Effects of Tumour Cell Lines on Endothelial Cell Survival

by

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A Thesis Submitted in Fulfilment of The Requirements for The Degree of Master of Science in Dentistry University of Sydney

Submitted March, 2001

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Acknowledgements

Firstly I would like to thank my supervisors Dr Hans Zoellner and Professor D. Murray Walker for their help and support throughout my candidature. I would like to thank Professor Walker for allowing me to join the Department of Oral Medicine and Oral Pathology at Westmead Hospital and allowing me use of all departmental facilities over the past five years. My principal supervisor Dr Zoellner deserves a special thank you for his constant encouragement and tireless effort, especially during the editing and formatting of this thesis.

I would also like to thank Ms Janice Matthews for her helpful and practical advice during many difficult situations encountered during this project. Mrs Mara Cvejic from the research support unit was particularly kind and helpful regarding Scanning Electron Microscopic analysis and was an invaluable assistance for computer software related problems.

A special thank you to Dr Ross Bodel and his friendly staff at the Electron Microscope Unit at Westmead hospital, for their unlimited patience and advice during the endless hours involved in the preparation of specimens and help during use of the Transmission Electron Microscope.

Dr Heather Medbury from the Department of Surgery at Westmead Hospital deserves thanks for her help in analysing FACS data. As does Dr John Gibbins from the Department of Pathology at Sydney University for allowing me to use his light microscope to take oil immersion photomicrographs.

My fellow postgraduate students should also be thanked for their support during the many long years of hard work. Their dedication to high achievement was a inspiration for my project.

Finally, I thank my wife Alexis and son Harrison for their understanding, love and support over the past five years.
Declaration

The work presented in this thesis is wholly the work of Alexander McEwen and was performed in the Department of Oral Medicine and Oral Pathology, at the Westmead Hospital Dental Clinical School, between March 1996 and March 2001 towards the degree of Master of Science in Dentistry (University of Sydney). To the best knowledge of the student, this work is original and has not been published or presented elsewhere by other workers unless otherwise stated.

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<td>American Type Culture Collection</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation (number)</td>
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<td>DAB</td>
<td>Diamino Benzidine Tetrahydrochloride</td>
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<td>DCC</td>
<td>Deleted in Colon Carcinoma</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>EC</td>
<td>Endothelial Cell</td>
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<td>ECGS</td>
<td>Endothelial Cell Growth Supplement</td>
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<td>BCM</td>
<td>Extracellular Matrix</td>
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<td>EDTA</td>
<td>Ethylene Diaminotetra-acetic Acid</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting Analysis</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>H5</td>
<td>Metastatic rat-carcinoma cell line</td>
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<tr>
<td>HBSS</td>
<td>Hanks Balance Salt Solution</td>
</tr>
<tr>
<td>HGF</td>
<td>Human Gingival Fibroblast</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan Sulphate</td>
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<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
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<td>ICAM</td>
<td>Intercellular Adhesion Molecules</td>
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<tr>
<td>LFA</td>
<td>Lymphocyte Function related Antigens</td>
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<td>M199</td>
<td>Medium 199</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>RER</td>
<td>Rough Endoplasmic Reticulum</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>SAOS-2</td>
<td>Osteosarcoma cell line</td>
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<td>SEM</td>
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<td>SPBS</td>
<td>Sorensons Phosphate Buffered Saline</td>
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<td>TEM</td>
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<td>Term</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase (TdT) – mediated dUTP Nick-End-Labelling</td>
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<td>UEA-1</td>
<td>Ulex Europeaus Lectin</td>
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<td>Y43BP</td>
<td>Non metastatic rat-carcinoma cell line</td>
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<tr>
<td>Zn$^{2+}$</td>
<td>Zinc ion</td>
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Summary

Benign tumours grow by expansion, displacing the surrounding parenchymal and stromal cells. This compresses adjacent tissues to produce a capsule separating the tumour from the normal cells. Malignant tumours on the other hand, tend to replace and destroy the surrounding tissues by invasion, increasing in size by both proliferation as well as by migration of tumour cells. The replacement of adjacent tissues by malignant tumour cells raises the possibility that one property of malignant cells is the ability to cause death of adjacent non-tumour cells. This may have a further role in facilitating metastasis, by aiding access of tumour cells to the blood. This study investigates the effects of tumour cells upon endothelial cells and additional experiments performed using human gingival fibroblast cells are also described.

The interactions between tumour and normal cells were examined using cell culture techniques. Cell numbers were analysed using direct cell counting and using FACS analysis making use of specific cell labelling characteristics. Cell morphology was analysed by Scanning and Transmission Electron Microscopy.

Human Umbilical Vein Endothelial Cells (HUVEC) and Human gingival fibroblasts (HGF) were co-cultured with three different tumour cell lines: a human osteogenic sarcoma cell line (SAOS-2), a non-metastatic rat-carcinoma (Y43BP) and a metastatic rat-carcinoma (H5) cell line. Three different co-culture protocols were used: Type A Co-culture involved establishing a monolayer of HUVEC or HGF prior to co-culture with a tumour cell line for 24 hours; Type B Co-culture involved similar establishment of a normal cell monolayer but this time on a porous transwell membrane, the adherent normal cells were then placed in close apposition to a separate established tumour monolayer for 24 hours; Type C Co-Cultures varied from Type B in that after establishment of a membrane bound monolayer of normal cells, the membrane was inverted and a tumour cell monolayer established on the direct underside of the initial normal cell layer, these monolayers being separated by the porous membrane itself. This variation in normal and tumour cell culture conditions allowed cell interactions at
varying proximity to be studied. In this way the roles of cell contact, soluble factors, medium depletion and gelatin matrix degradation were studied.

For Type A Co-cultures, monolayers were fixed and stained with the endothelium specific marker Ulex europaeus lectin-1 (UEA-1), and SAOS-2 were labelled for the enzyme alkaline phosphatase. It was noted that there was a significant reduction in HUVEC and HGF numbers when cultured with SAOS-2, while no significant reduction was seen for these normal cells under similar conditions for Y43BP and H5. These results were then replicated and analysed using flow cytometry, a similar significant reduction in HUVEC was seen in Type A Co-culture with SAOS-2.

A reduction in HUVEC number was not seen in Type B and Type C Co-culture conditions suggesting that intimate cell contact between SAOS-2 and HUVEC was required to induce cell death. This data was supported by media depletion experiments using serially diluted tumour extract medium. Results showed a slight increase in HUVEC and HGF with 100% tumour extract medium, suggesting that normal cell death seen in Type A Co-cultures was not due to medium depletion by tumour cells. Pre-treatment of the adherent Gelatin matrix with tumour cells prior to culture of HUVEC failed to effect HUVEC survival, suggesting that the effect of tumour cells was not due to matrix degradation.

Scanning electron microscopy of Type B and C Co-cultures was used to examine the role of cell contact in HUVEC cell death. Evidence for cell processes passing through pores in the membrane suggests the possibility of cell contact as the cause of normal cell death seen in Type A Co-cultures. Specific gold bound UEA-1 labelling of HUVEC allowed examination of Type A Co-cultures of HUVEC with SAOS-2 using Transmission electron microscopy. Transmission electron microscopy and silver-enhanced oil immersion light microscopy revealed detached populations of gold labelled HUVEC after 24 hours of co-culture with the ultrastructural features of apoptosis.

It is concluded that, SAOS-2 induce HUVEC and HGF death in a contact dependent manner and that cell death may be via induction of apoptosis.
Chapter 1

Tumour Growth, Necrosis and Apoptosis

1.1 Growth Patterns of Benign and Malignant Tumours

1.1.1 Malignant Tumours Have Invasive Margins While Benign Tumours Do Not:

An important clinical sign in the diagnosis of malignancy is fixation of the tumour mass to the surrounding tissues. This reflects the invasion of these tissues by strands of malignant tumour cells, which tether the tumour mass to the surrounding healthy tissues (Walter, 1996). The invasion is due to migration of tumour cells away from the main body of the tumour into the adjacent tissues. In this way, malignant tumours increase in size by both proliferation as well as by migration of tumour cells (Liotta, 1983).

This invasive pattern contrasts strongly with the presentation of benign tumours, which do not have invasive margins (Ruoslhahti, 1996). Because of this, benign tumours grow in size only through proliferation of the benign cells. Adjacent tissues become compressed with the formation of a capsule separating the benign tumour from the normal tissue. The clinical outcome of this pattern of growth is that benign tumours are usually mobile on palpation (Kumar, 1994).

1.1.2 Malignant Tumours Replace Adjacent Tissues While Benign Tumours Displace Them:

The invasive growth of malignant tumours has a further effect that is evident in examination of clinical specimens. Because the invasive cells migrate into the adjacent tissues and then proceed to proliferate, malignant tumours tend to replace the adjacent tissues. Benign tumours on the other hand push the adjacent normal tissue aside, often causing gross distortion of the tissues but not eroding into the affected organs (Enzinger, 1994). For example, in breast cancer, fibro-fatty tissue is replaced by the tumour mass that eventually may destroy the overlying skin to produce a malignant ulcer. Benign lesions such as fibroadenomas, however, displace the surrounding fibro-fatty tissue and do not cause
ulceration of the overlying skin (Rusin, 1994). This difference in the behaviour of malignant as compared with benign tumours is illustrated in Figures 1.1 and 1.2, which compare the macroscopic and histological appearance of endometrial carcinoma and Leimyoma of the uterine cervix.
Figure 1.1.a
Photograph of a leiomyoma of the uterus. Tumour mass (arrow) is well circumscribed and has grossly expanded and stretched the surrounding myometrium (M) to severely distort the overall shape of the uterus. These tumours are able to reach a great size but do not destroy the adjacent tissues. Instead, the myometrium is stretched around the expanding tumour mass.

Figure 1.1.b
Photomicrograph of a leiomyoma of the uterus. The tumour mass (T) is contained within a well defined capsule (C) consisting largely of compressed collagen fibres from the surrounding myometrium (M). The capsule is believed to form by collection of stromal collagen fibres around the expanding tumour mass along with pressure induced stromal cell death.
Figure 1.2.a
Macroscopic view of endometrial carcinoma of the uterus. Tissue replacement by invading tumour (T) is seen so that the lesion is poorly circumscribed and in places (arrows) is difficult to discern from the adjacent myometrium (M). Although the tumour has reached a large size, the uterus itself is not greatly dilated, indicating replacement of uterine tissues with malignant tumour.

Figure 1.2.b
Light micrograph of the invasive margin (IM) of an endometrial carcinoma. Although clearly defined areas of tumour (T) and myometrium (M) are seen, the boarder between these is poorly defined, as tumour cells (arrows) migrate between adjacent myometrial cells. It is clear that the myometrial cells are replaced by tumour cells during growth of the tumour.
1.2 Invasion and Metastasis

1.2.1 A Three Step Model for Invasion and Metastasis
Cell proliferation in most tissues is under strict control where cells respect specific boundaries and do not spread to distant sites (Folkman, 1978; Liotta, 1992). Invasion and metastasis define the essential difference between benign and malignant tumours. It is important to note, however, that malignant tumours show a spectrum of invasive and metastatic potential (Fearon, 1990; Pazouki, 1997).

A three-step hypothesis has been proposed to describe the sequence of biochemical events during tumour cell invasion of the extracellular matrix. The first step is attachment of tumour cells to the matrix. Attachment may be mediated by specific glycoproteins such as laminin (Terranova, 1982) or fibronectin (Humphries, 1989; Kosmehl, 1999). Following attachment, the tumour cell secretes hydrolytic enzymes, or induces secretion of enzymes by adjacent non-tumour cells (Tryggvason, 1987). The third step is tumour cell locomotion into the region of matrix modified by hydrolysis. The direction of locomotion may be influenced by chemotactic factors, and the chemotactic factors derived from serum or local parenchyma may influence the organ specificity of metastases (Nicolson, 1982). Continued invasion of the extracellular matrix may take place by cyclic repetition of these three steps (Boudreau, 1999; Koblinski, 2000).

Invasion from most primary epithelial tumours involves disruption of the basement membrane (Mareel, 1991; Miner, 1999), bringing tumour cells into contact with the underlying connective tissues containing extracellular matrix and other cells including, fibroblasts, macrophages, adipocytes, muscle cells, bone, cartilage and nervous tissue. Invasion of lymphatic or blood vessels is thought to be the basis for metastasis (Ruoslahti, 1996).

1.2.2 Adhesion during Invasion and Metastasis
Adhesion of tumour cells to the extracellular matrix substrate is important early in invasion, as the cells must bind to components of this matrix in order to move through the tissues (Curran, 1999). Similar binding events are also implicated in metastasis, when cells both pass through the basement membrane and escape
through the tissues (Foltz, 1982). Participating in this are components of the extracellular matrix such as collagens, fibronectin, laminin, elastin, proteoglycans, glycosaminoglycans, and the relevant cell surface receptors such as integrins and laminin receptors (Hansen, 2000).

1.2.3 Degradation and Synthesis of the Extracellular Matrix during Invasion and Metastasis
Parenchymal epithelial cells such as hepatocytes or salivary gland cells remain attached to their respective side of the basement membrane and do not normally cross to the interstitial side even during tissue remodelling (Vracko, 1974); (Lentsch, 2000). In contrast, during invasion, tumour cells must traverse two types of extracellular matrix; basement membranes and the interstitial stroma. Tumours have been shown to modify extracellular matrix in three ways: 1. Degradation of matrix components associated with invasion (Foltz, 1982), 2. Increased production of matrix components by host cells in response to the presence of the tumour, termed desmoplasia (Barsky, 1982) and, 3. Tumour cell synthesis of matrix components with the actual amount of matrix produced by tumour cells frequently being much less than from the normal tissues (Kramer, 1985; Ohtani, 1999).

The host extracellular matrix is a mechanical barrier that does not normally contain pre-existing passageways for cells. The matrix becomes focally permeable to cell movement only during specific conditions such as normal tissue remodelling, wound healing, inflammation, or neoplasia (Lentsch, 2000). Cell infiltration of the matrix depends on multiple factors including cell motility, the character of the specific tissue matrix, chemotactic factors and cell-cell interactions. Since it is unlikely that cell motility alone can provide the means to penetrate matrix barriers, it has been proposed that penetration is accomplished by the local release of hydrolytic enzymes which solubilize the matrix (Liotta, 1980; Kobliński, 2000).

1.2.4 Migration of Cells during Invasion and Metastasis
Migration of malignant cells is an essential physical aspect of invasion and metastasis, and this movement can best be explained as active translocation by alternating adhesion to and release from the extracellular matrix. Current
concepts about mechanisms of cell locomotion focus on the dynamic equilibria of actin, tubulin and their associated proteins, and on turnover of plasma membrane constituents (Russo, 1981; Groom, 1999; Orr, 2000).

1.3 Apoptosis and Necrosis
Apoptosis is the mechanism whereby excess cells are removed from the body. As such, it plays an important role in the embryogenesis, growth, development and remodelling of many tissues (Wyllie, 1987; Ross, 1997; Brill, 1999). Apoptosis is a word of Greek origins, describing the process of leaves falling from trees, and this word has been adopted in cell biology to describe a similar highly selective senescent behaviour (Wang, 1999). Apoptosis is a controlled process, where cells die following a programmed sequence of events without causing any damage to the surrounding tissues (Figure 1.3). Cells undergoing apoptosis loose water to the extracellular space leading to cell shrinkage (Fadeel, 1999). Since this water must move against an osmotic gradient, water transport in apoptosis must be fuelled by energy rich molecules like ATP, and this requires intact and functioning mitochondria which appear quite normal by TEM (Vieira, 1999). When apoptosis occurs in-vivo, cells fragment into small apoptotic particles which are then phagocytosed by adjacent cells (Duvall, 1985; Savill, 1993; Aderem, 1999). Apoptotic cells have intact membranes and organelles, as well as condensed nuclear fragments. The morphology of apoptotic cells is readily identified by both light microscopy and transmission electron microscopy (Kerr, 1972a). In the early stages of apoptosis, both mitochondria and plasma membranes remain intact and functioning, however, characteristic ‘blebbing’ or ‘boiling’ of the plasma membrane is observed generating apoptotic particles (Laster, 1996).

The gross changes in cell shape must involve cytoskeletal collapse or at least rearrangement. With regard to this, it has been shown that microtubule disrupting agents are potent inducers of apoptosis in a number of cell lines (Martin, 1993). Nuclear condensation parallels that of the cytoplasm and is associated with the activation of a specific endonuclease which cleaves DNA at internucleosomal sites (Duke, 1983). This enzyme is present in the nucleus endogenously and is activated by Ca$^{2+}$ and Zn$^{2+}$ions (Jackisch, 2000). This is the most widely
accepted biochemical marker of apoptosis, and internucleosomal DNA cleavage is readily seen as a ladder by agarose gel electrophoresis (Arends, 1990). DNA cleavage can also be detected by terminal deoxynucleotidyl transferase (TdT) -mediated dUTP Nick-End-Labelling (TUNEL) in histological sections or isolated cells (Gavrieli, 1992). The reduced DNA content and size of apoptotic particles is readily observed by Fluorescence Activated Cell Sorting (FACS) Analysis (Darzynkiewicz, 1992).

Nuclear fragmentation in cells undergoing apoptosis may produce up to 20 vesicles containing condensed chromatin (Ross, 1997). The cell itself eventually fragments into apoptotic bodies which are then specifically recognised and phagocytosed by neighbouring cells (Lang, 1994). Apoptotic neutrophils are readily recognised and phagocytosed by macrophages and this is mediated via the macrophage vitronectin receptor (Savill, 1990). Cells undergoing apoptosis in vitro, where there are no phagocytic cells to ensure their removal, ultimately swell before lysing. This is because the homeostatic capacity of the apoptotic particle is eventually overwhelmed by the progressive biochemical degradation. Because of the close morphological similarities with necrosis, this terminal phase of in vitro apoptosis has been termed “secondary necrosis” (Savill, 1993) (Figure 1.3).

Necrosis is separate to apoptosis, and occurs when cells fail to maintain homeostasis after major cellular insults, for example complement mediated cell lysis, severe hypoxia or hypothermia (Duvall, 1986). Necrosis can be distinguished from apoptosis on the basis of morphology, ultrastructure and the presence of random DNA fragmentation as seen by DNA gel electrophoresis gels (Searle, 1982; Compton, 1992). Unlike apoptosis, necrosis does not result in cellular shrinkage and the formation of discrete cellular fragments, but is first accompanied by swelling of cells due to loss of ion pump activity (Arends, 1994). Organellar structures also swell and loose integrity during necrosis (Trump, 1981), and this is readily seen in electron micrographs. In addition to this organellar swelling, ribosomes detach from the rough endoplasmic reticulum (RER) in necrosis, while the RER is intact during apoptosis (Putney, 2000). Also, nuclei fail to undergo condensation and fragmentation in necrosis, but instead
undergo more irregular degradation, eventually resulting in the formation of anucleate cellular structures (Nicotera, 1997). Necrotic cells are mechanically fragile, so that in culture they often degenerate into unstructured cellular debris. With the ultimate fragmentation of the cell, there is a release of cytoplasmic contents, including lysosomal enzymes, into the extracellular fluid (Arends, 1991). These destructive enzymes not only damage neighbouring cells and tissues, but also contribute to excitation of an inflammatory reaction (Figure 1.3) (Lentsch, 2000).
Figure 1.3 - Diagram outlining the main features of apoptosis and necrosis.
When cells are injured, homeostatic mechanisms are challenged so that DNA, ATP, and protein synthesis are reduced. The result of this is dysregulation of ion-pumps and normal cell organellar function, which is seen morphologically as cell swelling, organellar swelling and lipid accumulation. If the cell is unable to regain homeostasis, swelling continues with disruption of organellar and plasma membranes. There is also detachment of ribosomes from rough endoplasmic reticulum (RER) as well as activation of proteolytic and DNA degradative enzymes resulting in generalised biochemical degradation with random DNA fragmentation. In-vivo, this release of cellular contents results in inflammation. In apoptosis, however, cells undergo active shrinkage and condensation with maintained membrane and organellar integrity. There is also condensation and fragmentation of nuclear material, accompanied by internucleosomal DNA fragmentation, seen in DNA gel electrophoresis as a "DNA ladder". When apoptosis occurs in-vivo, adjacent cells phagocytose the resulting fragments so that there is no consequent inflammation. In cultured cells, however, adjacent cells are often unable to phagocytose apoptotic particles so that these particles accumulate in the culture medium. Because the apoptotic process results in progressive cellular and biochemical fragmentation, a point is reached where apoptotic particles are unable to maintain homeostasis. The outcome of this is that these particles undergo necrotic change and this "secondary necrosis" is a feature of apoptosis seen only in cultured cells (Wyllie, 1980; Amarante-Mendes, 1999; Benedetti, 1999; Hacker, 2000).
Necrosis

Apoptosis

Normal cell

Cell Swollen
Organelles damaged
Chromatin altered

Cell Shrunken
Organelles intact
Chromatin condensed

Cell lysed
Organelles degraded
Chromatin degraded
Random DNA fragmentation
Contents released

Apoptotic bodies formed
Organelles intact
Inter-nucleosomal DNA fragmentation
Contents retained

Inflammation

In-Vivo
Phagocytosis of particles
No inflammation

In-Vitro
Secondary
Necrosis
1.4 Apoptosis and Necrosis in Tumours

Apoptosis is commonly seen in growing tumour masses (Steel, 1968; Wyllie, 1999). These dying cells exhibit the classical characteristics of apoptosis including nuclear fragmentation, cytoplasmic condensation and internucleosomal DNA fragmentation (Saraste, 2000). It has been noted that many tumours grow at a much slower rate than would be predicted from the number of mitotic figures seen (Kerr, 1984). This appears to be due to apoptosis of tumour cells so that the net increase in the size of tumours represents a balance between proliferation and apoptosis (Kerr, 1972b). Some chemotherapeutic agents and therapeutic levels of irradiation have been found to induce apoptosis of tumour cells (Cotter, 1992) so that there is an extensive literature detailing the apoptotic behaviour of malignant cells (Arends, 1991; Duke, 1996).

Necrosis also occurs in many malignant tumours, and is often considered to be a sinister sign in histo-pathological diagnosis (Farber, 1994). This usually occurs in the central portions of malignant lesions and is thought to be due to the tumour outgrowing its vasculature (Folkman, 1996). In contrast, apoptosis has been observed in tumours from the outer rim through to the central core, also apoptosis is seen in both small and large tumours (Wyllie, 1999).

The incidence of apoptosis is markedly higher in tumours with extensive lymphocyte infiltration (Stambolic, 1999). In vitro studies have shown that both cytotoxic T lymphocyte cells and natural killer cells have the capacity to kill tumour cells by inducing apoptosis (Hameed, 1989). Both cell types contain the pore forming protein perforin, and this protein is able to induce apoptotic cell death in target tumour cells when purified (Corazza, 2000).

Because cells undergo apoptosis singly, apoptotic bodies are often unnoticed in tumours. It is interesting to note that the ratio of apoptotic to mitotic cells in untreated tumours can be as large as 1:1. In spontaneously regressing tumours or those responding to chemotherapy, the ratio can be even higher (Martin, 1991). Despite the importance of apoptosis and necrosis in tumour biology and the clearly destructive behaviour of tumour cells, it is surprising that at the time that the research described in this thesis began, there were no reports of the effect of tumour cells upon apoptosis and necrosis of adjacent stromal cells.
1.5 Tumour Angiogenesis and Desmoplasia

Despite the paucity of literature detailing the influence of tumours upon stromal cell death, there are several interactions between malignant tumours and the surrounding tissues, which have been described and require discussion.

*Desmoplasia*

Fibroblasts seem to be able to respond to tumours, as many tumours induce desmoplasia (Ohtani, 1999; Diaz-Cascajo, 2000), which is manifested by fibroblastic proliferation and dense fibrosis of the associated tissues (Liotta, 1983). This was first recognised as a phenomenon associated with malignant tissues by the ancient Greeks, who noted the hard dense tissue around some tumours (Kumar, 1994). Desmoplasia appears to be under the control of locally produced mediators, particularly Transforming Growth Factor-β. Some invasive tumours, such as melanoma, are seldom associated with a desmoplasia, whereas other tumours, such as infiltrating ductal breast carcinoma are almost always associated with a marked desmoplastic reaction (Barsky, 1982). In scirrhous breast carcinoma, for example, it is the desmoplastic response and not the clustering of neoplastic cells that is responsible for the diagnostic clinical “lump” (Jackson, 1957; Fenihalls, 1999). During invasion, the possible role of the desmoplastic response in limiting these invasive processes is unclear. It could be argued that the desmoplastic matrix represents an attempt by the host to ‘wall off’ the invading tumour and limit its progress. However, this equally may benefit the tumour by reducing access for host immune cells.

*Angiogenesis*

The term ‘angiogenesis’ refers to the development of new blood vessels and in tumour angiogenesis, there is directional sprouting of new vessels toward a solid tumour mass from pre-existing microvessels (Desai, 1999). Most solid human tumours exist in situ for a substantial length of time without the need for vascularisation (Folkman, 1995), however, vascularisation is necessary for progression of the neoplasm beyond a size of 2-3 mm³ (Weidner, 1991). The growth of solid tumours is reported to be dependent upon the growth of new blood vessels into the tumour mass (Folkman, 1963) and inhibition of this greatly reduces tumour expansion (O’Reilly, 1996). The first apparent sign of an
angiogenic stimulus from a tumour, is the dissolution of basement membrane surrounding pre-existing vessels, usually post-capillary venules (Koblinski, 2000). Next, endothelial cells begin to migrate out of these vessels, toward the tumour. This tumour angiogenesis is thought to be under the control of a variety of agents (Dawas, 1999) released by both tumour cells and adjacent stromal cells including Vascular Endothelial Cell Growth Factor (Cornali, 1996), Tumour Necrosis Factor (Le, 1987), Transforming Growth Factor-β (Höfler, 1993) and Platelet Derived Growth Factor (Fox, 1996). This is inhibited by some anti-angiogenic factors including angiostatin, endostatin, platelet factor-4 and interleukin-1 (Dawas, 1999). Tumour angiogenesis has become widely accepted as an important phenomenon in tumour biology (Kerbel, 2000).

It has been argued that a high degree of tumour vascularisation increases the opportunity for tumour cells to enter the circulation and so metastasise (Van der Pluijm, 2000). Entry of tumour cells into the bloodstream may take place through the tumour-induced capillaries or through existing small veins and venules (Blood, 1990). Although large veins may be invaded by highly aggressive tumours, arterial invasion rarely occurs (Komatsu, 2000). Direct invasion of tumour cells into the lumen of existing blood vessels requires that the neoplastic cells penetrate the sub-endothelial basement membrane of the vessel and then traverse the endothelial barrier.

Over the last 25 years, a dogma has been established that solid tumours are dependent on angiogenesis (Folkman, 1996). This premise, first proposed by Folkman, was based on several key observations. First it was shown that tumours grow slowly when implanted into isolated perfused organs, where blood vessels did not grow (Gimbrone, Jr. 1974). The tumours were able to survive for an extended period of time but were at first unable to expand and grow into adjacent tissue. However, once the advancing edge to the tumour approached adjacent microvessels, diffusible “angiogenic factors”, released from the tumour, stimulated endothelial cells to grow and migrate toward the tumour, eventually organising into a capillary network. This apparent switch from pre-vascular to vascular phase was accompanied by exponential growth of the tumour (Gimbrone, Jr. 1974; Bohle, 1999). Consistent with this idea, is the appearance
of neo-vascularization at the base of melanomas entering the vertical growth phase, and the red "blush" which signals the onset of rapid growth and increased metastatic potential of cervical and oral carcinomas (Penfold, 1996).

It is believed that tumour cells recruit new blood vessels by several different mechanisms. 1. They produce diffusible angiogenic factors that directly activate endothelial cells, stimulating them to sprout and grow toward the developing tumour (Folkman, 1995). 2. Also they elaborate cytokines which attract activated macrophages, mast cells, and neutrophils, which in turn release angiogenic factors (Polverini, 1984). 3. They are also able to block the production of inhibitors of angiogenesis (Kim, 1993). 4. Tumour cells also produce enzymes that release angiogenic factors sequestered in the extracellular matrix (Vindigni, 1997). 5. They can stimulate adjacent normal tissues to make enzymes such as stromelysin and collagenase (Fox, 1996) which can also promote angiogenesis.

1.6 The Extracellular Matrix
At early stages of invasion, malignant tumour cells detach from the tumour mass and start migrating into the adjacent tissue, guided by chemotactic factors (Varani, 1982). The invading cells may reach lymphatic or blood vessels, from which they can spread throughout the body, extravasate and initiate metastases (Ruoslahti, 1996; Orr, 2000). From a clinical point of view, metastasis is the most serious aspect of cancer, since it represents the extension of an essentially local disease to an increasing number of distant organs and tissues.

The process of metastasis is an extremely complicated phenomenon that involves a series of sequential steps. One of those events is the removal of the extracellular matrix to allow the spreading of tumour cells. It has become well established that removal of extracellular matrix in tumour invasion occurs through the action of a variety of degradative enzymes produced either by the tumour cells themselves or cells of the host tissue (Tryggvason, 1987; Curran, 1999; Ohtani, 1999).
1.6.1 Composition of the Extracellular Matrix
Components of the extracellular matrix that are degraded during invasion by
tumour cells can be divided into two major categories, basement membranes and
matrix of the interstitial connective tissues. Basement membrane has a uniform
acellular sheet like structure, providing a substratum for orderly growth of cells
and contributes to ordering tissue architecture (Miner, 1999). Basement
membrane consists of a number of glycoproteins, forming a highly cross-linked
matrix. Type IV collagen is basement membrane specific and is a major
structural component, assembling into a network-like structure which allows cell
attachment (Timpl, 1981). This protein also interacts with heparan sulfate,
proteoglycan and fibronectin (Rojas, 1999). Another basement membrane
specific component, Laminin, acts in concert with type IV collagen to facilitate
cell binding by attaching to specific cell membrane proteins and also other
basement membrane components such as Enactin or Nidogen (Terranova, 1982;
Tryggvason, 1987; Kosmehl, 1999). The interstitial connective tissue is
composed of cells such as fibroblasts, osteoblasts, chondrocytes and
macrophages, embedded in a matrix of collagen fibres, glycoproteins,
proteoglycans and hyaluronic acid ground substance (Hay, 1982; Liotta, 1986;
Yang, 1993; Hansen, 2000).

1.6.2 Matrix Degrading Enzymes
The presence of proteolytic enzymes during tumour invasion has been widely
documented (Liotta, 1983; Pauli, 1983; Noel, 1997). These enzymes are grouped
into four main classes depending on their catalytic site, pH optimum, cation
requirements and susceptibility to inhibitors. Serine-proteinases include plasmin,
plasminogen activitors and elastase. Plasminogen activators (PAs) are an
interesting group of enzymes that specifically convert plasminogen to the active
proteinase plasmin (Irigoyen, 1999). The best characterised substrate for plasmin
in vivo is fibrin, allowing dissolution of fibrin clots. PAs are often found at
increased levels in malignant cells and tissues (Tryggvason, 1987) and have been
associated with tumour invasion ( Koblinski, 2000). The cystiene-proteinase
casthepsin B has been suggested as another enzyme capable of degrading matrix
components such as type I collagen, laminin and proteoglycans in a similar
fashion to plasmin (Langer, 2000). Metallo-proteinases include collagenase,
gelatinase and stromelysin and are particularly involved in disruption of the basement membrane, specifically by type IV collagenase (Yip, 1999). It has been suggested that concomitant secretion of type IV collagenase with PA is a prerequisite for type IV collagen break down (Irigoyen, 1999).

The basement membrane produced by endothelial cells is rich in collagen, other proteins such as fibronectin and laminin, and proteoglycans. The major glycosaminoglycan of the endothelial matrix produced by bovine aortic endothelial cells in vivo is heparan sulphate with smaller amounts of chondroitin 6-sulphate and chondroitin 4-sulphate (Kramer, 1982). These authors found that B16F1 melanoma cells, which invade the subendothelial matrix, partially degrade some of its glycoproteins and proteoglycans, particularly fibronectin and heparan sulphate (HS) proteoglycan. Degradation was shown to be independent of serum plasminogen and probably involved two different enzymes, one of which has turned out to be a novel endo-β-D-glucuronidase (Evans, 1991). This enzyme cleaves β-D-glucuronosyl-N-acetylglucosaminyl linkages in HS, and has been called heparanase to distinguish it from heparitinase, which can also cleave HS, but with different site-specificity (Evans, 1991).

Many proteinases are produced as inactive precursors, which are converted to the active form by proteolytic cleavage, with plasmin thought to play a central role (Andreasen, 2000). In this way complex regulatory networks of proteinase and inhibitors are responsible for matrix degradation.

The origin of tumour proteinases has been traced to both the tumour cells, and the normal host tissue cells (Curran, 1999; Ellerbroek, 1999). Therefore extracellular matrix degradation in malignancies seems to be a highly complicated process involving abnormal expression of enzymes and their inhibitors, both by the neoplastic and normal cells. It has been demonstrated that, following malignant cellular transformation, secretion of certain proteinases is clearly increased in respective cells and tissues (Liotta, 1980; Zhivotovsky, 1997; Langer, 2000). The structure and molecular action of tumour associated-proteinases in vivo is important for understanding tumour invasion at the molecular level.
1.7 Cell Adhesion in Tumour Invasion

There are a number of cellular adhesion abnormalities in tumour cells, which are thought to contribute significantly to their ability to invade locally and metastasise (Loester, 2000). It seems that changes in adhesion in tumour cells are complex and involve many adhesion systems (Gonzalez-Amaro, 1999). There are five major families of cell adhesion molecules and these are grouped on the basis of chemical, structural or functional similarities. Three rely on interactions between proteins; integrins, cadherins, and the immunoglobulin super family and two rely on interactions between proteins and carbohydrates; selectins and hyaluronate receptors (Freemont, 1995; Mizejewski, 1999; Rojas, 1999; Zell, 1999). All of these cell surface associated molecules are transmembrane glycoproteins with extracellular binding regions and cytoplasmic functional domains, which may initiate intracellular signals triggered by binding events to extracellular domains (Juliano, 1993).

1.7.1 Integrins

Integrins are a large family of homologous cell surface receptors that mediate cell-matrix and cell-cell adhesion (Ruoslhti, 1991). Each integrin is composed of an α and a β sub-unit. Because of their structure and domain organisation, integrins serve as transmembrane linkers between the extracellular matrix and the cytoskeleton (Boudreau, 1999).

The rationale for an involvement of integrins in tumourigenesis is twofold. First, integrins mediate stable adhesion for migration on extracellular matrix components. Because of this, changes in levels of expression or function may contribute to tumour invasion (Giancotti, 1994). Steps of the metastatic process in which integrins are likely to play a role include: 1. detachment from the primary tumour and penetration of adjacent extracellular matrices and blood vessels (Luscinkas, 1994); 2. interaction with platelets and leukocytes in the circulation and; 3. arrest and extravasation in target organs (Albelda, 1993). Further, integrins transmit signals from the extracellular matrix to the cell interior and these signals affect cellular growth and differentiation (Ginsberg, 1992). Adhesion to an extracellular matrix is required for the progression of normal cells through the cell cycle, a phenomenon called anchorage dependence.
In addition, certain extracellular matrices can modulate gene expression either positively or negatively and these effects appear to be mediated by integrins. In this way extracellular matrices, through regulation of cellular growth and differentiation, are thought to have an important role in the invasive progression of tumours (Pauli, 1983; McDonald, 1991; Mizejewski, 1999). Therefore changes in integrins may contribute to the unrestrained growth and the lack of differentiation of neoplastic cells.

### 1.7.2 Immunoglobulin-like Super Family

This is a large and diverse family of molecules, which are so named because they have one or more immunoglobulin like domains (Johnson, 2000). Included in this group are molecules concerned with antigen recognition and adhesion by lymphocytes. These molecules include CD3, CD4 and CD8 which together recognise complexes of antigen peptide and the major histocompatibility complex on cells, as well as lymphocyte function related antigens (LFA) such as CD2 (Tangye, 2000). Other important molecules in this group are the intercellular adhesion molecules (ICAM) which are expressed on epithelial and endothelial cells (Rojas, 1999).

In an extensive study of genetic aberrations in colonic carcinomas, a common deletion was identified in a gene that encodes a protein with homology to the immunoglobulin-like superfamily of adhesion molecules (DCC - “Deleted in Colon Carcinoma”) (Fearon, 1990). Loss of expression of this protein is thought to allow reduced tumour adhesion, and so result in more effective invasion of the adjacent stromal tissue.

### 1.7.3 Cadherins

Cadherins are calcium dependent cell adhesion molecules, thought to bind in a homotypic way to other cadherins on adjacent cells (Rojas, 1999). Intracellularly, they attach to catenins, which link the cadherin molecule to intermediate filaments of the cytoskeleton. The best characterised member of this group of adhesion molecules is E-cadherin which is an important component of desmosomes (Juliano, 1993).
There is a strong correlation between the loss of E-cadherin expression and the acquisition of the undifferentiated state. In a series of human tumour cell lines, invasion into collagen gels was inversely related to expression of E-cadherin. Also, inhibition of invasion was achieved by transfection with a vector expressing E-cadherin, suggesting a metastatic suppressor function for this cell adhesion molecule (Vleminckx, 1991).

1.7.4 Selectins
Selectins have lectin-like carbohydrate binding regions in extracellular domains (Zak, 2000). There are three major groups of selectins: 1. The L-selectins are homing receptors for specific adhesion of lymphocytes to endothelial cells of peripheral lymph nodes (Freemont, 1995). 2. The E-selectins (endothelial leucocyte adhesion molecules) are important mediators of leukocyte binding in inflammation (Teddler, 1995). 3. P-selectin, which is contained in the Weibel-Palade bodies of endothelial cells and α-granules of platelets (Wagner, 1993) are released during platelet activation and neutrophil binding, and so mediates the adhesion of neutrophils and platelets.

Selectins may also play an important role in metastasis (Orr, 2000). Potential endothelial ligands for L-selectin are observed on venules in a high proportion of cutaneous lymphomas, suggesting they may be involved in the spread of these lymphomas (Michie, 1993). There is also evidence that expression of carbohydrate determinants for E-Selectin binding is related to the metastatic potential of colonic carcinoma cells (Nakamori, 1993).

1.7.5 Hyaluronate Receptors
Hyaluronate is an abundant saccharide component of extracellular matrices believed to be important in a variety of pathological processes, including inflammation and tumourigenesis (Lapcik, 1998). Physiologically, it has numerous other functions including the promotion of cell growth and migration (Crossin, 2000). Hyaluronate is hydrophilic and has the capacity to take on a macrostructural role characterised by hypocellular gels such as cartilage and synovial fluid. Several proteins have specific affinity for hyaluronate including matrix proteins such as cartilage link protein and aggrecan (Juliano, 1993). Cells
bind to hyaluronate through cell-surface receptor proteins. To date only two have been characterised, CD44 and a ‘receptor for hyaluronate mediated motility’ (Yang, 1993).

1.8 Difficulties Arising From the Literature and A Hypothesis to Account for these Discrepancies

Although there is general and widespread agreement on the important role of angiogenesis for tumour growth (Liotta, 1991; Abe, 1993; Folkman, 1995; Gasparini, 1995), there is equally widespread agreement with the idea that malignant tumours undergo necrosis due to growth beyond the capacity of the vasculature (Steel, 1968; Terz, 1971; Kalluri, 2000; Kerbel, 2000; Scoazec, 2000). These two concepts appear to be contradictory, as it would be expected that the angiogenic activity of tumours would make ischaemic necrosis impossible. An additional problem arises when studies of three-dimensional vascular perfusion castings in tumours are examined (Fox, 1996). Rather than finding increased vessel density, as expected from an angiogenic response, vessels appear to be sparse and poorly formed in tumours (Repesh, 1984) while adjacent tissue is much more densely vascularized (Blood, 1990). This seems inconsistent with an angiogenic response but rather would imply a destructive effect of malignant tumours upon the vasculature. Also, although there is ample evidence for degradation of basement membrane by tumour cells (Stetler-Stevenson, 1993), it is still not clear how tumour cells are able to pass through the significant endothelial barrier during metastasis (Orr, 2000).

Separate to these discrepancies between observed changes and the current dogma of tumour induced angiogenesis, is the question of the fate of stromal and parenchymal cells in tissues invaded by malignant tumours. In Figures 1.2.a and 1.2.b, it was seen that in malignant tumours, the neoplastic cells replace the adjacent normal tissues while in benign tumours, adjacent tissues are displaced and a capsule tends to form from the remnant stroma. This raises the question of what happens to normal stromal and parenchymal cells, replaced by the malignant tumour. These cells are not displaced, or else they would be
represented by swelling of affected tissues. Instead, they seem to disappear as part of the 'invasive' process.

These observations lead to the hypothesis addressed in this thesis, that "malignant tumour cells are able to induce apoptotic or necrotic death of normal stromal cells while benign tumour cells do not posses this property".

If this were the case, the destruction of the vasculature, the hypoxic death of malignant but not benign tumours, and the apparent disappearance of stromal cells adjacent to malignant tumours would be explained.

This hypothesis has particular relevance with regard to the possible death of endothelial cells in malignant tumours, as such an event would provide a mechanism whereby malignant but not benign tumour cells could pass through the endothelial barrier to effect metastasis.

In the course of performing this work, two papers (Kebers, 1998; Holash, 1999) were published supporting and in some aspects replicating data shown in this thesis. These will be discussed in detail after presentation of the results, as most of the laboratory work had been completed by the time these papers became available to the candidate.
Chapter 2

Material and Methods

2.1 Overview of Material and Methods

Most tissues are composed of specialised parenchymal cells supported by a connective tissue stroma. The principal stromal cells are fibroblasts and vascular endothelial cells. Vascular endothelial cells are of particular interest with regard to the hypothesis investigated in this thesis as it is possible that endothelial cell death induced by tumour cells contributes to vascular invasion and metastasis. Because of this, most experiments in this study have focused upon effects of tumour cell lines upon cultured umbilical vein endothelium (Jaffe, 1973) while some additional experiments, described in the appendix, have been performed with gingival fibroblasts (Freshney, 1994). The human osteosarcoma cell line SAOS-2 (Rodan, 1987) has been used in most experiments, although some further experiments have been performed with rat squamous cell carcinoma cell lines Y43BP and H5 (Gibbins, 1991).

Initial experiments defined culture conditions for the planned experiments, with the aim of developing a co-culture model to investigate the interaction of normal cells with tumour cells. Firstly the correct co-culture medium had to be determined to allow the stable interaction of at least two different cell types. Various combinations of Medium M199 (Morgan, 1950), Joklik’s Medium (Eagle, 1959), Bovine Calf Serum (BCS), Heparin, Endothelial Growth Supplement (ECGS) and Bovine Serum Albumin (Fraction V) were trialed to establish the final serum free co-culture medium used in experiments. Both M199 and Joklik medium were found to be sufficient, but only when supplemented with BSA (4%).

Cell density was found to be a critical factor in the co-culture model. Cells needed to achieve approximately one-third confluence for co-culture experiments to be interpreted. One-third cell confluence was then used as the cell culture density for co-culture experiments using what is defined latter in this chapter as “Type A” co-culture.
2.2 Materials

Cell culture medium 199 (M199) and Iron Fortified bovine Bovine Calf Serum (BCS), were obtained from JRH Biosciences (Lenexa, USA), as was Trypsin (0.25%)/ 1mM EDTA. Antibiotics added were Penicillin, Streptomycin (CSL Biosciences, Parkville, Victoria, Australia) and Amphotericin B (ICN Biomedicals Inc., Ohio, USA). Glutamine was purchased from CSL Biosciences (Parkville, Victoria, Australia), and added to culture medium as required. Bovine serum albumin (BSA) fraction V was from ICN Biomedicals Inc. (Ohio, USA). Tissue culture plastic ware including Transwell membranes were from Costar (Cambridge, USA) while Falcon centrifuge and FACS tubes were purchased from Becton Dickinson (Lincoln Park, New Jersey). Membrane filters were purchased from Satorius, Minisart (Gottingen, Germany). Heparin was purchased from Pharmacia and Upjohn, (Perth, Western Australia), while endothelial growth supplement (ECGS) was prepared from bovine hypothalamus using a method described by Maciag et al. (Maciag, 1979). Netwells and Cell Scrapers were purchased from Nunc Inc. (Naperville, Denmark). RNase A and propidium iodide were purchased from (Roche Diagnostics Australia, Castle Hill, Australia). Saline was from Baxter (Toongabbie, NSW, Australia). Phosphate Buffered Saline (PBS) tablets were from Oxoid (Hampshire, England). Aquamount (BDH, Laboratory Supplies, Poole, England) was used for coverslipping fixed cultured cells. *Ulex Europeaus Lectin (UEA-I)* was from DAKO (Botany, NSW, Australia). UEA-1 conjugated colloidal gold (10nm) and FITC conjugated UEA-1 were both from Sigma-Aldrich (Castle Hill, NSW, Australia). Hank’s Balanced Salts Solution (HBSS), crude collagenase, Dimethyl Sulfoxide (DMSO) and Joklik’s medium were also from Sigma-Aldrich (Castle Hill, NSW, Australia), as were the antibodies *Rabbit Anti- UEA-I Antibody* and *Goat Anti- Rabbit immunoglobulin conjugated with HorseRadish Peroxidase*. Glutaraldehyde (EM grade) 25% and Osmium Tetroxide were purchased from ProSci Tech (Qld, Australia). Alcohol and Acetone were from BDH, Laboratory Supplies (Poole, England). Spurrs resin (Spurr, 1969) was from Taab Laboratories (Berkshire, England), while LR white resin was purchased from ProSci Tech (Qld, Australia).
2.3 Cell Culture Methods:
The two stromal cell lines used in co-culture experiments were Human Umbilical Vein Endothelial Cells (HUVEC) and Human Gingival Fibroblasts (HGF). These cell lines were derived by collagenase perfusion and explant culture respectively as described below. Because these cells were not immortal it was necessary to frequently prepare new isolates for these cells. Three tumour cell lines were used; an osteosarcoma (SAOS-2), a non-metastatic rat-carcinoma (Y43BP) and a metastatic rat-carcinoma (H5) cell line. These cell lines were donated from other laboratories and due to their immortal nature, were subcultured repeatedly without detectable change in phenotype.

2.3.1 Stromal Cells:

2.3.1.1 Isolation of HUVEC from umbilical cords
Human umbilical cords from normal vaginal deliveries were obtained from the delivery ward at Westmead Hospital. Cords were stored at 4°C in HBSS prior to isolation. Isolation was performed using careful aseptic technique in a laminar flow hood. Blood clots were expressed from the vein with sterile gauze while to improve asepsis, both ends of the cord were clamped and dipped in 70% alcohol for 30 seconds before wiping with an alcohol soaked gauze.

A one-way cannula was inserted at one end of the umbilical vein, and then fixed with a clamp. To remove blood clots the umbilical vein was gently irrigated 3-5 times with 20 ml volumes of warm HBSS using a 20-ml disposable syringe. 7-10 ml of sterile crude collagenase solution (1mg/ml in HBSS) was injected into the vein via the one way cannula and the distal end of the cord clamped. The cord was then left in HBSS for 15-20 minutes, at room temperature, and was massaged gently to enhance the release of endothelial cells.

To collect the released cells, the vessel and cord were cut using sharp scissors above the clamp, draining the contents into a 50-ml centrifuge tube containing 5 ml BCS. Any remaining cells were collected by flushing the vein with 20-ml of warm HBSS, and this was added to the centrifuge tube. A cell pellet was observed at the bottom of centrifuge tubes after centrifugation for 5 minutes at 1000 rpm. After the supernatant was discarded, the cell pellet was resuspend in 5 ml of HUVEC culture medium comprising M199 with BCS (20%), ECGS (50μg/ml) and heparin (30U/ml); penicillin (100U/ml), streptomycin (100μg/ml)
and amphotericin B (2.5µg/ml). The resuspended cells were then seeded in 25 cm² culture flasks which had been previously coated with Gelatin (0.1%) in PBS. Flasks were then incubated at 37 °C in 100% relative humidity, under CO₂ (5%). Endothelial cells settled and attached to the surface of the flask within 1 to 2 hours. Initially, the medium was changed daily; although later, cells were fed every 3 to 4 days. HUVEC grew and became confluent within 5-7 days. Confluent flasks were then passaged to larger 75 cm² culture flasks, at a split ratio of 1:3, using Trypsin with EDTA to release adherent cells. Cells were stored frozen with DMSO (10%) in liquid nitrogen and used in 4ᵗʰ or 5ᵗʰ passage for experiments.

2.3.1.b Isolation and Culture of Fibroblasts from Human Gingiva

HGF were isolated by explant culture from human gingival biopsies taken from extraction sites. Small fragments of gingival tissue were harvested from extraction sites immediately prior to extraction from patients in the Department of Oral Surgery at the Dental Clinical School Westmead Hospital. Tissues were stored in medium M199 in sterile specimen jars before washing three times with M199 and the antibiotics - penicillin (100u/ml), streptomycin (100g/ml) and amphotericin B (2.5µg/ml). The gingival tissues were then cut into 5-mm pieces with a sterile scalpel blade and then placed into 12-well culture plates. Tissue pieces were incubated in separate wells with 1ml of Trypsin (0.25%) / 1mM EDTA for 15 mins at 37°C. After incubation with trypsin, tissue fragments were then transferred to fresh wells. The tissue was then washed three times in M199 in the fresh culture well.

To aid fragmentation, the tissue was squashed with the flat edge of a scalpel, after which cells could be seen floating from cut edge of tissue (Freshney, 1994). To each well, 1-ml of Medium M199 with BCS (20%) was added then the culture wells were then left in a humidified incubator overnight at 37 °C under CO₂ (5%).

After 2 days, cells could be seen starting to attach to the floor of each well. After 1 week, a healthy growth of fibroblasts was observed extending from margins of the tissue explants. During this time, the medium was changed at regular
intervals at least two times per week and each time, 1-ml of Medium M199 with BCS (20%) was added. After 2-3 weeks, fibroblasts from the explants were transferred to 25 cm² flasks. The tissue explants were filtered out from the trypsinized HGF using 74 μm Netwells (Jackson, 1990). HGF were subsequently cultured with Medium M199 + BCS (10%) and the antibiotics penicillin (100u/ml), streptomycin (100g/ml) and amphotericin B (2.5μg/ml). Once confluent, fibroblasts in 25 cm² flasks were passaged to 75 cm² flasks. Subsequent passages of human gingival fibroblasts were split at a ratio of 1 to 4. At 4th passage, the HGF were used experimentally or stored in liquid nitrogen with DMSO (10%).

### 2.3.2 Tumour Cells

#### 2.3.2.a SAOS-2 Osteogenic Sarcoma Cell Line
This cell line was obtained from the A.T.C.C. (HTB-85) and is one of an extensive series of human tumour lines isolated and characterised by J. Fogh and G. Trempe (Fogh, 1975; Fogh, 1977). The cell line was isolated from a primary tumour arising in an 11-year-old Caucasian female. In culture, this cell line establishes a monolayer with an epithelial morphology. SAOS-2 are hyperdiploid to hypopentaploid with chromosomal abnormalities including breaks and secondary constrictions, and have a modal number of 56 chromosomes per cell. These cells express elevated levels of alkaline phosphatase, secrete type I collagen, and produce mineralised matrix in Millipore filter chambers (Rodan, 1987).

In this laboratory, we have used Medium M199 with BCS (10%) and the antibiotics penicillin (100u/ml), streptomycin (100g/ml) and amphotericin B (2.5μg/ml) as a standard growth medium for these cells. Cells were stored in liquid nitrogen in this medium with DMSO (10%).

#### 2.3.3.b Y43BP and HS Rat Carcinoma Cell lines
These cell lines were developed by Dr John Gibbins in the Department of Pathology at the University of Sydney. The Y43BP cell line is a non-metastatic rat carcinoma, which has been cloned and has a stable benign phenotype (Gibbins, 1991). This cloned cell line was transfected by a ras oncogene to
produce the H5 malignant cell line, which fails to express keratin. Both cell lines were grown in Joklik's medium supplemented with proline (40 mg/l) and BCS (10%).

2.3.3 Identification of HUVEC and SAOS-2 in Culture

2.3.3.a Characteristics of Cell Differentiation Used for Identification
It was important to be able to reliably identify HUVEC and SAOS-2 in Co-culture experiments. HUVEC can be identified on the basis of their characteristic cobblestone morphology as well as by specific labelling for FVIII associated antigen and UEA-1 lectin binding (Holthöfer, 1982; Jaffe, 1973). SAOS-2 cells are strongly Alkaline Phosphatase positive (Rodan, 1987), and this can be used to detect these cells in culture.

2.3.3.b Immuno and Lectin Histochemistry
Cell monolayers were fixed with formalin (10%) in PBS before extensive washing with PBS. All lectin and antibody solutions were applied to these monolayers as dilutions in PBS with BCS (10%) and Tween-20 (0.5%) with antisera diluted 1/100 and lectin applied at a concentration of 0.01mg/ml. Before application of the primary label, endogenous peroxidase activity was inhibited by incubation for 5 minutes with H$_2$O$_2$ (3%) in H$_2$O. Primary label consisting of either UEA-1 or FVIII associated antigen, was then applied for 45 minutes at room temperature. Monolayers were then washed three times with PBS containing Tween-20 (0.5%) and then treated with either rabbit-anti-UEA-1 or peroxidase conjugated goat-anti-rabbit serum dependant upon the primary label. Monolayers were again washed with PBS, Tween (0.5%) and in the case of UEA-1 lectin histochemistry, treated with peroxidase conjugated goat-anti-rabbit serum before a final washing step. The peroxidase label was detected using diaminobenzidine (DAB) (0.6mg/ml) for up to 10 minutes before rinsing with water, counterstaining with haematoxylin and coverslipping using an aqueous mounting medium. Using this protocol, it was possible to identify HUVEC as both UEA-1 and FVIII associated antigen positive cells.

2.3.3.c Alkaline Phosphatase Histochemistry
SAOS-2 express the Liver-Bone-Kidney isoenzyme of alkaline phosphatase (Rodan, 1987), and this endogenous activity was detected using a chromogenic
labelling system. Briefly, monolayers fixed and washed as indicated in 2.3.3b were incubated with a solution of Napthol AS-MX phosphate (0.1mg/ml) and fast red violet (0.2mg/ml) in Tris HCl (10mM, pH 8.0) at 37°C for 1 hour. This procedure produces an intense red stain in cells expressing alkaline phosphatase. Once counter stained with haematoxylin and coverslipped, SAOS-2 could be readily identified on the basis of this histochemical technique while other cells studied failed to express this label.

2.3.4 Identification of a Culture Medium Suitable for Co-Culture of Tumour Cells with Stromal Cells

Cultured cells often have highly specific growth and culture requirements, with the specific formulation of the culture medium having a profound effect upon the survival of cells (Freshney, 1994). For this reason, it was necessary to perform a series of experiments to identify culture media in which both tumour and stromal cells could survive.

In these experiments, H5, Y43BP, SAOS-2, HUVEC or HGF were grown to confluence in 75 cm² flasks using known optimal culture media (Sections 2.3.1 and 2.3.2) and then seeded into 24 well tissue culture plates. Cells were then cultured with either M199 or Joklik’s medium with or without BSA (4% w/v) for 24 hours.

Serum free media were trialed because it was desired that cell proliferation be minimised in co-culture experiments. BSA was included in these serum free media because it was known that HUVEC undergo rapid apoptosis in the absence of serum and that BSA inhibits this without stimulating proliferation (Zoellner, 1996b).

Cell survival was assessed on morphological grounds, with clear reduction in cell confluence being readily apparent in culture media determined as inappropriate for co-culture. Media for experimentation were selected on the basis that both tumour and stromal cell types survived without proliferation in the respective medium. M199 with BSA (4%) as well as Joklik’s medium with BSA (4%) were both found to be suitable for co-culture experiments. M199 with BSA (4%) was
used in most experiments, as this widely used culture medium was most readily available.

2.3.5 Co-Culture of Stromal Cells with Tumour Cells

Three co-culture methods were devised and used in this study.

2.3.5a Co-Culture Type A

Co-culture Procedure:
Stromal cells were grown to one-third confluence in complete growth media in triplicate 25 cm² culture flasks, 75 cm² culture flasks or 6 well tissue culture plates. After washing with M199, tumour cells were applied, also at one-third confluence in serum free medium with BSA (4%). Controls consisted of stromal cells not exposed to tumour cells as well as tumour cells seeded in the absence of stromal cells. The number of stromal cells present at the beginning of each experiment was determined by fixation of triplicate wells with formalin (10%) in PBS before addition of tumour cells. Co-cultures were incubated for up to 24 hours at 37°C under CO₂ (5%) before washing with M199 and fixation.

Quantification of Cell Density:
Cell density was determined for Type A co-cultures in 6 well tissue culture plates. HUVEC were labelled by lectin peroxidase-histochemistry for UEA-1 binding (as outlined in Section 2.3.3) followed by counter staining with haematoxylin. It should be noted that HUVEC labelled strongly and unambiguously with the lectin marker while tumour cells were uniformly negative for this label, but could be identified by their positive nuclear haematoxylin labelling. After labelling, a 1 cm² photographic graticule using a 4 x objective in a 10 x inverted microscope (Olympus, Tokyo, Japan) was used to count all cells in five representative microscopic fields selected as indicated in Figure 2.1. All cells in these five fields were counted as either lectin positive or negative and totalled for calculation of means and standard deviations. In some experiments, HGF were co-cultured with SAOS-2 cells using an identical approach, with the difference however, that SAOS-2 were labelled for Alkaline Phosphatase activity (see section 2.3.3) while HGF were identified as alkaline phosphatase negative, nuclear haematoxylin positive cells.
**Figure 2.1.**
Diagram showing the way in which fields were selected for determination of cell density in Type A co-cultures. Stromal cells co-cultured with tumour in 6 well culture plates were fixed with formalin (10%) in PBS, and then stained for either UEA-1 binding or alkaline phosphatase activity. Monolayers were then coverslipped in wells with 15-millimeter diameter circular cover slips and aqua mount. Cells were counted at a magnification of x 400 at 5 different positions using an eye-piece mounted graticule, using an Olympus inverted microscope. The areas represented by the graticule eyepiece were calculated as being 1/16 mm$^2$, and cell density counts were expressed as cells/mm$^2$. 
Transwell Co-Culture Methods
Changes in stromal and tumour cell density were seen in Type A co-cultures. To further study the possible role of cell contact in this, additional experiments were performed using Transwell culture inserts. These were designated: Type B co-cultures in which stromal cells grown on transwell surfaces were introduced to tumour cell cultures in 6 well culture plates; and Type C co-cultures in which stromal and tumour cells were grown on opposite sides of the shared microporous membrane.

2.3.5.b Co-Culture Type B
Stromal cells were grown to near confluence on transwell surfaces while tumour cells were grown to confluence in separate 6 well tissue culture plates. Transwells were then transferred to culture plates containing tumour cells and incubated at 37°C under CO₂ (5%) for up to 24 hours (Figure 2.2). Trypsin / EDTA was used to release both stromal and tumour cells for quantification of cell numbers using a Neubauer haemocytometer (Hawksley, London). Briefly, medium was removed and the wells washed once with 1-ml of M199. The transwell, was then placed on a sheet of dental base-plate wax before adding 200 μl of Trypsin / EDTA for 3 minutes. Released cells were then collected and counted using a haemocytometer. Means and standard deviations were calculated for triplicate wells.

2.3.5.c Co-culture Type C
A modified transwell system was developed in which polypropylene centrifuge tubes were cut and trimmed to adapt 12-well plate transwell culture wells for culture on both sides of the well surface (Figure 2.3). Firstly, stromal cells were cultured on one side of the well to near confluence. After this, transwells were inverted and tumour cells seeded on the reverse surface. Any trapped air bubbles were removed (Figure 2.4) and transwells cultured for up to 24 hours in serum free medium. Stromal cell number was determined by releasing cells with 200 μl of Trypsin / EDTA (see section 2.3.5.b) and counting with a haemocytometer. Means and standard deviations were calculated from triplicate wells in these experiments.

2.10
Figure 2.2
Diagram illustrating Type B Co-Cultures. Stromal cells were grown to near confluence on the transwell surface while tumour cells were grown to confluence in separate 6 well tissue culture plates. Transwells were then transferred to culture plates containing tumour cells and incubated at 37°C under 5% CO₂ for up to 24 hours. Trypsin / EDTA was used to release both stromal and tumour cells for determination of cell number.
Figure 2.3
Diagram illustrating Type C Co-Cultures. These cultures were established in two discrete steps, using 12-well plate transwell culture wells which had been modified by application of a cut centrifuge tube to create two separate culture compartments on either side of the membrane (A). Stromal cells were seeded at confluence onto the upper surface of this modified transwell.
After one day of culture, transwells were inverted (B), and tumour cells seeded at confluence on the newly established upper, and previously lower, transwell membrane surfaces. In this way, stromal and tumour cells were cultured close to each other (10 µm), but separated by a porous membrane for up to 24 hours. Stromal cell number was determined by haemocytometer counts of cells released by Trypsin / EDTA. In establishing these cultures, air bubbles were removed using a curved syringe as indicated in Figure 2.4.
A

Tissue Culture Plate Cover

Transwell for 12 well plate

Upper compartment of Co-Culture

Lower compartment of Co-Culture

Stromal cells cultured on upper surface of modified transwell

8 mm

Modified centrifuge tube to create new transwell compartment

Microporous membrane

6 well cluster plate

B

Tissue Culture Plate Cover

Transwell

New Upper compartment of co-culture

New Lower compartment of co-culture

Tumour cells seeded on upper surface of inverted modified transwell

Medium Level

Stromal cells adherent to lower surface of inverted modified transwell culture wells

Microporous membrane

6 well cluster plate
Figure 2.4
Diagram illustrating the removal of air bubbles using a curved needle and syringe in Type C Co-Cultures. Any trapped bubbles under modified transwells in type C co-cultures were readily removed using a curved needle as indicated. This was important to prevent drying of the cells in the lower chamber as well as to avoid problems due to floating of modified transwells.
2.3.6. Medium Transfer Experiments
Dose response experiments with tumour cell conditioned medium were performed to determine the effect of such conditioned medium upon stromal cell survival. Confluent Tumour cells were cultured with serum free medium (M199 with 4% BSA) for 24 hours in 75 cm² flasks. The resultant tumour cell conditioned medium was then diluted with fresh serum free medium (4% BSA with M199) at concentrations ranging from 0 to 100% and then applied to stromal cells, at one-third confluence. Experiments were in 24 well tissue culture plates, while the stromal cells were incubated with tumour cell conditioned medium for up to 24 hours at 37°C under CO₂ (5%). Cell counts of trypsin/EDTA released cells were then performed for triplicate wells using a Haemocytometer, as described in 2.3.5.

2.3.7 Tumour Cell Conditioned Gelatin Matrix
HUVEC growth and differentiation is strongly dependant on matrix binding (Caveda, 1996). To determine if degradation of the gelatin matrix by tumour cells was a likely cause of reduced HUVEC survival, a series of experiments was performed in which SAOS-2 were allowed to interact with gelatin matrix prior to seeding of the endothelial cells.

Tumour cells were seeded at confluence on an identical 0.1% gelatin matrix to that used in the HUVEC cultures. After 24 hours, the medium was changed to serum free medium, M199 with BSA (4%) and after a further 24 hours, cells were lysed using sterile MilliRO 12 plus water, from a MilliQ PF water system (Millipore). Complete cellular lysis was confirmed by phase microscopy. HUVEC were then seeded at one third confluence upon the remaining gelatin matrix and cultured in serum free medium for 24 hours. After washing with M199, final HUVEC numbers were determined by releasing the cells using Trypsin / EDTA and counting with a haemocytometer. Means and standard deviations were calculated from triplicate wells in these experiments.
2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to further investigate the possible role of cell contact in interactions between stromal and tumour cells. Type C co-cultures were established as indicated in 2.3.5. Early experiments were with transwells with 3 μm membrane pores. However, tumour cells were found to migrate through these large pores, so that further experiments were with 0.4 μm pores.

After culturing HUVEC and SAOS-2 for 24 hours in Type C co-cultures (2.3.5), membranes were fixed for 10 minutes with 2.5% glutaraldehyde. Dehydration with graded alcohols was followed by critical point drying using a semi-automatic critical point drying apparatus (Balzers Union, Leichtenstein). Some samples were then fractured after freezing in liquid nitrogen (Bozzola, 1991), to permit examination of cell processes passing through the membrane. All samples were sputter coated with gold using a Balzers Union sputter coater (Balzers Union, Leichtenstein). Samples were examined using a Philips CM-10 scanning electron microscope (The Netherlands).

2.5 FACS Analysis

Fluorescence Activated Cell Sorting Analysis (FACS) was performed with cells in Type A co-cultures in 25 cm² flasks. Briefly, HUVEC and SAOS-2 were cultured over a 24 hour period in 25 cm² tissue culture flasks using serum free medium as described in 2.3.5a. Fluorescein Isothiocyanate (FITC) conjugated UEA-1 was used to label HUVEC, while Propidium Iodide/RNase-A (PI/RNase) (Propidium Iodide (50μg/ml), RNase (0.1mg/ml), Sodium Citrate (0.1% w/v), Triton X-100 (0.1%)) solution was used to label DNA (Darzynkiewicz, 1997).

In performing these experiments, HUVEC and SAOS-2 cells were first established in 25 cm² flasks. The SAOS-2 cells were then released with Trypsin / EDTA, pelleted with serum (5ml) and then seeded over the HUVEC in M199 with BSA (4%). Parallel cultures of HUVEC not exposed to tumour cells as well as of SAOS-2 seeded on gelatin matrix were performed as controls. Flasks were then incubated at 37°C under CO₂ (5%) for 24 hours. Detached cells were then collected by washing, while adherent cells were released with trypsin and added
to the detached population. After washing, cells were treated with 100 μl of UEA-1 FITC (1mg/ml) in PBS with BCS (10% v/v) for 30 minutes. Cells were then washed three times with PBS before fixation with ethanol (70%) at -20°C for 2 hours. PI/RNAse was then added over night to label DNA. Cell population analysis was performed using a Becton-Dickinson flow cytometer with FACScan and Lysis II software.

2.6 Transmission Electron Microscopy and Silver Enhanced Staining

2.6.1 Exploitation of UEA-1 Labeling to Characterize Cells in Type A Co-Cultures

Transmission Electron Microscopy (TEM) was performed on cells in Type A co-cultures of HUVEC with SAOS-2. This was correlated with silver-enhanced light microscope sections of UEA-1 gold labelled cells. It was possible to identify HUVEC in transmission electron micrographs of mixed populations of HUVEC with SAOS-2 cells, by labelling these populations with UEA-1 gold particles (10nm). This was extended to the level of the light microscope using silver-enhancement techniques on acrylic semi-thin sections. To prepare cells for TEM, floating detached cells from both Type A co-cultures as well as from control SAOS-2 and HUVEC cultures were collected by washing with PBS. Adherent cells were harvested by scraping, in order to avoid ultrastructural changes found in preliminary experiments to be due to exposure to Trypsin / EDTA. Cells were seeded into 75 cm² flasks and cultures were identical to those described in 2.3.5 a.

Floating and attached cells were washed in Sorensons Phosphate Buffered Saline (SPBS). After fixing in Gluteraldehyde (1%) in PBS pH 7.2 for 10 minutes, the cells were washed in SPBS and then incubated in SPBS with Tween-20 (0.05%) BSA (0.5%) and of UEA-1 labelled colloidal gold particles (0.2 mg/ml) for 30 minutes. To facilitate handling, pelleted cells were encapsulated in BSA (10%) in PBS and then fixed with Karnovsky’s fixative for 6 hours at 4°C. The resulting pellet was divided in two using a scalpel, so that one half could be embedded in Spur resin for further processing for TEM, leaving the other half for embedding in LR White resin and silver-enhancement staining.
2.6.2 Transmission Electron Microscopy
After fixation with Karnovsky’s and washing with SPBS (Section 2.6.1), albumin pellets were post-fixed with Osmium Tetroxide (2%) in SPBS for 1 hour. Specimens were then rinsed with distilled water and then dehydrated in graded alcohols before transfer to acetone (100%). Finally, acetone was replaced with spurs resin and polymerised at 70°C for 10 hours. Semi-thin and ultra-thin sections were prepared, using a Reichert Ultracut-E ultramicrotome (Leica, Vienna). 70 nm ultra-thin sections, were double stained on 400 bar copper grids with uranyl acetate (2%) in ethanol (50%) and Reynold’s lead citrate. Sections were examined using a Philips CM-10 Transmission Electron Microscope (The Netherlands) operating at 80kV.

2.6.3 Silver Enhancement Microscopy
Silver Enhancement was performed using the kit available form British Biocell International (Cardiff, U.K.). Since osmium fixation results in significant background precipitation of silver, samples were not post-fixed with osmium. The albumin pellet was dehydrated with graded alcohols, and then infiltrated with LR White resin and polymerised at 60°C for 20 hours. Semi-thin LM sections (0.5μm) were then prepared, and silver enhancement of gold label performed by addition of one drop of initiator solution to one drop of accelerator solution to sections on glass slides. After 10 minutes of this treatment, sections were rinsed with water and stained with Toluidine Blue. Sections were then cover-slipped and examined by light microscopy (Olympus, Tokyo, Japan. Model. Vanox AHB-LB. Also Carl Zeiss, Germany) under oil immersion.
Chapter 3

The effect of Tumour Cell Lines upon HUVEC Survival

3.1 HUVEC Could be Distinguished Form Tumour Cells on the Basis of UEA-1 labelling

HUVEC labelled intensely for UEA-1 while SAOS-2, H5 and Y43BP cells failed to bind this label (Figure 3.1). Controls in which HUVEC were not treated with UEA-1, but were exposed to the other reagents used, failed to demonstrate any labelling, confirming dependence of the staining method upon UEA-1.

This highly specific and unambiguous labelling of HUVEC permitted the use of this lectin-histochemical method for quantification of cell density in Type A co-cultures of HUVEC.

The use of UEA-1 labelling to identify HUVEC and tumour cells is illustrated in Figure 3.2. It is seen that HUVEC are readily distinguished from tumour cells, which are both morphologically and histochemically different.

3.2 HUVEC Density was Reduced in Type A Co-Cultures with SAOS-2 Cells

Figure 3.3 shows the results of a typical experiment in which HUVEC were exposed to tumour cells in Type A co-cultures. In the absence of tumour cells, there was no significant change in HUVEC density at any time point sampled. In the presence of SAOS-2 cells, however, there was a significant reduction in HUVEC density by 12 hours of co-culture (p<0.02) and this became more pronounced by 24 hours (p<0.01). Interestingly, there appeared to be fewer HUVEC in SAOS-2 co-cultures by the 2 hour time point. In contrast to SAOS-2, no significant reduction upon HUVEC density was seen when endothelium was exposed to H5 or Y43BP cells (Figure 3.1). Similar observations were made in a further 4 experiments with HUVEC obtained from different donors.
Figure 3.1
UEA-1 lectin histochemistry of HUVEC, SAOS-2, Y43BP and H5 cells. Cultured cells were fixed with formalin, washed and then treated with UEA-1 as described in section 2.3.3. Bound lectin was then detected with a specific polyclonal antiserum and peroxidase histochemistry (Section 2.3.3). Only HUVEC labelled for UEA-1 using this technique. (Bar = 25 μm)
Figure 3.2
Photomicrograph of UEA-1-peroxidase labelled HUVEC cultured with SAOS-2, Y43BP and H5 cells 2 hours and 24 hours after seeding tumour cells.
HUVEC labelled intensely and specifically for UEA-1 (arrows), permitting differentiation from unlabelled tumour cells, in which only the nuclear counter-stain was seen (arrow heads). It was possible to quantitate HUVEC and tumour cell density in these cultures, by counting UEA-1 labelled and unlabelled cells. [Bar = 50 μm]
Figure 3.3
No reduction in HUVEC density was seen in the absence of tumour cells. However, in the presence of SAOS-2 cells, a slight reduction in HUVEC density was seen at 2 hour as compared with the absence of SAOS-2. By 12 hours, HUVEC density in the presence of SAOS-2 was lower than at 2 hour (p<0.02) and this was also seen at the 24 hour time point (p<0.01). In contrast to this, no clear effect of Y43BP or H5 cells was seen upon HUVEC density.
3.3 HUVEC Stimulate Tumour Cell Growth in Type A Co-Cultures

Figure 3.4 shows the changes in tumour cell density over time in the same experiment as that shown in Figure 3.3. HUVEC stimulated tumour cell growth in all cell lines studied (p<0.01). This contrasted with cultures of tumour cells seeded in the absence of HUVEC, where no increase in tumour cell density was seen in the absence of serum (Figure 3.4) with the exception of Y43BP cells in which cell density increased over time. Similar results were obtained in 4 further separate experiments. From this, it was concluded that HUVEC stimulate the proliferation of the three tumour cell lines studied.
Figure 3.4 – The density of tumour cells in Type A co-cultures in the absence (□) and presence (■) of HUVEC. In the absence of HUVEC there was a slight reduction in SAOS-2 density (p<0.01) and an increase in that for Y43BP (p<0.01) while no change in H5 density was seen. However, when HUVEC were included with each of the three tumour cell lines studied, a significant increase in tumour cell density occurred (p<0.01). It is important to note that in the case of Y43BP cells, although these cells increased in density in the absence of HUVEC, co-culture with these stromal cells increased Y43BP density significantly more than in the absence of stromal cells (p<0.01).
Chapter 3 – Effect of Tumour Cells on HUVEC Survival

![Graphs showing tumour cell density over time for SAOS-2, Y43BP, and H5.](image)

**Tumour Cell Density (Cells x 10^3/mm^2)**

**Time (Hours)**
3.4 Serum Free Tumour Cell Conditioned Medium Stimulates HUVEC Proliferation

Examination of Figure 3.3 reveals rapid reduction of HUVEC density 12 hours after co-incubation with SAOS-2 cells followed by more modest changes at 24 hours. This suggested that proliferation of HUVEC during the experiment complicated HUVEC density data, with cell density reflecting a balance between proliferation and HUVEC death. To further investigate the possibility that tumour cells release growth factors for HUVEC in this experimental system, further experiments were performed testing the effect of tumour cell conditioned serum free medium upon HUVEC number (see section 2.3.6).

Figure 3.5 shows the result of a typical experiment testing the effect of serial dilutions of serum free medium, pre-conditioned by tumour cells, upon HUVEC density. It is seen that medium conditioned by all 3 tumour cell lines, increased HUVEC number at concentrations ranging from 1.2% to 100% (p<0.05). Similar observations were made in 4 separate experiments. This indicated the release of soluble growth factors for HUVEC by tumour cells. On this basis, it is concluded that HUVEC data for Type A co-cultures is complicated by HUVEC proliferation, and that this masks much of the HUVEC death in these experiments. Importantly, this data indicates that reducing HUVEC density was not due to medium exhaustion by tumour cells.
Figure 3.5
The effect of tumour cell conditioned medium upon HUVEC number.
Serum free medium conditioned by 24 hour incubation with SAOS-2 (▲),
Y43BP (□) or H5 (○) tumour cell lines significantly increased HUVEC number
(p<0.01) and this activity was reduced by serial dilution in fresh serum free
medium.
3.5 Reduced HUVEC Density in Type A Co-Cultures Could Not be Attributed to Tumour Cell Degradation of HUVEC Supportive Matrix

Results in Figure 3.5 indicated that reduced HUVEC density in Type A co-cultures was not due to medium depletion by tumour cells. Since HUVEC require a gelatin matrix for survival, the possibility was considered that tumour cells degraded this matrix, making adhesion and survival of HUVEC impossible. To test this possibility, gelatin coated culture surfaces were pre-treated with tumour cells or HUVEC before lysis of the cells with sterile distilled water and re-seeding with HUVEC (see section 2.3.7). Figure 3.6 shows that this pre-treatment had very little effect upon HUVEC survival. Instead of reducing HUVEC survival, SAOS-2 pre-conditioned gelatin surfaces appeared to slightly increase HUVEC number (p<0.05). Similar results were obtained in one further experiment with HUVEC from a different donor. From this, tumour cells either failed to degrade the gelatin matrix or deposited fresh matrix suitable for HUVEC survival. In either case, it was not possible to attribute reduced HUVEC density in Type A co-cultures to matrix degradation by tumour cells.
**Figure 3.6**

The effect of tumour cell conditioning upon the ability of the gelatin matrix to support HUVEC survival. Pre-treatment of gelatin coated matrix with HUVEC, SAOS-2 or Y43BP followed by cell lysis with distilled water did not reduce HUVEC survival. However, a slight increase in HUVEC number was seen when gelatin matrix was pre-conditioned by SAOS-2 cells (p<0.05)
3.6 Experiments with Transwell Co-Cultures Support a Role for Cell Contact in SAOS-2 Induced HUVEC Death

3.6.1 Scanning Electron Microscopy indicated that 3.0 μm Transwells Were Inappropriate for Co-Culture Experiments

Difficulties were encountered in scanning electron microscopy, such that the integrity of plasma membranes was often lost during processing. Nonetheless, when this occurred it was still possible to clearly see the underlying cytoskeletal structures so that the relationship of cells and their processes to transwell pores was still readily seen (Figure 3.7).

Importantly, when SAOS-2 were seeded onto 3.0 μm transwell membranes, broad cell processes were seen passing into the membrane pores (Figure 3.7). Passage of these tumour cell processes through the transwell was confirmed in scanning electron micrographs of fractured membranes (Figure 3.8), as well as of the intact surfaces of Type C Co-cultures (Figure 3.9). HUVEC failed to penetrate membranes in this way, so that broad SAOS-2 processes were seen to emerge adjacent to HUVEC (Figure 3.9), but HUVEC were never seen emerging adjacent to SAOS-2 cells. This suggested that SAOS-2 cells may have migrated completely through 3.0 μm pore transwell membranes.

When SAOS-2 were seeded onto 0.4μm pore membranes in Type C Co-cultures, cells seemed unable to pass across the membrane, although very occasional cell processes appeared to enter pore openings (Figure 3.10). No cell processes were seen emerging on the opposite side of the 0.4 μm membranes seeded with either SAOS-2 or HUVEC alone.

HUVEC appeared to have more extended and elongated cell processes than SAOS-2 (Figure 3.12), but did not show any sign of trans-cellular migration in the manner seen in SAOS-2. Because SAOS-2 appeared to readily pass across the 3.0 μm transwell membranes while cells were unable to pass across 0.4 μm transwell membranes, it was decided to confine quantitative studies of changes in HUVEC or tumour cell number in Type B and C Co-cultures to 0.4 μm transwells.

3.12
3.6.2 Apoptotic Features Were Seen in Occasional HUVEC in Type C Co-Cultures

Scanning electron microscopy of HUVEC in Type C Co-cultures with SAOS-2 cells revealed occasional cells with cell processes entering 0.4 μm pores (Figure 3.13). Amongst these cells were very occasional HUVEC with an obvious blebbing morphology, typical of that expected for apoptosis. In these cells, occasional cell processes entering transwell pores were seen (Figure 3.14).

3.6.3 Quantification of HUVEC and SAOS-2 in Type B and C Co-Cultures

Cell counts of HUVEC 24 hours after Type B co-culture with tumour cells failed to reveal any significant differences between HUVEC in the presence or absence of SAOS-2 (Figure 3.15). Similar observations were made in 5 separate experiments with SAOS-2 cells. Also in Type C co-cultures, there was no consistent change in HUVEC number (Figure 3.16) and this was seen in 2 further experiments.

These data suggested that HUVEC were unable to proliferate on the transwell culture surface, and that no soluble death inducing factor could explain the reduced cell density of HUVEC in Type A co-culture.

However, it should be noted that in Type B and Type C co-cultures, the stromal and tumour cells were separated by 1mm and 10 μm respectively. The possibility remains that over this distance, a highly labile soluble factor may have become inactive, although it is difficult to conceive of a soluble factor unable to survive the short 10 μm passage across the transmembrane in Type C Co-cultures.
Figure 3.7
Scanning electron micrograph of a SAOS-2 cell on the tumour cell surface of a 3.0 μm transwell in Type C Co-culture. Although the plasma membrane has been damaged during preparation to expose the underlying cytoskeletal structure (Arrow head), the emergence of broad cell processes penetrating into the 3.0 μm transwell pore is easily seen (Arrows). (Bar = 10 μm).

Figure 3.8
Scanning electron micrograph of 3.0 μm pore transwell membrane with SAOS-2 and HUVEC in Type C Co-culture subjected to fracture. SAOS-2 processes are clearly seen passing through 3.0 μm transwell pores (Arrows). HUVEC failed to penetrate membranes in this way. (Bar = 10 μm)
Figure 3.9
Scanning electron micrograph of the HUVEC side of a 3.0 μm pore transwell membrane with a Type C Co-culture of SAOS-2 with HUVEC. 24 hours after seeding tumour cells, SAOS-2 processes are seen protruding through the pores (Arrows) as is a nearby HUVEC (Arrow head). From this, SAOS-2 appeared capable of migration through transwells while HUVEC were not. (Bar = 10 μm)

Figure 3.10
Scanning electron micrograph of SAOS-2 cell seeded onto a 0.4 μm transwell membrane in Type C Co-cultures with HUVEC for 24 hours. Very occasional fine cell processes appear to enter some pores (Arrows), although shrinkage artefact has disrupted some of the cell processes (Arrowhead). (Bar = 10 μm)
Figure 3.11
Scanning electron micrograph of the lower surface of a 0.4 μm transwell membrane, above which HUVEC have been cultured for 24 hours. No cell processes are seen passing through the transwell pores.

Figure 3.12
Scanning electron micrograph of HUVEC cultured for 24 hours on a 0.4 μm transwell membrane. HUVEC appear to have more extended and elongated cell processes than SAOS-2. (Bar = 10 μm)
**Figure 3.13**
Scanning electron micrograph of HUVEC in Type C Co-culture with SAOS-2 for 24 hours on a 0.4 μm transwell membrane. Despite some artifactual degeneration of the plasma membrane (Arrowhead), the location of cell processes is clearly seen. These processes appeared to occasionally pass over pores (Long arrow), but also seemed to sometimes enter pores (Short arrow). (Bar = 1 μm).

**Figure 3.14**
Scanning electron micrograph of HUVEC in Type C Co-culture with SAOS-2 cells in a 0.4 μm transwell membrane. The cell has fragmented into multiple smaller blebbing bodies, while numerous cell processes are seen attaching the cell to the matrix. A single cell process is seen entering a 0.4 μm membrane pore (Arrow), while occasional membrane pores consistent with canalicular fragmentation are seen (Arrow head). (Bar = 10 μm)
Figure 3.15
Cell counts of HUVEC 24 hours after Type B co-culture with SAOS-2. No significant difference was seen in the number of HUVEC in the presence or absence of tumour cells.
Figure 3.16
HUVEC number 0 and 24 hours after incubation with SAOS-2 in Type C Co-cultures. No significant change in HUVEC number was seen over the 24-hour culture period, suggesting the absence of a labile soluble pro-apoptotic factor released by SAOS-2.
3.7 Discussion

Co-culture Type A experiments demonstrated reduced HUVEC density in the presence of SAOS-2 cells. Because HUVEC cell density levels were lower than in the starting population, it was concluded that this was due to loss of HUVEC by cell death. Tumour cell conditioned media failed to reduce HUVEC number but instead stimulated HUVEC proliferation. All tumour cell media increased HUVEC number to a similar extent. Considered together, these data suggested that tumour cells differed with regard to their ability to cause HUVEC death, with SAOS-2 being particularly potent in this regard. Contrasting with SAOS-2, the Y43BP and H5 cell lines appeared not to significantly affect HUVEC density in Type A co-cultures due to a relatively weak death inducing activity balanced by secreted proliferative activity. Experiments with Type B transwell cultures further supported the absence of any soluble death-inducing factor released by tumour cells. While the possibility that a highly labile soluble factor released by SAOS-2 appeared to be excluded in experiments with Type C Co-cultures. Together, these data suggest cell contact dependant death inducing activity for HUVEC in all three-tumour cell lines studied.

In view of the proliferation of HUVEC in response to tumour cell conditioned medium, it was surprising that HUVEC number did not increase in Type B Co-cultures. This was thought to reflect either an inability of HUVEC to proliferate on the transwell surface, or alternatively, accumulation of insufficient proliferative factors from tumour cells in the culture medium in these experimental conditions, to simulate HUVEC proliferation. It seemed likely that in the absence of any obvious cytotoxic agent or medium depletion, that HUVEC death was not due to non-specific necrotic effects, but reflected activation of apoptosis, while occasional HUVEC with seemingly apoptotic morphology were seen by scanning electron microscopy. Interestingly, cells with this apoptotic appearance had occasional cell processes passing into transwell pores. This appears to be a separate phenomenon to the trans-membrane migration of SAOS-2 on 3.0 µm transwells, as the processes involved were very different in size and the large cell processes seen crossing 0.4 µm transwells were seen only in SAOS-2 and never in HUVEC which had fine cell processes. Although no HUVEC processes were seen on the opposite surface of 0.4 µm transwells.
seeded with HUVEC alone, it is possible that in Type C Co-culture, HUVEC processes were attracted by SAOS-2, and cell contact occurred on the basal SAOS-2 surface. This would be consistent with a cell contact dependant HUVEC death activating activity in SAOS-2 which may not have been detected in quantitative studies due to an insufficient number of cell contacts permitted by the limited number of cell processes passing through 0.4 μm transwells. The apoptotic appearance of occasional HUVEC in Type C Co-cultures required further investigation of the mechanism of cell death in Type A Co-cultures, the subject of the next chapter.
Chapter 4

Demonstration of Apoptotic Death of HUVEC Co-Cultured with SAOS-2 cells

4.1 Introduction
Previous experiments demonstrated contact dependent HUVEC death in the presence of SAOS-2 in Type A Co-cultures. It was suggested that apoptosis was responsible for this, and this chapter describes work investigating this possibility.

4.2 FACS Analysis Revealed Characteristics of HUVEC Apoptosis in Type A Co-Culture with SAOS-2

FACS analysis of Type A co-cultures of HUVEC and SAOS-2 (see section 2.5) as compared with isolated cells alone revealed specific labelling of HUVEC with UEA-1 as well as DNA in both cell types with Propidium iodide (Figure 4.1). All stages of the cell cycle were seen in DNA profile histograms, including the G0/G1, S and G2/M regions, with the expected aneuploid population of SAOS-2 presented as additional G0/G1 and G2/M peaks. A small sub-diploid apoptotic population was seen in HUVEC cultured alone, while these cells labelled strongly for UEA-1 as compared with SAOS-2 cells, in which labelling was approximately two orders of magnitude less intense. When HUVEC were co-cultured together with SAOS-2 cells, it was possible to exploit the intense labelling of HUVEC with UEA-1 to gate the HUVEC population for assessment of the DNA profile. This revealed a significant increase in the magnitude of the sub-diploid peak as compared with HUVEC cultured in the absence of SAOS-2. Similar results were obtained in a total of 4 separate experiments. These data are consistent with apoptosis of HUVEC but not SAOS-2 in Type A co-cultures.
Figure 4.1 – FACS analysis of UEA-1 (i) binding and DNA content (ii) in Type A co-cultures of HUVEC with SAOS-2 (C) as compared with SAOS-2 (A) and HUVEC (B) cultured alone.

All stages of the cell cycle were identified in DNA profiles of HUVEC (Bii) as well as in SAOS-2 cells. It was noted, however, that SAOS-2 contained a distinct aneuploid population generating further supra-diploid G0/G1 and G2/M populations (Aiii). HUVEC also contained a small sub-diploid population, suggestive of low levels of apoptosis (Bii). HUVEC in all stages of the cell cycle were found to label intensively for UEA-1 binding (Bi) while SAOS-2 cells bound UEA-1 only very weakly (Ai). Because of this, it was possible to gate HUVEC in co-cultures with SAOS-2 on the basis of UEA-1 binding and this is illustrated by the polygon surrounding strongly UEA-1 positive cells in Ci. The DNA fluorescence profile of this gated population (Cii) includes a significantly larger sub-diploid peak as compared with HUVEC alone (Bii). These data are consistent with increased HUVEC apoptosis in Type A co-cultures with SAOS-2 as compared with HUVEC cultured alone.
4.3 Detached Floating Cells in Type A Co-Cultures are HUVEC

Examination of sections of silver-enhanced UEA-1 / gold labelled HUVEC revealed labelling of almost all cells (Figure 4.2A) while SAOS-2 failed to label under these conditions (Figure 4.2B). Adherent cells from Type A co-cultures consisted mainly of UEA-1 / gold negative SAOS-2 cells (Figure 4.2C) while detached cells were almost all HUVEC on the basis of UEA-1 labelling. These detached cells displayed all of the morphological features expected of apoptotic HUVEC including; cellular shrinkage, fragmentation into small apoptotic bodies and the formation of condensed, round nuclear particles (Figure 4.2D).

It was noted that the gold label was often apparently internal in both adherent and non-adherent HUVEC. In the case of non-adherent cells, this was attributed to canalicular fragmentation, while for adherent cells, seemingly cytosolic labelling was attributed to deep folds of plasma membrane into cells as a result of the scraping cell harvesting technique used.
**Figure 4.2**
Black and white photomicrographs of silver-enhanced UEA-1 / gold labelled HUVEC and SAOS-2 alone and in Type A Co-cultures. Adherent HUVEC alone (A) labelled intensely with UEA-1 (Arrows), with seemingly cytoplasmic labelling reflecting cytoplasmic folds formed during cell harvesting. SAOS-2 (B) failed to label with UEA-1, while in Type A Co-cultures, detached cells (C) had the morphological appearance of apoptosis including fragmented and condensing nuclear material (☆) and what appeared to be canicular fragmentation (C), evidence of secondary necrosis (SN) is seen and all cells clearly labelled for UEA-1 (Arrows). Adherent cells in Type A Co-cultures (D) were primarily UEA-1/gold negative (S), suggesting that the HUVEC (H) have mostly detached and joined the floating cell population (C). (Bar = 5 μm)
4.4 UEA-1 Labelled Detached HUVEC from Type A Co-Cultures had the Ultrastructural Features of Apoptosis.

Transmission electron microscopy with UEA-1 gold labelling was used to study the ultrastructure of Type A co-cultures of HUVEC with SAOS-2. HUVEC could be reliably identified as separate to SAOS-2 by the presence of membrane bound 10nm gold particles.

As indicated in Figure 4.2 UEA-1 was bound by HUVEC membranes exclusively, allowing accurate identification of HUVEC in co-culture with SAOS-2. The gold particles were seen in electron micrographs of HUVEC as distinct membrane associated particles (Figure 4.3). Deep folds of plasma membrane (Figure 4.4) were found to be responsible for the seemingly intracellular location of UEA-1 / gold particles seen in silver enhanced light microscope sections (Figure 4.2). SAOS-2 were negative for the UEA-1 label (Figure 4.5)

Canalicicular fragmentation characteristic of apoptosis in endothelium (Figure 4.6 and 4.7) was the basis for similar labelling in detached cells. These cells displayed other features of apoptosis including nuclear fragmentation and condensation, increased cytoplasmic electron density, and intact organellar structure (Figure 4.6 and 4.8)

UEA-1 negative SAOS-2 were, however, not found in the floating apoptotic population of cells in Type A co-cultures, indicating that not only were detached cells apoptotic, but they were also HUVEC.

Some of the cells in the floating population were highly degenerate, but of similar size to apoptotic particles, despite swelling due to loss of membrane pump activity. These were interpreted as having undergone secondary necrosis (Figures 4.9). Examination of adherent cells by transmission electron microscopy revealed that both SAOS-2 and HUVEC were healthy without any clear sign of cytotoxicity and few apoptotic cells.
Figure 4.3
Transmission Electron Micrographs of adherent HUVEC, harvested by cell scraping and labelled with UEA-1 gold. (A) The nucleus is large and vesicular (N) while RER (Arrowheads) and Mitochondria (M) are readily apparent. This cell displays the highly convoluted folds of plasma membrane (F), which may create the impression of intracellular plasma membrane labelling in light micrographs. (B) Examination of the plasma membrane at higher magnification reveals numerous membrane bound UEA-1 / gold particles (Arrows), consistent with the endothelial identity of the cell. (Bar = 5 μm for A, and 1 μm for B)
Figure 4.5
Transmission electron micrographs of SAOS-2 treated with UEA-1 / gold particles. (A) Cells have large vesicular nuclei (N) and deep folds of plasma membrane (F) presumed to represent artefactual membrane folding during cell harvesting. (B) At higher magnification UEA-1 / gold particles were not seen attached to the plasma membrane. Occasional lipid vacuoles (L) were seen due to cell stress. (Bar = 5 μm for A, and 1μm for B)
Figure 4.6
Transmission electron micrographs of detached cells in Type A Co-culture of HUVEC with SAOS-2, labelled with UEA-1 / gold. (A) Advanced canalicular fragmentation (C) appears to have displaced cytoplasmic remnants to the periphery of the cell and UEA-1 / gold particles (Arrows) are found associated with these membranes. (B) Membrane labelling with UEA-1 / gold (Arrows) consistent with the seemingly intracellular location of this plasma membrane marker in silver-enhanced light microscope sections (Figure 4.2). (Bar = 5 μm for A, and 1 μm for B)
Figure 4.7
Transmission electron micrographs of detached cells labelled with UEA-1 / gold from Type A Co-cultures of HUVEC with SAOS-2.
Numerous cells with the features of apoptosis are seen (A). These characteristics include; condensed nuclear material (☆), electron dense cytoplasm, small size and canalicular fragmentation (C) commencing at the surface of the cell (Arrowhead) and seeming to progress to honeycomb the cell (Large Arrow).
Examination at higher magnification reveals UEA-1 / gold particles associated with these canalicular fragmented membranes (B and C, small arrows)
(Bar = 5 μm for A, 5 μm for B, and 1 μm for C)
Figure 4.8
Transmission electron micrographs of detached cells from Type A Co-cultures of SAOS-2 with HUVEC, labelled with UEA-1 / gold. (A) Features typical of endothelial apoptosis are seen including; nuclear fragmentation and condensation (†), canalicular fragmentation (C), and increased cytoplasmic electron density. (B) Intact organellar integrity is seen at higher magnification, where mitochondria (M) and RER (Arrowhead) are readily seen. UEA-1 / gold particles (Arrows) are seen attached to these apoptotic HUVEC membranes. (Bar = 5 µm for A, and 1µm for B)
Figure 4.9
Transmission electron micrographs of detached cells from Type A Co-cultures. (A) Features typical of endothelial cell apoptosis are seen including; nuclear fragmentation (☆), canalicular fragmentation (C) and golgi (g). However, some cells have relatively electron lucent cytoplasm and are degenerate in appearance. Retained organellar structure, condensed nuclear material and the small size of particles suggest that these represent secondary necrosis (SN), an artefact of apoptosis in cultured cells. Some cells with obvious secondary necrosis had some condensed electron dense material, remnants of the apoptotic process (Arrows). High power view of HUVEC undergoing apoptosis. (B) Morphological structures such as endoplasmic reticulum (Arrowheads) and canalicular fragmentation (C) can be seen, consistent with apoptosis. (Bar = 10 μm for A and B)
4.5 Discussion
Co-Culture experiments described in Chapter 3 suggested that HUVEC undergo apoptosis when exposed to SAOS-2 cells. One of the features of apoptosis is the formation of a sub-diploid peak in FACS analysis (Darzynkiewicz, 1992), and this was exploited in conjunction with the specific labelling of HUVEC with UEA-1 to determine if there was an increase in apoptotic HUVEC in Type A SAOS-2/HUVEC co-cultures. Highly specific labelling of HUVEC was demonstrated while SAOS-2 failed to label with UEA-1. Importantly for the hypothesis tested in this thesis, there was a marked increase in the relative magnitude of the sub-diploid apoptotic peak in Type A SAOS-2/HUVEC co-cultures, and these particles were essentially all HUVEC.

To further confirm the apoptotic status of cells in Type A co-cultures, the rapid detachment of HUVEC during apoptosis (Zoellner, 1996b) was exploited along with UEA-1 / gold labelling. Silver enhancement was used to identify gold labelled cells in light microscope sections. In this way, it was possible to confirm that in Type A co-cultures, detached cells were HUVEC while surviving adherent cells were primarily SAOS-2 cells. The morphology of the detached cells was typical of apoptosis and the intracellular location of many gold particles could be accounted for on the basis of canalicular fragmentation, an ultrastructural feature of apoptosis unique for endothelial cells (Zoellner, 1996a). Further ultrastructural features of apoptosis were seen including nuclear condensation, and maintained organelar structure.

Some detached cells were, however, clearly necrotic and these were interpreted as representing secondary necrosis (Wang, 1996). Their small physical size as compared with non-apoptotic HUVEC and SAOS-2 was consistent with this interpretation, as cells undergoing necrosis independent of previous apoptosis are swollen to a greater size than the original cell, and viable HUVEC were larger than the necrotic cells found in Type A co-cultures. Supporting this was the presence of some intact organelles and condensed nuclear material.

This, when considered together with the results of the FACS, light microscope and TEM observations, indicated that the reduction in HUVEC number seen in Type A co-cultures described in Chapter 3, was due to apoptosis.
Put into biological context, these data suggest that malignant tumour cells may be able to gain access to the microcirculation during metastasis by causing apoptosis of the constituent endothelial cells.
Chapter 5

General Discussion

Experimental Models Used in this Thesis
It was hypothesised that malignant tumour cells could have a death inducing activity for the surrounding normal stromal cells. In this thesis, models using cultured tumour and stromal cells were used to explore this possibility. Endothelial cells and fibroblasts are the most prevalent and ubiquitous cell type found in stromal connective tissue, and for this reason were used in this study. Most work focused upon endothelial cells for which there are strong and convincing markers (Holthöfer, 1982) and involvement of which has special relevance for metastasis. Tumour cells used were derived from both non-metastatic and metastatic carcinomas and an osteosarcoma. These cell lines were selected to permit comparison of the three major types of malignant tumour cell types.

The co-culture models used permitted isolation of cell types to facilitate analysis of complex interactions. In the experiments described, only two different cell types were allowed to interact at any one time, so that any confounding effects by other cell types normally present in tumours such as inflammatory cells were eliminated. Although facilitating experimentation and interpretation of data, the possible role of these other cell populations could not be studied using the described systems and would require a different and more complex approach.

Reproducible data was obtained, showing cell death in HUVEC and HGF co-cultured with SAOS-2. This phenomenon was further investigated and identified apoptosis as the mode of cell death in HUVEC. A confounding effect was noted in which tumour cells induced stromal cell proliferation so that the final surviving cell number represented a balance between proliferation and cell death. Since stromal cell proliferation occurred in response to all tumour cells studied, but no increase in stromal cell number occurred in Type A co-cultures it was concluded that all tumour lines studied can cause HUVEC death but that this

5.1
is most pronounced for SAOS-2 cells. For the sake of clarity, further experiments characterising this phenomenon used SAOS-2 cells, although H5 and Y43BP cells would be expected to have similar but less pronounced activity. Cell counts were used in this study as a simple and direct means of quantitating stromal cell survival. As mentioned above, this had the disadvantage of being sensitive to any proliferative effect, however, it is felt that this very direct approach is superior to more indirect methods such as release of radioactive label or ELISA techniques, as altered metabolic turnover, phagocytosis of apoptotic debris or modified antigen expression could occur using these alternative methods, and not only confound data but also be difficult to control for. Using the approach in this thesis, however, it was possible to control for the effect of cell proliferation by i) comparing cell density with that before seeding tumour cells and ii) determining the effect of tumour cell conditioned medium upon stromal cell number. It is noted that the latter of these two does not allow for any cell contact dependent proliferative effect of tumour cells. However this seems unlikely in view of the clearly destructive effect of SAOS-2 upon HUVEC and the absence of HUVEC proliferation in H5 and Y43BP Type A co-cultures, despite proliferation in tumour cell conditioned medium. Also, earlier work has shown that HUVEC fail to proliferate in the co-culture medium alone (section 3), so that there was no proliferative effect of the medium alone upon HUVEC. This is important as increased proliferation from serum contaminants would likely have obscured the effect of SAOS-2 cells.

Apoptosis was verified on the basis of morphology, FACS analysis and TEM. These techniques are widely accepted as specific for apoptosis, with TEM being considered the “gold standard” because the features of apoptosis are clearly distinct from those of necrosis (Wyllie, 1980) and TEM was used in the earliest studies characterising and defining apoptosis (Kerr, 1972a). By combining TEM with UEA-1 / gold labelling it was possible to identify HUVEC as apoptotic at the level of individual cells.

Canaliclar fragmentation is a feature of apoptosis unique for endothelium and was also seen by TEM, providing a further method for identifying the origin of
apoptotic cells. In FACS analysis, reduced DNA content of apoptotic particles was noted, also characteristic of apoptosis.

Rapid cell detachment is a feature of apoptosis, which can be exploited to provide an indirect measure of the extent of apoptosis in a cell population (Jaffe, 1984). This measure is potentially confounded by cell proliferation, but as outlined above, HUVEC proliferation does not occur in the culture medium used (section 3). However, it is conceded that increased HUVEC proliferation due to soluble tumour cell derived factors may have obscured any less potent soluble apoptotic factor released by the cells.

An important consideration for the co-culture models used in this project was the medium used. Serum free conditions were used for a number of reasons. Firstly, it was necessary to find a medium that was capable of supporting both stromal and tumour cells at once. This can often be a difficult task as many cultured cells lines have strict nutrient requirements. Fortunately all five cell types used were supported when cultured in either Joklik’s or M199 medium supplemented with 4% BSA. Serum free medium allowed cell survival while preventing proliferation of all cells other than Y43BP, providing a stable stromal cell number that could be assessed statistically. Supplementation of medium with 4% BSA was necessary to protect HUVEC form apoptosis, known to be activated by serum deprivation. It should be noted that earlier work has shown that BSA specifically inhibits endothelial apoptosis in serum free conditions without stimulating proliferation (Zoellner, 1996b).

Despite these concerns, and the clearly artificial nature of the cell culture models in which macrovascular isolated cells are grown on plastic surfaces, it is felt that the co-culture models used were appropriate for the biological question addressed in this thesis, and permitted performance of experiments from which data could be meaningfully interpreted.
**Interpretation of Data**

SAOS-2 clearly and reproducibly reduced HUVEC survival in Type A Co-cultures. In contrast, the two carcinoma cell lines (Y43BP, H5) had little or no effect on HUVEC number. This may make some biological sense, as sarcomas tend to invade via the blood stream while carcinomas have a preference for lymphatic invasion and metastasis (Enzinger, 1994; Kumar, 1994). However, it is possible that this is a species specific phenomenon as SAOS-2, HUVEC and HGF are human cell lines, while Y43BP and H5 are derived from rats.

The fundamental reduction in HUVEC density in the presence of tumour cells clearly supports the hypothesis. However, further work is needed to clarify the pathway that leads to this fundamental observation. In examining the mode of cell death, various factors were considered including; soluble death factors, medium depletion, dissolution of the gelatin substrate, and cell contact.

Type B co-culture was specifically designed to examine the effects of separating two cell populations by a short distance (1mm) and allowing communication only via the shared culture medium. Since no significant reduction in HUVEC number was seen, it was concluded that soluble death factors did not induce the HUVEC death seen.

It should, however, be noted the distance of 1 mm may be too great for any highly labile soluble factors to be still active. Although the existence of such a highly labile pro-apoptotic factor would be inconsistent with the absence of HUVEC death in Type C Co-cultures where cells were separated by a 10 μm space. Interestingly, experiments with tumour cell conditioned medium demonstrated the presence of soluble proliferative factors for HUVEC. This stimulating activity was stable for at least 24 hours and capable of inducing linear cell growth of HUVEC. From this, medium depletion could not account for reduced HUVEC density in these experiments. Opposing forces of cell proliferation and cell death appear to be present and this would mirror the complex interactions seen clinically. Similarly, pre-treatment of gelatin matrix with SAOS-2 cells failed to prevent subsequent HUVEC adhesion and survival,
so that it was not possible to account for data from Type A Co-cultures of the basis of substrate destruction.

Since medium depletion, soluble death factors and gelatin substrate dissolution seem not to contribute to HUVEC death in Type A Co-cultures, intimate cell contact appears to be necessary for the HUVEC death. Scanning Electron Micrographs demonstrated fine cytoplasmic extensions present on SAOS-2 and HUVEC. Although there was little evidence for the passage of these processes through the transwell membranes, it is possible that occasional cell processes were able to establish cell contact between SAOS-2 and HUVEC, and that this contributed to the presence of occasional isolated HUVEC with an apoptotic morphology, despite the absence of statistically significant reduction in HUVEC number in Type C Co-cultures. It should be noted that occasional HUVEC cell processes were seen passing into membrane holes from apoptotic HUVEC, while SAOS-2 were seen passing through large 3 μm holes in some experiments.

As discussed in earlier chapters all three tumour cell lines possessed the ability to stimulate HUVEC proliferation while a clear reduction in HUVEC number was seen only in SAOS-2 Type A Co-cultures. This, together with results of Type B and C Co-cultures suggests that all of the tumour cells studied are able to kill HUVEC in a cell contact dependent manner, although it was only in SAOS-2 cultures where this activity was sufficient to overcome the proliferative effect of the tumour cells.

This may reconcile the hypothesis tested in this thesis, that tumour cells cause endothelial cell death during intravascular invasion, with the established dogma that tumours cause angiogenesis (Kerbel, 2000). On the basis of these data, it is proposed that tumour cells cause angiogenesis at sites some distance from the cells themselves, through the release of soluble factors, but that once tumour cells actually contact these perhaps newly formed vessels, apoptosis is induced, destroying the vasculature and facilitating intravascular invasion. This would be consistent with the reduced vascularity within tumours leading to necrosis (Enzinger, 1994) despite angiogenesis at the invading edge of the tumour.
(Scoazec, 2000). The active nature of this tumour cell induced endothelial cell death is illustrated by the apoptotic rather than necrotic pattern of death seen in these co-culture experiments.

**Data in Relation to Literature:**

Enzyme degradation of basement membranes and other extracellular matrix components, followed by intravascular invasion by tumour cells (Liotta, 1992; Stetler-Stevenson, 1993; Bernstein, 1994) is currently thought to be the basis for metastasis. Supporting this idea are experiments in which protein cleaving metalloproteinase enzymes are selectively inhibited by antibodies, totally abolishing the invasive action of tumour cells (Liotta, 1992). The findings of this thesis are consistent with this, but also indicate a role for endothelial cell apoptosis.

Much work has focused on new blood vessel formation, with workers such as Folkman demonstrating the angiogenic capacity of tumour masses (Gimbrone, Jr. 1974; Folkman, 1996). The proliferation of HUVEC seen in this thesis is again consistent with this widely accepted finding. However, the concomitant endothelial cell apoptosis seen here introduces a previously unrecognised complication to this predominantly “angiogenic” view of interactions between tumour cells and the vasculature. In the course of performing the experimental work described in this thesis, manuscripts were published which support and in one instance reproduce aspects of this work, in experimental tumours in rodents (Holash, 1999). Tumours grew by coopting pre-existing blood vessels from surrounding tissue. However, these coopted vessels latter regressed, leading to transient tumour regression, only to be replaced by newly made vessels that facilitated tumour growth. The angiogenic antagonist angiopeptin-2 and pro-angiogenic vascular endothelial growth factor (VEGF) were suggested as critical regulators of this balance between vascular regression and growth. This *in-vivo* observation followed an earlier report in which tumour cells induced apoptosis of HUVEC in co-culture experiments similar to those in this thesis (Kebers, 1998; Lewalle, 1998). It was suggested that interruption of intercellular adhesion was important in this. These reports essentially reproduced and so supported this thesis’s observations. Ways in which these studies differ from the currently
described observations described are: Adenocarcinoma and Fibrosarcoma cell lines rather than Osteogenic Sarcoma (SAOS-2) were found to induce HUVEC apoptosis, and this apoptotic increase was observed in the presence or absence of serum. Identification of apoptosis in the published paper described used TUNEL labelling and Caspase-3 activity along with DNA gel electrophoresis to identify apoptotic cells. The differences in the experimental protocol used suggest that endothelial apoptosis may occur after co-culture with different types of tumour cells than those described.

These observations invert the focus of earlier studies in which the apoptosis of tumour cells (Schatten, 1962; Steel, 1968; Steel, 1977; Kerr, 1994) and degradation of the surrounding extracellular matrix were studied (Liotta, 1986; Pauli, 1983; Stettler-Stevenson, 1993). In the 1970s and 1980s Nicolson and Kramer and co-workers performed a number of tumour invasion studies utilising endothelial cell monolayers and reconstituted basement membrane (Kramer, 1979; Kramer, 1980; Kramer, 1981; Kramer, 1986). Scanning Electron Micrographic studies pointed towards tumour invasion by attachment to sub-endothelial matrix, leaving the endothelial layer more or less undisturbed as migration was via broken intercellular junctions which later reformed. It is possible that endothelial apoptosis occurred in these earlier studies, but was not observed because of the attention paid at the time to matrix effects.

**The Direction of Future Work**

Work described in this thesis illustrates a seemingly contact dependant pro-apoptotic activity in SAOS-2 cells for endothelium, and this is now supported by several other studies (Kebers, 1998; Lewalle, 1998). Unfortunately, the further characterisation of this and extension of these studies to other cell types has been limited by the time restrictions of this thesis. There are several aspects of the currently described work, which should be extended. One important criterion for apoptosis is internucleosomal DNA fragmentation. However, attempts to observe this feature were unsuccessful due to technical difficulties and are described briefly in the Appendix. This is one aspect of this project which should be completed but which unfortunately could not be done during the time.
of this thesis due to the requirement for submission by a specific date. Also, interactions between tumour cells and HUVEC in Type C Co-cultures are ambiguous, as the presence and significance of cytoplasmic processes crossing the transwell membrane was not determined. Further experiments in which transwells with a pore size intermediate to those used should be performed while confocal microscopy of Type C Co-cultures, in which tumour and endothelial cell plasma membrane are labelled with fluorescent surface markers would permit proper assessment of any tumour-membrane processes as well as their relationship to morphologically apoptotic cells. Although no reduction in HUVEC density in Type A Co-cultures was seen for two tumour cell lines, indicating that mere co-culture was not sufficient to account for the pro-apoptotic effect of SAOS-2, further experiments of Type A Co-cultures mixing HUVEC with non-tumour cells such as fibroblasts of smooth muscle cells should be performed. Similarly, all of the tumour cell lines used were locally invasive and it would be interesting to determine the effects of cells from non-invasive tumours forming clinically apparent capsules. Since HUVEC are essentially macrovascular it would be interesting to extend these studies to cultured microvascular endothelium.

Attention in this thesis was focused upon endothelium, but as indicated in Chapter 1, it is possible that the apoptotic inducing activity seen for endothelium reflects a more general effect of tumour cells in causing apoptosis of stromal cells including fibroblasts. Some preliminary experiments investigating this possibility are described in the Appendix and appear to indicate a similar response of fibroblasts to SAOS-2 cells as was seen in HUVEC. Further extension of these studies to “ring type” cultures, in which stromal cells are separated from tumour cells by a removable ring would also be valuable in characterisation of the contact dependent death inducing activity. In-vivo experiments characterising these observations should also be performed while the actual biochemical mechanism through which the pro-apoptotic activity is mediated needs to be elucidated. From this, it seems that this thesis opens multiple new lines of investigation, which would be interesting to pursue.
Chapter 6

References


Eagle H. *Science* 1959; 130:432.


Kerr JFR, Searle J. A suggested explanation for the paradoxically slow growth rate of basal-cell carcinomas that contain numerous mitotic figures. *J Pathol* 1972b; 107:41-44.


Kramer RH, Fuh GM, Karasek MA. Type IV collagen synthesis by cultured human microvascular endothelial cells and is deposition into the subendothelial basement membrane. *Biochemistry* 1985; 24:7423-7430.


Liotta LA. Cancer Cell Invasion and Metastasis. *Sci Amer* 1992; 266:54


Rusin E, Faber JL. *Pathology*. 2nd ed. J.B. Lippincott Company, 1994:


Chapter 7
Appendix I

HGF Displayed Similar Changes to HUVEC in Co-Culture Experiments with SAOS-2

7.1 Introduction
Experiments described in Chapters 3 and 4 indicated that SAOS-2 cause apoptosis is HUVEC and support the idea that malignant tumour cells may induce endothelial cell apoptosis contributing to metastasis. As outlined in 1.8, it is difficult to explain the way in which malignant tumour cells replace normal stromal cells on the basis of currently widely accepted models of tumour growth. The increased endothelial apoptosis seen in Type A / SAOS-2 co-cultures raises the possibility that other stromal cells also become apoptotic in response to tumour cells, potentially explaining the way in which malignant tumour cells replace stromal cells.

Fibroblasts together with endothelial cells are the major cell type in most connective tissues and appear to play a critical role in formation of extracellular matrix and tissue organisation (Freshney, 1994). Although lacking any specific marker for isolation, fibroblasts are readily cultured from a variety of tissues and are widely accepted as representative of fibroblasts from their respective parent tissues (Mukaida, 1991). Because of this, fibroblasts cultured from human gingiva were used to determine if SAOS-2 were able to induce similar changes in cells other than endothelial.

7.2 The Effect of SAOS-2 upon HGF in Type A, B and C Co-Cultures
The protocols used in Type A, B and C co-cultures of HGF were similar to those used for HUVEC described in Chapters 2, 3 and 4. An important difference, however, was that in co-culture Type A, SAOS-2 were identified on the basis of Alkaline Phosphatase histochemistry while HGF were identified as Alkaline Phosphatase negative cells with nuclear haematoxylin stain only.
Figure 7.1
Type A co-culture of HGF with SAOS-2 cells 24 hours after seeding tumour cells and stained for alkaline phosphatase activity. SAOS-2 (red arrows) were readily identified on the basis of their intense labelling for alkaline phosphatase activity while HGF (black arrows) were found to have a more elongated and spindle shaped morphology as compared with the tumour cells. SAOS-2 appeared to increase in density while HGF density reduced over the 24 hour period. (Bar = 20 μm)
Figure 7.2
The effects of Type A Co-culture with SAOS-2 upon HUVEC and HGF density. Similar changes were seen in HGF as for HUVEC (See Figure 3.3). HGF were seeded and cultured 24 hours prior to addition of SAOS-2. Type A Co-cultures were then grown under serum free conditions, using an identical protocol to that in section 2.3.5. Quantification of cells was dependent on labelling of alkaline phosphatase activity of SAOS-2, as HGF have no specific marker.
Figure 7.3
The effect of SAOS-2 upon HGF number in Type B Co-cultures at 0 and 24 hours. There was no effect of tumour cells upon HGF number in this experiment, similar to earlier experiments with HUVEC (Figure 3.15). HGF were first grown on 0.4 μm pore transwell membranes and then cultured 1mm above a monolayer of SAOS-2 cells for 24 hours (see figure 2.2) in serum free conditions. Cell interaction was allowed via the shared co-culture medium entering the porous membrane. HGF density was determined by releasing the cells using trypsin / EDTA and counting directly using a haemocytometer.
Figure 7.4
Scanning electron micrograph of HGF grown on 0.4 μm pore transwell membranes in Type B Co-culture. Fine cytoplasmic processes (Arrowhead) can be seen spreading out from the cells across the transwell membrane, these processes appear to avoid the 0.4 μm pores and in some places pass directly over the pores without entering (Short Arrow). Some artefactual degeneration can be seen in the cell cytoplasm (Long Arrow) due to cell dehydration. (Bar = 10 μm)
Figure 7.5
SAOS-2 conditioned medium increased HGF number, similar to events with HUVEC (See Figure 3.5). As with HUVEC the higher the concentration of SAOS-2 conditioned medium the greater the HGF density. From this data it was apparent that the reduction in HGF density seen in Type A co-cultures was not due to tumour cell depletion of the co-culture medium.
7.3 Discussion of Work with HGF
The preliminary experiments outlined in this appendix for HGF provide similar data to that obtained for HUVEC. Experiments conducted in Type A, B and C Co-cultures produced very similar results as with HUVEC. Further experiments examining medium depletion further duplicated results seen with HUVEC. This would suggest that the cell contact dependent death inducing activity described is not confined to endothelium, but applies to stromal cells in general. HGF deserve closer examination using similar protocols used in this project, such as FACS and TEM analysis, however a specific label would be required for HGF to allow accurate interpretation of any data.

From the scanning electron micrograph (Figure 7.4) of HGF fine cytoplasmic processes could be seen extending from the cells, this was also seen for HUVEC (Figure 3.12). Both HGF and HUVEC form this fine meshwork in an attempt to form a stable interface with the underlying substrate, allowing survival. As cell contact seems the mostly likely mode of cell death these cytoplasmic processes may play a significant role in the process of contact-dependent-death seen in this project.

If with further work, contact dependent cell death proves to be the case, the activity described may help to explain the way in which malignant tumour cells replace tissue, a biological problem outlined in the introduction to this thesis. It appears that further work investigating this possibility is warranted.
Appendix II

DNA Gel Electrophoresis

7.4 Introduction
An important biochemical property of apoptosis is the internucleosomal fragmentation of DNA to produce a laddered effect in DNA gel electrophoresis. In this thesis, the criteria for apoptosis used were primarily ultrastructural and on the basis of FACS analysis. Although ultrastructural identification of apoptosis is considered to be the "gold standard" and FACS analysis does provide evidence for fragmentation of DNA, since it depends upon the escape of small DNA fragments from apoptotic particles, it was considered worthwhile obtaining further verification of apoptosis in the Type A co-cultures of HUVBC by DNA gel electrophoresis. Unfortunately, the few experiments performed to assess this were unsuccessful, probably due to contamination of samples or reagents with DNAase, and for this reason were not shown in the body of the thesis. Also, the time constraints of this work precluded the successful completion of this particular series of experiments. This appendix briefly describes attempts to demonstrate internucleosomal DNA fragmentation by DNA gel electrophoresis by the student.

7.5 Materials
Tris, Tris-HCl, Tris-borate, EDTA, Sodium Acetate, ethyldium bromide, bromophenol blue, RNAase A and proteinase K were from Roche Molecular Biochemicals (Nutley, New Jersey, USA). Glycerol was obtained from BDH Laboratory Supplies (Poole, England). Ajax Chemical (Auburn, NSW, Australia) supplied phenol, absoluted thanol, sodium chloride and potassium chloride. Molecular biology grade agarose was purchased from BioRad (Hercules, Ca, USA).
7.6 Methods
Type A co-cultures of HUVEC and SAOS-2 cells were established in 225 cm$^2$ tissue culture flasks as indicated in Section 2.3.5. Also, control cultures of SAOS-2 and HUVEC were established. Cells were co-incubated for 24 hours, at which time detached floating cells were collected by gentle washing and adherent cells were collected using either Trypsin-EDTA or a cell scraper. Cells were pelleted by centrifugation at 2000 rpm and pellets resuspended in 1 ml of Tris-buffered saline (TBS) followed by further pelleting by centrifugation. The pellets were then resuspended in 50 µl of lysis buffer comprising Tris (50 mM), EDTA (10mM), SDS (0.5%) and RNAase (0.1 mg/ml). The lysis solution was then incubated for 1 hr at 37°C before adding proteinase K to a final concentration of 0.5 mg/ml and further incubation for 3 hr or overnight at 55°C. After this, the solution was made up to 500 µl with Tris-EDTA (TE) buffer, consisting of Tris HCl (10 mM) with EDTA (1mM) at pH 8.0, and 500 µl of Tris-buffered phenol added for phenol extraction. The solution was mixed well by vortexing before centrifugation at 13,000 rpm, 4°C for 15 min. The aqueous layer was retained and subjected to a further round of phenol extraction. The resulting aqueous layer was then collected and 1/10 volume (50 µl) of sodium acetate (0.3 M) added followed by twice the volume of ice cold absolute ethanol (1 ml). The solution was mixed by inversion before being stored at -40°C for at least 30 Min. Prior to electrophoresis, the solution was centrifuged at 13,000 rpm for 15 Min to pellet precipitated DNA. Pellets were washed with ice-cold ethanol (70%) and recentrifuged before removal of the supernatant and allowing pellets to dry at room temperature. The dried pellets were resuspended in 15 µl of TE buffer before adding 5 µl of loading buffer consisting of bromophenol blue (0.25%) and glycerol (30%) in water. DNA was subjected to electrophoresis using a 2% agarose gel with 0.5% ethidium bromide at 709 V for from 30 to 45 min and the results visualised by UV light. Results were recorded photographically using a DS34 Polaroid Direct Screen Instant Camera and Polaroid 667 Black and White film.
7.7 Results
Although some large molecular mass DNA was found to remain intact, there was significant smearing of DNA from both adherent and detached cell populations, indicating non-specific fragmentation of the material. Because DNA from adherent HUVEC does not normally present as a smear in DNA gel electrophoresis, it was clear that there had been significant non-specific cleavage as a result of processing. Because of this, it was decided that the results were uninterpretable, and no conclusion could be made regarding the possible internucleosomal fragmentation of DNA in Type A co-cultures. Despite this, some apparently apoptotic banding was seen in the detached SAOS-2 population. This presumably represented a minor population of cells which was not readily observed by FACS and other methods used, but in which low levels of apoptosis produced some laddering of the DNA. The bright low molecular bands sometimes seen in the detached population of HUVEC and co-cultured cells may represent the terminal phases of late internucleosomal fragmentation (Figure 7.6), although further experiments collecting material at earlier time points would be necessary to be certain of this.

7.8 Discussion of Work with DNA Gel
It is clear that further experiments are required to improve the quality of these gels and determine with more certainty if internucleosomal fragmentation occurs in Type A co-cultures. However, in view of the clearly apoptotic ultrastructure and FACS analysis data, it seems very unlikely that this biochemical feature of apoptosis will prove not to be present in this co-culture model.
Figure 7.6
DNA gel electrophoresis of material collected from adherent (A) and detached (D) cells after 24 hours from: co-cultures of HUVEC with SAOS-2, HUVEC alone or SAOS-2 alone. Significant non-specific fragmentation and smearing of DNA was seen in the attached cell populations, as well as in the detached cell populations from co-cultures and HUVEC. Some apoptotic laddering was noted in the detached SAOS-2 population (arrows), while strong low molecular mass bands may represent the terminal internucleosomal fragments of prolonged apoptosis. However, this and other gels obtained were unconvincing as to definite internucleosomal fragmentation in co-cultures.