (arrowhead). Magnification x 4,155.
Figure 3.14 - telangiectatic "altered" tumour

Figure 3.14a - photomicrograph of "altered" telangiectatic area showing numerous vascular elements (v), large areas of haemorrhage (H), and large, irregular cells (arrows). Haematoxylin and eosin. Magnification x 350.

Figure 3.14b - electronphotomicrograph of cells comprising a vascular element. Mitochondria (m) and RER (arrowhead) are evident in the cytoplasm. Primitive tight cell junctions (arrows) are seen between adjoining cells. Magnification x 5,654.

Figure 3.14c - electronphotomicrograph of enlarged "altered" cell. Cytoplasm shows large areas of dilated RER (arrowhead). Magnification x 2,078.

Figure 3.14d - electronphotomicrograph of enlarged "altered" cells. Cytoplasm shows vacuolation (V). The cytoplasm of the cell in the top left hand corner (X) is invaginated into villous-like projections (arrows). Magnification x 2,078.
Chapter 3

3.4 - DISCUSSION

The aims of this chapter were to characterise the various cell types and morphologies found in different histological sub-classification areas of osteosarcoma after pre-operative chemotherapy, since very little has so far been reported on the assessment of such tissue. Unique to this study, enlarged, irregular "altered" cells seen at the light microscope level were further characterised ultrastructurally.

From figure 3.2 it can be seen that sub-classification of osteosarcoma at the light microscopical level shows a broad range of histological sub-classes, each with fairly distinct and recognisable properties. The most frequent histological type seen was the osteoblastic phenotype. These findings of differing phenotypes, and a predominance for the osteoblast-like appearance, concur with the view that osteosarcoma may be derived from a pluripotent stem cell with the potential to differentiate into osteoblastic, chondroblastic, fibroblastic, telangiectatic or epithelioid cells, or alternatively, remain undifferentiated. Also, although there is differentiation potential in several directions, there is a preference for the osteoblastic phenotype (Grundmann et al., 1981), which may reflect the presence of various humoural factors within the local environment of tumour growth.

The light microscopical and ultrastructural descriptions given in this chapter for viable tumour cells in osteoblastic, fibroblastic, chondroblastic, undifferentiated and telangiectatic areas conform with those in the literature (Williams et al., 1976, Grundmann et al., 1981, Hirohata et al., 1981, Aho and Aho, 1982, Shapiro, 1983, Povysil, 1986, Dickman, 1987). The only sub-classification described here not previously described was the "epithelioid" type. The distinct appearance of areas sub-classified in this way, compared to other areas, do however, warrant a separate sub-classification. Osteosarcoma cells with epithelioid features have been observed before at the light microscopic level (Hasegawa et al., 1993), and their description of enlarged polygonal cells concur with the findings made here. An interesting feature of most of the epithelioid areas was the presence of intracytoplasmic desmosome-like structures in some of the tumour cells. These did not appear to be membrane bound, and hence it could be assumed that these were not internalised material from other sources. The possibility of these bodies being artefactual would appear to be negated by the fact that they were only seen in epithelioid cells, and not in cells from other sub-classes of osteosarcoma. To confirm if these bodies were actually desmosomes, sections of epithelioid areas could be assessed.
immunohistochemically using antibodies to desmosomal plaque proteins such as desmoplakin (Cowin et al., 1985).

With increasing chemotherapy effect, there appeared to be an increasing alteration of the "viable" pattern for cellular and matrix components, as seen at light microscopical level. These changes have previously been observed (Huvos et al., 1977, Ayala et al., 1980, Delling et al., 1983, Raymond and Ayala, 1987), and the degenerative changes seen in the regular-sized tumour cells would naturally be expected for any tumour which has been shown to be responsive to therapy. Accordingly, most areas displayed a lowered cell density when compared to viable areas.

What is different in osteosarcoma, compared to other tumour types, is the presence of enlarged, "altered", "bizarre" (Huvos et al., 1977, Ayala et al., 1980) cells. That these cells were osteosarcoma cells was confirmed on the tumour imprints using alkaline phosphatase histochemistry. The possibility of these enlarged cells being osteoclasts was negated by the fact that osteoclasts were alkaline phosphatase negative, and upon ultrastructural observation these cells were different in morphology to osteoclasts. Also, it was seen that some of these enlarged cells had the same morphology and structure as the "viable" tumour cells. However, many of these large cells demonstrated classical degenerative changes found in cell death, such as irregular clumping of nuclear chromatin, disintegration of organelles, and mitochondrial swelling (Walker et al., 1988).

In the areas where some chemotherapy effect was in evidence, i.e. class D3 and D2 areas, "normal, viable" tumour cells were evident among the "altered" cells. Combined with the presence of the greatly enlarged but normal-looking "altered" cells, this would suggest a progression in response to chemotherapy of some of the tumour cells to enlarge without any obvious pathology, before cell death changes start to occur. Whether all of the enlarged but structurally normal cells become committed to cell death, or have the potential to continue to propagate the tumour is investigated further in other sections of this thesis.
CHAPTER 4 - FURTHER CHARACTERISATION OF OSTEOSARCOMA -
THE DISTRIBUTION PATTERN OF GLYCOSAMINOGLYCANS IN
HISTOLOGICALLY DIFFERENT AREAS OF OSTEOSARCOMA
4.1 - INTRODUCTION

From the previous chapter, it can be seen that cytologically, osteosarcoma presents as a very heterogeneous tumour. From what little has been reported on the morphological differentiation of osteosarcoma, it is evident that differences in morphology are partially responsible for differences in response to therapy, and consequently the prognosis of patients. A group in Hamburg (Kersjes et al., 1987) found that tumours with little nuclear polymorphism and those with a chondroblastic appearance in greater than 20% of the biopsy material, showed a poor response to chemotherapy. Thus, they concluded, that patients with a large amount of chondroblastic ground substance in the biopsy probably required more aggressive chemotherapy. Hence it would be useful to obtain a profile of the composition of the tumour connective tissue to see if there are any differences in distribution of certain molecules which could be exploited in therapy, or whose expression could be a specific marker for a specific disease state.

One such group of candidate molecules are glycosaminoglycans (GAGs). GAGs are the determinant, component molecules of proteoglycans. Proteoglycans are ubiquitous, and are located on cell surfaces, within intracellular vesicles, and incorporated into extracellular matrices. These proteoglycans differ markedly in their overall molecular size, carbohydrate composition, size of their constituent GAG chains, and physiological function.

Unlike other proteins that are grouped into families on the basis of amino acid similarities, the proteoglycans are defined by a common type of post-translational modification: the GAG moiety (Lander, 1993). GAGs are protein-binding polysaccharide moieties, and in proteoglycans are arranged as side chains bound to a central polypeptide core at serine residues. GAGs are linear polymers, synthesized from only two monosaccharides, strung together in strictly alternating fashion. There are only three such disaccharide repeat units that can be polymerised onto proteins in this fashion, giving rise to three basic "parent polymers" from which all protein-bound GAGs are fashioned. Following polymerisation of these simple chains (the lengths of which are variable), certain types of enzymatic modifications are carried out on the sugars themselves, leading to the variation seen in GAGs and proteoglycans.

The development of monoclonal antibody techniques, in relation to carbohydrates, has led to a means of detecting specific structural or functional domains in
connective tissue GAGs and proteoglycans (Caterson et al., 1985). Using these techniques, it has already been shown that in certain disease processes such as osteoarthritis, the expression of certain native and novel GAG epitopes greatly increases (Caterson et al., 1990, Ratcliffe et al., 1993). It would be potentially useful for the purposes of therapeutic manipulation and prognosis, to see if these findings of high levels of native and novel epitope expression also occur in osteosarcoma, and to investigate any differential distribution of GAG epitopes within the histological sub-classes of osteosarcoma.

In this chapter, the spatial patterns of GAGs commonly found in osteo-articular connective tissue were characterised for different areas of osteosarcoma using an immunogold silver-enhancement technique on acrylic resin embedded specimens.
4.2 - MATERIALS AND METHODS

Tumour samples for this study were taken upon resection or amputation from 24 patients with clinically diagnosed high grade malignant osteosarcoma treated at the Royal National Orthopaedic and Middlesex Hospitals. Their clinical data are shown in figures 4.1a and 4.1b.

The tissues were fixed and processed for embedding in the acrylic resin, LR White as described in Chapter 2 (section 2.5). For each patient, four blocks of tissue were selected, and 1μm sections were cut and transferred to a drop of water on chrome-alum gelatin coated slides (Chapter 2, section 2.6), and sections were air dried at 45°C for 1 hour.

Sections were then subjected to the immunogold silver enhancement procedure described in Chapter 2 (section 2.9), with some of the primary antibodies used requiring an enzymatic pre-digestion step of either chondroitinase AC or chondroitinase ABC (both from Sigma, and used at 0.25 Units ml⁻¹) for 20 minutes at 37°C, with subsequent washes in PBS, before applying a 1:20 normal rabbit serum block for 20 minutes, and then the primary antibody for 2 hours.

The four primary monoclonal antibodies used were:

5D4 - which recognises a native epitope of keratan sulphate (KS). Used at a dilution of 1:2500.

7D4 - which recognises novel non-terminal over-sulphated epitopes of chondroitin sulphate (CS), and is associated with elevated GAG synthesis. Used at a dilution of 1:500.

3B3 - recognises native epitopes at the non-reducing terminus of the CS GAG chain when no pre-digestion is performed, and neo-epitopes of chondroitin-6-sulphate (C-6-S) when pre-digestion with chondroitinase ABC is carried out. Used at a dilution of 1:300.

2B6 - recognises chondroitin-4-sulphate (C-4-S) and dermatan sulphate (DS) with chondroitinase ABC predigestion, and C-4-S only with chondroitin AC. Used at a dilution of 1:1000.
Chapter 4

Figure 4.1a:-
Age-sex distribution for patients in this study

Figure 4.1b:-
Site-sex distribution for patients in this study

Key: fem = femur, tib = tibia, hum = humerus, pms = pulmonary metastases, ilm = ilium, scp = scapula, calc = calcaneus
The secondary antibody used was a rabbit anti-mouse 5nm gold-conjugated antibody, at a dilution of 1:50 for 90 minutes. The controls used in this technique were primary and secondary controls as described in Chapter 2 (section 2.9), with and without enzymatic pre-digestion.

Sections were examined and photographed on a Leitz DM/RB microscope using Kodak Gold 100 colour 35mm negative film at 100 ASA. Positive staining was visualised as black granules. Antibody labelling was assessed semi-quantitatively, and for each zone of tissue per section a grading was assigned as follows:

- no evident antibody labelling (score value 0)
+ \(- weakly positive antibody labelling (score value 0.5)
+ positive antibody labelling (score value 1)
++ very strongly positive antibody labelling (score value 2)

Using the above grading system, approximate mean scores were made for each sub-classification area of osteosarcoma as assessed according to Chapter 3.
4.3 - RESULTS

Ninety-six sections from the 24 specimens were assessed for histological sub-classification, and for either no, or minimal response to therapy (V or D3 from previous chapter), grouped together here as viable, or a more discernible response to therapy (D2 or D1 from previous chapter), grouped together here as "altered". Five separate histological areas were classified: osteoblastic, chondroblastic, fibroblastic, anaplastic/undifferentiated and epithelioid. None of the sections exhibited the telangiectatic profile as described in the previous chapter. Figure 4.2 shows the frequency distribution of the histological areas and therapeutic responses seen for the assessed sections:
Figure 4.2:-

Histological sub-classification of osteosarcoma by evaluation of 1μm sections

Key: osteo = osteoblastic, fibro = fibroblastic, chond = chondroblastic, telan = telangiectatic, epith = epithelioid, undif = undifferentiated
Tables 4.1 - 4.9 show the combined mean labelling profiles for each of the five sub-classifications in 4 different zones of tissue: cellular, pericellular, extracellular matrix (ECM) and perivascular. For all histological sub-classifications except chondroblastic, where no viable areas were observed, there is a table each for viable and responsive tissue.

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### Table 4.2: Osteoblastic - "altered" areas

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### Table 4.3: Fibroblastic - viable areas

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Chapter 4

### Table 4.5: Chondroblastic - "altered" areas

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Chapter 4

For osteoblastic areas (Tables 4.1 and 4.2), viable tissue showed a slightly different localisation pattern of the GAG epitopes to "altered" tissue. For DS (see Materials and Methods section for explanation of abbreviations and which GAGs are recognised by which antibody), "altered" tissue showed strong positive labelling in the ECM compared with viable tissue. Weak C-4-S labelling was detected in "altered" tissue ECM, but was absent from viable tissue. Neo-epitopes of C-6-S as determined by the 3B3 antibody with enzymatic pre-digestion, were detected in the ECM of both response types of tissue, and pericellularly for "altered" areas. Weak immunopositivity for the native CS 3B3 epitope was also seen in the ECM of viable areas. Using 7D4, novel epitopes were also detected in the ECM, with higher levels in "altered" compared with viable areas. In "altered" areas, weak cellular immunopositivity for 7D4 was also noted. Weak KS immunopositivity was seen in the ECM for both tissue types. Figure 4.3(a-f) shows the immunolocalisation patterns of the GAG antibody panel in an "altered" osteoblastic area of tumour.

For fibroblastic areas (Tables 4.3 and 4.4), the GAG distribution pattern in the ECM was similar to that seen in osteoblastic areas, except that the native CS 3B3 epitope was not evident. None of the antibodies used exhibited any immunopositivity in the perivascular regions of either "altered" or viable tissue.

For chondroblastic areas (Table 4.5), no viable sections were seen, but in "altered" areas there were strong positivities seen for pericellular DS, C-4-S, novel C-6-S epitopes with 7D4, neo-epitopes of C-6-S with 3B3, and KS. In many instances pericellular immunopositivity was seen surrounding the enlarged "altered" chondroblast-like cells. Weak immunopositivity for the native CS 3B3 epitope was also seen. A similar pattern was seen for the ECM, except the native 3B3 epitope was not detected. There was weak cellular KS immunopositivity, but no perivascular immunopositivity. Figure 4.4(a-f) shows the immunolocalisation patterns of the GAG antibody panel in an "altered" chondroblastic area of tumour.

For undifferentiated areas (Tables 4.6 and 4.7), there were hardly any detectable GAG epitopes. As for the ECM in osteoblastic and fibroblastic areas, more epitopes were detected in "altered" areas than in viable areas, with weak DS and C-6-S (neo-3B3 and novel-7D4) epitope immunopositivity in the ECM in "altered" areas. Weak novel C-6-S positivity was also detected cellularly in both viable and "altered" areas.
For epithelioid areas (Tables 4.8 and 4.9), very strong immunopositivity was seen in the ECM in "altered" areas and less stronger immunopositivity in viable areas, with both showing immunopositivity for DS, novel- and neo-C-6-S, and KS epitopes. Weak C-4-S immunopositivity was also seen in the ECM in "altered" areas. The native 3B3 epitope was not detected in epithelioid areas. In "altered" areas, as well as in the ECM, DS was also detected pericellularly, and weak immunopositivity was seen in cells. In viable areas, there was weak DS immunopositivity in cells and perivascularly. Novel 7D4 epitopes were also weakly positive in pericellular and perivascular areas.

In the negative control sections used in this study, no immunopositivity was evident (figure 4.3g and h).
Chapter 4

Figure 4.3 - an example of immunolocalisation of GAGs in an "altered" osteoblastic area and negative control sections

Figure 4.3a - 2B6 antibody with chondroitinase ABC pre-digestion: weak immunopositivity is seen perivascularly (arrow) and also within the bone spicules (B). Magnification x 353.

Figure 4.3b - 2B6 antibody with chondroitinase AC pre-digestion: as with figure 4.3a, weak immunopositivity is seen within the bone spicules, but there is no perivascular positivity. Magnification x 353.

Figure 4.3c - 3B3 antibody with chondroitinase ABC pre-digestion: again weak immunopositivity is seen in the bone spicules. Magnification x 353.

Figure 4.3d - 3B3 antibody: there is no visible immunopositivity. Magnification x 353.

Figure 4.3e - 7D4 antibody: weak immunopositivity is seen in the ECM (arrowhead), and there is some evidence of cellular positivity (arrow). Magnification x 353.

Figure 4.3f - 5D4 antibody: very weak immunopositivity is evident in the ECM near to the bone spicules (arrowhead). Magnification x 353.

Figure 4.3g - negative control section for silver enhancement where no primary or secondary antibody has been added before the silver step. No evidence of positivity. Magnification x 353.

Figure 4.3h - negative control section for silver enhancement where non-immune mouse IgGs have been added before the gold-bound secondary antibody and silver step. No evidence of positivity. Magnification x 353.
Chapter 4

**Figure 4.4 - an example of immunolocalisation of GAGs in an "altered" chondroblastic area**

Figure 4.4a - 2B6 antibody with chondroitinase ABC pre-digestion: strong immunopositivity is seen in the ECM, particularly pericellularly (arrow). Magnification x 353.

Figure 4.4b - 2B6 antibody with chondroitinase AC pre-digestion: immunopositivity is seen pericellularly (arrow), but the strong ECM positivity seen in figure 4.4a is lost. Magnification x 353.

Figure 4.4c - 3B3 antibody with chondroitinase ABC pre-digestion: very strong immunopositivity is seen in the ECM (arrowhead), particularly pericellularly (arrow). Magnification x 353.

Figure 4.4d - 3B3 antibody: strong immunopositivity is seen pericellularly, but the ECM positivity seen in figure 4.4c is lost. Magnification x 353.

Figure 4.4e - 7D4 antibody: immunopositivity is seen throughout the ECM, with some strong pericellular positivity. Magnification x 353.

Figure 4.4f - 5D4 antibody: immunopositivity is seen in the ECM, and cells have strong pericellular positivity. Magnification x 353.
4.4 - DISCUSSION

The aims of this chapter were to assess the spatial pattern of GAGs commonly found in osteo-articular connective tissue, in histologically different areas of osteosarcoma.

It was seen that a range of patterns of GAGs occurred over the 5 recognised histological sub-classes, ranging from unclassified areas with hardly any immunopositivity for GAGs, to chondroblastic and epithelioid areas where strong immunopositivity was seen for most of the epitopes in pericellular and ECM areas.

Where both viable and "altered" areas where seen for a particular histological sub-class, the expression of epitopes was stronger in "altered" areas compared with viable areas, especially in the ECM. This would suggest that in the chemotherapy affected areas, the matrix was denser than in the unaffected areas, and indeed it has been previously shown that the detection of GAG epitopes is semi-quantitative (Ratcliffe et al., 1984). The finding of increased epitope expression in "altered" areas is in opposition to findings by Kersjes et al. (1987), who found that the greater the ground substance (matrix), the poorer the response to therapy. Hence it would be expected that therapy-effected tumour would show a decrease in matrix components. However, the presence of reactive and repair tissue in the therapy-effected areas could lead to an increase in matrix components being produced and deposited into the matrix. High epitope expression in chondroblastic areas was also seen pericellularly to enlarged, "altered" cells, suggesting that the effects of chemotherapy may be to upregulate GAG synthesis in these cells.

Interestingly, the ECM in almost all the tumour sub-class areas was positive for the 7D4 monoclonal antibody. Previous work has indicated that the epitope recognised by 7D4 is an oversulphated region on C-6-S, and that expression of this epitope has been associated with elevated GAG synthesis in cartilage during either development in the chick embryo (Fernandez-Teran et al., 1993) or pathology, such as in osteoarthritis (Caterson et al., 1990) and the degenerate intervertebral disc (Roberts et al., 1994). The fact that in "altered" chondroblastic areas, high expression of pericellular 7D4 was seen around enlarged cells would hence suggest that these cells were showing elevated levels of GAG synthesis, and hence were highly metabolic.

In a similar vein to 7D4, the detection of the native 3B3 epitope has also been associated with pathologies not only in cartilage (Ratcliffe et al., 1993), but also in other tissues, e.g. granulosa cell tumour of the ovary (Meng et al., 1994). The
significance of these findings remain to be determined. However, it is clear that the use of serum detection of the 3B3 or 7D4 epitopes as markers for joint degeneration in diagnosis can not preclude the presence of osteosarcoma.
CHAPTER 5 - ASSESSMENT OF THE METABOLIC STATUS OF OSTEOSARCOMA CELLS FOLLOWING NEOADJUVANT THERAPY
Chapter 5

5.1 - INTRODUCTION

As has been shown in the previous chapters, osteosarcoma presents a unique profile in its response to chemotherapy when compared to other tumour types. Samples of tumour examined upon resection or amputation following pre-operative chemotherapy show areas which are "histologically altered" and are neither totally necrotic (i.e. responsive to therapy) nor viable (i.e. resistant to therapy) (Raymond and Ayala, 1988). It has been shown that alkaline phosphatase positive cells are present in these "altered" regions (Pringle, personal communication, Chapter 3, figure 3.3), indicating that cells in these regions are exhibiting an osteogenic phenotype, and are metabolically active. What is not known, however, is whether these cells are in the initial stages of programmed cell death or are mitotically quiescent, or are in an extended cell cycle, and thus could re-enter the proliferative phase of the cycle once chemotherapy has been removed.

Since proliferation and death are multi-step processes, there are several time-frames within these processes at which detection of an event within the proliferation or death processes in a cell can be evaluated. Several techniques have been used for the assessment of cell proliferation and cell death as described in Chapter 1 (sections 1.5 and 1.6).

With cell proliferation, the cell cycle may be assessed on fixed, embedded tumour material at the commitment stages of S-phase (DNA synthesis) and M-phase (mitosis) using immunohistochemical detection of cell markers, and counts of mitotic figures in sections stained with routine histological dyes, such as haematoxylin.

Proliferating Cell Nuclear Antigen (PCNA), also known as cyclin, is an intranuclear peptide whose synthesis reaches its maximum during S-phase of the cell cycle (Takasaki et al., 1984). A monoclonal antibody, PC10, which has been shown to detect an epitope of PCNA in formalin-fixed, paraffin-embedded human material has been developed and characterised (Waseem and Lane, 1990). Thus, this antibody is useful for the retrospective analysis of cell cycle states in processed tissue.

The presence of cytosolic acid phosphatase has been associated with cell death (Sylven and Niemi, 1972), and has been used as a marker as such in many biological areas including tumour biology (Sarraf and Bowen, 1986), using an azo-
Chapter 5

dye histochemical method. This histochemical method has been used to detect cell death successfully in methacrylate-embedded tumour specimens, which gives good tissue resolution and preservation of enzyme. Potential problems arise with osteosarcoma tumour tissue, however, in that this method does not comprehensively discriminate between some positive tumour cells, and reactive cells such as macrophages and osteoclasts, thus making analysis of the degree of cell death difficult. One way of discriminating between positive tumour cells and reactive cells, would be to use an antibody to an epitope which is found exclusively on either the tumour cells, or the reactive cells. One such epitope is the CD68 molecule which is expressed on the cell membrane of reactive cells including osteoclasts, and a monoclonal antibody, KP1 (DAKO) has been raised against the CD68 molecule (Micklem et al., 1989). Thus, by combination of the histochemical detection method for acid phosphatase, and an immunohistochemical detection method for the CD68 molecule, a more precise picture of tumour cell death could potentially be achieved.

However, with the methacrylate embedding technique, there is a lack of preservation of epitopes for immunohistochemical studies, and hence a different embedding medium needs to be used. Although the antibodies used in this section would work on paraffin-embedded material, the relatively high temperature at which paraffin-embedding takes place would destroy the acid phosphatase activity. The acrylic resin, LR White, has been shown to preserve tissue epitopes for immunohistochemical detection, when tissue is embedded at low temperatures (<4°C) (Newman and Hobot, 1987), and one could assume, although nothing has been reported, that at the low temperatures involved in the embedding procedure, the acid phosphatase enzyme would be preserved for histochemical analysis.

In this chapter, the metabolic status of tumour cells in "altered" areas of osteosarcoma tumour specimens was assessed and compared with cells in viable areas of tumour, by investigating cell proliferation and cell death using histological, histochemical, and immunohistochemical techniques.
5.2 - MATERIALS AND METHODS

5.2.1 - Tumour Material
Tissue samples were taken upon resection or amputation from 41 patients with clinically diagnosed high-grade malignant osteosarcoma, who had received pre-operative chemotherapy. Selective samples of tissue were taken from areas of tumour which macroscopically appeared to the pathologist to be "altered" or viable (where present). Figures 5.1a and b present the clinical data for the patients whose material was embedded in HEMA for cell death studies. The clinical data of patients whose material was embedded in LR White for the cell death and proliferation studies is shown in figures 5.2a and b.

5.2.2. - Tissue Processing and Embedding
Initially, tissues were fixed and processed for embedding in hydroxyethyl methacrylate (HEMA) resin as described in Chapter 2 (section 2.4) for histochemical studies. As it was not possible to carry out immunohistochemical studies on sections embedded in HEMA resin, LR White acrylic resin was then utilised for specimen embedding. The procedure for LR White processing was as described in Chapter 2 (section 2.5).
Chapter 5

Figure 5.1a:-
Age-sex distribution for patients in this study (HEMA embedding)

![Bar chart showing age-sex distribution for patients in this study.](chart1)

Figure 5.1b:-
Site-sex distribution for patients in this study (HEMA embedding)

![Bar chart showing site-sex distribution for patients in this study.](chart2)

Key: fem = femur, tib = tibia, hum = humerus, pms = pulmonary metastases, ilm = ilium
Figure 5.2a:-
Age-sex distribution for patients in this study (LR White embedding)

Figure 5.2b:-
Site-sex distribution for patients in this study (LR White embedding)

Key: fem = femur, tib = tibia, hum = humerus, ilm = ilium, pms = pulmonary metastases, scp = scapula, calc = calcaneus
Chapter 5

5.2.3 - Cell Proliferation Studies
LR White embedded material was used for these studies. For each patient, four blocks of tissue were selected, and 1μm sections were cut and transferred to a drop of water on chrome-alum gelatin coated slides (Chapter 2, section 2.6). Sections were then air dried at 45°C for 1 hour.

Sections were then subjected to the gold-silver enhancement immunocytochemical procedure described in Chapter 2 (section 2.9). In order to assess the optimum conditions for the immunohistochemical procedure, a range of primary and secondary antibody concentrations and incubation times was evaluated using LR White embedded human tonsil (high cell proliferation in germinal centres) as a positive control tissue. The primary antibody used was the mouse anti-human monoclonal antibody to PCNA, PC10 (DAKO), and the concentration used was 0.25mg IgG/ml, with the incubation time being 2 hours at room temperature.

After silver enhancement, sections were counterstained with Harris' haematoxylin for 5 minutes. Examination and cell counts were made on a Zeiss Photomicroscope III under water immersion at an overall specimen magnification of X500. Cell counts were made using a 10 x 10 square eyepiece graticule (area = 0.2mm²), with cells (excluding clearly reactive cells, haemocytes and those in vascular elements) in 10 fields being counted per section. PC10 immunopositive cells, mitotic figures visualised with the applied counterstain, and negative cells were tallied for each field. Photomicrographs were taken with Ilford PAN F 50 ASA film used at 100 ASA to increase contrast.

5.2.4 - Cell Death Studies
Initial studies of the histochemical assessment of cell death were carried out on HEMA embedded material. Where blocks has successfully polymerised, 2-3μm thick sections were taken dry and floated out on cold water before transferring to uncoated slides.

Air-dried sections were then subjected to the histochemical method for the detection of acid phosphatase as described in Chapter 2 (section 2.7). Initially, sections of HEMA embedded mouse thymus (which contains many cells undergoing programmed cell death) were used as a positive control, in order to optimize the incubation time for the assay. For HEMA embedded material, this was found to be 2 hours. Owing to the problems of distinguishing reactive cells from tumour cells,
and lack of epitope preservation in HEMA described in section 5.1 above, further
tumour material was processed in LR White.

Sections, as prepared in the previous sub-section 5.2.3, were subjected to a
combination of the immunogold silver enhancement method (Chapter 2, section 2.9)
followed by the histochemical procedure (section 2.7) with some modifications.

The primary monoclonal antibody, mouse anti-human macrophage CD68, KP1
(DAKO) was applied to the sections at a concentration of 0.19mg IgG/ml, overnight
at 4°C. The following morning, sections were subjected to immunogold-silver
enhancement of the primary antibody as described in Chapter 2 (section 2.9), and
after the silver enhancement was carried out, the sections were given three 5 minute
washes in distilled water before addition of the acid phosphatase incubation mixture.
After initial runs to assess the optimum time for incubation on slides with sections
from several tumours, it was found that the optimal incubation time for acid
phosphatase on LR White sections was 5 hours. After 5 hours the mixture was
washed off and the sections counterstained and processed as described in Chapter 2
(section 2.7).

Examination and cell counts were made on a Zeiss Photomicroscope III under water
immersion at an overall specimen magnification of x500. Cell counts were made
using a 10 x 10 square eyepiece graticule (area=0.2mm²), with cells (excluding
those in vascular elements, and haemocytes) in 10 fields being counted per section.
For the HEMA processed sections, counts were made of acid phosphatase positive
cells and non-positive cells. A further differentiation was made between positive
"tumour cells" (diffuse red staining), and positive "osteoclasts and reactive cells"
(enclosed, dense granular staining). For the LR White processed sections, counts
were made of diffuse acid phosphatase positive cells, cells which had granular acid
phosphatase positivity alone or combined with KP1 immunopositivity, solely KP1
positive cells, and non-positive cells.

Photographs were taken on a Zeiss Photomicroscope III or Leitz DM/RB
microscope using either Kodak Gold 100 colour 35mm negative film used at 100
ASA, or Kodak EPY64T colour transparency film at 64 ASA.

5.2.5 - Statistical Analysis
For all the studies carried out in this chapter, total cell category counts for a given
section were converted to percentages. Upon histological examination by the
pathologist, sections were assigned to one of the 4 categories of response to therapy as described in Chapter 3: V, D3, D2, or D1. In order to have a more substantial sample base for statistical analysis to compare the cell death/proliferation figures for "altered" compared with viable area cells, the 4 categories were grouped into viable (sections of V and D3 areas), and "altered" areas (sections of D2 and D1 areas).

Since it was not possible to assess whether the results for both areas fell within a normal population distribution, the results obtained for each cell category (e.g. mitotic figures, reactive cells, etc.) for viable and "altered" sections were compared using a non-parametric statistical test, the Mann-Whitney U-test. Computation was carried out using Graphpad Instat2 software (Graphpad Software, San Diego, CA).
5.3 - RESULTS

5.3.1 - Cell Proliferation
One hundred and twelve sections from the 28 cases of osteosarcoma embedded in LR White were examined of which 32 were subsequently classified as viable tissue (12 V, and 20 D3), and 80 as "altered" (27 D2, and 53 D1). PC10 immunopositivity (figure 5.3a-d) was seen in the positive control sections of tonsil almost exclusively in the germinal centres of the organ, and was evident in most of the test tumour sections, as distinct black grains overlying cell nuclei. In some sections, there was also a small amount of cytoplasmic localisation of the black granules, and localisation in cells undergoing mitosis. In "altered" sections of tumour, PC10 immunopositivity was mainly seen in small cells, and not in enlarged, irregular cells. As well as small regular mitotic figures, large, irregular ones were found in both "altered" and viable tissue sections (figure 5.4a + b).

Figure 5.5a shows stacked bar charts of the median and quartile percentages of PC10 positive cells in "altered" and viable areas. Using a Mann-Whitney statistical test (figure 5.6a), it was found that at the 95\% confidence level, there was a significant difference in the percentage of cells immunopositive for PC10 between viable and "altered" areas (p=0.013), with a higher percentage of PC10 positive cells in "altered" sections. Figure 5.5b shows stacked bar charts of the median and quartile percentages of mitotic figures in "altered" and viable areas. Applying the same statistical analysis (figure 5.6b) to the mitotic figure counts however, there was a significantly higher percentage of mitotic figures in viable areas compared with "altered" (p<0.0001).
Chapter 5

**Figure 5.3 - PC10 immunolocalisation**

Figure 5.3a - photomicrograph of PC10 immunolocalisation in human tonsil positive control: immunopositive cells (arrows) seen only in cells of the lymph nodule germinal centre (central area of photomicrograph), and not in the surrounding nodular tissue. Magnification x 242.

Figure 5.3b-d - photomicrographs of PC10 immunolocalisation in tumour cells in 3 different cases of osteosarcoma. Immunopositivity is seen as black granules overlying the cell nucleus (arrowheads). In c + d there is also cytoplasmic localisation of the antibody (arrows). Magnification x 616.
Figure 5.4 - mitotic figures

Figure 5.4a - photomicrograph of small "regular" mitotic figures (arrows) in a viable area of tumour. Magnification x 616.

Figure 5.4b - photomicrograph of large, irregular mitotic figures (arrowheads), and regular mitotic figures in an "altered" area of tumour. Magnification x 616.
Figure 5.5a:-
Median-quartile plot for the percentage of PC10 positive cells

(Arrowheads denote the median values)

Figure 5.5b:-
Median-quartile plot for the percentage of mitotic figures

(Arrowheads denote the median values)
Figure 5.6a:

Mann-Whitney Test
Are the medians of viable - pcl0 and "alt" - pcl0 equal?
Mann-Whitney U-statistic = 895.00
U' = 1665.0
Sum of ranks in viable - pcl0 = 1423.0. Sum of ranks in "alt" - pcl0 = 4905.0.
The two-tailed P value is 0.0130, considered significant.
(The P value is an estimate based on a normal approximation.)

Summary of Data
Parameter: viable - pcl0 "alt" - pcl0
Mean: 0.9709 2.077
# of points: 32 80
Std deviation: 1.805 2.562
Std error: 0.3190 0.2864
Minimum: 0.000 0.000
Maximum: 7.350 13.640
Median: 0.2750 1.335
Lower 95% CI: 0.3202 1.506
Upper 95% CI: 1.622 2.648

* * *

Figure 5.6b:

Mann-Whitney Test
Are the medians of viable - mfs and "alt" - mfs equal?
Mann-Whitney U-statistic = 598.50
U' = 1961.5
Sum of ranks in viable - mfs = 2489.5. Sum of ranks in "alt" - mfs = 3838.5.
The two-tailed P value is < 0.0001, considered extremely significant.
(The P value is an estimate based on a normal approximation.)

Summary of Data
Parameter: viable - mfs "alt" - mfs
Mean: 0.7528 0.2428
# of points: 32 80
Std deviation: 0.9925 0.7495
Std error: 0.1755 0.0838
Minimum: 0.000 0.000
Maximum: 4.950 4.880
Median: 0.4700 0.000
Lower 95% CI: 0.3949 0.0757
Upper 95% CI: 1.111 0.4098

* * *
5.3.2 - Cell Death

i - HEMA-embedded material

Thirty-five sections from the 13 cases of osteosarcoma, where material was embedded in HEMA, were examined. Twelve of the sections were subsequently classified upon histological examination as viable tissue, and 23 as "altered". A variety of patterns of acid phosphatase positivity were seen (figure 5.7a-c) in both the thymus control and in the tumour test sections, including diffuse staining (positivity of free cytosolic acid phosphatases due to processes of cell death) mainly in tumour-like cells, and also in enlarged cells in "altered" areas. Small, dense apoptotic bodies, and osteoclast-like and macrophage-like cells containing dense, granular staining (positivity of lysosomal acid phosphatase, and in the reactive cells, positivity due to engulfed material from dead tumour cells) were also seen in viable and "altered" tumour areas. It was not always easy to clearly distinguish between positive diffuse cells and granular cells, since some of the diffuse cells contained a few dense granulations.

Figure 5.8a shows stacked bar charts of the median and quartile percentages of cells undergoing cell death, and reactive cells in "altered" and viable areas. Statistical analysis using a Mann-Whitney test (figure 5.9a) showed that for tumour cells undergoing cell death there was a significant difference at the 95% confidence level in the percentage of positive cells seen in "altered" areas compared with viable areas (p<0.0001). Figure 5.8b shows stacked bar charts of the median and quartile percentages of reactive cells in "altered" and viable areas. For detection of reactive cells (figure 5.9b), it was also found that there were significantly more reactive cells in the "altered" specimens compared with the viable ones (p<0.0001).
Figure 5.7 - cell death in HEMA-embedded material

Figure 5.7a - photomicrograph of viable area of tumour showing a single tumour cell with diffuse acid phosphatase positivity (arrowhead). Magnification x 353.

Figure 5.7b - photomicrograph of "altered" area of tumour showing an enlarged irregular tumour cell with diffuse acid phosphatase positivity (arrowhead). Also present are macrophage-like cells with dense granular staining (arrow). Magnification x 353.

Figure 5.7c - photomicrograph of "altered" area of tumour showing dense granular acid phosphatase positivity in osteoclast-like cells (arrowhead), as well as cells demonstrating diffuse positivity (arrows). Magnification x 353.
Chapter 5

Figure 5.8a:
Median-quartile plot for the percentage of cells undergoing programmed cell death for HEMA-embedded specimens

(Arrowheads denote the median values)

Figure 5.8b:
Median-quartile plot for the percentage of reactive cells for HEMA-embedded specimens

(Arrowheads denote the median values)
Chapter 5

Figure 5.9a:

Mann-Whitney Test
Are the medians of "alt" - ap and viable - ap equal?
Mann-Whitney U-statistic = 22.000
U' = 254.00
Sum of ranks in "alt" - ap = 530.00. Sum of ranks in viable - ap = 100.00.
The two-tailed P value is < 0.0001, considered extremely significant.
(The P value is an estimate based on a normal approximation.)

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Figure 5.9b:

Mann-Whitney Test
Are the medians of "alt" - reac and viable - reac equal?
Mann-Whitney U-statistic = 9.000
U' = 267.00
Sum of ranks in "alt" - reac = 543.00. Sum of ranks in viable - reac = 87.000.
The two-tailed P value is < 0.0001, considered extremely significant.
(The P value is an estimate based on a normal approximation.)

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<td>Upper 95% CI</td>
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ii - LR White embedded material

One hundred and twelve sections from the 28 cases of osteosarcoma embedded in LR White were examined of which 32 were subsequently classified as viable tissue (12 V, and 20 D3), and 80 as "altered" (27 D2, and 53 D1). A similar pattern of diffuse acid phosphatase positivity was seen in the tumour sections as was seen for the HEMA embedded material, with diffuse staining seen in tumour-like and enlarged cells. Cells were present in most sections demonstrating immunopositivity to the KP1 antibody alone, without any suggestion of acid phosphatase positivity. These were mainly small, roundish cells with the immunopositivity seen as black granularities associated with the cell membrane. Combined granular acid phosphatase and KP1 positivity was mainly seen in large, multinucleated osteoclast-like cells, and also in small roundish cells (figure 5.10a-c).

Figure 5.10a shows stacked bar charts of the median and quartile percentages of cells undergoing programmed cell death in "altered" and viable areas. Statistical analysis using a Mann-Whitney test (figure 5.11a) showed that there were significantly higher levels of tumour cells undergoing cell death (diffuse acid phosphatase positivity) in "altered" areas compared with viable areas (p=0.0116). Figure 5.10b shows stacked bar charts of the median and quartile percentages of reactive cells in "altered" and viable areas. For detection of reactive cells (cells which had granular acid phosphatase positivity alone or combined with KP1 immunopositivity, or solely KP1 positive cells) (figure 5.11b), it was found that there was no significant difference in the levels of reactive cells seen in the "altered" sections compared with the viable ones (p=0.7885).
Figure 5.10 - cell death in LR White-embedded material

Figure 5.10a - photomicrograph of "altered" area of tumour. Diffuse acid phosphatase positivity is seen in regular tumour cells (arrow), whilst macrophage-like and osteoclast-like cells exhibit combined KP1 immunopositivity and granular acid phosphatase positivity (arrowhead). Also present are large irregular mitotic figures (M). Magnification x 681.

Figure 5.10b - photomicrograph of "altered" area of tumour with cells showing KP1 immunolocalisation as black granules superimposed over the cell membrane (arrow). Magnification x 681.

Figure 5.10c - photomicrograph of "altered" area of tumour. Macrophage-like and osteoclast-like cells exhibit combined KP1 immunopositivity and granular acid phosphatase positivity (arrowheads). Magnification x 681.
Chapter 5

Figure 5.11a:-
Median-quartile plot for the percentage of cells undergoing programmed cell death for LR White-embedded specimens

(Arrowheads denote the median values)

Figure 5.11b:-
Median-quartile plot for the percentage of reactive cells for LR White-embedded specimens

(Arrowheads denote the median values)
Figure 5.12a:

**Mann-Whitney Test**
Are the medians of viable - ap and "alt" - ap equal?
Mann-Whitney U-statistic = 887.50
$U' = 1672.5$
Sum of ranks in viable - ap = 1415.5. Sum of ranks in "alt" - ap = 4912.5.
The two-tailed P value is 0.0116, considered significant.
(The P value is an estimate based on a normal approximation.)

**Summary of Data**

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**Figure 5.12b:**

**Mann-Whitney Test**
Are the medians of viable - reac and "alt" - reac equal?
Mann-Whitney U-statistic = 1238.0
$U' = 1322.0$
Sum of ranks in viable - reac = 1850.0. Sum of ranks in "alt" - reac = 4478.0.
The two-tailed P value is 0.7885, considered not significant.
(The P value is an estimate based on a normal approximation.)

**Summary of Data**

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Chapter 5

5.4 - DISCUSSION

The aims of the work in this chapter were to assess the metabolic status of cells in "altered" areas of osteosarcoma tumour specimens, and compare them with cells in viable areas of tumour, by investigating cell proliferation and cell death using histological, histochemical, and immunohistochemical techniques.

Due to the size of the sections used in these studies, i.e. very small, it could not be assumed with total confidence that the areas that were chosen for study were representative of the tumours of the patients involved. This was mainly due to the fact that it was only possible to obtain small samples of tissue from the tumour specimens. However, at the same time, these specimens were somewhat larger in size than the tissue obtained at biopsy for initial diagnosis. Previous studies looking at the response of tumours to chemotherapy (Picci et al., 1985, Misdorp et al., 1988) have used large cross-sections of the whole tumour to assess the degree of necrosis, or other characteristics. However, a large variety of histological sub-classes and responses to chemotherapy was seen, and so any analysis should account for the metabolic status of the range of tumour cells normally seen after chemotherapy.

It was decided to link together the sub-categories V with D3, and D2 with D1 since categorising tumour sections into these 4 groups was highly subjective, and the difference between a V and a D3 section, and a D2 and a D1 section were sometimes quite minimal, mainly due to the small size of the sections which were being assessed. Also, any irregular cells or cell death seen in the D3 group could be due to spontaneous necrosis and programmed cell death, and so tumours classed as D3 could actually be V. Previous studies have suggested that less than 10% necrosis (which includes the groups V and D3) is prognostically a bad sign (Rosen et al., 1979, Ayala et al., 1980, Rosen et al., 1982, Salzer-Kuntschik et al., 1983, Kempf et al., 1991, Petrilli et al., 1991).

For the studies on cell proliferation, two parameters of the cell cycle were investigated: S-phase using an antibody to PCNA (PC10), and mitosis by counting mitotic figures. PC10 was the antibody of choice for localisation of PCNA, since it has been found to give the most intensive staining of the antigen in formalin-fixed tissue (Wolf and Dittrich, 1992).
Chapter 5

As for previous studies carried out in this thesis, it was seen that a high resolution, low background and highly sensitive immunohistochemical localisation method was provided by the combined use of acrylic resin embedding, and gold-silver enhancement immunocytochemical techniques, in this case with the PC10 antibody.

In accordance with other reported work using the PC10 antibody, localisation of the antibody was generally confined to the nucleus with a spectrum of intensity of the granular staining (Hall et al., 1990, Wilkins et al., 1992, Wolf and Dittrich, 1992). Rare cytoplasmic localisation has previously been reported (Hall et al., 1990, Connolly and Bogdanffy, 1993), particularly associated with mitotic cells. The nature of this localisation is unclear, but may represent mitochondrial synthesis or breakdown of the PCNA. The fact that cytoplasmic localisation was seen in mitotic figures is not totally surprising, since the nuclear membrane is lost during mitosis (Hall et al., 1990).

PC10 localisation was not observed in the large, irregular cells in sections of "altered" areas, which would suggest that these cells are not cycling. However, large mitotic figures were rarely seen in "altered" areas, which would suggest that there is some proliferative activity in these irregular cells, but this may just be due to "regular" cells aborting mitosis and becoming swollen and enlarged.

Using the Mann-Whitney U-statistical test it was seen that there was a significant difference between the mean percentage of cells in S-phase, by PC10 immunolocalisation, in "altered" and viable areas. Surprisingly there was a higher mean PC10 positivity in "altered" areas (2.08%) compared with viable (0.97%). This could be due to the fact that there were a considerably larger number of "altered" sections reviewed than viable, and although the figures were deemed to be statistically significant, by looking at the ranges of percentage figures for PC10 positivity (Figure 5.5a), there seems to be no real difference between "altered" and viable areas.

There were considerably significantly higher mean percentage mitotic figure counts in viable areas compared with "altered", with most "altered" areas showing no mitotic figures.

The above data on the proliferative status of cells in different chemotherapy-affected areas of osteosarcoma suggests that cells within the "altered" areas of tumour may be in a prolonged cell cycle, or alternatively that the cells cannot proceed to mitosis,
and cells may be blocked in S-phase, suggesting DNA damage, which may account for the surprisingly higher levels of PC10 positive cells, or blocked in G2-phase.

Consequently, although chemotherapy may have changed the phenotype of the tumour cells, it has not resulted in the removal of them from the cell cycle. It must also be taken into account that within the "altered" areas were clusters of "normal", viable tumour cells, and these would have an influence on the results seen for the "altered" areas.

In order to assess cell death, an enzyme histochemical technique using the hydrolase enzyme acid phosphatase was employed. Acid phosphatase has been used to characterise lytic activity within cells in many systems and circumstances, including embryological or larval development (Lockshin and Williams, 1964, Allenspach, 1976, Clarke, 1990), starvation in planaria (Bowen and Ryder, 1976), mouse thymus involution (Bowen and Lewis, 1980), and tumour cell apoptosis (Sarraf and Bowen, 1986).

The role ascribed to acid phosphatase in characterising lytic activities mainly falls into two basic types: lysosomal (heterophagic and autophagic), and non-lysosomal free hydrolase which acts freely on organelles in the cytoplasm. These types correspond respectively to the granular and diffuse patterns of acid phosphatase activity seen in this study.

There is some controversy surrounding the precise role of acid hydrolases such as acid phosphatase in cell death. Autophagy and free hydrolases may well feature in the initial processes leading to cell fragmentation during morphogenetic apoptosis (Hurle and Hinchcliffe, 1978), although Wyllie et al. (1981) believe that hydrolases play a secondary role during final cell degradation. There is some evidence for the involvement of acid hydrolases in the process of phagocytosis and digestion by neighbouring cells or macrophages, which follows cell fragmentation (Bowen and Bowen, 1990). Bowen and Ryder (1974) have suggested that extracisternal free acid phosphatase may represent nascent enzyme newly formed at the ribosomes which occur outside the endoplasmic cisternae during apoptosis. This infers that enzymes produced specifically for cell lysis do not need to pass into the Golgi apparatus or lysosomes. This hypothesis goes against traditional thought that free hydrolase results from broken or fragile lysosomes. However, the work from Bowen and co-workers support the idea of a more direct and economic distribution of destructive acid hydrolase. Where programmed cell death occurs, it would make greater
economic sense to produce active hydrolases *de novo* in the cytosol, rather than expend energy synthesizing and transporting enzyme for storage in lysosomes for eventual release into the cytosol (Bowen and Bowen, 1990).

The initial cell death studies were carried out on HEMA-embedded specimens, since the resin could ostensibly be polymerised at room temperature, so allowing for preservation of enzyme activity within tissue specimens, without losing cellular detail and resolution, which occurs with the usual tissue handling for enzyme histochemistry, namely frozen section techniques.

One of the main problems with this technique was being able to clearly distinguish between acid phosphatase positive diffuse cells which also exhibited slight lysosomal positivity, and some of the reactive cells which had only very few positive lysosomes. Because of this, it was decided to conduct assessment of cell death in further specimens using an immunological marker against the intracytoplasmic membrane glycoprotein CD68, which would distinguish cells of macrophage lineage, which includes reactive macrophages and osteoclasts, from the tumour cells which may show slight granular acid phosphatase positivity.

However, due to the lack of retention of antigenicity in HEMA embedded material, it was decided to embed further tumour material in the acrylic resin LR White, which would preserve both antigenicity and enzyme activity, due to the low temperature and partial dehydration techniques employed in embedding (Newman and Hobot, 1987).

It was found that for both the HEMA and LR White embedded series of material, there were significant differences in the amount of tumour cells in the process of programmed cell death, with "altered" areas having a significantly higher percentage of tumour cells undergoing apoptosis than viable areas.

Although both procedures for assessing apoptosis showed statistically significantly higher death in "altered" areas than viable, the magnitude and range of tumour cells with cell death varied greatly between HEMA and LR White methodologies.

For the HEMA protocol, the mean percentage of cells in "altered" areas was 20.1% (range 1.0-59.9%), whilst for LR White the mean was 6.8% (0.0-34.1%). For viable areas, this difference was also evident, with a mean of 2.5% (0.0-10.0) for HEMA processed tissue, and 4.5 (0-23.5) for LR White processed tissue.
A reason for the disparity in these figures could be down to the fact that slightly different methods were used for analysing cell death, and the fact that cells deemed positive by the purely histochemical method were elicited as reactive-type cells once the combined immunohistochemical/histochemical method was utilised. However, these methodological alterations alone would not account for differences, and it can be seen that the total of acid phosphatase positive cells (including both positive tumour and reactive cells) for the combined method is still lower than the figures seen for the HEMA method.

Another reason could be that there are differences in the degree of enzyme preservation, with preservation of enzyme better in HEMA-embedded material than in LR White. This could be assessed by embedding material from the same specimen by both procedures, and using the acid phosphatase histochemical method, carrying out positive cell counts. Unfortunately, due to changes in working circumstances, this was not possible to carry out.

Despite the disparities in the quantity of positive apoptotic cells between the HEMA and LR White processing techniques, the trend of increased levels of programmed cell death in "altered" areas was evident for both methodologies.

When assessing the results for reactive cells, it was seen that for HEMA-embedded specimens there was a significantly higher number of reactive cells in the "altered" areas compared with viable areas. This would be expected since there would be a higher amount of debris from the higher amount of dead or dying cells in the "altered" areas.

In contrast, for the LR White embedded specimens where the KP1 antibody, which localises the CD68 antigen on the membrane of macrophage-lineage cells was used, it was found that there was no significant difference between the number of reactive cells in viable and "altered" areas. This finding may differ from the results for HEMA-embedded material, in that the KP1 antibody used in this study also detected macrophages which had no discernible acid phosphatase positivity, which were not yet activated and synthesising acid phosphatase. These solely KP1 positive (inactivated?) cells were seen to be present in similar numbers in both "altered" and viable areas, and thus contributed to there being no significant difference between the amount of reactive cells in these areas. The inference for these findings is that in "altered" areas macrophages are being preferentially activated.
Chapter 5

For both methods it was found that the large, irregular cells which were characteristic to the "altered" areas demonstrated diffuse acid phosphatase positivity. None of these cells expressed proliferative activity, and hence it could be assumed that these enlarged cells are committed to cell death, and pose no threat for the propagation of the tumour. These histochemical findings concur with those from the ultrastructural studies seen in Chapter 3, that these enlarged cells appeared to be showing cell death changes.

Combining the proliferative and cell death studies carried out in this chapter, some conclusions about the status of cells in "altered" areas may be drawn. Since the number of proliferating cells (in terms of mitotic figures) in the "altered" areas is significantly lower than that in the viable areas, and that there are significantly higher number of tumour cells in programmed cell death, it may be concluded that the presence of "altered" areas suggest that chemotherapy has had a positive cell killing effect on the cells in these "altered" areas. However, cells with proliferative potential are present in these areas, which would suggest that any remnant tumour in the body that has shown an "altered" response to chemotherapy may still contain cells which may proceed to propagate the tumour once chemotherapy is withdrawn.

Thus, in terms of prognosis for the patient, although one can not be certain without taking into account the actual data for patient follow-up post-surgery, an "altered" area response to neoadjuvant chemotherapy is better than a viable one. However, one must still be aware that there may be cells around with the potential to propagate the tumour if "altered" areas remain post-surgery.
CHAPTER 6 - EXPRESSION OF THE MULTI-DRUG RESISTANCE MARKER P-GLYCOPROTEIN IN OSTEOSARCOMA
Chapter 6

6.1 - INTRODUCTION

Failure to respond to, or relapse from drug therapy are among the most common causes of death in patients with osteosarcoma. While tumour unresponsiveness to drug therapy may be related to many factors, multiple-drug resistance (MDR) may prove to be the most important obstacle to successful treatment (Chabner, 1986).

A recently discovered, and most frequently described, model of drug resistance is the P-glycoprotein-specific MDR-1 gene phenotype (See Chapter 1, section 1.4). Increased levels of P-glycoprotein (P-gp) are thought to confer MDR to tumour cells by decreasing the net intracellular accumulation of unrelated lipophilic cytotoxic agents such as doxorubicin and vincristine, which are commonly used in the treatment regimens for high grade malignant osteosarcoma.

For an assessment of MDR in tumours, studies have either used methods for detecting the mRNA for the MDR-1 gene, or raised monoclonal antibodies against epitopes on the P-gp molecule (See Chapter 1, section 1.4). One such monoclonal antibody is C219, which was developed by a group in Toronto (Kartner et al., 1985). C219 is not species-specific, and recognizes a small, highly conserved epitope of P-gp in the cytoplasmic domain of the molecule (Weinstein et al., 1990). C219 has the advantage over other antibodies in being able to detect P-gp in formalin-fixed, paraffin-embedded material, and hence it may be used on routinely prepared pathological material. This would obviously have great advantages for modulation of therapy, if in the future it is concurred that P-gp expression is a useful prognostic indicator for therapeutic outcome.

Preliminary studies carried out on osteosarcoma have shown a correlation between P-gp expression at biopsy, and response to chemotherapy (Chan et al., 1991; Rosier et al., 1992; Wunder et al., 1993), but none of these studies investigated the alterations in P-gp expression before and after neoadjuvant chemotherapy. This would give an indication of the extent that P-gp, and hence drug resistance levels are altered after drug exposure, and just how important P-gp is in relation to drug resistance in osteosarcoma.

In this chapter, the expression of P-gp in biopsy and post-surgical material from patients who had received neoadjuvant chemotherapy, including agents involved in MDR (e.g. doxorubicin), was assessed using immunohistochemical techniques, with an antibody to a cell membrane epitope of P-gp, C219.
6.2 MATERIALS AND METHODS

Biopsy and resection or amputation material from 19 patients with clinically diagnosed high grade malignant osteosarcoma treated at the Royal National Orthopaedic and Middlesex Hospitals was used in this study. For the thirteen patients in the study for which treatment protocol data were available, all had received pre-operative combination chemotherapy including an MDR agent (either doxorubicin or vincristine), and at least one non-MDR alkylating agent (cisplatinum and/or ifosfamide). The clinical data of the patients are shown in table 6.1.

The biopsy material was formalin-fixed and paraffin embedded as described in Chapter 2 (section 2.2). 8μm thick sections were cut dry, placed on a water bath, and collected on glycerol-albumin coated slides.

The post-surgical material was subjected to the LR White processing procedure as described in Chapter 2 (section 2.5). For each patient, four blocks of tissue were selected, and 1μm sections were cut and transferred to a drop of water on chrome-alum gelatin coated slides (Chapter 2, section 2.6), and sections were air dried at 45°C for 1 hour.

Sections were then subjected to the immunogold silver enhancement procedure described in Chapter 2 (section 2.9). The biopsy sections were first de-paraffinised, and rehydrated by passing through the following series of solutions for 5 minutes in each: 3 x xylene; 2 x 100% IMS; 90% IMS; 75% IMS; 50% IMS; 2 x distilled water, and PBS, after which the sections were processed as for the LR White sections.

The primary antibody used was the monoclonal mouse anti-human P-gp marker C219 (CIS (UK) Ltd.), and the antibody was applied for 2 hours at room temperature, at a protein concentration of 5μg/ml.

After silver enhancement, sections were counterstained with Harris' haematoxylin for 5 minutes. The LR White sections were then differentiated and mounted as described in Chapter 2 (section 2.9). The biopsy sections were differentiated for 3 minutes in running tap water, and then a cytoplasmic counterstain of eosin was applied for 20 seconds. After rinsing off the excess eosin in running tap water, the sections were then dehydrated through a series of alcohols, and xylene, before being mounted in DPX mounting medium (BDH).
Table 6.1 - clinical data and chemotherapy regimens for patients used in study:

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Key:-
* - no therapy data available for patient
M - male; F - female
fem - femur; pms - pulmonary metastases; tib - tibia; scp - scapula; calc - calcaneus;
ilm - ilium
DXR - doxorubicin; CPL - cisplatinum; DEX - dexamethasone; IFOS - ifosfamide;
MTX - methotrexate; VCR - vincristine
+ - compound used in pre-operative chemotherapy protocol
Negative controls were as described in Chapter 2 (section 2.9). As a positive control, paraffin sections containing areas of normal dermis, in which sweat glands have shown to be positive for P-gp (Van der Valk et al., 1990) were used. Also, in order to assess whether there were differences in the antibody detection levels between paraffin and resin-embedded tissue, samples from the same area of a post-surgical specimen were embedded in either paraffin or LR White, and sections were assessed for C219 immunolabelling as described above.

Examination and cell counts were made on a Zeiss Photomicroscope III under water immersion at an overall specimen magnification of X500. Cell counts were made using a 10 x 10 square eyepiece graticule (area = 0.2mm²), with 20 fields (excluding clearly reactive cells, haemocytes and those in vascular elements) being counted for both biopsy and post-surgical material per patient. Photographs were taken on a Leitz DM/RB microscope using Kodak Gold 100 colour 35mm negative film used at 100 ASA.

The percentage P-gp immunopositivity counts obtained for biopsy and post-surgical material were compared using a paired student’s t-test. Computation of the statistical test was carried out using Minitab software (Minitab Inc., PA, USA).
6.3 - RESULTS

For each of the 19 patients, 1 biopsy section and 4 sections of post-surgical tissue were examined. C219 immunopositive cells were seen in positive control sections and most biopsy and post-surgical sections examined, with the positivity evident as dense black granules at the periphery of the cell (figure 6.1a-f). In the positive control sections (figure 6.1g), cells in the sweat glands of the normal dermis were the only cells which were consistently immunopositive. No differences were seen in the levels of P-gp expression in the specimen whose tissue was embedded in both paraffin and LR White.

When C219 immunopositive cells were counted, for most of the specimens it was seen that there were generally higher percentages of cells in the biopsy specimens as opposed to the post-surgical ones, with 12 patients having greater than 10% of cells exhibiting C219 immunopositivity at biopsy, compared with 2 after surgery. Figure 6.2 shows the relationships between biopsy and post-surgical tissue for percentage C219 immunopositive cells for the 19 patients included in this study. A paired student’s t-test was performed upon the data, and it was found that the amount of C219 immunopositive cells was significantly greater at biopsy compared with postsurgery (p=0.003). From this group of 19 patients, however, 3 showed a reverse of the general trend, with the percentage of cells expressing C219 increasing slightly from biopsy to post surgery, though by conducting a t-test on the figures for these 3 patients, it was found that the increase was not significant (p=0.076).
Figure 6.1a - immunolabelling for C219 antibody in a biopsy section from a 16-year old male with osteosarcoma. Immunopositivity is seen as black granules towards the periphery of positive cells (arrowheads). Haematoxylin and eosin counterstain. Magnification x 882.

Figure 6.1b - immunolabelling for C219 antibody in a post-surgical section from the same patient as in figure 6.1a. Immunopositivity is seen as black granules towards the periphery of positive cells (arrowhead). Weak haematoxylin counterstain. Magnification x 882.

Figure 6.1c - immunolabelling for C219 antibody in a biopsy section from a 16-year old female with osteosarcoma. Immunopositivity is seen as black granules towards the periphery of positive cells (arrowheads). Haematoxylin and eosin counterstain. Magnification x 882.

Figure 6.1d - immunolabelling for C219 antibody in a post-surgical section from the same patient as in figure 6.1c. Immunopositivity is seen as black granules towards the periphery of positive cells (arrowhead). Weak haematoxylin counterstain. Magnification x 882.

Figure 6.1e - immunolabelling for C219 antibody in a biopsy section from a 35-year old female with osteosarcoma. Immunopositivity is seen as black granules towards the periphery of positive cells (arrowheads). Haematoxylin and eosin counterstain. Magnification x 882.

Figure 6.1f - immunolabelling for C219 antibody in a post-surgical section from the same patient as in figure 6.1e. Immunopositivity is seen as black granules towards the periphery of positive cells (arrowhead). Weak haematoxylin counterstain. Magnification x 882.

Figure 6.1g - immunolabelling for C219 antibody in a paraffin-embedded section of normal dermis used as a positive control section for the antibody. Sweat gland cells show strong immunopositivity towards their periphery (arrowheads). Toluidine blue counterstain. Magnification x 882.
Figure 6.2:-
The expression of C219 immunopositivity in biopsy and post-surgical sections for the 19 patients used in this study.
6.4 - DISCUSSION

From this study, as with the previous gold-silver enhancement immunocytochemical studies carried out for this thesis, it can be seen that the immunogold-silver enhancement method provides a highly sensitive methodology for visualising P-gp epitopes in osteosarcoma tissue which has been paraffin or acrylic resin embedded.

It was found that there was a statistically significant decrease in the percentage of cells expressing the P-gp epitope in the post-surgical specimens compared with the biopsy specimens. The pre-operative combination chemotherapy regimens for these patients included not only agents involved in MDR, such as doxorubicin and vincristine, but also cisplatin. The actions of cisplatin on tumour cells are not affected by expression of P-gp, and thus, it may be that a significant amount of the population of P-gp resistant cells in the tumour were killed off by the action of cisplatin, leading to lower P-gp expression levels observed post-surgery.

For most of the sections of post-surgical specimens examined, there were cells in "altered" areas which were included in the cell counts for the section. It could be that in the "altered" cells the expression of the epitope on the P-gp molecule that is recognised by the C219 antibody may have been transformed due to action of chemotherapeutics upon the cell genotype. This transformation of the C219 epitope may not have had any effect on the function of the P-gp membrane pump, and thus, although the P-gp molecules are not being recognised by the antibody detection system used in this study, these cells may be still be expressing the MDR phenotype. To check for this possibility, antibodies which recognise different epitopes on the P-gp molecule could be applied. Probably a more foolproof way, if it was possible to obtain unfixed material, would be to assess the MDR-1 gene expression levels. If the P-gp is still being produced normally, but being physically altered in situ, the levels of the encoding mRNA should be little different post-surgery compared with at biopsy.

Ideally, material assessed post-surgically would be from viable, non-chemotherapy affected areas, if they still existed after pre-operative chemotherapy. This would reduce the potential problem of the "altered" cells. However, sampling by the pathologist does not allow for this in the present study.

The theory that it is cisplatin that is circumventing the MDR mechanism, and so killing the P-gp positive cells, may be proved by looking at the mechanisms within
the cell for exclusion of metal compounds, such as cisplatin. These include metallothionein (MT), which is part of a family of low molecular weight cytosolic proteins that are rich in cysteine and bind heavy metals, such as zinc, copper, cadmium and mercury. MT appears to have a physiological role in the absorption, transport and metabolism of the important trace metals, as well as a role in heavy metal detoxification. MT also seems to affect the cellular sensitivity to electrophilic anti-cancer agents, such as cisplatin. Cells with acquired resistance to heavy metals such as cadmium, overexpress MT, and are cross-resistant to the alkylating agents and cisplatin. The mechanisms by which MT produces cisplatin resistance is not known, although some investigators have suggested that MT may bind cisplatin (Bahnson et al., 1991). Antibodies are available against MT, and these could be applied to the biopsy and post-surgical material using the method described in this chapter. If the hypothesis is right that cisplatin is killing the MDR cells, and that the cells remaining post-surgery are drug resistant by other mechanisms except from MDR, especially the cisplatin resistant mechanism, then there should be significantly higher levels of metallothionein expression in the post-surgical sections than in biopsy ones.

In this study it was not possible to acquire sufficient follow-up data on the patients involved in the study to investigate any correlation between levels of P-gp expression and response to therapy. A retrospective study is in the process of being carried out in which biopsy specimens from a large number of patients with known long-term follow-up data will be assessed using immunohistochemical techniques for levels of P-gp and MT expression at biopsy.

Thus, it may be concluded that the P-gp phenotype for MDR in osteosarcoma may be circumvented by use of appropriate agents unaffected by the P-gp mechanism.
CHAPTER 7 - IN VITRO ASSESSMENT OF THE RECOVERY POTENTIAL OF OSTEOSARCOMA CELLS AFTER CHEMOTHERAPY
7.1 - INTRODUCTION

From Chapter 5 it was concluded that although there was a proportion of the "altered" cells in the process of programmed cell death, there was still evidence that cells were present which were either in a prolonged cycle, or could not proceed from S-phase or G$_2$-phase to mitosis.

Two questions arise from these findings. Firstly, are cells which are acid phosphatase positive irreversibly committed to programmed cell death, or can they recover once a change of environment, such as the withdrawal of chemotherapeutic agents has occurred? The second question is, can cells which were detected with the PC10 antibody to be in S-phase proceed through the cell cycle once chemotherapy is withdrawn?

Although these questions would appear irrelevant, in that the assessment of metabolic status was carried out on primary tumour tissue removed from the patients, and hence these samples no longer represent a threat to the patients, there is still the problem of cells in secondary tumour deposits, and also any remnant material at the site of the primary tumour. Assuming that these cells have been affected by therapy in the same way as those seen in "altered" areas of the primary tumour, an idea of the way to proceed with post-operative chemotherapy could be gathered by an assessment of the response of the "altered" cells to the removal of chemotherapy.

One method of observing how the cells react once the chemotherapeutic agent is withdrawn, would be to transfer them into an in vitro environment, and see if tumour cell differentiation and proliferation can occur. Although primary culture and in vitro manipulation of cells from human osteosarcomas has been carried out before (Fournier and Price, 1991, Kawai, 1990, Fodstad et al., 1986, Levine et al., 1980), there is no mention in the literature of anyone attempting to culture cells from "altered" areas of tumours from patients who had received pre-operative chemotherapy.

In this preliminary study, an attempt was made to grow up tumour cells from explants of human osteosarcoma taken from surgical specimens which had been subjected to pre-operative neoadjuvant chemotherapy. Outgrowing cells were then assessed to see if they were bone precursor cells or not using a histochemical detection method for alkaline phosphatase. The efficacy of the alkaline phosphatase
methodology was confirmed using an immunofluorescence technique with antibodies to two bone matrix proteins. The amount of tumourous cells present with time in culture was thus monitored in order to assess whether cells in "altered" areas could recover and proliferate in response to a change in environment lacking the presence of chemotherapeutic agents. Where present, explants were taken from viable areas of tissue to see if there was any differences between the levels of alkaline phosphatase expression in viable and "altered" tissue.
7.2 - MATERIALS AND METHODS

Freshly excised tissue was collected from eight patients with high grade malignant osteosarcoma who had received pre-operative chemotherapy. Selective samples of tissue were taken from areas of tumour which macroscopically appeared to the pathologist to be "altered" or viable (where present). Figure 7.1a and b present the clinical data for the patients used in this study. Tissue was then processed for tissue culture as explants, as described in Chapter 2 (section 2.10).

At varying time intervals, once outgrowth of cells in substantial numbers from the explants had been observed, dishes of cells were fixed, air dried and stored as described in Chapter 2 (section 2.10). Cells were then assessed histochemically for the presence of alkaline phosphatase, a marker for osteoblast differentiation, using the procedure outlined in Chapter 2 (section 2.11).

Dishes were examined using a Zeiss Axiovert 35 inverted microscope, and photomicrographs taken using Ilford PAN F 50 ASA film at 100 ASA. Two hundred cells were counted per explant, and the number of alkaline phosphatase positive cells (a red membrane/cytoplasmic staining) was noted. Up to 6 explants were assessed for each time point, and the mean percentage of alkaline phosphatase positive cells was calculated.

In order to further confirm that the outgrowing cells were of osteoblastic lineage, immunofluorescence was carried out according to the procedure described in Chapter 2 (section 2.12). The primary antibodies used were:

**AON-1** - which recognises human bone osteonectin. Ascitic fluid used at a dilution of 1:100.

**VD12** - which recognises human bone osteopontin. Tissue culture supernatant used neat.

The primary antibody was applied for 45 minutes. The secondary antibody used was a rabbit anti-mouse fluorescein-conjugated antibody, at a concentration of 1:50 for 45 minutes. The negative controls used in this technique were PBS and mouse non-immune immunoglobulins (Sigma) at comparable protein concentrations to the primary antibody, which were applied instead of the primary antibody.
Assessment of immunopositivity was carried out using confocal laser scanning microscopy. This was performed using a Molecular Dynamics Sarastro 2000 Confocal Scanning Microscope. Specimens were scanned using a 25mW argon laser with appropriate excitation and emission filters for fluorescein (488/510 nm). Specimens were examined using a x40 oil immersion objective lens (50 μm confocal aperture). Series of optical sections (512 x 512 pixels; approximately 0.5 μm thick) were taken through the entire cell monolayer at a spacing of 1-2 μm. Three dimensional projections were prepared from the section series using Molecular Dynamics "Imagespace" volume rendering software running on Silicon Graphics UNIX workstations. Projections were made using a look-depth reconstruction method. With this method, optical section layers are added together and deeper layers are attenuated proportionally to their distance from the viewer before the addition to the reconstruction. Section series were filtered using 3D Gaussian (smoothing or noise removal), or 3D Gradient (edge definition) filters. The three-dimensional projections were printed using a Shinko CHC-S446i dye sublimation colour printer.
Chapter 7

Figure 7.1a:-
Age-sex distribution for patients in this study

Key: fem = femur, tib = tibia, hum = humerus, pms = pulmonary metastases

Figure 7.1b:-
Site-sex distribution for patients in this study

Key: fem = femur, tib = tibia, hum = humerus, pms = pulmonary metastases
Chapter 7

7.3 - RESULTS

Of the specimens used in this chapter, examination by the pathologist deemed 3 of the tissue samples to be from viable areas of tissue, and 7 from "altered" areas. Outgrowth of cells was seen for all of the specimens which were cultured. Table 7.1 shows the outgrowth rates and the days of harvesting for the specimens. However, the rate of outgrowth, and the number of explants demonstrating outgrowth varied greatly between specimens. It can also be seen that there was no discernible increase in the number of explants showing cell outgrowth with increasing time in culture.

There was a large variation in the cell size and morphologies observed, many explants having mixed populations of cells of different shapes and sizes. Figure 7.2a-d shows some of the different morphologies which were seen.

Alkaline phosphatase positivity was seen in cells of varying morphologies and sizes in the majority of explants which were assessed (figure 7.3a + b). There was also a variance in the number of positive cells seen. Figures 7.4a and 7.4b show plots of alkaline phosphatase positivity against days in culture for the examined viable and "altered" specimens. For most of the specimens examined, it can be seen that there was an increase in the proportion of alkaline phosphatase positive cells with time in culture.

In order to further confirm that the outgrowing cells were of osteoblastic lineage, immunofluorescence was performed using antibodies to osteonectin and osteopontin. Cytoplasmic immunopositivity for both antibodies was detected in the cytoplasm of both normal and enlarged tumour cells (figure 7.5a-f). There was no immunopositivity detected in the negative controls.
Chapter 7

Table 7.1:

The outgrowth rates and harvesting times of explant cultures from 8 osteosarcoma specimens

<table>
<thead>
<tr>
<th>SPECIMEN NUMBER</th>
<th>VIABLE (V)/ &quot;ALTERED&quot; (A) TUMOUR RESPONSE</th>
<th>DAYS IN CULTURE</th>
<th>NUMBER OF EXPLANTS WITH CELL OUTGROWTH</th>
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<td>V</td>
<td>17</td>
<td>4</td>
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<td>21</td>
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<td></td>
<td></td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>91/1A</td>
<td>A</td>
<td>17</td>
<td>1</td>
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<td>21</td>
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<td>24</td>
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<tr>
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<td>6</td>
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<td>17</td>
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<td>91/4</td>
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Figure 7.2 - explant cultures of osteosarcoma: cell morphology

Figure 7.2a - phase contrast photomicrograph of cells adjacent to tissue explant (X) from an "altered" tissue sample after 16 days growth in culture. A variety of cell morphologies are evident. Amongst the mainly spindle-shaped regular cells (arrows), some enlarged irregular cells are seen (arrowheads). Magnification x 384.

Figure 7.2b - phase contrast photomicrograph of cells adjacent to tissue explant (X) from a viable tissue sample after 9 days growth in culture. As with figure 7.2a, a variety of cell morphologies are evident. Most of the cells have a round or spindle-shape (arrows). Some enlarged irregular cells are also evident (arrowheads). Magnification x 384.

Figure 7.2c - phase contrast photomicrograph of outgrowing cells away from the tissue explant from an "altered" tissue sample after 17 days growth in culture. More of the cells are enlarged and irregular (arrowheads), than in the areas adjacent to the explant. Also visible are regular spindle-shaped cells (arrows). Cytoplasmic vacuolation is evident in many of the cells. Magnification x 384.

Figure 7.2d - phase contrast photomicrograph of outgrowing cells away from the tissue explant from a viable tissue sample after 17 days growth in culture. Most of the cells are enlarged and irregular (arrowheads), and many are binucleate (arrows). As with the "altered" area cells in figure 7.2c, cytoplasmic vacuolation is evident in many of the cells. Magnification x 384.
Figure 7.3 - explant cultures of osteosarcoma: alkaline phosphatase positivity

Figure 7.3a - photomicrograph of outgrowing cells from a viable tissue sample after 17 days growth in culture. Alkaline phosphatase positivity is seen in some of the enlarged cells (arrowheads), and regular spindle cells (arrows). Magnification x 384.

Figure 7.3b - photomicrograph of outgrowing cells from an "altered" tissue sample after 17 days growth in culture. Alkaline phosphatase positivity is seen in some of the enlarged cells (arrowheads), and regular spindle cells (arrows). Magnification x 384.
Figure 7.4a:-
Percentage of alkaline phosphatase positive cells in explant cultures from viable osteosarcoma tissue

Figure 7.4b:-
Percentage of alkaline phosphatase positive cells in explant cultures from "altered" osteosarcoma tissue
Figure 7.5 - explant cultures of osteosarcoma: osteonectin and osteopontin immunopositivity

Figure 7.5a - negative control where phosphate buffered saline was applied instead of the primary antibody. No evident immunopositivity.

Figure 7.5b - negative control where mouse immunoglobulins were applied instead of the primary antibody. No evident immunopositivity.

Figure 7.5c - immunolabelling for AON-1 antibody in outgrowing "regular" tumour cells away from a tissue explant. Immunopositivity is seen in the cell cytoplasm.

Figure 7.5d - immunolabelling for AON-1 antibody in outgrowing enlarged irregular, and "regular" tumour cells away from a tissue explant. Immunopositivity is seen in the cell cytoplasm.

Figure 7.5e - immunolabelling for VD12 antibody in outgrowing "regular" tumour cells away from a tissue explant. Immunopositivity is seen in the cell cytoplasm.

Figure 7.5f - immunolabelling for VD12 antibody in an outgrowing enlarged irregular tumour cell away from a tissue explant. Immunopositivity is seen in the cell cytoplasm.
7.4 - DISCUSSION

The aims of this chapter were to assess whether tumourous cells in "altered" areas of osteosarcoma could continue to grow and differentiate in an in vitro culture system, once they were removed from their local environment and the presence of chemotherapeutic agents.

The in vitro system chosen for culture and propagation of cells was an explant culture method. This was preferred to freeing cells from the surrounding extracellular-matrix using proteases such as collagenase, as it was feared that this system could not be adequately standardised and controlled, in that each tissue sample would have a differing thickness of matrix, which would mean varying digestion times for each specimen. Estimating digestion times would be difficult just on sight of the tissue alone. Hence there would be the potential problem of overdigestion of tissue, and as a consequence damage to liberated cells being exposed to the proteases for long periods of time, although carrying out sequential enzyme digests with associated cell harvesting rather than just one digest might alleviate some of the problems of tissue damage.

With explant culture, the main disadvantage was that with the osteosarcoma tissue, outgrowth of sufficient cells to assay took a long period of time to occur (usually 7-14 days), and until the alkaline phosphatase histochemistry was carried out, it was not possible to tell if the outgrowing cells were of osteoblastic lineage and hence most probably tumourous or were stromal cells from repair and reactive tissue.

It was seen that for all tissue samples, regardless of chemotherapy effect, outgrowth of alkaline phosphatase positive tumour cells was seen for some or all of the explants assessed. There was no recognisable increase in the number of explants showing cell outgrowth with time in culture, or any relationship between the number of explants from viable and "altered" regions showing outgrowth. It was also seen that there was no difference in the trends of tumour cell differentiation as assessed by alkaline phosphatase between viable and "altered" samples.

In order to assess the efficacy of using alkaline phosphatase methodology as a marker for osteoblastic lineage, an immunofluorescence technique with antibodies to two matrix proteins which are commonly associated with bone cell phenotype, osteonectin (Serra et al., 1992), and osteopontin (McCabe et al., 1994) was carried out. It was seen that both regular-sized and enlarged irregular cells demonstrated
positivity for the osteonectin and osteopontin antibodies, which would suggest that these cells were of osteoblastic lineage and not contaminant stromal fibroblasts or reactive cells.

All samples, except for 91/11V, showed an increase in the percentage of cells showing osteoblastic differentiation with increasing time in culture. This could be put down to many factors. Firstly, normal cells, such as fibroblasts, may be the first to migrate away from the explant, since perhaps the matrix surrounding them will be less dense than that surrounding the tumour cells, thus their movement will be less impeded. With time, an increasing number of tumour cells will migrate, and also proliferate at a higher rate than the non-tumourigenic cells which would be reflected in an increase in the proportion of cells showing alkaline phosphatase positivity, with increasing time in culture. Secondly, in connection with the first factor, for each specimen the rate of outgrowth of cells varied from dish to dish. Thirdly, but less likely, it could be that the normal fibroblasts are differentiating into osteoblastic cells and hence will start to express alkaline phosphatase positivity.

Of all the samples studied, the only exception to the trend of increased alkaline phosphatase activity was specimen 91/11V, which showed an increase in alkaline phosphatase activity from day 7 to 10, and then a decline in activity thereafter. This could be due to there being a large number of fibroblasts in the dishes selected for assessment for the later time-points, which would decrease the percentage of alkaline phosphatase positive cells seen.

Enlarged irregular cells showing alkaline phosphatase positivity were seen throughout the duration of culture, in both viable and "altered" explant cultures, suggesting that these cells are still metabolically active. However, it was not possible from this study to assess the proliferative potential of these enlarged cells \textit{in vitro}, but this could have been done using immunohistochemical markers for PCNA, or radioactive isotopes, which would indicate if the cells were in cycle. Also, to assess if these were migratory cells from the explant or progeny cells, the cells in the explant could be tagged with a vital intra-cytoplasmic dye, e.g. di-I, whose presence could be monitored by assessing intensity in the outgrowing cells, with progeny cells demonstrating a weaker intensity of staining than the original explant cells.

When the proportion of alkaline phosphatase cells in "altered" and viable areas was compared, it was seen that the trends of outgrowth were very similar. This would
suggest that the tumour cells within the "altered" area explants are migrating out and proliferating at a similar rate to those in the viable area explants.

These data would tentatively suggest that cells in the "altered" areas have the capacity to continue growth once they are removed from the *in vivo* environment, and hence from the influence of chemotherapeutic agents. Thus, "altered" areas of tissue remaining in the body after surgery would still contain cells which would have the potential to further propagate the tumour, once chemotherapy is withdrawn. Hence, as far as prognosis is concerned, the presence of "altered" areas is not a good sign, and probably continuation and modification of aggressive chemotherapy post-surgery would be recommended. However it must be emphasised that this is a preliminary study, and due to the heterogeneous nature of osteosarcoma (as reported in previous chapters) in both its morphology and response to chemotherapy, it would be necessary to culture material from several tumours in order to obtain a more accurate picture.
CHAPTER 8 - GENERAL DISCUSSION
Osteosarcoma is a cancer which causes a lot of concern in that the age at which it affects people is during the phase of greatest growth (i.e. second decade of life). Hence, although it is not high in the league table of population rates of cancer, there are many attempts being made to improve the survival rate of people with the disease. This has led to a change in the attitudes to treatment of the disease within the last twenty years, from amputation to a less aggressive protocol where combination chemotherapy is given to reduce the tumour load before the tumour is excised, and a prosthesis put in its place in order to preserve function to the affected limb. With this treatment protocol now firmly in place as the preferred mode of treatment for patients with high-grade malignant osteosarcomas, there have been several attempts made to give a prognosis for the patient, and modulate post-operative chemotherapy, by examining the tumour at resection, and assessing the response of the tumour to the pre-operative chemotherapy. As yet no single method has proved successful in giving an irrefutable prediction for tumour behaviour post-surgery, and this is probably due to the heterogeneity of the disease in terms of appearance. It has been almost universally accepted though, that a reduction of the viable tumour burden to less than 10% of the original tumour mass, may be considered a good sign (Rosen et al., 1979, Ayala et al., 1980, Rosen et al., 1982, Salzer-Kuntschik et al., 1983, Kempf et al., 1991, Petrilli et al., 1991). But what is confusing with osteosarcoma, is that areas are seen in resected or amputated specimens which are neither totally viable (i.e. resistant to the pre-operative chemotherapy), nor necrotic (i.e. responsive to therapy), but are "histologically altered", and are known to contain cells which are alkaline phosphatase positive, which suggests that these cells are still metabolically active. Hence "altered" cells remaining in the body after surgery may potentially pose a threat to the patient in terms of tumour propagation. However, the small amount that has been written in the literature about these "altered" cells has just been to acknowledge the presence of these cells, and nothing has been published about their tumourigenic potential, and part of the aims of this thesis have been to acquire more information about these cells, to see if their character may have any prognostic significance for the patient.

This characterisation took many aspects, with work sub-divided in terms of: the morphology of chemotherapy-affected cells; differences in extracellular matrix molecules; metabolic status of affected cells; changes in expression of the multi-drug resistance molecule P-glycoprotein (P-gp), and in vitro assessment of the recovery potential of affected cells.
Most of the previous investigations which have histologically examined tumour tissue after neoadjuvant chemotherapy have noticed the presence of "bizarre" cells in areas where there has not been total tumour response to the chemotherapy (Huvos et al., 1977, Grundmann et al., 1983, Raymond and Ayala, 1988). The work in this thesis has concurred with these findings of "bizarre" cells, and has taken this work a step further by ultrastructurally examining the cells. Upon ultrastructural examination, some of these cells had the same structural composition as "regular" sized cells of the same histological sub-class, and did not demonstrate any signs of degeneration, which would suggest that these cells are still in a "regular" functioning state. This hypothesis is backed up in some way by the fact that in the in vitro studies carried out, enlarged cells were also evident from an early stage in explant culture, and hence were not a result of frequently observed hypertrophism due to long term cell culture. These cells were alkaline phosphatase positive which suggests that they were metabolically active, and hence if these cells correspond to the cells seen in vivo, then this would also infer that the in vivo cells are metabolically active.

The next question posed then is whether these cells have any potential to contribute to further tumour propagation. When metabolic studies were carried out, it was seen that none of these enlarged cells were immunopositive for the S-phase cell-cycle marker PCNA. Enlarged cells were seen undergoing mitosis, but these mitoses were highly irregular, and it was likely that "regular" cells had aborted in mitosis due to the action of cytotoxic agents, and had become swollen and enlarged.

This finding would suggest that these enlarged "altered" cells do not impose a threat in terms of tumour propagation, and this is further confirmed by the histochemical marker studies on cell death, where many of these cells were shown to be undergoing programmed cell death.

In vitro however, it was seen that enlarged cells were evident in subsequent passages of the tumour cells. These hypertrophic cells may be the result of prolonged cell culture, and using markers for cell proliferation and cell death, the status of these cells could be determined.

Also seen within the "altered" areas were "normal" looking tumour cells, which probably correspond to the small clumps of unaffected tumour cells reported by many authors in chemotherapy-affected areas (Rosen et al., 1979, Salzer-Kuntschik et al., 1983, Misdorp et al., 1988). Some of these cells did show positivity to the
PCNA antibody, and regular mitotic figures were evident, which would suggest that these cells have the potential to further propagate the tumour. Thus if "normal" looking cells are seen within "altered" areas of tumour, then from the studies carried out in this thesis, one would be wary of assigning these "altered" areas as any better a response to neoadjuvant therapy in terms of potential tumour propagation, than for viable areas.

In terms of the extracellular matrix (ECM), little could be concluded about alterations in the ECM due to chemotherapy from Chapter 3. Hence, this was one of the reasons for looking at alterations in glycosaminoglycan (GAG) matrix molecules in Chapter 4. It was found in "altered" areas that expression of the GAG epitopes was more pronounced than in viable areas for each of the histological sub-classes examined. This could possibly be due to the increased repair mechanisms being carried out in the areas where necrotic tumour was being replaced by reactive connective tissue. However, as with most other results in this thesis, these trends of higher GAG expression in "altered" areas was very subjective, due to the low sampling sizes for some of the sub-classes.

Where "regular" osteosarcoma tissue was histologically examined, i.e. viable unaffected material in surgical specimens, findings agreed with previous reports. In this study, it was seen that osteosarcoma is an incredibly heterogeneous tumour in terms of histological appearance, with a variety of distinct cell based sub-classifications. Differences in cell types were seen at light microscope level, and were confirmed by differences seen at the ultrastructural level.

Furthermore, the investigation of GAG composition of the ECM and of the cells, also showed a range of patterns for different epitopes between the different sub-classifications. This study of the ECM in osteosarcoma appears to be the first time that anyone has analysed specific components of the ECM in untreated or treated osteosarcoma, though it has been previously reported that the presence of certain histological sub-groups (chondroblastic) in the composition of a tumour is a sign of poor response to chemotherapy (Kersjes et al., 1987). Although the study in this thesis was focussed on just a very small component group of the ECM, the fact that differences were seen would suggest that this area may be worth investigating at further depth, by looking at the levels of other components of the ECM, such as collagens, to see if similar patterns are observed. Another group of matrix-associated molecules which would be important to characterise in terms of levels in samples from the different histological sub-classes would be the matrix
metalloproteinases and their inhibitors (Murphy et al., 1989). The matrix metalloproteinases are a group of enzymes which break down ECM components such as collagens which have been implicated in the process of tumour cell metastasis. Thus, the levels seen in cells and ECM in different sub-groups of osteosarcoma and in areas of differing chemotherapy affect may be a predictor as to the aggressiveness of the particular manifestation of osteosarcoma.

When untreated tumour was assessed for expression of the multi-drug resistance marker, P-glycoprotein, it was seen that varying levels of the marker could be detected in biopsy samples using the chosen immunohistochemical technique. This study did not agree with previous reported studies that there was a correlation between P-gp expression levels in biopsy samples and response of the patient to chemotherapy (Chan et al., 1991; Rosier et al., 1992; Wunder et al., 1993). However, the small sample size used for this study, with a larger number of cases than would be expected either dead or showing tumour recurrence, may account for this disparity.

Novel work carried out in this thesis in terms of drug resistance, was the investigation of P-gp expression in surgical specimens post-chemotherapy. As for biopsy material, no correlation was seen with expression and response of the patient to treatment, but when P-gp levels before and after neoadjuvant chemotherapy were compared, it was seen that there was a significant decrease in the levels of P-gp expression after therapy. This would suggest that the multi-agent approach for treatment of osteosarcoma is circumventing the P-gp resistance mechanism.

When the levels of expression of P-gp were compared with the levels of cell death and PCNA expression seen in the post-surgical tumour specimens, no correlations were evident. This again suggests that P-gp drug-resistance mechanisms are not playing a definitive role in whether or not a cell is being affected by the multi-agent chemotherapy.

The above findings, along with the lack of correlation between expression of P-gp and clinical outcome, would suggest that the future proposed use of the P-gp phenotype as a definitive predictor for response to chemotherapy in osteosarcoma is a method which would have to be treated with extreme caution. A more reliable prediction system would perhaps be the use of P-gp detection systems in combination with those for other drug resistance mechanisms, such as the presence
of metallothionein which has been implicated in the exclusion of metal compounds such as cisplatin from cells.

Potential refinements of the procedures used within this thesis have emerged since the work was carried out in terms of detection of the cell metabolism characteristics, and the recovery potential of chemotherapy-affected cells.

The use of PCNA as a marker for cell proliferation has been called into question. It is known that the molecule has a very long half-life such that it is still detectable in cells for prolonged periods of time after undergoing mitosis (Bravo and Macdonald-Bravo, 1985). Also PCNA can be synthesised by cells which are not cycling, and is induced by growth factors (Baserga, 1991). Thus, it is only in long-term quiescent cells where PCNA is totally downregulated, and hence these are potentially the only cells where PCNA is not detected by the immunohistochemical technique used in this study. One way of assessing whether the PCNA immunolabelling seen in this study was only of cells in the S-phase of the cell cycle would have been to carry out a dual labelling methodology combining the antibody technique with tritiated thymidine incorporation.

The controversies surrounding the use of acid phosphatase as a marker for cell death have already been discussed in Chapter 5, and the problem of using the enzyme to distinguish between positive tumour cells and reactive cells has also been touched on. In embedded tissue, an ideal way to assess cell death would be to utilise antibodies to molecules expressed in detectable levels solely during the process of programmed cell death, which would not be expressed by reactive cells present at the tumour site. Several molecules have been proposed as markers for programmed cell death, including the presence of histones (Duke and Cohen, 1986), the co-expression of the tumour suppressor genes bcl-1 and p53 (Shaw et al., 1992, Lane, 1993), the Rb-1 tumour suppressor gene (Clarke, 1994), the early response genes c-fos and egr-1 (Walkinshaw & Waters, 1994).

The question of whether cells in "altered" areas which were shown with acid phosphatase histochemistry to be undergoing programmed cell death were irreversibly committed to the process, or could recover and proceed to proliferate once chemotherapeutic agents were removed, was addressed briefly in this thesis by explant culture in vitro of surgical material. From examination of the small number of samples used in this study, the trend appeared to be that once chemotherapy was withdrawn, cells from "altered" areas could continue to proliferate. However this in
vitro system is not entirely ideal for assessing tumour cell propagation in that many of the growth and proliferation control factors found in the natural in situ environment of the tumour in the patient, are not present in the in vitro system. A way of circumventing this problem would be to cultivate the tumour samples to an in vivo environment. Models have already been established for the growth of human tumour cell lines in both nude mice (Bell et al., 1988, Sturtz et al., 1992, Berlin et al., 1993) and rats (Witzel et al., 1992), and these could be manipulated for cell recovery potential studies. The most practical way of doing this would be to inject tumour fragments or cells into immunocompromised animals, and monitor the animals for signs of tumour propagation, in terms of growth at the primary tumour site, and evidence of metastases to recognised secondary sites such as the lungs. If tumour growth occurred, the primary tumour or metastases could be removed and characterised in terms of cell morphology and cell metabolism, using the methods described for looking at surgical material described in this thesis. This data could then be compared with that obtained from the initial surgical material to confirm the lineage of any arising tumours in the animals. In order to continue the work done in this thesis, I have started to use the orthotopic transplantation model described by Berlin et al. (1993) in order to investigate the recovery potential of "altered" tumour in vivo in the fashion described above.

In conclusion, the main concern of the work carried out for this thesis was to address the question of what prognostic significance the presence of "altered" areas of tissue seen in surgical material after neoadjuvant chemotherapy has in terms of modulating post-surgical chemotherapy. The answer, from the work carried out in this thesis, is that although the enlarged irregular cells seen in these "altered" areas do not provide a threat in terms of tumour propagation, any "regular" cells present in these areas may have the potential to propagate the tumour. Thus on the basis of these findings, a similar aggressive chemotherapy regimen to that used on patients with little response to pre-operative therapy would be recommended in patients with a mainly "altered" appearance of tumour after neoadjuvant chemotherapy.

It may also be concluded from the work carried out in this thesis that P-gp alone should not be used as a predictive marker for response to chemotherapy.
Appendices

A1 - Buffers

ALL BUFFERS STORED AT 4°C

A1.1 - 0.1 M cacodylate
To make a 500 ml stock solution of 0.1 M cacodylate, add 10.7 g of sodium cacodylate (BDH) to 500 ml of distilled water.

A1.2 - 0.1 M phosphate buffer
Make up 500 ml stock solutions as follows:
1. Add 7.8 g of sodium dihydrogen orthophosphate (BDH) to 500 ml of distilled water.
2. Add 17.6 g of disodium hydrogen orthophosphate (BDH) to 500 ml of distilled water.
To make up 100 ml of 0.1 M phosphate buffer at pH 7.2 add 10 ml of 1 to 90 ml of 2.

A1.3 - 0.1 M acetate buffer
To make up a 1 litre solution, add 13.61 g of sodium acetate tri-hydrate (BDH) and 2.1 ml of glacial acetic acid (BDH) to 950 ml of distilled water. Using 0.1 M NaOH or HCl adjust the pH of the solution to 4.8, and then top up to 1 litre using distilled water.

A1.4 - 0.75 M tris acetate
To make up a 100 ml solution, add 9.07 g of trizma base (Sigma) to 80 ml of distilled water. Add concentrated acetic acid until pH 7.5 is reached. Top up to 100 ml using distilled water.
Appendices

A2 - Fixatives

A2.1 - 2% glutaraldehyde used as fixative for electron microscopy
To make up a 25 ml solution, add 2 ml of 25% glutaraldehyde solution (Agar) to 23 ml of 0.1 M cacodylate buffer. Store at 4°C.

A2.2 - 1% osmium tetroxide
To make up a 2 ml working solution, add 1 ml of cacodylate to vial containing 1 ml of 2% osmium tetroxide (Agar). Use immediately.

A2.3 - 10% neutral buffered formalin
To make up a 1 litre solution, add 4 g of sodium dihydrogen orthophosphate and 6.5 g of disodium hydrogen orthophosphate to 100 ml of 40% formaldehyde solution (w/v) (BDH) and 850 ml of distilled water. Using 0.1 M NaOH or HCl adjust the pH of the solution to 7.0, and then top up to 1 litre using distilled water. Store at room temperature.

A2.4 - 1% glutaraldehyde solution used as fixative in immunogold silver enhancement method
To make up a 25 ml solution, add 1 ml of 25% glutaraldehyde solution (Agar) to 24 ml of distilled water. Store at 4°C.
Appendices

A3 - Histological Stains and Mountants

A3.1 - toluidine blue
To make up a 500 ml solution, add 5 g toluidine blue powder (BDH) and 5 g of disodium tetraborate (borax) to 500 ml of distilled water. Store at room temperature.

A3.2 - uranyl acetate
To make up a 50 ml solution, add 2.5 g of uranyl acetate (Agar) to 50 ml of distilled water. Store at 4°C.

A3.3 - Reynold's lead citrate
To make up a 50 ml solution, add 1.33 g of lead (II) nitrate (BDH) and 1.76 g of tri-sodium citrate (BDH) to 30 ml of water. Shake vigorously and allow to stand for 30 minutes before adding 8 ml of 1M NaOH. Make up to 50 ml with distilled water. Store in the dark at 4°C.

A3.4 - Harris' haematoxylin
(All reagents from BDH)
To make up a 500 ml solution, dissolve 2.5 g of haematoxylin in 25 ml of absolute ethanol. Add this solution to a solution of 50 g ammonia alum dissolved in 475 ml of warm distilled water. Bring to the boil, add 1.25 g of mercuric oxide and dissolve whilst still boiling. Cool rapidly and filter, and add 8 ml of glacial acetic acid. Store at room temperature.

A3.5 - Mayer's haematoxylin
(All reagents from Fisons, Loughborough)
To make up a 1 litre solution, dissolve 1 g of haematoxylin, 50 g of potassium alum, and 0.2 g of sodium iodate in 1 litre of distilled water overnight. Add 50 g of chloral hydrate and 1 g of citric acid then heat to the boil, and boil for 5 minutes and then cool to room temperature.

A3.6 - Aqueous eosin
To make up a 500 ml solution add 5 g of eosin to 500 ml of distilled water.

A3.7 - Haematoxylin and eosin histological staining procedure
Dewax and rehydrate paraffin sections by dipping slides in the following solutions in order: 3 changes of xylene for 5 minutes each; 2 changes of 100% IMS for 5 minutes each; 90% IMS for 5 minutes; 70% IMS for 5 minutes; 50 % IMS for 5
minutes; running tap water for 5 minutes. Then place in Mayer's haematoxylin for 5 minutes followed by 5 minutes in running tap water to "blue" the stain. Place in eosin for 2 minutes and then wash for 3 minutes in running tap water. Then dehydrate and clear sections as follows: 70% IMS for 1 minute; 90% IMS for 2 minutes; 2 changes of 100% IMS for 5 minutes; 3 changes of xylene for 5 minutes. Finally mount in a suitable non-aqueous mounting medium such DPX (BDH).

A3.8 - Aqueous mountant medium for immunofluorescence
To make up a 100ml solution, add 33 ml of glycerol (BDH) to 67 ml of 0.1M Tris buffer, pH 8.5. Add 15 g of polyvinyl alcohol (Sigma), and heat at 60°C until completely dissolved. Cool to 40°C, and add 3 g of DABCO (1,4-diazabicyclo[2.2.2]octane) (Sigma), stirring until fully dissolved. Aliquot and store at -20°C until use.
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