BIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF A SERIES OF HUMAN UVEAL AND CUTANEOUS MELANOMAS.

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by

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Aims

Metastasis is a complex multistep process and it is important to elucidate the underlying molecular mechanisms in this process. The proposed aim of this study will be to identify similarities and/or differences between uveal and cutaneous melanoma. Characteristics such as ploidy values and rates of proliferation of cells within the primary uveal melanoma will be compared with established clinical and histological prognostic criteria. A culture system for uveal melanomas will be developed to allow the in vitro study of karyotypic abnormalities. The expression of degradative enzymes such as tissue-type plasminogen activator, matrix metalloproteinase-2 and -9 (72 and 92 kDa gelatinase respectively) will be studied in vitro using a combination of gelatin, casein and casein/plasminogen zymography; levels of plasminogen activator expression will be quantitated using a chromogenic assay specific for plasminogen activators. The expression of these enzymes will be compared with patient status.

Further studies will be undertaken to derive cutaneous melanoma cell subpopulations having different experimental metastatic potentials in congenitally athymic nude mice. These cell lines will be analysed in vitro to determine the characteristics responsible for the alteration in experimental metastatic potential. The effect of host derived factors on melanoma cell cultures will be studied in vitro, particularly emphasis will be given to the cytokines TNFα and IL-1. Their effect upon in vivo characteristics will be correlated with changes in melanoma associated markers in vitro.

The role of the cytokines TNFα, TGFβ and IL-1 in modulation of cutaneous melanoma gelatinase will be analysed to determine synergistic interactions between these factors. Comparable studies will be undertaken to determine similarities or differences in the control of these enzymes in uveal melanoma. These studies will determine the importance of the expression and modulation by host factors of gelatinase in the metastatic process.
Summary

Cutaneous and uveal melanomas display different patterns of metastatic spread in patients. This study has compared these two melanoma types and investigated parameters identifiable with the metastatic phenotype. A number of investigations were performed, and it was determined that most uveal melanomas were diploid and displayed high proliferation rates; high levels of plasminogen activator activity and the expression of the 92 kDa gelatinase were associated with tumors most likely to invade the sclera and cause metastatic disease. In addition, unique patterns of chromosomal alterations were associated with this tumor type.

Further studies were undertaken using the cutaneous melanoma cell lines, A375 and its related sub-line A375/NUPR1, which was derived from A375 following subcutaneous passage in nude mice. A375/NUPR1 showed an increased “experimental” metastatic potential in congenitally athymic nude mice, but expressed similar levels of a number of melanoma associated markers: MHC class I and II and ICAM-1, and a similar proliferation rate in vitro. The 92 kDa gelatinase was found to be expressed by A375/NUPR1 but not the parental A375 line. Doubling times for tumors implanted at subcutaneous and intradermal sites were also similar, as was the spontaneous metastatic potential for both cell lines.

Modulation of these melanoma associated markers by TNFα and IL-1 showed that whilst MHC expression was unaltered, proliferation rates were reduced and ICAM-1 expression increased by both cytokines on both cell lines, IL-1 having the greater effect. Induction of 92 kDa gelatinase was observed in both cell lines following 24 hour incubation with TNFα, but only A375/NUPR1 responded to IL-1 stimulation. The alterations in A375 cell phenotype following TNFα treatment did not alter the experimental metastatic potential of this cell line.

In depth studies of 92 kDa gelatinase modulation identified synergy between TNFα and TGFβ; this was observed in melanoma cell lines of both cutaneous and uveal origin.
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CHAPTER 1
AN INTRODUCTION TO CANCER INVASION
AND METASTASIS
1. UVEAL MELANOMA.

Uveal melanoma is a rare cancer (Deves et al., 1987), but it holds considerable interest because of the rapid and unexplained rise in incidence of the related but more common melanomas of the skin (Cutler and Young, 1975). Although uncommon in the context of general neoplasia, uveal melanomas (those located in the ciliary body and choroid), are the most common primary adult intraocular malignancy, they frequently metastasize, and approximately half of the patients die from their disease within 15 years of enucleation (Egan et al., 1988). Metastatic disease is responsible for virtually all tumor-related deaths. However tumor metastatic potential varies; some tumors have little or no metastatic capabilities whereas others metastasise readily. Once metastases intervene the prognosis is extremely poor with a median survival time of six months (Seddon et al., 1983). Despite recent advances in treatments, including many directed at preserving the eye, the overall prognosis has remained unaltered (Zimmerman and McLean, 1984). The lack of an economically acceptable culture system that permits the routine \textit{in vitro} growth of uveal melanomas has hindered the study of the basic properties of this tumor type. Albert et al. (1984) reported a culture protocol for uveal melanomas involving the use of a fibroblast feeder layer and high concentrations of epidermal growth factor (10 \( \mu \text{g/ml} \)), this protocol was prohibitively expensive, we developed a less expensive short term culture protocol which to date has a 80% success rate, the established cells were verified as abnormal by cytogenetic analysis (Sisley et al., 1990). This has given us the ability to examine a number of different phenotypic and genotypic parameters associated with this tumor type with the aim of both investigating possible new prognostic indicators for this malignancy and studying the biology of this tumor type.

Traditional prognostic indicators for uveal melanoma rely heavily on the assessment of the morphological and histological appearances of the tumor. The tumor location and size are of prognostic significance. Generally "posterior" uveal melanomas located anterior to the equator have a less favorable prognosis than ones located posteriorly (Shammas and Blodi, 1977). In addition, large tumors (tumor diameter
greater than 10 mm) have a less favorable prognosis than small tumors (Shammas and Blodi, 1977). Histologically, uveal melanomas may be classified as: spindle cell, epithelioid cell or mixed cell tumors. Pure spindle cell tumors generally do not metastasise as frequently as epithelioid tumors; the five year survival rate for these tumors is approximately 80%, whereas epithelioid tumors have a survival rate of only 40% (Paul et al., 1962). Calculation of the inverse standard deviation of the nucleolar area has been assessed as an indicator of survival (John et al., 1977). More recently flow cytometry has been evaluated as a method of predicting patient survival; it has been demonstrated that the presence of a large aneuploid population of cells in the tumor may infer a poor prognosis (Meecham and Char, 1986). We have used this technique to investigate cell cycle kinetics in fresh uveal melanoma tissue (Rennie et al., 1989).

1.2. CUTANEOUS MELANOMA.

The incidence of, and number of deaths due to, malignant cutaneous melanoma are rising at a rapid rate. Malignant melanoma accounts for less than 5% of all cancers but it is among the most lethal because of its extremely high metastatic propensity. The incidence of cutaneous melanomas, particularly in the sunbelt, is increasing (Glass and Hoover, 1989). Cutaneous melanoma shows a high frequency of metastases to the brain or central nervous system which result in clinical symptoms in up to 20% of patients with melanoma (Amer et al., 1978; Akslen et al., 1987; McCreany and Balch, 1987). Additionally, skin metastases are observed in more than half of melanoma patients (Akslen et al., 1987).

1.3. TUMOR PROGRESSION.

Neoplasms are heterogeneous and contain sub-populations within them which have different metastatic propensities. It is thought that both naturally occurring and induced neoplasms may develop from a single transformed cell (Fialkow, 1979; Nowell, 1976; Vogelstein et al., 1985). During the course of disease the characteristics of the tumor change from a benign into a malignant phenotype. Studies by Foulds (1969; 1975) using murine mammary tumors led to the description of this
phenomena as 'neoplastic progression' which he defined as 'the acquisition of permanent, irreversible qualitative changes in one or more characteristics of a neoplasm'; the host influences tumor progression by introducing microenvironmental selection pressures (Prehn, 1976). Studies by Fidler and Hart (1981) provided evidence that tumors of unicellular origin are capable of developing subpopulations of cells with altered metastatic propensities within 6 weeks. This rapid process of tumor progression is considered to be due to both the genetic instability of tumor cells and the host imposed selection pressures which continually select for clones exhibiting an increased ability to grow.

Analysis of tumor cell karyotypes has shown that cells exhibit increasingly altered karyotypes and ploidy values as they progress from benign to malignant neoplasms (Yunis, 1983; Nowell, 1989). Therefore as the tumor progresses it should exhibit an increased rate of genetic mutation since cellular homeostatic mechanisms are likely to become altered or inactivated. Experimental evidence for this was provided by Cifone and Fidler (1982) who showed that highly metastatic cells were both phenotypically and genotypically more unstable than non-metastatic cells. Furthermore, since tumors are composed of a number of subpopulations, which were phenotypically distinct, it is to be expected that these subpopulations will interact not only with the host but with each other. Miller et al. (1981) were able to show that subpopulations within mammary tumors could influence the growth characteristics, immunoreactivity and responsiveness to chemotherapy of each other. This idea was developed further (Fidler and Kripke, 1980; Miller and Heppner, 1979) who showed that isolated clonal populations behaved differently in polyclonal combinations than in clonal isolation. This polyclonal stabilisation of tumor cell subpopulations within a tumor would create an equilibrium of these subpopulations, until a host selective pressure occurred, at which time the clone most able to survive this pressure would eventually dominate the tumor and due to genetic instability, would diversify producing subclones which would again reach an equilibrium until another selective pressure occurred and the process was repeated, this is the idea behind Kerbel's clonal
dominance theory (Kerbel et al., 1988). If the tumor is continually experiencing selective pressures the rate of diversification will be higher than if it were not since the selection pressures remove the polyclonal stabilisation, potentially resulting in greater genetic instability and metastatic potential.

1.4. CANCER METASTASIS.

The main problem in cancer treatment is the prevention or destruction of metastases. In 1928 Ewing proposed that metastasis formation at regional and distant sites is primarily due to lymphatic and circulatory anatomy and the mechanical lodgement of malignant cells at the first barrier encountered. Therefore the sites of metastasis for a particular tumor are dependent upon its primary site, the properties of the surrounding tissues, the locations of mechanical barriers to the passage of malignant cells consequently lymph nodes and blood capillaries were considered important in determining the location of metastases. Thus, the distribution of metastases is related to the lymphatic and circulatory anatomy and to mechanical lodgement properties, this hypothesis is referred to as the anatomical-mechanical theory of metastasis.

Two basic hypotheses have been proposed to explain patterns of hematogenous metastases (i) the one step or (ii) the multistep process. In a study by Bross et al. (1975) 4,278 autopsies were analysed for the pattern of metastases and the multi-step process was favoured, these authors suggest that the final site of metastasis involves a series of intermediate sites, typically the lung or liver, and subsequent metastatic spread occurs by the metastasis of metastases. The process of cancer metastasis consists of a series of sequential interrelated steps, each of which can be rate limiting, since a failure to complete any single step aborts the process (Poste and Fidler, 1979). Therefore the outcome of metastasis depends upon both the tumor cell phenotype and the host's reaction to the tumor cell.
Metastasis is complex and a scheme of events called "the metastatic cascade", has a number of components, these are listed below.

1) The initial transforming event and growth of the neoplastic cells.

2) Neovascularisation of the tumor leading to growth greater than 2 mm (Folkman, 1984).

3) Local invasion of the host extracellular matrix by peripheral tumor cells.

4) Escape of the tumor cell(s) from the primary tumor as either homo or heterotypic emboli or single cells, into either the lymphatic system or the vasculature. Heterotypic emboli may be formed by tumor cells interacting with lymphocytes, macrophages and/or platelets and may be formed either before escape or during haematogenous transit.

5) Survival of the tumor cells in the circulation.

6) Specific or non-specific arrest in the capillary beds of organs.

7) Extravasation.

8) Proliferation within the organ parenchyma completes the process.

9) Subsequent metastasis of metastases is thought to occur in the same way.

The formation of multicellular emboli is considered to be an important event in determining the site of arrest since passage through lymph nodes and capillaries will be increasingly difficult as emboli size increases. The ability of a tumor cell to undergo homotypic and heterotypic adhesion during their transport to a distant site is potentially important. In addition to homotypic adhesion of tumor cells (Fidler, 1973), heterotypic adhesion of circulating malignant cells with platelets (Gasic, 1984), lymphocytes (Fidler, 1975), and monocytes (Starkey et al., 1984) can modify bloodborne arrest of circulating tumor cells and lead to an increased number of metastases. However, this anatomical-mechanical hypothesis of cancer metastasis does not explain the unique organ colonisation patterns observed in some cancers, ocular melanoma amongst others (Nicolson, 1982). Zeidman and Buss, (1952) demonstrated that circulating tumor cells or their multicell emboli were not always arrested in the first capillary bed encountered, and that these cells could be deformed and released to
recirculate to other organs. Initial attempts to explain metastatic colonisation patterns that cannot be due to mechanical lodgement and anatomical considerations, were made by Paget (1889) who proposed a "seed and soil" hypothesis. This hypothesis proposes that the microenvironment of each organ (the soil) influences the implantation, invasion, survival and growth of particular tumor cells (the seeds). Obviously organ specific metastasis is dependent upon both tumor and host properties. It is highly likely that most malignant diseases spread by a combination of both of these mechanisms. In addition to these two theories another explanation for distant metastasis put forward by Bross and Blumenson (1976) suggests that organs become sequentially involved by a stepwise metastatic spread. Their concept of a metastatic cascade considers that metastasis occurs first to a "generalising site" and then further dissemination occurs to other sites, whilst this may be the situation in certain cancers (Bross and Blumenson, 1976), it may be that during the malignant progression of cancers they acquire the capability of colonising additional organ sites even in cancers that spread initially to only one distant site; these secondary tumors may therefore be composed of unique malignant cell subpopulations capable of metastasising to different specific sites (Nicolson, 1982; Nicolson and Poste, 1982). Alternatively, as the tumor progresses, the malignant cells may acquire additional properties that allow them to colonise many organ sites simultaneously (Nicolson, 1987).

Since it is not ethical to investigate experimental cancer metastasis in patients, animals have been used to a large extent, particularly in mice and other rodents. Tarin et al. (1984) have studied site specific metastasis in humans by the introduction of peritoneovenous shunts for palliation of malignant ascites. Patients with malignant ascites had their ascitic fluid drained into the circulation via the jugular vein. In this study it was reported that in 15 of the 29 patients studied the shunts did not increase the risk of metastasis; in 8 patients, micrometastases were found in extra-abdominal organs, mainly the lungs, and in 7 patients, no evidence of tumor colonies could be detected other than in the abdomen. These negative finding are supportive of the seed-soil hypothesis.
1. 4. 2. Experimental Models of Metastasis.

The development of animal models to study human malignant melanoma may be important in understanding of the biology of the disease, especially the metastatic properties, both from the point of improving therapeutic regimes and to further the understanding of both regional and site specific metastasis. The use of both congenital athymic 'nude' and/or 'SCID' (severe combined immunodeficiency) mice has proved popular since these immunologically deficient animals allow the growth of xenografted human tumor tissue. In nude mice successful growth of fresh tumor tissue is rare (Fogh et al., 1979; Neulat-Duga et al., 1984; Philips et al., 1989), however s.c. growth of cultured cell lines has been found to occur more frequently and occasionally metastasis is observed (Fogh et al., 1977; Sharkey and Fogh, 1979).

Xenograft growth in nude mice was considered to be inhibited by residual natural immunity (Fidler, 1986) and young or immunosuppressed mice have been reported to be better hosts for tumor growth and metastasis (Ferrick et al., 1989). Developing this idea, it would be logical that a mouse with a broader immune deficiency would be an improved vehicle for these studies, and the use of SCID mice has proved this hypothesis. The SCID mouse lacks both functional B- and T- cells due to multifunctional rearrangements of immunoglobulin and T-cell receptor genes (Bosma et al., 1983; Schuler et al., 1986;). Natural killer cells and macrophages are apparently unaffected by the autosomal recessive SCID mutation (Dorshkind et al., 1985; Czitrom et al., 1985; Dorshkind et al., 1984). Using the SCID mouse it has been reported that both fresh tumor tissue (Bankert et al., 1989) and cell lines (Reddy et al., 1987; Ghestie et al., 1990) display improved growth, whilst fresh tumor tissue did not metastasise when injected subcutaneously. (Reddy et al., 1987; Ghestie et al., 1990), if the tissue was dissociated a high frequency of spontaneous metastasis was observed (Hill et al., 1991).

Many cancers show characteristic patterns of metastatic spread, and both uveal and cutaneous melanoma are no exception. Uveal melanoma shows a high incidence of metastasis to the liver (Nicolson, 1982) and cutaneous melanoma to the central
nervous system, liver and bowel (Nicolson, 1982). The relevance of experimental metastasis to the elucidation of the relative importance of the anatomical-mechanical and the seed-soil hypotheses is debatable and may depend upon the tumor system under examination and the method of administering the tumor cells. Intravenous injection of tumor cells, the experimental metastasis assay, measures only the terminal steps of metastasis, whereas, subcutaneous injection of tumor cells and measurement of subsequent spontaneous metastasis, although more time consuming, is perhaps a more appropriate assay for the metastatic process.

The seed-soil hypothesis has been studied using organ-specific variant cell lines which have been selected from the parent population by showing alterations in their sites of metastasis (Nicolson, 1982). Whilst site specific metastasis has been explained by tumor cell immune system interactions (Reif, 1978), stimulation of proliferation due to organ specific factors (Sargent et al., 1988), or specific adhesion to a particular organ endothelium (Tohgo et al., 1986) are essential events in this process. Alternatively, adhesion, invasion and proliferation are inhibited at other sites within the body, thus the process is one of negative rather than positive selection. Bearing these factors in mind it is possible that by using a different species the investigator may introduce an error, that of possible mismatch between receptors and their ligands across a species barrier.

1. 4. 3. Lymphatic Metastasis.

During tumor cell invasion, tumor cells can easily penetrate small lymphatic vessels and be passively transported in the lymph to be trapped in the first draining lymph node, or they may bypass these regional lymph nodes to form distant nodal metastases ("skip metastases"); the lymphatic and vascular systems have numerous connections (Fischer and Fischer, 1966), which allow tumor cells to pass from one system to the other (Carr, 1983). The role of regional lymph nodes as temporary barriers to cell dissemination is an area of controversy (Black et al., 1971; Crile, 1969). Removal of regional lymph nodes, which are considered to be the first site of metastasis, is thought to reduce the chance of tumor cells reaching the circulation and
hence spreading to distant organs. In melanoma there is evidence that some patients, those with intermediate thickness melanomas (1-4 mm), benefit from elective lymph node dissection (Balch et al., 1985).

1.4.4. Vascular Metastasis.

Although most solid tumors are highly vascular, these tumor vessels are not identical to normal vessels in mature tissues. This distinction between tumor and normal blood vessels includes differences in the cellular composition of tumor vasculature and the basement membrane structure leading to alterations in permeability and vessel stability. The vessels that grow in response to a tumor stimulus are different from normal capillaries in that they may contain only endothelial cells rather than endothelial cells and vascular pericytes; recent evidence indicates that pericytes act as a maturation signal that prevents endothelial cell proliferation (Orlidge and D'Amore, 1987). The mechanism for this effect involves the expression of activated transforming growth factor-β, a inhibitor of endothelial cell proliferation (Antonelli-Orlidge et al., 1989).

As mentioned previously, a solid tumor is angiogenesis-dependent and as such there is a prerequisite that the tumor must produce factors which induce angiogenesis if it is to grow to a size greater than 2 mm (Folkman, 1984). Angiogenesis is a process dependent upon endothelial cell proliferation and migration and is controlled by a number of soluble factors and matrix components (Risau, 1990). Tumor cells are capable of using several different pathways for the induction of angiogenesis:

(i) direct synthesis of angiogenic factors by the tumor cell (Klagsbrun et al., 1986).

(ii) Production of macrophage chemoattractants to cause migration of macrophages to the tumor site and, upon activation, the release of angiogenic activity (Polverini and Leibovich, 1984).

(iii) Release of vascular permeability factors that cause leakage of fibrinogen from the post capillary venules resulting in the build up of a fibrin gel causing the accumulation of a capillary bed due to endothelial migration (Dvorak, 1986).
(iv) two or more of the above acting in sympathy.

Endothelial cells produce a variety of extracellular matrix components which form a basement membrane. The quantity of basement membrane surrounding new tumor blood vessels is less than is found in normal blood vessels, whilst most of the basement membrane components are present in reduced quantity, Auspunk et al. (1981a and b) have reported that there are changes in the glycosaminoglycan species present in the membrane. Tumor vasculature has been reported to have an increased permeability relative to normal blood vessels (Underwood and Carr, 1972), which may potentiate tumor cell escape from the primary tumor, however, although escape from the primary tumor may not pose a major problem to successful metastasis, it may be that extravasation of the tumor cell into the organ parenchyma at a distant site represents the crucial importance of degradative enzyme expression, since at this stage the basement membrane may act as a major obstacle to tissue invasion.

1.4.5. Cell-Cell/Matrix Adhesion In Metastasis.

The ability of a tumor cell to detach from other tumor cells in the primary tumor, form homotypic and/or heterotypic emboli, adhere to and migrate through the endothelial cell layer and the basement membrane into the organ parenchyma are all mediated by cellular adhesion molecules. A number of different families of adhesion molecules exist which facilitate interactions between other cells and extracellular matrix components (For recent reviews: Albela and Buck, 1990; Lasky, 1991; Mecham, 1991; Humphries, 1990). Briefly, the major families of adhesion receptors are:

(i) the integrins, are calcium dependent heterodimeric molecules assembled from α and β subunits; 12 α and 7β chains are currently known which are capable of mediating cell-cell and cell-matrix interactions.

(ii) the immunoglobulin superfamily of receptors which are primarily involved in cell-cell interactions.

(iii) the cadherins, a developmentally regulated family of calcium dependent receptors involved in cell-cell interactions.
(iv) the selectins or LEC-CAMs a family of receptors, containing lectin, EGF and complement binding protein-like domains involved in endothelial leukocyte adhesion.

(v) lymphocyte homing receptors.

1. EXTRACELLULAR MATRIX: COMPOSITION AND STRUCTURE.

1. 2. Interstitial Connective Tissue.

Single tumor cells or groups of cells must separate from the primary tumor mass in order to form metastatic colonies at anatomically distant sites. As tumors progress toward malignancy, the surrounding extracellular matrix is slowly degraded and the strength of the adhesive interactions between tumor cells is diminished. As enzymatic destruction of the extracellular matrix continues (Goldfarb and Liotta, 1986), migratory tumor cells may effectively traverse tissue barriers, and increased interstitial pressures within the tumor may also facilitate tumor expansion into adjacent tissues and into blood vessels within the tumor.

The components of these barriers will now be discussed, their physiological functions described and their relative resistances to degradation commented upon prior to a detailed description of two groups of enzymes thought to be important in tissue invasion.

1. 3. Collagen type I.

Fourteen types of collagen have been reported to date (Mayne and Burgeson, 1987; van der Rest and Garrone, 1990). Collagen is the most abundant protein of the matrix and may account for up to 90% of the dermis, bone or submucosa. The collagens may be divided into two groups, the fibrillar collagens and the non-fibrillar collagens (Mayne and Burgeson, 1987). Fibrillar collagens consist of at least 5 major types, I, II, III, V and XI (Miller and Gay, 1987). The triple helical form of type I collagen is the prevalent form and is a heterotrimer composed of two identical $\alpha_1(I)$ chains and an $\alpha_2(I)$ chain. In addition to the heterotrimer form a homotrimer composed of $\alpha_1(I)$ chains has been found to be synthesised by cells in culture (Mayne et al., 1975) and in certain tumors (Moro and Smith, 1977). The chain sequence is
composed of a Gly-X-Y repeating triplet (where X and Y represent amino acids) which forms a left-handed helix. Each of the 3 chains revolves around a common central axis in a right-handed superhelix (Mayne and Burgeson, 1987).

The triple helical region of the fibrillar collagens is highly resistant to proteolysis. At neutral pH the major collagen degrading enzymes are the interstitial collagenases (MMP-1 and 8) (Woessner, 1991). A collagenase is defined as an enzyme capable of cleaving the collagen triple helix at approximately the \( \frac{3}{4} : \frac{1}{4} \) locus between the NH2 and COOH termini of the molecule. This releases two fragments the \( \frac{3}{4} \) (TCA) and the \( \frac{1}{4} \) (TCB) components which are unstable at 37°C and denature into gelatin (Barrett, 1979); this gelatin is then further degraded by a number of enzymes (Werb, 1990).

Interestingly, although there are many reports of interstitial collagenase correlating with the invasive ability of tumor cells (Woolley, 1984). Greater than 95% of all interstitial collagens are found in a fiber structure which is resistant to degradation by interstitial collagenases (Weiss et al., 1980; Barrett, 1981). This structure is both highly stable and bound to proteoglycans, hyaluronate and other glycoproteins forming a structure extremely resistant to degradation. Barrett (1981) suggested that broad specificity enzymes like plasmin, elastase, stromelysin and cathepsins are in fact responsible for degrading the links between these fibers known as telopeptide regions.

1.5.4. Collagen Type III.

Collagen type III has been reported to be found in association with collagen type I in distensive tissues, this type of collagen has a homotrimer α1 (III) (Chung and Miller, 1974). In vitro studies using mixtures of collagen types I and III have shown an inverse relationship between fibril size and the quantity of type III collagen present (Lapiere et al., 1977), suggesting a mechanism for controlling the size and properties of the collagenous component of a fibrous stroma.
1. 5. 5. Collagen Type V.

Collagen type V has been identified in a number of tissues but only as a minor component, it has been reported to have a number of structures, both homotrimeric and heterotrimeric: \( \alpha_1(V) \alpha_2(V), \alpha_1(V) \alpha_3(V), \) and \( \alpha_1(V) \alpha_2(V) \alpha_3(V), \) the synthesis of which may be cell type or tissue specific, possibly reflecting its physiological role (Miller and Gay, 1987). Although isolated from placenta, it is now known to be widely distributed in pericellular zones (Martinez-Hernandez et al., 1982) in association with basal lamina (Martinez-Hernandez et al., 1982) and may have the function of linking dissimilar connective tissues. Type V collagen molecules contain high amounts of noncollagenous sequences which make the molecule susceptible to nonspecific proteolysis.

1. 5. 6. Fibronectin.

Fibronectins are a family of closely related glycoproteins, the molecule is a virtual homodimer, disulphide-bonded close to the carboxy termini (Petersen et al., 1989). Both chains have molecular weights of approximately 250 kDa, and are composed of a number of domains joined by flexible regions. Virtually all the carbohydrate (5%) is present in the collagen binding domain. Differential slicing of the mRNA results in a large number of variants from a single gene (Petersen et al., 1989; Hynes, 1985), the soluble forms of fibronectin found in the plasma and in the migration pathways of chick embryos (Ffrench-Constant et al., 1988) appear to have distinctive splicing patterns which may influence their functional properties due to the presence or absence of specific domains. Fibronectin is both an important cell-binding extracellular matrix protein and is also important in the binding of other extracellular matrix components such as collagen (Engvall and Ruoslahti, 1977), glycosaminoglycans (Stathakis and Mosesson, 1977) and fibrinogen (Edsall et al., 1947). Much of the work on the cell binding properties of fibronectin has involved the study of integrin receptor binding sequences in the molecule. Integrins are heterodimeric proteins consisting of an \( \alpha \) and \( \beta \) subunit (Review: Albelä and Buck, 1990). The integrin receptors may form part of a transmembrane linkage between cytoskeletal and extracellular matrix components,
evidence for this hypothesis is that the intracellular domains of certain integrins have the ability to interact with the microfilament associated protein talin, and the extracellular domains interact with fibronectin and other extracellular matrix proteins (Horwitz et al., 1986). The presence of fibronectins as basement membrane proteins has been an area of debate, since it appears that embryonic or immature basement membranes are enriched in fibronectin, and this amount decreases with maturity (Gibson et al., 1983; Abrahamson, 1986; Thiery et al., 1985). The role of fibronectin in the modulation of cell migration is being elucidated and it has been demonstrated that both epidermal cell migration (Donaldson and Mahan, 1988) and mesenchymal cell migration (Thiery et al., 1985; Woods and Couchman, 1988) can be promoted by fibronectin.

1. 5. 7. The Basement Membrane.

In the pathology of tumor invasion the basement membrane is regarded as a major barrier to the invasion of normal tissues by cancer cells (Burtin et al., 1982; Liotta et al., 1983), fragmentation of the basement membrane has been correlated with invasion, metastasis and prognosis (Daher et al., 1987). Following the early studies of Sylven and Bois (1960) there have been numerous reports of a correlation between invasive capacity and enzyme expression by cancer cells (Liotta et al., 1980; Nakajima et al., 1986; Niedbala et al., 1987) and enzyme inhibitors (Cawston et al., 1983; Halaka et al., 1983). Invasive capacity has been shown to increase with type IV collagenase (gelatinase) activity in some studies (Eisenbach et al., 1985), in others it has not (Warburton et al., 1987).

The basement membrane is a continuous quasi-elastic heteropolymeric mesh-like extracellular matrix containing laminin, type IV collagen, heparan sulfate proteoglycan, and other minor macromolecules which is produced by epithelial and endothelial cells. This membrane has many functions including cell and tissue support, compartmentalisation, providing an interactive surface for cellular regulation and molecular sieving (Farquhar, 1981), and many of these functions are dependent upon cell attachment to matrix determinants (Timpl, 1989). Heterogeneity of structure
and function occur in different tissues, in development (Form et al., 1986), and in response to different physiological needs.

The sheet-like structure, typically between 100-200 nm, is organised morphologically into three major zones:

a) The lamina lucida externa or lamina rara which is an electron lucent region 20-40 nm wide located just below or adjacent to the epithelial or endothelial.

b) The lamina densa or basal lamina is the middle layer, 20-100 nm wide, which is electron dense and contains a meshwork of fibrils.

c) The lamina lucida interna is an electronlucent region of variable width located at the interface between the lamina densa and the underlying connective tissue.

Epithelial and certain other basement membranes contain filaments (2-8 nm thick) which bind the epithelial tonofilament-desmosome complex to the lamina densa. On the opposite side of the basement membrane, anchoring fibers with a periodic binding pattern, smaller anchoring fibrils, and tubular microfilaments run between the lamina densa and the interstitial collagens of the connective tissue stroma.

1.5.8. Collagen Type IV.

The type IV collagen monomer is derived from three polypeptide chains [α1 (IV)2 α2 (IV)], and in contrast to the interstitial collagens is a flexible thread-like molecule about 400 nm in contour length and possessing a distinctive globular domain at its carboxy terminus. Interruptions in the human α1 (IV) and α2 (IV) triple helical domains coincide to produce 26 irregularly spaced sites that impart the increased flexibility to the molecule (Brazel et al., 1988). The amino terminal 30 nm segment is referred to as the 7S domain (Timpl et al., 1981) and homologous COOH-terminal segments of the three chains (227-229 amino acid residues), which each possess six cysteines, form a disulphide-stabilised globular domain (Weber et al., 1988). Type IV collagen can self assemble into a stable three dimensional basement membrane network by association at the carboxy termini globules to form linear dimers (Timpl et al., 1981), association of four amino-terminal ends to form a 28 nm, end-overlapped (7S) domain leading to antiparallel dimeric and trimeric intermediates followed by the
stable tetramer structure (Yurchenco and Furthmayr, 1986). Finally type IV collagen dimers self-interact through lateral association (Yurchenco and Furthmayr, 1984) to form the chicken wire structure associated with basement membrane collagen. Type IV collagen contains a cell attachment site in the triple helical region of the molecule (Timpl, 1989). Type IV collagen, presumably due to the interruptions in the helical structure, is degraded by a number of proteinases at neutral pH which include stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), PUMP-1 (MMP-7) 72 and 92 kDa gelatinases (MMPs -2 and -9) and plasmin.

1.5.9. Collagen Type VII.

Type VII procollagen is a 450 nm long monomer with a carboxy-terminal globular domain that dimerizes through a disulphide-linked, 60 nm overlapped, amino terminal interaction (Timpl, 1989). The carboxy-terminal domain is proteolytically cleaved after alignment of the dimers, which associate to form anchoring fibrils. In epithelial tissues these fibrils connect foci of basement membrane components called anchoring plaques, which are located within the stroma, to each other and to the lamina densa of the basement membrane (Keene et al., 1987). This network is thought to anchor the basement membrane to the stroma and to stabilise the stroma (Briggaman et al., 1971). The ability of type IV collagenase/gelatinase to cleave type VII collagen (Seltzer et al., 1989), and in doing so weaken the integrity of the epidermal-dermal junction, could augment the invasive process. This may be the case with basal cell carcinoma invasion since the basement membrane integrity is found to be disrupted and loss of anchoring fibrils has been observed (Lane et al., 1985).

1.5.10. Laminin.

Laminin is a glycoprotein found in the extracellular matrix which is known to mediate cell attachment, movement, differentiation, and growth (Reviews: Yurchenco and Schittny, 1990; Mecham, 1991; Mercurio and Shaw, 1991)

Laminin is a flexible four-armed glycoprotein with a molecular weight of approximately 1000 kDa, it consists of three short arms (220 kDa) and one long arm (440 kDa) (Engel et al., 1981), which are linked via disulphide bonds to form the
characteristic asymmetric cross-structure as seen by electron microscopy. Laminin possesses a number of sites which mediate its interactions with cells and other extracellular matrix molecules. During tumor metastasis it is necessary for tumor cells to cross the basement membrane; an important step in this process may be attachment to the basement membrane via cell-surface receptors for laminin. The first to be identified was a 65 to 70 kDa protein that bound to laminin with high affinity. The expression of this receptor was first found on tumor cells (Rao et al., 1983; Malinoff and Wicha, 1983), and later on a wide variety of cell types (Reviews: Mercurio and Shaw, 1991; Mecham, 1991). An amino acid sequence which can act as a binding site for the 67 kDa receptor is present in the B1 chain and has the peptide sequence YIGSR (Graf et al., 1987). Both the YIGSR sequence and another sequence, PDSRG, found in the same region, promote cell attachment, migration and melanoma colonisation of the lungs following tail vein injection (Kleinman et al., 1989). Three other peptide sequences, VGVAPG, LGTIPG and IKVAV, have been shown to induce chemotaxis in the melanoma cell lines, A2058 (VGVAPG and LGTIPG) and B16F10 (IKVAV) (Mecham et al., 1989; Tashiro et al., 1989). The 67 kDa receptor may also bind to the carbohydrate side chains of the laminin molecule (Arumugham et al., 1986; Fujiwara et al., 1988).

1. 6. TUMOR CELL INVASION.

Tumor invasion may be considered as the primary step in a multistep process which, if successfully completed, leads to the formation of metastases. During the metastatic cascade cellular invasion is important in at least three places:

1) local invasion at the tumor-host interface, allowing enlargement of the tumor.

2) Escape from the primary tumor into the vasculature or lymphatics (intravasation)

3) Escape from the vasculature due to invasion at a secondary site (extravasation)

The extracellular matrix, which is probably the commonest barrier to invasion, is comprised of the connective tissue stroma and the basement membrane. Tumor cell invasion of the adjacent host tissues may occur by a variety of mechanisms. The first
mechanism is dependent on proliferation rate, the actively growing tumor expands along the pathway of least resistance; the second mechanism is based on active locomotion of tumor cells through the host tissues. In addition to these two basic mechanisms, invasion is promoted by the degradation of the tissue through which it occurs. The invasive process has been divided into three sequential steps (Liotta, 1985) (i) tumor cell attachment to the extracellular matrix components laminin and fibronectin (ii) lysis of matrix due to the production of hydrolytic enzymes by the tumor cells (iii) tumor cell locomotion into the region of lysis modified by proteolysis. Later studies have closely linked these three steps and shown the stimulation of degradative enzyme production following attachment to laminin (Turpeenniemi-Hujanen et al., 1986), and chemotaxis towards degraded extracellular matrix components (Terranova and Lyall, 1986; Mundy et al., 1981; Nakeshima et al., 1986) and certain proteinases (Terranova et al., 1989). Furthermore, it is possible, if not probable, that tumor cells may escape from the primary tumor site during the angiogenic process, when extracellular matrix is being degraded and cellular migration is stimulated by a number of cytokines and growth factors. This controlled process involves the co-ordinated invasion and migration of endothelial cells and is proteinase dependent. The similarities between tumor invasion and angiogenesis are reviewed by Moscatelli and Rifkin (1988).

Human cutaneous melanoma invasion is thought to occur by repetitive cycles of active locomotion followed by cessation of movement and proliferation (Suh and Weiss, 1984), although little is known about uveal melanoma invasion processes but Char et al. (1983) suggest that choroidal melanomas frequently grow rapidly after a period of relative inactivity.

In relation to the majority of the experimental work presented in this thesis, enzymes thought to be involved in the invasive component of the metastatic cascade will be discussed in detail. Due to the diversity of the components of the basement membrane and the interstitial matrix which may act as barriers to invasion, the invading tumor cell may require the expression of an array of degradative enzymes,
some highly specific and some with broader substrate specificities, rather than merely the expression of a single multifunctional enzyme. Likewise the expression of both membrane bound and secreted proteinases may be essential for invasion of certain tissues but not for others. A specific set of enzymes may be required for the degradation of different tissues, each of which has a unique composition of extracellular matrix components.

1. 7. DEGRADATIVE ENZYMES.

The enzymes which are typically associated with matrix hydrolysis are proteases and glycosidases (Goldfarb and Liotta, 1986). Proteases have been classified into exopeptidases (peptidases) and endopeptidases (proteinases) (Review: McDonald, 1985). Exopeptidases are active upon the amino and carboxy termini of proteins in contrast to proteinases which cleave within the protein chain. Exopeptidases are classified as amino-, carboxy-, dipeptidyl-, tripeptidyl-, peptidyl di-, tri-, omega-peptidases. Their direct importance in extracellular matrix invasion is debatable, but they are thought to modify peptide regulatory factors involved in tumor growth and angiogenesis. They have not been shown to directly degrade the macromolecular matrix structure. The enzymes which are primarily involved in degradation of the extracellular matrix macromolecules are the proteinases. This group of enzymes has been detected at intracellular, cell surface and extracellular locations. Intracellular enzymes are frequently found in lysosomes, and these enzymes are active against proteins taken up during endocytosis, extracellular enzymes have been detected in either soluble or vesicle associated forms.

There are currently four categories of proteinases determined by the catalytic mechanism of the active site: aspartic and cysteine proteinases, typically active at acid pH, and serine and metalloproteinases, active at neutral and slightly alkaline pH (Review: McDonald, 1985). Proteinases are involved in a variety of physiological and pathological processes (Table. 1. 1).
Table 1. 1.
Functions of proteinases in physiological processes.

Emigration of inflammatory cells out of blood vessels, through the basement membrane and underlying connective tissue.
Migration and proliferation of microvascular endothelial cells and fibroblasts during neovascularisation.
Editing of excess matrix components during rapid connective tissue synthesis and assembly.
Removal of obsolete matrix components from migration, signalling and assembly pathways.
Breakdown of connective tissue components coupled to synthesis during tissue expansion and growth.
Destruction of extracellular matrix during inflammation, activation of connective tissue cells, and fibrosis.

Taken from Werb, 1990.

Some of these actions may have relevance to tumor growth and invasion, for example stimulation of endothelial cell migration may augment neovascularisation of the tumor and hence increase the growth of the tumor.
1. **PROTEINASES IN INVASION AND METASTASIS.**

The increased proteolytic activity of malignant cells was noted by Fisher in 1925, and its correlation with neoplastic transformation has been extensively studied (Reviews: Mullins and Rohrlich, 1983; Dano et al., 1985; Goldfarb and Liotta, 1986). Elevated levels of a number of proteinases have been correlated with tumor invasiveness. These include plasminogen activators, interstitial collagenase, type IV collagenase, cathepsins, and proteoglycanases (Review: Tryggvason et al., 1987). Recent studies propose that the following factors are important in the invasive process: (i) the localisation of the enzyme at the cell surface, (ii) the synergistic action of two or more proteinases working in concert and (iii) the balance of enzyme to inhibitor expression. The first of these two concepts will be discussed here and the third will be illustrated in the section concerning tissue inhibitors of metalloproteinases (TIMPs).

Evidence for the first concept was presented by Chen et al. (1984) who grew transformed chicken embryo fibroblasts and mouse BALB/c 3T3 cells on radiolabelled fibronectin covalently linked to the surface of a gelatin film. The fibronectin was found to be degraded only at the points of cell-matrix contact. This degradation could be inhibited by 1, 10-phenanthroline but not by a number of other proteinase inhibitors, subsequently a fibronectin-degrading metalloproteinase was identified in membrane fractions from these cells (Chen and Chen, 1987). Additional evidence was obtained by Sas et al. (1986), who observed that when a number of tumor cell lines were grown on glass coverslips coated with purified fibronectin, laminin, or type IV collagen, degradation of the substratum began at the sites of initial cell contact and was restricted to the paths of cellular migration. Similar studies by Jones and DeClerk (1980) showed that HT 1080 human fibrosarcoma cells degraded the extracellular matrix produced by rat smooth muscle cells in a similar cell associated manner. In addition this group showed that when this extracellular matrix was floated in the medium above the cells, no degradation occurred. Studies involving the degradation of basement membrane using human breast cancer cells seeded onto endothelial cell-derived matrix, showed that they degraded both the glycoprotein and collagen.
components. However, the conditioned medium had no degradative ability presumably due to either a catalytic ability of the cell surface or inactivation due to inhibitor binding once released from the cell surface (Yee and Shiu, 1986). Further studies on invasion using HT 1080 cells showed that during invasion of a reconstituted basement membrane, clearing was observed only in the vicinity of invading pseudopodia (Kramer et al., 1986). Binding sites for a number of degradative enzymes have been identified on the cell surface, urokinase-type plasminogen activator (UK) is a prominent example of this phenomena. Following the identification of a high affinity cell-surface receptor for UK (Vassalli et al., 1985; Stoppelli et al., 1985), this receptor was found on a number of tumor cell types (Stoppelli et al., 1986). The receptor allows UK to bind to the cell surface via an amino-terminal domain which has partial sequence homology with epidermal growth factor (Stoppelli et al., 1985). Tissue-type plasminogen activator (tPA) also has a binding site on cultured fibroblasts (Hoal et al., 1983), platelets (Vaughan et al., 1989) and human umbilical vein endothelial cells (Hajjar et al., 1987). Recently tPA has been shown to bind to the man nose receptor which is expressed on macrophages and liver sinusoidal cells (Otter et al., 1991), however it is not known if the enzyme retains activity when bound as is the case for the UK receptor.

The cell surface may play an important role in degradation by (i) protecting the enzyme from inhibitors due to steric hindrance, (ii) causing bound enzyme to be more readily activated and have a higher specific activity than soluble enzymes, (iii) acting as a catalyst by bringing the enzyme and its substrate into close proximity and (iv) localising the degradative event so as to concentrate upon a small area and tunnel through the matrix.

In contrast the role of secreted enzyme may be equally important since these molecules may (i) cause a generalised lysis of large areas of matrix, (ii) bind to inhibitors present in the microenvironment in doing so stabilise the enzyme and remove inhibitors from the immediate vicinity of the tumor, (iii) locally activate cytokines or growth factors (iii) act as chemotactic agents or growth promoting factors in their own
right and (iv) bind to the surface of other cells, causing them to gain an altered cellular phenotype.

Metastatic tumor cells produce a variety of degradative enzymes that weaken or destroy extracellular matrix molecules (Tryggvason et al., 1987). In addition to enzymes produced by the tumor cells, an advancing front of invasive tumor cells can also induce secretion of hydrolytic enzymes from adjacent non-tumor tissue. Extracellular matrix destruction at the tumor periphery may be facilitated by degradative enzymes produced by host cells, such as neutrophils and monocytes, that are attracted to the tumor site (Gabbert, 1985). Tumor angiogenesis may further promote matrix degradation because the migrating endothelial cells (Kalebic et al., 1982) and co-migrating mast cells (Dabbous et al., 1986) modify the extracellular matrix via the production of hydrolytic enzymes or by the deposition of new matrix molecules (Risau and Lemmon, 1988). The extracellular matrix components potentially representing barriers to tumor invasion and the proteinases capable of degrading them are shown in table 1.2.
Table 1.2.
Proteinase susceptibility of selected extracellular matrix proteins.

<table>
<thead>
<tr>
<th>Matrix protein</th>
<th>Proteinases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interstitial connective tissue</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen type I</td>
<td>MMP-1, MMP-8, Cathepsins B and L</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>MMP-1, MMP-3, MMP-8, MMP-10, plasmin</td>
</tr>
<tr>
<td>Collagen type V</td>
<td>MMP-2, MMP-3, MMP-9, MMP-10</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>MMP-2, MMP-3, MMP-7, MMP-10, Cathepsin G, UK, Plasmin, PMN elastase, macrophage elastase, cathepsin D</td>
</tr>
<tr>
<td>Elastin</td>
<td>PMN elastase, macrophage elastase, MMP-2, MMP-3 and cathepsin D</td>
</tr>
<tr>
<td><strong>Basement membrane</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, plasmin</td>
</tr>
<tr>
<td>Collagen type VII</td>
<td>MMP-1, MMP-2</td>
</tr>
<tr>
<td>Heparan sulphate proteoglycan</td>
<td>Mast cell chymase, PMN elastase</td>
</tr>
<tr>
<td>Laminin</td>
<td>Plasmin, MMP-3 and PMN elastase</td>
</tr>
<tr>
<td>Denatured collagen (Gelatin)</td>
<td>MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, Cathepsins B and L, PMN elastase</td>
</tr>
</tbody>
</table>

Adapted from Werb, 1990.

MMP Matrix metalloproteinase

In respect to the experimental investigations performed for this thesis two groups of enzymes will be introduced in additional detail, these are the metalloproteinase family also known as the matrixins and the plasminogen activators, tissue-type plasminogen activator and urokinase-type plasminogen activator.
1. 9. 1. METALLOPROTEINASES AND THEIR NATURAL TISSUE INHIBITORS.

1. 9. 2. Metalloproteinases.

Metalloproteinases are a group of enzymes which are involved in both normal remodelling processes such as embryonic development, post partum involution of the uterus, bone and growth plate remodelling, ovulation, and wound healing and in pathological processes including rheumatoid arthritis, tumor invasion and periodontitis (Mullins and Rohrlich, 1983).

Recently, the matrix metalloproteinases, which are commonly referred to as the collagenase gene family have had a systematic series of names proposed for them, the family has been tentatively renamed the matrixins. The matrixins have been previously named on the ability of an enzyme to degrade a single substrate e. g. type IV collagenase, collagenase etc, however "type IV collagenase" has the ability to degrade a number of substrates.

The enzymes present in the matrixin family have a number of properties which characterise them:

(i) Dependence on zinc at the catalytic site (Whitham et al., 1986; McKerrow, 1987; Jongeneel et al., 1989).

(ii) Secretion in a zymogen form.

(iii) Activation of the zymogens by proteinases or organomercurials (Review: Van Wart and Birkedal-Hansen, 1990).

(iv) Cleavage of the enzyme during activation which results in a loss of approximately 10 kDa (Review: Van Wart and Birkedal-Hansen, 1990).

(v) homology in the cDNA with interstitial collagenase (Reviews: Docherty and Murphy, 1990; Matrisian, 1990).

(vi) Cleavage of extracellular matrix components by the enzyme.

The matrixins are systematically named as matrix metalloproteinase n, where n is a number; this is typically abbreviated to MMP-1, MMP-2 etc. The matrixin family of enzymes is rapidly expanding, however, many matrixins have been characterised in only a single laboratory and await confirmation or sequence data. Three such enzymes are MMP-4 (Azzo and Woessner, 1986), MMP-6 (Nakano and Scott, 1987) and Stromelysin-3 (Basset et al., 1990). The nomenclature, previous names, molecular weights of the respective enzymes and the natural substrates of the matrixins (matrix metalloproteinases) is shown in table 1.3.
Table 1. 3.
Nomenclature and natural substrates of the matrix metalloproteinases$^a$.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Enzyme names</th>
<th>Molecular mass (kDa)</th>
<th>Matrix substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Interstitial collagenase</td>
<td>52 Deduced</td>
<td>Collagen types I, II, III,VII, X Gelatins</td>
</tr>
<tr>
<td></td>
<td>MMP-1</td>
<td>(57/52) Latent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vertebrate collagenase</td>
<td>(48)/42 Active</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibroblast collagenase</td>
<td>24 Modified</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neutrophil collagenase</td>
<td>53 Deduced</td>
<td>Collagens I, II, III</td>
</tr>
<tr>
<td></td>
<td>MMP-8</td>
<td>85 Latent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>65 Active</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>72-kDa gelatinase</td>
<td>72 Deduced</td>
<td>Gelatin type I</td>
</tr>
<tr>
<td></td>
<td>MMP-2</td>
<td>72 Latent</td>
<td>Collagens, IV, V, VII, X</td>
</tr>
<tr>
<td></td>
<td>Type IV collagenase</td>
<td>66 Active</td>
<td>Fibronectin, elastin</td>
</tr>
<tr>
<td></td>
<td>92-kDa gelatinase</td>
<td>80 Deduced</td>
<td>Gelatins I, V</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>92 Secreted</td>
<td>Collagens IV, V</td>
</tr>
<tr>
<td></td>
<td>Type V collagenase</td>
<td>84 Active</td>
<td></td>
</tr>
</tbody>
</table>

$^a$This table presents recommended names for the matrixins, together with synonyms. The molecular weights are those deduced from cDNA (minus the signal peptide) and those of the secreted form (variably glycosylated) and active form (or forms). Minor forms are in parentheses. Taken from Woessner, 1991.
Table 1.3 continued.

Nomenclature and natural substrates of the matrix metalloproteinases\(^a\).

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme names</th>
<th>Molecular mass (kDa)</th>
<th>Matrix substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>Stromelysin</td>
<td>52 Deduced</td>
<td>Proteoglycan, link protein</td>
</tr>
<tr>
<td></td>
<td>MMP-3</td>
<td>(60)/57 Secreted</td>
<td>Fibronectin, laminin</td>
</tr>
<tr>
<td></td>
<td>(EC 3. 4. 24. 17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transin</td>
<td>(50)/48 Active</td>
<td>Gelatins I, III, IV, V</td>
</tr>
<tr>
<td></td>
<td>Proteoglycanase</td>
<td>28 Active</td>
<td>Collagens III, IV, V, IX</td>
</tr>
<tr>
<td></td>
<td>Procollagenase activator</td>
<td></td>
<td>Procollagen peptides</td>
</tr>
<tr>
<td></td>
<td>Collagenase activator protein</td>
<td></td>
<td>Activates procollagenase</td>
</tr>
<tr>
<td></td>
<td>Stromelysin-2</td>
<td>53 Deduced</td>
<td>Gelatins I, III, IV, V</td>
</tr>
<tr>
<td></td>
<td>MMP-10</td>
<td>53 Secreted</td>
<td>Weak on collagens III, IV, V</td>
</tr>
<tr>
<td></td>
<td>Transin-2</td>
<td>47 Active</td>
<td>Activates procollagenase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28 Active</td>
<td>Fibronectin</td>
</tr>
<tr>
<td></td>
<td>Uterine metalloproteinase</td>
<td>28 Deduced</td>
<td>Gelatins I, III, IV, V</td>
</tr>
<tr>
<td></td>
<td>MMP-7</td>
<td>28 Secreted</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(21)/19 Active</td>
<td>Activates procollagenase</td>
</tr>
</tbody>
</table>

\(^a\)This table presents recommended names for the matrixins, together with synonyms. The molecular weights are those deduced from cDNA (minus the signal peptide) and those of the secreted form (variably glycosylated) and active form (or forms). Minor forms are in parentheses. Taken from Woessner, 1991.
The matrix metalloproteinases are frequently compared using structural criteria and have been divided into a number of domains. MMP-9, the largest matrixin, has 7 domains (Table 1.4). The other members have fewer domains, the smallest matrix metalloproteinase, MMP-7, having only four domains. The domains are typically:

(i) a signal peptide of between 17 and 29 amino acids.

(ii) a propeptide sequence of between 77 and 87 amino acids which is cleaved from the enzyme during activation.

(iii) an amino-terminal latent proteinase typically either 112 or 113 amino acids.

(iv) a domain with homology to the gelatin binding region of fibronectin present only in gelatinase species.

(v) a putative zinc binding region of between 50 and 60 amino acids.

(vi) a domain with homology to a 54 amino acid segment of the $\alpha_2$ (V) collagen chain present in only the 92 kDa gelatinase.

(vii) a carboxy-terminal hemopexin-like domain which is typically between 202 and 213 amino acids and thought to be involved in determining substrate specificity (Clark and Cawston, 1989).

The distribution of these domains in the human enzymes of the matrixin family is shown in table 1.4.
Table 1.4.

Domain structure of matrixins of human origin.

The number of amino acids is shown within each domain. Where domains are left blank this indicates that the domain is absent in the enzyme.

<table>
<thead>
<tr>
<th>MMP</th>
<th>Signal peptide</th>
<th>Propeptide</th>
<th>Active enzyme</th>
<th>Fibronectin</th>
<th>Zinc</th>
<th>α2(V) collagen</th>
<th>Hemo-plexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>19</td>
<td>87</td>
<td>112</td>
<td>176</td>
<td>50</td>
<td>53</td>
<td>210</td>
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<tr>
<td>2</td>
<td>29</td>
<td>80</td>
<td>112</td>
<td>175</td>
<td>50</td>
<td></td>
<td>202</td>
</tr>
<tr>
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<td>19</td>
<td>80</td>
<td>112</td>
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<tr>
<td>7</td>
<td>17</td>
<td>77</td>
<td>113</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Woessner, 1991.
1. 9. 3. Activation of Metalloproteinases.

As mentioned earlier the matrixins are secreted in a latent form and activated in situ by physiological mechanisms which are at present unclear. Two amino acid sequences, one in the propeptide (P^{71} R C G V/N P D V/L A/G) and the other in the zinc binding region (V/Y^{196} A A H E L/F G H S/A L/M G) are highly conserved in all 11 enzymes for which the cDNA sequences are available. These two sequences are thought to form the "cysteine switch". The current hypotheses put forward to explain the activation process involves the "cysteine switch". Activators of the matrixins may be separated into four groups (i) proteases (trypsin, autolysis), (ii) conformational perturbants (SDS, NaSCN), (iii) reversible sulfhydryl-group modifiers (Hg(II), organomercurials), and (iv) irreversible sulfhydryl-group modifiers (oxidants, alkylating agents). The high level of conservation of these sequences lead Van-Wart and Birkedal-Hansen (1990) to suggest that latency of the matrixins was due to complex formation between the propeptide cysteine and the active-site zinc atom and that this represents a previously unknown mechanism for the latency of this family of enzymes. The complex is thought to both block the active site and exclude water from the coordination sphere of the zinc atom. Water has been found to be essential for the catalytic activity of zinc hydrolases and is thought to act as a fourth ligand (Vallee and Auld, 1990). It is likely that all modes of activation of the matrixin zymogens involve dissociation of this complex and and the replacement of the cysteine ligand by water.

1. 9. 4. Gelatinases and Tumor Invasion and Metastasis.

There is a large body of literature correlating the expression of proteinases with tumor invasion and metastasis. Proteinases such as gelatinases act in synergy with interstitial collagenase in the degradation of collagen types I, II and III. Interstitial collagenase cleaves these collagen to 3/4 and 1/4 collagen fragments (McCroskery et al., 1975; Yamanashi et al., 1973), which are denatured to gelatin at body temperature and become susceptible to gelatinolytic proteinases (Netheray and O'Grady, 1989; Zucker et al., 1987). The synergistic action of these two groups of enzymes appears
to play an important role in the penetration of the connective tissue surrounding the
tumor.

The 72 and 92 kDa gelatinases have been reported to share many common features
including close substrate specificities (Mackay et al., 1990; Murphy et al., 1991).
Recent studies by Murphy et al., (1991) have determined the relative activities of the
two gelatinase species against several substrates. The 92 kDa gelatinase was found to
have a higher activity than 72 kDa gelatinase when type IV collagen was the substrate,
however, the 72 kDa gelatinase exhibited higher activities against elastin and
proteoglycan. A study performed by Mackay et al. (1990) using 64 and 92 kDa
gelatinases purified from tumor conditioned medium demonstrated these enzymes had
little or no ability to degrade native type IV collagen under conditions reported to
preserve the tertiary structure of type IV collagen and that degradation of collagen
types IV and V occurred at the telopeptide region and these workers suggest that these
enzymes are not true type IV collagenases.

There are a number of studies which have correlated expression of interstitial
collagenase and/or gelatinase and the malignant potential of in vitro cultured tumor
cells (Ballin et al., 1988; Liotta et al., 1980; Nakajima et al., 1987; Yamagata et al.,
1988; Yamagata et al., 1989). Collagenase activity has been correlated with the grade
of histological differentiation of tumor tissue (Van Der Stappen et al., 1990), extent of
host tissue invasion (Wirl and Frick, 1979) and invasion of lymphatics and veins in vivo
(Kubochi et al., 1986) by tumor cells. In addition it has been proposed that
secretion of a 92 kDa species of gelatinase (possibly MMP-9) by carcinoma or
oncogene transfected cells correlates with increased metastatic potential (Ballin et al.,
1988; Bernhard et al., 1990; Wilhelm et al., 1989; Yamagata et al., 1988; Yamagata et
al., 1989).
1. 9. 5. Regulation of Metalloproteinase Production.

Both the 72 kDa and 92 kDa gelatinase genes contain 13 exons which coincide (Huhtala et al., 1990; Huhtala et al., 1991). Whilst the genes for interstitial collagenase (Collier et al., 1988), rat stromelysin-1 and 2 (transin-1 and 2) (Matrisian et al., 1986; Breathnach et al., 1987) and 92 kDa gelatinase (Huhtala et al., 1991) are of similar size (approx 7-8 kb), the gene for 72 kDa gelatinase is about 27 kb (Huhtala et al., 1990). The interstitial collagenase and stromelysin genes contain 10 exons (Collier et al., 1988; Matrisian et al., 1986; Breathnach et al., 1987), the exons present in these genes correspond with those present in the type IV collagenase genes. The 92 kDa gelatinase promoter is closely related to those of human interstitial collagenase and human stromelysin. The TATA-like sequence (TTAAA) is present in the same region in both the human stromelysin (Quinones et al., 1989) and 92 kDa gelatinase promoters (Huhtala et al., 1991). Rat (Matrisian et al., 1986) and rabbit (Fini et al., 1987) stromelysin and human interstitial collagenase (Collier et al., 1988) however contain true TATA boxes at these locations (Fini et al., 1987), but the 72 kDa gelatinase does not contain a TATA box. In addition the TPA responsive elements (TRE) located at -79 to -73 are present in the promoters of human 92 kDa gelatinase, rat stromelysin, and both rabbit and human interstitial collagenase but not in the human 72 kDa gelatinase promoter. In contrast the 72 kDa gelatinase promoter contains two GC consensus sequences (GC boxes), for binding the transcription factor Sp1, which are not found in the other metalloproteinase promoter sequences studied to date. One GC box starts at -89 and the other at -69. 92 kDa gelatinase contains only one GC box at -533 to -527. Furthermore, the 72 kDa gelatinase promoter contains a potential binding site for the transcription factor AP-2. All metalloproteinase promoters studied to date have lacked the presence of a CAAT box. Curiously the sequence between positions -131 to -90 in the 92 kDa gelatinase promoter consists of alternating C and A residues. In common with the stromelysin, urokinase, elastase, interstitial collagenase and c-myc genes (Kerr et al., 1990) there is
a FOS-binding sequence (GNNTTGGNGN) in the promoter of the 92 kDa gelatinase; this sequence is not found in the 72 kDa gelatinase promoter.

1. 9. 6. Oncogene Induction and Adenovirus E1A Repression of Metalloproteinase Gene Expression.

Initial studies by Thorgeirsson et al. (1985) showed that ras oncogene transfection induced the metastatic phenotype and an increase in type IV collagenolytic activity in NIH/3T3 fibroblasts. In further studies by this group, Ballin et al. (1988) demonstrated that ras transfection induced a 92 kDa gelatinase in NIH/3T3 fibroblasts which is thought to be responsible for the increase in collagenolytic activity. Comparable studies by Collier et al. (1988) showed induction of a 65 kDa enzyme capable of degrading gelatin and type IV collagen from human bronchial epithelial cells transfected with ras. However, more recent studies (Ballin et al., 1991) using N-nitrosomethylurea induced rat mammary carcinomas found no direct relationship with between levels of ras expression and neoplastic changes. The levels of ras expression were examined in both primary tumors, metastases and metastatic and nonmetastatic tumors and no consistent differences were found.

In a series of co-transfection experiments Garbisa et al. (1987) correlated type IV collagenolytic activity with metastatic ability in rat embryo fibroblasts transformed with either c-Ha-ras alone or co-transfected with c-Ha-ras plus v-myc, and in those co-transfected with c-Ha-ras and E1A. Cells transfected with c-Ha-ras alone or in combination with v-myc displayed high spontaneous metastatic ability, however, when co-transfected with Ad-2 E1A the cells although still highly tumorigenic were nonmetastatic and did not express type IV collagenase.

Related studies by Frisch et al. (1990) demonstrated that following stable transfection of HT 1080 with Adenovirus E1A the expression of a number of proteinases including 72 kDa type IV collagenase, interstitial collagenase and urokinase was repressed, and correlated with decreased invasion of reconstituted basement membrane in vitro and a reduction in metastatic potential in vivo. In addition phorbol ester tumor promoter induction of interstitial collagenase was found to be
blocked in the E1A transfected HT 1080 cells. Transient transfection of HT 1080 with plasmids containing the 5' flanking regions of either interstitial collagenase or type IV collagenase linked to the chloramphenicol acetyl transferase (CAT) coding sequence demonstrated that gene transcription was repressed, this repression was investigated using a TPA responsive element (TRE) driven thymidine kinase promoter and found to be due to inhibition of the activator protein-1 (AP-1) transcription pathway; this effect was not due to repression of the c-jun gene which was found by this group to be hypersensitive to TPA induction. TPA modulation occurs via a TRE (Angel et al., 1987). AP-1, binds to this element. AP-1 is the protein complex formed by the c-jun and c-fos oncogene products, JUN is the DNA binding component of this complex, the binding of which is enhanced by FOS (Angel et al., 1987; Lee et al., 1987; Bohmann et al., 1987; Angel et al., 1988; Chiu et al., 1988; Curran and Franza, 1988). In contrast, De Groot et al. (1991) demonstrated that in three different cell types: P19, JEG-3 and HeLa, E1A induced activity of jun/AP-1 and that binding of jun/AP-1 to a TRE is induced when E1A is expressed.

Conca et al. (1991) demonstrated that, using mutant IL-1 (Arg^{127}-Gly), expression of the immediate early genes c-fos, c-jun and junB following stimulation with IL-1 was not sufficient to lead to the expression of TRE containing genes. The PEA3 binding (c-ets) transcription factor which binds immediately upstream of AP-1 (Gutman and Wasylyk, 1990; Wasylyk et al., 1990) could be regulated by IL-1 and lead to transcription, alternatively junB has been reported to be capable of negative regulation (Chiu et al., 1989).

Collagenase gene expression is modulated by IL-1 (Stephenson et al., 1987), TPA (Stephenson et al., 1987) and TNFα (Dayer et al., 1985). IL-1 has been reported to induce c-fos, c-myc (Lin and Vilcek, 1987), and c-jun (Conca et al., 1989) expression in human foreskin fibroblasts, and IL-1 together with TNFα could trigger expression by similar mechanisms to TPA.
1. 9. 7. PEA3 and AP-1 Binding Sites In the Collagenase Promoter.

AP-1 has been shown to regulate highly expressed genes present in transformed cells such as stromelysin, c-fos and collagenase (reviews: Herrlich and Ponta, 1989; Imler and Wasylyk, 1989), NF-κB (a transcription factor expressed by B cells and activated T-cells and involved in regulation of immunoglobulin kappa light chain and IL-2α-receptor, (Sen and Baltimore, 1986a; Nabel and Baltimore, 1987) (Leonardo and Baltimore, 1989) and PEA3 (Wasylyk, 1989). PEA3, a factor which binds to the polyoma virus enhancer (Martin et al., 1988) is induced by serum, TPA and the non-nuclear oncogenes v-src, Py middle T, Ha-ras, v-mos and v-raf (Wasylyk, 1989). Gutman and Wasylyk, (1990) demonstrated that PEA3 binds to the collagenase promoter and acts synergistically with AP-1 to achieve maximal induction of transcription by TPA and several oncogenes. Angel et al. (1987a and b) reported that collagenase contains a TPA-responsive element that binds AP-1. Analysis of deletion mutants of the collagenase promoter determined the existence of additional inducible elements upstream of the AP-1 site (Angel et al., 1987a; Schontal et al., 1988), Gutman and Wasylyk (1990) showed that the PEA3 and AP-1 motifs participate in the response of the collagenase promoter to TPA stimulation, thereby delimiting a TPA and oncogene responsive unit (TORU). AP-1 is essential to confer inducibility, but maximal levels of expression can only be obtained with the co-operation of PEA3. Ptashne (1988) proposed that two eukaryotic activators can work synergistically, not by direct contact, but because they touch a third protein (promiscuous cooperativity). NF-κB may be regulated by similar factors to AP-1 suggesting that these factors are the nuclear targets of a common pathway. A number of different types of TORU are thought to exist: (i) simple, formed by AP-1 or NF-κB alone. (ii) Composite, formed by two or more interacting elements. Collagenase promoter is an example of the second type formed by AP-1 and PEA3.
1. 9. 8. Tissue Inhibitors of Metalloproteinases.

The activity of the matrixins in tissues is regulated by a family of inhibitors, the TIMP family (Cawston, 1986), this family is composed of at least three members, TIMP-1 (Sellers et al., 1977), TIMP-2 (Stetler-Stevenson et al., 1989) and LIMP (Cawston et al., 1990). Another inhibitor IMP is identical to TIMP-2 (Herron et al., 1986; Stetler-Stevenson et al., 1989).

TIMP-1 is a glycosylated protein of $M_r$ 28,500 that inhibits all activated members of the matrixin family. There are 12 highly conserved cysteine residues which form 6 disulphide bonds, producing the characteristic double domain structure of the molecule (Williamson et al., 1990). TIMP-2 is a 21 kDa nonglycosylated protein which is about 40% homologous with TIMP-1, however the 6 disulphide bonds are conserved as are other important residues, which has lead to the suggestion that they may act in a similar manner (Williamson et al., 1990).

MMP-9 is secreted as a complex of zymogen with TIMP-1 (Wilhelm et al., 1989) and MMP-2 is found as a complex with TIMP-2 in the conditioned medium of the human melanoma cell line A2038 and bovine aortic endothelial cells, this time as a 27.5 kDa protein (DeClerk et al., 1989). Both TIMP-1 and TIMP-2 bind at a site other than the active site and upon activation, both the MMP-2 and MMP-9 can be inhibited by binding a second molecule of TIMP-1 or TIMP-2 (Goldberg et al., 1989).
1. 9. 9. TIMP's and Invasion and Metastasis.

Further evidence for the role of matrix metalloproteinases in tumor invasion has come from studies involving TIMPs. Matrix degradation is thought to occur only when activated enzyme levels exceed the levels of inhibitor expression and availability. Initial studies demonstrated that both natural and recombinant TIMP-1 inhibited invasion of the human amniotic membrane and metastasis as determined by lung colonisation of the B16 melanoma following tail vein injection (Mignatti et al., 1986; Schultz et al., 1988), amnion invasion by the sarcoma cell line M5076 was decreased by adding excess TIMP-1 (Thorgeirsson et al., 1982). rTIMP-1 has also been shown to reduce lung colonisation by c-Ha-ras-transfected rat embryo fibroblasts in nude mice (Alvarez et al., 1990). Studies by Kokha et al. (1989) using antisense strand constructs to decrease the level of TIMP-1 mRNA and hence the level of TIMP-1 protein so shifting the enzyme to inhibitor ratio towards active enzyme resulted in enhancement of the invasive phenotype. Recent studies by DeClerk et al. (1991) reported that rTIMP-2 inhibited invasion of smooth muscle cell multilayers by HT-1080 cells and ras-transformed rat embryo fibroblasts. Degradation of glycoprotein and collagen components of these matrices was also inhibited by rTIMP-2. In addition Albini et al. (1991) have reported that exogenous TIMP-2 inhibits invasion in the Membrane Invasion Culture System assay.

1. 10. 1. PLASMINOGEN ACTIVATORS AND PLASMIN.

Two forms of plasminogen activator have been identified, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (UK). These two PAs are products from different genes and may be distinguished by a number of biological, molecular biological and immunological factors.

Tissue type plasminogen activator is synthesised as a 72 kDa single chain polypeptide that is proteolytically cleaved into a double chain form by a variety of proteinases, including plasmin, tissue kallikrein and activated factor X (Ichinose et al., 1984). Two-chain tPA contains a heavy (40 kDa) and a light (30 kDa) chain linked by a single interchain disulfide bond. The heavy chain contains two "kringle regions" a
"finger region" which resembles a domain found in fibronectin, a domain with homology with epidermal growth factor (Gerard et al., 1986); the light chain contains the active site (Rijken and Groeneveld, 198). The single and two chain forms of tPA are equally effective in activating plasminogen (Rijken et al., 1982). The enzymatic activity of tPA is greatly enhanced in the presence of fibrin (Ranby, 1982; Hoylaerts et al., 1982). It is the formation of a trimolecular complex of fibrin, plasminogen and tPA which results in the activation of plasminogen to plasmin (Collen, 1987). tPA is directly inhibited by the plasminogen activator inhibitors 1 and 2 (Blasi et al., 1987; Medcalf et al., 1988).

UK and related plasminogen activators compose the second major class of PA, urokinase-type PAs. The enzyme is synthesised and secreted as a single chain glycoprotein. Single chain UK must undergo a proteolytic cleavage to yield a disulphide linked two chain form which displays a high level of activity in comparison with the single chain form (Pannell and Grewich, 1987). The two-chain species (54 kDa) has a similar catalytic domain to t-PA (Gerard et al., 1986), only one kringle domain (Gerard et al., 1986) and no finger domain (Collen and Lijnen, 1986). Urokinase has a cell binding region (Fibbi et al., 1986; Appella et al., 1987) in the amino terminus consisting of 18-32 amino acids (Appella et al., 1987).

Plasminogen activators of both higher and lower molecular weights have been reported. A low molecular weight species (31-33 kDa) of urokinase was sequenced by Steffens et al. (1982), which was found to be the product of proteolysis and was unable to bind to the cell surface receptor (Vassali et al., 1985); a 110 kDa species has been reported to be the product of tPA binding to an inhibitor (Dano et al., 1985) and a 105 kDa species expressed by thymocytes is possibly a stable enzyme-receptor complex (Fulton and Hart, 1980; Fulton and Hart, 1981).

Plasminogen is a 90 kDa single chain glycoprotein produced in the liver (Raum et al., 1980). The native form of plasminogen has an amino-terminal glutamic acid which is converted to lysine following digestion by plasmin (Saskela, 1985). Binding of plasmin to fibrin and extracellular matrix components has important implications for
enzyme activity. Binding of plasminogen to fibrin produces marked stimulation of the
activation by tPA (Takada et al., 1989). Binding to the extracellular matrix is saturable
and reversible (Knudsen et al., 1986); matrix bound plasminogen is a good substrate
for activation by tPA or UK. The plasmin formed whilst bound to the matrix is
protected from the inhibitor α2-antiplasmin (Knudsen et al., 1986).

Plasmin is a trypsin like serine protease which is activated following the
proteolytic cleavage of plasminogen at the Arg 560-Val 561 peptide bond (Robbins et al.,
1967). In addition to its role in fibrinolysis (Astrup, 1978), plasmin is able to
degrad a number of extracellular matrix proteins, such as fibronectin, laminin,
collagen types III, and IV, gelatin and cartilage proteoglycans (Review: Werb, 1990)
and to activate latent enzymes such as metalloproteinases (Mignatti et al., 1986).

1. 10. 2. Plasminogen Activators In Invasion and Metastasis.

Plasminogen activators/plasmin have been repeatedly linked with both neoplastic
growth and tumor cell invasion (Markus, 1988). The activation of plasminogen is
potentially able to produce large scale extracellular proteolytic activity, since the
zymogen plasminogen is found at high concentrations (2 μM) in the body fluids and
plasmin has a broad range of substrates both protein and proteoglycan in nature;
proteoglycan degradation involving hydrolysis of the protein portion of the molecule
(Liotta et al., 1981). The role of plasminogen activators in matrix invasion is
potentially three fold: (i) they represent proteinases which convert plasminogen to
plasmin, which has a broad range of matrix substrates, (ii) plasmin thus produced can
act as a catalyst in the activation latent enzymes and (iii) plasmin can act in synergy
with other active proteinases both to clear protected substrates allowing specific
enzymes access to their substrates. Studies using HT 1080 showed that inhibition of
PA activity not only inhibited the lysis of plasmin-sensitive glycoproteins but also
plasmin resistant substrates such as elastin and interstitial collagen, highlighting the
importance of plasmin in the turnover of a number of extracellular matrix components
(Bogenmann and Jones, 1983; Bergman et al., 1986; Heisel et al., 1983). This
indirect effect of plasmin on matrix components is perhaps best understood in the
activation of interstitial collagenase and subsequent collagen degradation (Paranjpe et al., 1980). Furthermore B16 melanoma cell invasion of the amnion basement membrane in vitro was blocked by inhibitors of plasmin and collagenase and antibodies to collagenase and UK (Mignatti et al., 1986). Involvement of plasminogen activators in metastasis has been demonstrated using epidermal carcinoma cells in a chick embryo chorioallantoic membrane model of metastasis. The invasion of the carcinoma cells through the allantoic membrane and subsequent metastasis to the embryonic lung was blocked following injection of antibodies against human urokinase into the blood stream of the embryo (Ossowski, and Reich, 1983). Further in vivo studies were performed by Ossowski (1988) who showed a requirement for plasminogen activators in both amnion invasion and intravasation. Although uncommon, UK production has been recently been associated with human melanomas which were also shown to produce tPA. Matrix degradation by UK expressing cell lines was shown to be mediated by UK rather than tPA. These cell lines were also spontaneously metastatic to the lung following s.c. growth (Quax et al., 1991).

1. 11. Tumor-Host Interactions During Metastasis.

Malignant tumor tissues frequently contain high proteinase activities, which may be dependent upon either tumor derived proteinases or host derived proteinases. The level of proteinase activity is dependent upon the balance of enzyme to inhibitor, an excess of enzyme compared to inhibitor results in active enzyme conversely an excess of inhibitor would lead to an absence of active enzyme. Tumor tissues frequently contain host cells, usually fibroblasts, macrophages and monocytes. All these types of cells have been reported to be able to express a number of proteinases and inhibitors. It is therefore conceivable that in some cases the host cells themselves may play a significant role in the degradation of the extracellular matrix during tumor invasion, either due to endogenous host cell proteinase or inhibitor expression, stimulation of tumor cell proteinase or inhibitor expression due to either soluble intercellular communication mediated by cytokines or growth factors or by the expression of specific extracellular matrix components capable of affecting tumor cell phenotype.
Alternatively tumor cells may stimulate host cell proteinase or inhibitor expression by similar mechanisms. The total enzyme: inhibitor balance of both tumor and host cells needs to be further established.

1.11.2. Interactions Between Tumor Cells and Leukocytes.

Polymorphonuclear neutrophils (PMNs) are the most abundant form of circulating white blood cell and may play an important role in regulating the viability and invasiveness of circulating tumor cells. The effects of PMNs on tumor cells are varied; some reduce experimental metastatic potential (Glaves, 1983) whilst others may potentiate experimental metastasis (Aeed et al., 1988). PMNs have been shown to increase the attachment of rat hepatocarcinoma cells to endothelial cells suggesting that they possibly increase the rate at which arrest and extravasation takes place (Orr and Warner, 1987). Current scenarios envisage that this phenomena may be due to ICAM-1: LFA-1 interactions which may facilitate metastatic spread to lymph nodes due to tumor cell: host cell adhesion and subsequent lymphocyte homing carrying bound tumor cells to the regional lymph nodes.

Intercellular adhesion molecule 1 (ICAM-1) is frequently found on cells of the melanocytic lineage, malignantly transformed cells showing a higher incidence of expression (Johnson et al., 1989; Natali et al., 1990). The expression of ICAM-1 in primary lesions from stage 1 melanoma patients has been shown to correlate with tumor thickness (Johnson et al., 1989; Natali et al., 1990), which is itself an indicator of prognosis. The frequency of expression is higher in metastases than in primary lesions, which may indicate a selective process, or alternatively because of the modulatory effects of various cytokines, possibly due to immunocyte derived cytokines (Matsui et al., 1987; Pober et al., 1986; Dustin et al., 1986; Rothlein et al., 1986; Dustin et al., 1988; Maio et al., 1988). The high frequency of ICAM-1 expression in metastases is interesting, since ICAM-1 has been shown to be a ligand for lymphocyte function antigen-1 (LFA-1) in T cell mediated cytotoxicity (Makgoba et al., 1988). However, LFA-1, LFA-3 and ICAM-1 were reported not to be the major adhesion ligands for LAK cell mediated lysis on a series of target tumor cell
lines, none of which were melanoma cell lines (Quillet-Mary et al., 1991). Current scenarios envisage that this phenomena may be a differentiation dependent event reflecting the stage of differentiation of the melanocytes from which the melanoma cells originated. Alternatively it may be that ICAM-1: LFA-1 interactions facilitate metastatic spread to lymph nodes due to tumor cell: host cell adhesion and subsequent lymphocyte homing carrying bound tumor cells to the regional lymph nodes. In addition, cytokines produced by tumor cells may act to modulate tumor and host cell behaviour; for example, recent work has shown that human melanoma cells produce IL-1 which stimulates their own ICAM-1 expression and affects adhesion to endothelial cells. This cytokine may also alter leukocyte cell behavior (Burrows et al. 1991).

1. 12. CYTOKINES.

1. 12. 2. Tumor Necrosis Factor Alpha.

Tumor necrosis factor/cachectin (TNFα) is a polypeptide hormone with a broad range of biological activities (Beutler and Cerami, 1987). Initially described as a component of the serum of endotoxin-treated mice with the ability to cause necrosis of some transplantable tumors (Carswell et al., 1975) it was later found to be capable of having a cytostatic or cytocidal effect upon some tumor cells (Williamson et al., 1983). In physiological situations it has now been found to be a mediator in the processes of host defence, immunity and tissue homeostasis, and is important in the pathogenesis of infection, tissue injury and inflammation (Tracey et al., 1989). Tumor necrosis factor and cachectin were found to be identical (Caput et al., 1986; Pennica et al., 1985). Active TNFα is composed of 3 subunits, each of which is a 157 amino acid peptide (17 kDa). Cleavage of the molecule at the cell surface is thought to be essential for the secretion of the molecule. TNFα and the related molecule (30% homology) lymphotoxin (TNFβ) bind to the same receptor and share several biological activities. TNFα is synthesised by a number of different cell types, including monocytes/macrophages, lymphocytes, natural killer cells, glomerular mesangial cells, astrocytes and microglial cells of the brain, and kupffer cells of the
liver (Tracey et al., 1989). TNFα does not exist in a stored form but is synthesised *de novo* following cell activation. The biosynthesis of TNFα is tightly regulated by transcriptional and post-transcriptional mechanisms (Beutler et al., 1986). However, Beutler et al. (1986) demonstrated that macrophages contain a pool of TNFα mRNA that is not expressed as protein.

Perhaps the most relevant activities, to this study, of TNFα are the role it plays in inflammation, tissue remodelling and cytotoxicity. TNFα has been shown to be involved in the release of other cytokines such as IL-1 (Nawroth et al., 1986) and TGFβ (Pober and Cotran, 1990). IL-1 and TNFα have the ability to alter endothelial cell phenotype, and activation of these cells results in vascular leakiness by causing a structural reorganisation of the endothelial cell layer (Brett et al., 1989). TNFα causes increased expression of intercellular adhesion molecules (ICAMs) (Munro et al., 1989), and the *de novo* expression of endothelial leukocyte adhesion molecule-1 (Bevilacqua et al., 1988), together with an increase in the expression of a number of other adhesion molecules such as inducible cell adhesion molecule or vascular cell adhesion molecule (Osborn et al., 1989). In addition TNFα, acting in concert with IL-1 and IFNγ, alters the proteinase to inhibitor balance, resulting in the degradation of basement membrane proteins by endothelial cells (Stolpen et al., 1986). TNFα may also be involved more deeply in tissue remodeling since it stimulates bone (Bertolini et al., 1986) and cartilage (Saklatvala, 1986) resorption and inhibits the synthesis of proteoglycans (Saklatvala, 1986). Furthermore, TNFα can act as a growth factor, by stimulating fibroblast and mesenchymal cell proliferation (Vilcek et al., 1986) directly, and indirectly by stimulating the production of other cytokines that mediate cell proliferation and matrix production (Tracey et al., 1989). TNFα may increase the mitogenic effect of epidermal growth factor by increasing the expression of the cell surface receptors for this cytokine (Palombella et al., 1987), and is capable of promoting neovascularisation (Frater-Schroder et al., 1987).

The TNFα receptor is a protein of molecular mass 300 kDa (Creasy et al., 1987) composed of two different subunits (Urban et al., 1986). Two different types of
TNFα receptor may exist (Imamura et al., 1987), a myeloid cell-type receptor and an epithelial cell type receptor (Hohmann et al., 1989; Yonehara et al., 1989). Soluble forms of the TNFα receptor have been detected in urine (Engelmann et al., 1990) and in sera of cancer patients (Schall et al., 1990), which may be a 'shed' form of the cell receptor which could compete with the cell surface receptor in the binding of TNFα thus acting as an inhibitor.

1. 12. 3. Transforming Growth Factor Beta.

The transforming growth factor-β family is composed of a minimum of five members showing a high degree of homology (Review: Massague, 1988). Transforming growth factor β is a multifunctional, hormone-like peptide that controls cell proliferation and differentiation in several cell lines (Sporn et al., 1986; Massague, 1987; Lyons and Moses, 1990). TGF β has been found to be expressed by a wide variety of cell types (Derynk et al., 1985) and has been detected at high levels in platelets (Assoian et al., 1983) and in bone (Seyedin et al., 1985). It is considered to be an important factor in embryogenesis, wound healing, and bone remodeling and has been shown to induce a tumorigenic phenotype in mesenchymal cells when they are co-stimulated with epidermal growth factor (Roberts et al., 1981). The effects of TGF-β are cell-type specific, it is mitogenic for fibroblasts (Leof et al., 1986) but inhibits the growth of keratinocytes and endothelial cells (Muller et al., 1987; Pietenpol et al., 1990) via the suppression of c-myc expression (Shipley et al., 1985) or dephosphorylation of the retinoblastoma gene product (Laiho et al., 1990). TGF-β stimulates wound healing processes (Sporn et al., 1986; Mustoe et al., 1987; Pierce et al., 1989), and in vitro it has been shown to increase the synthesis of extracellular matrix components, namely, fibronectin (Ignatz et al., 1987; Nickoloff et al., 1988; Wikner et al., 1988), type I collagen (Ignatz et al., 1987) and proteoglycan (Edwards et al., 1987) in mesenchymal and epithelial cells. TGF-β is known to inhibit the expression proteinases involved in degradation of the extracellular matrix, such as plasminogen activators (Laiho et al., 1986), interstitial collagenase (Overall et al., 1989), stromelysin (Matrisian et al., 1986; Kerr et al., 1988), cathepsin L (Chiang
and Nilsen-Hamilton, 1986) and elastase (Redini et al., 1988), and to modulate the action of growth factor-induced expression of interstitial collagenase (Edwards et al., 1987) and stromelysin (Kerr et al., 1990), possibly due to Fos binding (Kerr et al., 1990). In contrast, TGF-β has been reported to increase synthesis of 72 kDa type IV collagenase in gingival fibroblasts (Overall et al., 1989; Overall et al., 1991) and in primary cultures of rat bone cells (Overall et al., 1989) and both the 72 kDa and 92 kDa type IV collagenase in keratinocytes (Salo et al., 1991) and fibrosarcoma cells (Kubota et al., 1991). Furthermore, TGF-β has been shown to decrease the level of proteinase activity by increasing the expression of TIMP-1 (Overall et al., 1989; Edwards et al., 1987) and plasminogen activator inhibitor-1 (Overall et al., 1989; Kubota et al., 1991).

1.12.4. Interleukin 1.

Interleukin 1 (IL-1) is a pluripotent peptide hormone which is produced by a variety of cell types including macrophages (Gery et al., 1972; Blyden and Handschumacher, 1977; Lachman et al., 1977; March et al., 1985), lymphoid (Scala et al., 1984; Matsushima et al., 1985) and non-lymphoid cells (Sauder et al., 1982; Fontana et al., 1982). Two molecular species of IL-1 are known, IL-1α and β (March et al., 1985); IL-1β is preferentially produced by human cells and the two species share 26% homology and have similar activities operating via the same receptor (Maeger, 1990).

IL-1 is both involved in the generation of antigen specific immune responses (Mizel, 1989), inflammation and in pathological conditions of excess connective tissue degradation, including rheumatoid arthritis (Henderson et al., 1987). In vitro, IL-1 stimulated cells synthesise a number of proteinases capable of degrading extracellular matrix such as, 92 kDa gelatinase (Wilhelm et al., 1989), plasminogen activators (Mochan et al., 1986) and stromelysin (Quinones et al., 1989).
1. 13. CYTOKINE INDUCED TRANSCRIPTION FACTORS.

Changes in the transcriptional expression of genes in eukaryotic cells in response to peptide regulatory factors are mediated through DNA sequences called enhancers and promoters. These contain multiple binding sites for sequence-specific DNA-binding proteins. The occupation of cis-acting elements by trans-acting factors determines the transcriptional activity of both constitutive and inducible genes. The action of certain cytokines has been linked with the modulation of specific DNA-binding proteins.

1. 13. 2. Interleukin-1.

Shirakawa et al. (1988) and Osborn et al. (1989) found that IL-1 treatment of 70Z/3 cells, YT cells or several T-cell lines would induce the activation of NF-κB, a DNA binding protein with specificity for an 11-base pair sequence found in the regulatory elements of the genes for the κ immunoglobulin light chain (Review: Lenardo and Baltimore, 1989). IL-1 has also been reported to induce the AP-1 transcription factor (Muegge et al., 1989).

1. 13. 3. Tumor Necrosis Factor α.

TNFα has been shown to induce the transcription factor AP-1 (Brenner et al., 1989) and a NF-κB-like DNA binding protein (Lowenthal et al., 1989; Osborn et al., 1989). Induction of NF-κB binding is independent of new protein synthesis and involves dissociation of NF-κB from a cytosolic complex with an inhibitor (I-κB) and subsequent translocation from the cytoplasm into the nucleus (Baeuerle and Baltimore, 1988a; 1988b). TNFα stimulates the genes for the transcription factors c-fos (Haliday et al., 1991) and c-jun (Brenner et al., 1989; Haliday et al., 1991), and decreases the expression of c-myc in HL-60 cells (Robinson-Benion et al., 1991).

1. 13. 4. Transforming Growth Factor β.

Comparatively little is known about the tertiary messengers which are regulated by TGFβ, although recent work by Robinson-Benion et al. (1991), found a downregulation of c-myc associated with cellular responses to both TGFβ and TNFα treatments.
CHAPTER 2
MATERIALS AND METHODS
CHAPTER 2.

MATERIALS AND METHODS.

2. 1. MATERIALS.

2. 1. 1. General Laboratory Equipment and Reagents.

Chemicals.

All chemicals used in the preparation of solutions, buffers or stains were obtained from FSA Laboratory Supplies (Laboratory Grade) unless otherwise stated.

Glassware.

Detergent washed pyrex glassware was rinsed in de-ionized water and dried in a hot air oven at 80°C followed by sterilisation at 160°C for 2 hours.

Plastics and Disposable Equipment.

Plastic and disposable equipment was purchased from the suppliers listed below:

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bijoux, 10ml and 30ml centrifuge tubes</td>
<td>Sterilin Ltd.</td>
</tr>
<tr>
<td>Disposable filters</td>
<td>Schleicher and Schuell.</td>
</tr>
<tr>
<td>10ml and 1ml plastic pipettes and tissue culture flasks.</td>
<td>Costar.</td>
</tr>
<tr>
<td>Syringes and needles, Falcon bottles,</td>
<td></td>
</tr>
<tr>
<td>Flexible microtitre assay plates and Tissue culture plates</td>
<td>Becton Dickenson Ltd.</td>
</tr>
<tr>
<td>Pico Provials</td>
<td>Canberra Packard Ltd.</td>
</tr>
</tbody>
</table>
Phosphate Buffered Saline.

Phosphate Buffered Saline tablets, purchased from Oxoid Ltd., were prepared as directed, prior to sterilisation by autoclaving at 15 lb/sq in for 15 mins.

Scintillation Liquid.

Scintillation liquid was purchased from Merk Ltd. as a stock solution and stored at room temperature.

$^{3}$H-Thymidine.

$^{3}$H-Thymidine was purchased from Amersham International Ltd. as a stock solution with an activity of 37 MBq/ml and was stored at $4^\circ$C.

Water.

De-ionized water for rinsing glassware was obtained by passing tap water through an Elgastat de-ionizer resulting in a specific conductivity of $>10^6$ Ohm/cm$^3$.

Distilled water for all other purposes was purified to a specific conductivity of 5mS by reverse osmosis using a Milli-Ro 6 Plus system (Millipore (UK) Ltd.). This distilled water was then sterilised by autoclaving at 15 lb/sq in for 15 mins, and for the remainder of this study termed simply water.
2. 1. 2. Tissue Culture Reagents.

Stock Solutions, Buffers and Supplements.

EDTA used to remove adherent tumour cell lines from plastic bottles was purchased from BDH made to 0.02 % (w/v) in PBS, autoclaved at 10 lb/sq in for 10 min and stored at 4°C.

Fetal calf serum, (FCS), purchased from Northumbria Biologicals Ltd. was stored at -20°C in 50 ml aliquots. All FCS used throughout this work was heat inactivated at 56°C for one hour and stored at -20°C until use.

Fungizone, was purchased from Gibco Chemical Co. was divided into 1 ml aliquots and stored at -20°C until use.

Glucose, purchased from Sigma Chemical Co. and dissolved in sterile water at a concentration of 200 g/l and stored at -20°C until use.

L-Glutamine, purchased from Flow Laboratories Ltd., as a 200mM solution and was stored at -20°C in 1 ml aliquots.

New born calf serum, (NBCS), purchased from Life Technologies Ltd. was stored at -20°C in 50 ml aliquots. All NBCS used throughout this work was heat inactivated at 56°C for one hour and stored at -20°C until used.

Non-essential amino acids, (NEAA) were purchased from Life Technologies Ltd. and stored at 4°C until use.

Penicillin, purchased from Glaxo Laboratories Ltd. and dissolved in sterile distilled water at a concentration of 100,000 U/ml prior to storage in 2 ml aliquots at -20°C until use.
Streptomycin, purchased from Glaxo Laboratories Ltd. and dissolved in sterile distilled water at a concentration of 100 U/ml prior to storage in 2 ml aliquots at -20°C until use.

Sodium bicarbonate solution, final concentration of 7.5% (w/v) in water and sterilised by autoclaving at 10 lb/sq in for 10 min and stored at 4°C until use.

Thioglycollate broth tablets, purchased from Oxoid Ltd., were prepared as per instructions and sterilised by autoclaving at 15 lb/sq in for 18 mins before storage at room temperature.

Trypan blue powder was prepared to a 0.1% (w/v) solution in PBS, residual, undissolved powder was removed by filtration before sterilisation by autoclaving at 15 lb/sq in for 15 mins and storage at room temperature.

Trypsin, purchased from Life Technologies Ltd. as a sterile 2.5% (v/v) (x10) solution, was diluted to a 0.25% (v/v) solution with sterile PBS, aliquoted and stored frozen at -20°C.
Media.

RPMI 1640 medium, purchased from Life Technologies Ltd. as a 1 x concentrate without glutamine, 10 ml of L-glutamine (200 mM) was added immediately prior to use.

Dulbecco's medium, purchased from Life Technologies Ltd. as 10 x concentrate, was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco's (10 x)</td>
<td>50 ml</td>
</tr>
<tr>
<td>Water</td>
<td>405 ml</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>25 ml</td>
</tr>
<tr>
<td>NEAA</td>
<td>5 ml</td>
</tr>
<tr>
<td>1M NaOH</td>
<td>5 ml</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
Posterior uveal melanoma medium, was composed of the following: Hams F12 basal medium containing l-glutamine, purchased from Sigma chemical Co. Ltd as a 1x media was supplemented as follows:

- Hams F12 500 ml
- FCS 25 ml
- Donor horse serum 75 ml
- Glucose 6 ml of 200 g/l stock
- EGF 60 μl of 100 μg/ml stock
- Penicillin 0.6 ml
- Streptomycin 0.6 ml
- Fungizone 6 ml

All media was stored at 4°C until use.

**Sterility Check.**

The sterility of each batch of media was checked before use. 1ml of medium was used to inoculate each of two universals of thioglycollate broth, whereupon they were incubated for 1 week at 32 and 37°C respectively after which time the media was considered sterile if the broth had remained clear.

**Mycoplasma Test.**

Cell lines were tested for contamination with a mycoplasma tissue culture rapid detection system (Laboratory Impex Ltd.) on a regular basis.
2. 1. 3. Electrophoresis Reagents.

All reagents were of electrophoresis grade and once prepared were kept at 4°C in the dark for not longer than 4 weeks, reagents such as ammonium persulphate were prepared fresh daily. Water used for electrophoresis applications was of the purest grade available.

2. 1. 4. Cytokines and Growth factors.

Recombinant human interleukin 1 alpha (IL-1α) was a generous gift of NIBSC with specific activity of 1x10^8 U/mg and Hoffman La Roche with specific activity of 2-3x10^8 U/mg. Dilution with RPMI 1640 medium, 2 % (v/v) BSA to 1x10^6 U/ml occurred prior to storage at -80°C.

Recombinant human interleukin 2 (IL-2) was a generous gift from the Glaxo Institute for Molecular Biology, Geneva, Switzerland with a specific activity of 1.6 x 10^6 U/mg and was reconstituted in RPMI 1640 and 0.2 % BSA prior to storage at -80°C.

Recombinant human interleukin 4 (IL-4) was generous gift from Sandoz Pharmaceuticals Ltd. Basel, Switzerland and was reconstituted in PBS prior to storage at -80°C.

Recombinant human interleukin 6 (IL-6) was a generous gift from ICI Pharmaceuticals with a specific activity of 4.1x10^7 U/mg and was reconstituted in PBS prior to storage at -80°C.

Recombinant human interferon alpha (IFNα) was a generous gift from Wellcome Foundation Ltd., Beckenham, Kent, England. It was supplied at 6x10^7 U/mg and made up to 1x10^5 U/ml in PBS containing 1 % BSA prior to storage at -80°C.

Recombinant human interferon gamma (IFNγ) was a generous gift from Dr. G. R. Adolf, Boehringer-mannheim, Vienna. Purified protein (>99 %, <2.5 EU/mg by L.A.L.) derived from Escherichia coli was supplied lyophilised with a specific activity of 2x10^7 IU/mg. Reconstitution with sterile 0.9 % (w/v) NaCl and dilution to
1x10^6 IU/ml with RPMI 1640, 0.2 % (v/v) BSA, occurred immediately before storage in aliquots at -80°C. 

Recombinant human tumor necrosis factor-alpha (TNFα) was a generous gift from Strangeways Laboratory, Cambridge. The specific activity was 2.7x10^8 U/mg. Dilution with RPMI, 0.2 % (v/v) BSA to 1x10^7 or 1x10^6 U/ml occurred immediately before storage in aliquots at -80°C. Alternatively, recombinant human Tumor necrosis factor alpha was a gift from Boehringer-Mannheim, specific activity of 6x10^7 U/mg.

Recombinant human transforming growth factor beta 2 (TGFβ2) was a generous gift from Sandoz pharmaceutical division, Basel, Switzerland with a specific activity of 2.8x10^4 U/mg.

Epidermal growth factor (EGF) was a generous gift from ICI and was reconstituted in water to give a stock solution of 1 mg/ml, aliquotted and stored at -80°C until use.

Insulin like growth factor 1 (IGF 1) was purchased from Kabi Pharmacia and was reconstituted in PBS to give a stock solution of 100 µg/ml, aliquotted and stored at -80°C until use.

Insulin like growth factor 2 (IGF 2) was purchased from Kabi Pharmacia and reconstituted in 0.5 % acetic acid to give a stock solution of 100 µg/ml, aliquotted and stored at -80°C until use.

Platelet derived growth factor (PDGF) was purchased from British Biotechnology and was reconstituted in 4 mM HCl + 1 mg/ml BSA to give a stock solution of 2 µg/ml which was aliquotted and stored at -80°C until use.

Acidic fibroblast growth factor (aFGF) was purchased from British Biotechnology with a specific activity of 1-5x10^7 U/mg and was reconstituted in PBS + 0.1 % BSA, aliquotted and stored at -80°C until use.

55
2. 1. 5. Antibodies.

FITC. Fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse F(ab')₂ antibodies was purchased from Caltag Laboratories Inc. 100 μl of 1/60 dilutions were used in MoAb staining tests as determined by titration assays.

HLA-DR. The monoclonal antibody against class II MHC antigens was purchased from Dakopatts, Denmark, and stored at 4°C. Aliquots of 5 μl were used in MoAb staining tests as determined by titration assays.

ICAM-1. The monoclonal antibody against ICAM-1 was a generous gift of Dr. Nancy Hogg, Imperial Cancer Research Fund Laboratories. 20 μl aliquots were used in MoAb staining tests as determined by titration assays. Aliquots were stored at -80°C.

IL-1α. Sheep anti IL-1α polyclonal antibody was a generous gift from NIBSC and was aliquotted and stored at -80°C.

TNFα. Goat anti TNFα polyclonal was a generous gift from NIBSC and was aliquotted and stored at -80°C.

W6/32. The monoclonal antibody against class I MHC antigens was a generous gift of Dr. Keith Gelsthorpe, Blood Transfusion Centre, Sheffield. After titration, aliquots of 10 μl were used in MoAb staining tests. Aliquots were stored at -80°C.

2. 1. 6. Maintenance of the Nude Mouse Colony.

Male and female, 4 to 6 week old, athymic Balb/c nude mice were bred and maintained under sterile conditions. Animals were maintained under the guide lines of the Home Office. All protocols were reviewed and approved by the Home Office. Mice were fed sterilised rodent chow and tap water ad libitum.
2. 2. METHODS: IN VIVO PROCEDURES.

2. 2. 1. Preparation of Cells for Subcutaneous and Intradermal Injection.

Subconfluent cultures were detached using 0.02 % EDTA, resuspended in Dulbecco's medium containing 10 % FCS, centrifuged at 400 g for 6 minutes and then resuspended in ice-cold PBS. Cell counts were done using a hemocytometer. Cell cultures were used that were >95 % viable as determined by trypan blue exclusion. Cells were diluted such that the required number of cells were present in 100 μl of ice-cold PBS. Cells were placed on ice until use.

2. 2. 2. Experimental Metastasis Assay.

Subconfluent cultures were detached using 0.02 % EDTA, resuspended in Dulbecco's medium containing 10 % FCS, centrifuged at 400 g for 6 minutes and then resuspended in ice-cold PBS. Cell counts were done using a hemocytometer. Cell cultures were used that were >95 % viable as determined by trypan blue exclusion. Cells were adjusted to a final concentration of 1×10^7 cells/ml by the addition of ice-cold PBS; mice received 1×10^6 viable cells in 0.1 ml into the lateral tail vein. Animals were killed 12 weeks post inoculation by cervical dislocation following etherisation and subjected to gross necropsies. Lungs were either fixed in neutral buffered formal saline and the extent of lung colonisation determined or establishment of cell cultures was attempted.

2. 2. 3. Spontaneous Metastasis Assay.

Subconfluent cultures were prepared as described above except that cells were titrated prior to subcutaneous injection such that the final number injected was in the range 1×10^6-1×10^4 in a volume of 0.1 ml. Tumors were resected when they reached approximately 0.8 cm^2, the wound was sealed either with clips or sutured, invasion of the body wall was rare. The animals were then observed for greater than 12 weeks to determine if spontaneous metastasis had occurred. Animals were killed by cervical
dislocation following etherisation and subjected to gross necropsies. Lungs and abdominal organs were visually examined for the presence of tumors. Tumors, if detected, were established in vitro as cell cultures

2.2.4. In Vivo Growth Kinetic Calculations.

To determine in vivo growth kinetics, tumors were measured weekly at orthogonal angles. Mean tumor diameter was calculated:

\[
\text{Mean tumor diameter} = \sqrt{\frac{X}{(Y)}}
\]

where \(X\) and \(Y\) are the diameters (mm) of two orthogonal measurements.

Doubling time was calculated according to the following equation (Welch et al., 1991):

\[
DT = \frac{0.1 \cdot (t_x - t_0)}{\log(diam_x) - \log(diam_y)}
\]

where \(t_x-t_0\) is the difference in time (in days) between tumor measurements, and \(diam_x\) is the mean diameter of the tumor at time= \(x\)

2.3. IN VITRO PROCEDURES.

2.3.1. Cutaneous Melanoma Tissue Culture.

Human cutaneous melanoma cell lines A375, DX3, SK23 and LT5.1 were a generous gift from Dr I. R. Hart (Imperial Cancer Research Fund, London). Cells were routinely grown in vented tissue culture flasks (Costar, Cambridge, MA, U.S.A) in Dulbecco's medium (Gibco, Paisley, Scotland) supplemented with 10 % FCS (Sera-lab, Sussex, England) and no antibiotics at 37°C in a humidified incubator (5 % CO₂ and 95 % air).
2. 3. 2. Establishment of Nude Mouse Metastatic Lesions *In Vitro.*

Tumor tissue derived from growth in nude mice was processed as follows: the tissue was excised; if the tissue was from either a subcutaneous or intradermal tumor, it was cut free of the surrounding host tissue, and divided into a number of aliquots; typically three, one aliquot was immediately snap frozen in liquid nitrogen vapour, another was formalin fixed and the remaining tissue was placed in sterile PBS prior to transport to the tissue culture laboratory. Lung derived colonies were treated in a similar manner, however, the quantity of tumor tissue was typically much smaller, and in consequence, storage in nitrogen vapour and formalin fixation was infrequent. The lungs as a whole were washed in sterile PBS prior to transport, in PBS, to the laboratory. Tumor tissue derived from subcutaneous or intradermal lesions was mechanically disaggregated into fragments of 1mm³ with scalpels, and seeded onto 75 cm² vented tissue culture flasks in Dulbecco's medium containing 20 % FCS and antibiotics (streptomycin, penicillin and fungizone).

Discrete lung lesions were dissected from the lung tissue by a variety of techniques. Dependent upon the size of the tumor colony, the neoplasm was excised with either a scalpel or picked off with the aid of a sterile needle. Disaggregation of the nodule was achieved either by mincing, as described previously, if the tumor was relatively large, or by gentle rubbing of the nodule against the growth surface of a 12 well tissue culture treated plate. Culture were established in Dulbecco's medium containing 20 % FCS and antibiotics at 37°C in a humidified incubator (5 % CO₂ and 95 % air).
2. 3. 3. Establishment and maintenance of Posterior Uveal Melanoma Cell Lines \textit{in vitro}.

Human posterior uveal melanoma cell lines were established from fresh biopsy tissue as short term cultures. After enucleation, the globe was bisected and samples of tumor tissue removed and placed in growth medium. The tumor tissue was minced into fragments of 1mm$^3$, and digested using a cocktail containing collagenase type II (0.025 %), pronase E (0.05 %) and dithiothreitol (0.5 μM) (Sigma, Poole, England) for 2 hours at 37°C. The tumor cell suspension was washed twice with growth medium and pelleted by centrifugation at 400 g for 6 minutes. The washed cell suspension was seeded onto 75 cm$^2$ vented tissue culture flasks in posterior uveal melanoma medium and cultured at 37°C in a humidified incubator in an atmosphere of 5 % CO$_2$ and 95 % air.

2. 3. 4. Cytogenetic Analysis of Melanoma Cell Cultures.

Cell cultures were harvested after 1 to 6 weeks in culture by the addition of 0.05 mg/ml colcemid for four hours, treated with 0.0375 M KCL for 15 minutes, spun down and and fixed immediately with 3: 1 methanol: acetic acid in water. Chromosome preparations were made using cold wet slides. Slides were G banded using trypsin and stained with Leishmans. Where a single abnormal or extra chromosome was observed or a missing chromosome was seen in only two cells, they were considered non-clonal and excluded

2. 3. 5. Coating of Tissue Culture Surfaces with Collagen type I.

Collagen (Sigma Chemical Co.) was added to sterile filtered 0.1 M acetic acid to obtain a 0.01 % collagen solution and stirred at room temperature until dissolved, typically 3 hours. Culture vessels were coated at concentrations of 5, 10, and 15 μg/cm$^2$ and allowed to dry for 48 hours at 37°C. The culture surface was rinsed with PBS prior to the addition of cells and/or medium.
2. 3. 6. Coating of Tissue Culture Surfaces with Collagen type IV.

Collagen (Sigma Chemical Co.) was reconstituted to a concentration of 2 mg/ml in filter sterilised 0.25 % acetic acid and allowed to dissolve overnight with gentle stirring at 4°C. Culture vessels were coated at concentrations of 5, 10, and 15 μg/cm² and allowed to dry overnight at 37°C. The culture surface was rinsed with PBS prior to the addition of cells and/or medium.

2. 3. 7. Coating of Tissue Culture Surfaces with Fibronectin.

Fibronectin (Sigma Chemical Co.) was reconstituted with ice cold sterile water to a concentration of 1 mg/ml and allowed to dissolve for 30 minutes at room temperature. The fibronectin was diluted with sterile PBS to a final concentration of 1 mg/ml and used to coat culture vessels at final concentrations of 1, 5, and 10 μg/cm² and allowed to dry at room temperature for 1 hour.

2. 3. 8. Coating of Tissue Culture Surfaces with Laminin.

The laminin (Sigma Chemical Co.) was thawed slowly at 4°C to avoid the formation of a gel. Laminin was diluted in PBS to a concentration of 1 mg/ml and used to coat the culture vessel surface at concentrations of 1, 2.5, and 5 μg/cm² and allowed to dry at room temperature for 1 hour prior to the addition of cells or medium.

2. 3. 9. Collection of Serum Free Melanoma Cell Conditioned Medium.

Human cutaneous and posterior uveal melanoma cells were seeded at a concentration of 2×10⁵ cells/well in 12 well tissue culture plates and cultured either overnight or for three days respectively in their usual growth medium. The serum containing medium was removed and the cells washed three times with PBS for one hour at 37°C and then further cultured in 1 ml of serum free medium in the presence or absence of cytokines or growth factors; for posterior uveal melanoma cultures serum free medium contained neither serum, antibiotics nor EGF. Cell cultures were
examined microscopically at both the beginning and the end of the experiment, to determine if the cytokines or growth factors under investigation were affecting the cellular morphology, culture appearance, or having a cytotoxic effect on the melanoma cell cultures. The serum free culture supernatants were collected and centrifuged at 15000 g for 5 minutes to pellet any non-adherent cells or cell debris. The supernatants were then aliquotted and stored at -80°C. As a control the medium which the cytokine was reconstituted in was placed in an empty well and incubated under identical conditions as the experimental wells, this medium was used as a blank in all subsequent analyses.

2. 3. 10. Preparation of Cell Lysates.

Cell lysates were prepared by a modification of the method of Murphy et al. (1989). Cell extracts in Triton X-100 (Sigma, Poole, England) were prepared to examine total cellular enzyme activities. Cell cultures were treated in an identical manner, however after removal of the conditioned medium, the cells, 1x10⁶ per 100 μl of lysis buffer, were solubilised at 4°C for 30 minutes using lysis buffer (0.1 % Triton X-100, 5 mM CaCl₂ in 50 mM Tris-HCl buffer pH 7.4), scraped with a rubber policeman, and stored at -80°C after centrifugation at 15000 g for 5 minutes.

2. 3. 11. Triton X-114 Phase Separation of Membrane Proteins.

Separation of integral membrane proteins was attempted using the method of Bordier (1981). Cell extracts in Triton X-114 (Sigma, Poole, England) were prepared as for Triton X-100 cell extracts, however the cells were incubated in a salted ice bath for 30 minutes rather than at 4°C in lysis buffer containing Triton X-114 not Triton X-100. The cell lysate, typically 100 μl, was layered on a 150 μl cushion of 6 % (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.06 % Triton X-114 placed at the bottom of a 1.5 ml conical eppendorf tube. The clear sample was incubated for 5 minutes at 37°C, causing clouding of the solution. The tube was centrifuged for 5
minutes at 400 g at room temperature in a swinging bucket rotor. Following centrifugation the detergent phase was identified as a pellet at the base of the tube. The separation was then repeated, following addition of of 0.5 % Triton X-114 to the aqueous phase. After dissolution of the Triton X-114 by placement in a salted ice bath for 15 minutes the mixture was again overlayed on the sucrose cushion used previously and incubated for 5 minutes at 37°C and centrifuged as described previously.

The final separation, to extract any remaining lipophilic proteins from the remaining aqueous phase, involved rinsing with 2 % Triton X-114 in a separate tube without a sucrose cushion. The detergent phase of this extraction was discarded. Finally buffer or Triton X-114 was added to the respective detergent and aqueous phases to ensure equal volumes, salt and surfactant concentrations. As a control, ovalbumin (1 mg/ml), a hydrophilic protein, was extracted in a parallel tube and its presence in the detergent and aqueous phases monitored by coomassie brilliant blue R 250 staining of the gel.

2.3.12. Antibody Inhibition of Cytokine Mediated Effects.

To determine the specificity of the cytokine mediated effects on gelatinase expression TNFα and IL-1α were preincubated with neutralising antisera to the respective cytokines. One neutralising unit of antisera will neutralise 10 units of cytokine. Preincubation, of 100 Units of TNFα and IL-1α, with 11 neutralising units and 110 neutralising units of specific antiserum for 1 hour at 40°C was used to confirm the role of these cytokines in the biological effects previously demonstrated. Controls were the medium which the cytokine was reconstituted in as a blank, cytokine alone as a positive control and antisera alone as a negative control. The resulting conditioned medium was analysed by zymography for the presence of gelatin degradation.
2. 3. 13. Concentration of Melanoma Cell Conditioned Medium.

Melanoma cell conditioned medium was concentrated using either Amicon centricom centrifugal concentrators or if the volume was greater than 15 ml with an Amicon stirred cell, both membrane types had molecular weight cut off values of 10 kDa. The conditioned medium was concentrated by following the manufacturers instructions.


Identification of gelatinolytic enzymes expressed by human cutaneous and human posterior uveal melanoma cells was performed by electrophoresis of conditioned medium or cell lysate in a gelatin containing SDS polyacrylamide gel, followed by washing of the gel in a 2 % solution of Triton X-100, incubation in a buffer appropriate for enzyme activity for 48 hours and finally staining with either amido black 10 B or coomassie brilliant blue R 250, based on the method of Heussen and Dowdle (1980). Electrophoresis was carried out by the method of Laemmli (1970) in polyacrylamide gels containing 1mg/ml gelatin. In some experiments casein hydrolysate was incorporated into SDS containing polyacrylamide gels.
Preliminary experiments determined that degassing of the gels was not required for consistently reproducible results and the following gel recipes were used. Polyacrylamide resolving minigel slabs measuring 0.75 x 85 x 45 mm were cast from a mixture prepared from stock solutions as follows:

(i) Acrylamide (29 %) and bisacrylamide (1 %) in water 875 µl

(ii) 1.5 M Tris-HCl, pH 8.8, containing 0.4 % SDS 875 µl

(iii) Gelatin: 1 % in water 350 µl

(iv) Ammonium persulphate: 100 mg/ml in water 30 µl

(v) TEMED 3 µl

(vi) Water 1400 µl

Volume used for the resolving gel was 3 ml.

The resolving gels were carefully overlaid with 100 µl of butanol and allowed to polymerise at room temperature. When polymerisation was complete, the butanol was removed by washing with water, typically three times, the glass plates were carefully dried with blotting paper and a stacking gel with castellated sample wells was cast on top of the resolving gel from the following mixture of stock solutions:

(i) Acrylamide (30 %) and bisacrylamide (1 %) in water 330 µl

(ii) 0.5 M Tris-HCl, pH 6.8, containing 0.4 % SDS 690 µl

(iii) Ammonium persulphate: 100 mg/ml 30 µl

(iv) TEMED 3 µl

(v) Water 1730 µl
Volume used for the stacking gel was 2 ml.

800 ml of Running buffer (0.25 M Tris, 0.2 M glycine, pH 8.5, 0.1% SDS) was added to the top and bottom reservoirs of a Biorad Mini Protean II electrophoresis tank and 20 μl of a mixture of 100 μl sample plus 33 μl of non-reducing sample buffer (0.5 M Tris-HCl, pH 6.8, 10 % SDS, 32 % glycerol, 0.1 % bromophenol blue) was loaded in the sample wells.

Electrophoresis was performed at room temperature at a constant voltage of 200 V. When the tracking dye front had reached the bottom of the gel (after 36 minutes) the gel was removed from its cassette and shaken gently at room temperature for 1 hour in 50 ml of 2 % Triton X-100, and then rinsed three times in 50 ml of 50 mM Tris-HCl pH 7.4, containing 200 mM NaCl and 5 mM CaCl2 for 5 minutes and incubated for 48 hours at 37°C in 50 ml of the same buffer. Gels were fixed and stained for 15 minutes in either a solution of 0.1 % amido black or coomassie brilliant blue R 250 in 30 % methanol and 10 % acetic acid in H2O and destained in an identical solution containing no stain. Gelatinolytic enzymes were detected as transparent bands on the blue background of the stained gel (reagents from BDH, Poole, England).
Alternatively resolving gels would contain an identical concentration of casein hydrolysate in place of gelatin and would be treated in the same manner to determine the presence of caseinolytic enzymes. Plasminogen activators could be detected in a similar manner, however the gel recipe had the following modifications:

(i) Acrylamide (30 %) and bisacrylamide (0.8 %) in water 1060 μl

(ii) 1.5 M Tris-HCl, pH 8.8, containing 0.4 % SDS 780 μl

(iii) Casein 2 % in 0.375 M Tris-HCl, 0.1 % SDS, pH 8.8 350 μl

(iv) Plasminogen: 1mg/ml in 0.375 M Tris-HCl, 0.1 % SDS, pH 8.8 20.5 μl

(v) Ammonium persulphate: 100 mg/ml in water 30 μl

(vi) TEMED

(vii) Water 1230 μl

Control gels did not contain plasminogen, the volume was made up with 0.375 M Tris-HCl, 0.1 % SDS, pH 8.8 buffer. Gels were treated in an identical manner but were incubated in 0.1 M Tris-HCl, pH 8.1 for 48 hours. Differences in band position between casein and casein/plasminogen gels could be used to identify plasminogen dependent lysis of casein. Reduced molecular weight markers were used to estimate apparent molecular weight values for bands of substrate degradation on all types of substrate gels.
The molecular weight marker solution was composed as follows:

(i) phosphorylase b 300 μl of a stock solution of 0.5 mg reconstituted in 300 μl of 50 % glycerol.

(ii) bovine serum albumin 10μl of a stock solution of 25 mg reconstituted in 500 μl of 50 % glycerol.

(iii) ovalbumin 10μl of a stock solution of 5 mg reconstituted in 100 μl of 50 % glycerol.

(iv) carbonic anhydrase 10 μl of a stock solution of 5 mg reconstituted in 100 μl of 50 % glycerol.

(v) 60 μl of 50 % glycerol.

(vi) 125 μl of reducing sample buffer

This gives a final protein concentration of 1 mg/ml.

Reduced standards were boiled for 5 minutes with reducing sample buffer (0.5 M Tris-HCl, pH 6.8, 10 % SDS, 32 % glycerol, 0.1% bromophenol blue containing 2.5 % mercaptoethanol), 20 μl of the reduced standard solution were run on each gel. For each sample analysed control serum free medium was run in parallel to ensure that bands of degradation were not due to medium contaminants.

2. 3. 15. Activation of Latent Metalloproteinases using p-APMA.

Activation of latent metalloproteinases was achieved using a modification of Stetler-Stevenson et al. (1989). Stock solutions of 10 mM para-aminophenylmercuric acetate (p-APMA) (Sigma, Poole, England) in 0.05 M NaOH were prepared fresh daily. Conditioned medium was incubated with a final concentration of 1 mM p-APMA for varying times (0-24 h) at 37°C. Control tubes
contained the same volume of 0.05 M NaOH as the experimental tubes. Following incubation with p-APMA samples and controls were analysed by zymography.

2. 3. 16. Use of Proteinase Inhibitors.

Proteinase inhibitors were prepared fresh daily and used as follows:

(i) Ethylenediaminetetraacetic Acid (EDTA): a stock solution of 100 mM EDTA in water was prepared. 5 ml of this solution was added to 40 ml of incubation buffer; the pH of the buffer was determined and adjusted to its original pH, the final volume was adjusted to 50 ml with water. Control gels were incubated in incubation buffer alone.

(ii) 1, 10 Phenanthroline: a stock solution of 100 mM 1, 10 phenanthroline in dimethyl sulphoxide (DMSO) was prepared. 5 ml of this solution was added to 45 ml of incubation buffer; and no alteration of pH was observed. Control gels were incubated in (i) 50 ml of buffer containing 5 ml of DMSO and (ii) incubation buffer alone.

(iii) Phenylmethylsulfonyl Fluoride (PMSF): a stock solution of 10 mM PMSF in isopropanol was prepared. 10 ml of this solution was added to 40 ml of incubation buffer, no alteration of pH was observed. Control gels were incubated in (i) 50 ml of buffer containing 10 ml of isopropanol and (ii) incubation buffer alone.

(iv) trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64): a stock solution of 10 mM E-64 in DMSO was prepared. 10 ml of this solution was added to 40 ml of incubation buffer, no alteration of pH was observed. Control gels were incubated in (i) 50 ml of buffer containing 10 ml of DMSO and (ii) incubation buffer alone.

(v) N-Ethylmaleimide (Net-Mal): a stock solution of 100 mM N-ethyl maleimide in DMSO was prepared, 10 ml of this solution was added to 40 ml of incubation
buffer, no alteration of pH was observed. Control gels were incubated in (i) 50 ml of buffer containing 10 ml of DMSO and (ii) incubation buffer alone.

2. 3. 17. Plasminogen Activator Activity Determination.

The activity of UK and tPA in conditioned media collected from melanoma cell cultures was determined using the chromogenic assay of Leprince et al. (1989). This coupled enzyme reaction is reported to be capable of distinguishing between UK and tPA activity, since tPA activity requires the presence of soluble fibrinogen fragments for activity (Lee et al., 1988). Step 1 of the assay allows plasmin to be generated from plasminogen due to the action of plasminogen activators, which are active at pH 8.5, the pH is decreased to 7.5 in step 2, thereby inhibiting the activity of plasminogen activators but promoting the activity of plasmin. Step 2 of the assay allows detection of plasmin due to cleavage of the synthetic substrate N αCBZ-1-Lysine thiobenzyl ester (Sigma Chemical Co.). The cleaved substrate then acts upon 5,5'-Dithiobis-(2-nitrobenzoic Acid) (DTNB) (Sigma Chemical Co.) to produce a yellow colour, the intensity of which is dependent upon the quantity of DTNB cleaved, which may be measured using a spectrophotometer.

A solution containing 5 µl of sample, 5 µl of 0.4 mg/ml plasminogen (Kabi Vitrum), reconstituted in assay buffer (0.1 M Tris-Glycine buffer, pH 8.5 containing 0.5 mg/ml bovine serum albumin (Sigma, Poole, England)) and where appropriate, to measure tPA activity, 10 µl of fibrinogen fragments (0.5 mg/ml in assay buffer) (American diagnostics) was made up to 30 ml with assay buffer and the plates incubated for 2 hours at 37°C (Step 1). 270 µl of a reagent mixture containing 100 parts 0.2 M sodium phosphate/sodium chloride pH 7.5, 1 part each of 0.1 % Triton X-100 in water, 22 mM DTNB in 50 mM sodium phosphate (Sigma, Poole, England) and 20 mM N αCBZ-1-Lysine thiobenzyl ester in water (Sigma, Poole, England) was added to each well and the reaction allowed to proceed for 4 hours at 37°C (Step 2). Optical density was measured at 405 nm in a Titertek multiscan microplate spectrophotometer.
Fig. 2. Arrangement of samples for the simultaneous measurement of UK and tPA activity on a 96 well plate.

- **10 µl of fibrinogen fragments**
- **5 µl of plasminogen**

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<tr>
<td>B</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>5 mIU UK</td>
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<td>C</td>
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**B-A = TOTAL PA ACTIVITY**

**C-A = UK ACTIVITY**

**B-C = tPA ACTIVITY**

Seven samples were measured on the same 96 well plate. 5 µl of sample is added to each of the wells 1-9. The first three wells (A) receive no plasminogen and measure plasminogen independent lysis of the substrate due to other proteases present in the conditioned medium. The first 6 wells receive fibrinogen fragments and are used for the determination of total PA (tPA and UK) activity (B-A). The last six wells receive no fibrinogen fragments and the values of wells 7 to 9 (C) minus the values of wells 1 to 3 (A) represents endogenous UK activity (C-A). TPA activity is the difference between total PA activity and UK activity (B-C). The bottom row receives no sample (1 to 6) and is used to determine spontaneous hydrolysis of the substrate or 5 mIU of UK which is used as an internal control between plates.
PA activities are the mean value of triplicate sample determinations deduced from a standard curve titration using UK and tPA standards assayed in triplicate. All measurements of standards and samples were performed such that PA determinations were in the linear range of the standard curve. All reagents were from BDH Chemical Co Ltd, Poole, England) unless otherwise stated. Storage of UK and tPA standards, plasminogen, fibrinogen fragments, DTNB and NαCBZ-L-Lysine thiobenzyl ester was at -80°C prior to assay. All buffers were prepared fresh on the day of use. Control wells were set up to measure plasminogen independent lysis of the substrate (no plasminogen added) and spontaneous lysis (5 µl of assay buffer instead of sample).

2. 3. 18. Proliferation Assays.

Melanoma cells were seeded onto flat-bottomed 96 well plates in 200 µl of Dulbecco's medium containing 10% FCS at a concentration of 4x10⁴ cells/well, and allowed to equilibrate for 24 hours at 37°C in an atmosphere of 5% CO₂ and 95% air. Following two 1 hour washes with PBS at 37°C, the cells were incubated in serum free medium containing cytokines, either used alone or in combination, for 24 hours in the conditions described previously. After this initial incubation, the cultures were pulsed with 0.5 µCi ³H-thymidine/well for 4 hours. Cellular ³H-thymidine incorporation was determined following the addition of 100 µl of concentrated trypsin/well for 20 minutes; and harvesting of this suspension onto filter paper using a Skatron cell harvester (Skatron, Suffolk, England). The dried filter papers, each corresponding to a single well, were counted in scintillation fluid, using a betaspectrophotometer (Canberra Packard Ltd., Bedfordshire, England).

The cell cycle kinetics of cutaneous melanoma cell cultures maintained in vitro was determined using a DNA analysis kit (Becton Dickinson Ltd., Oxford, England). Alterations in cell cycle kinetics due to cytokine treatment was studied by the following method; the cells were detached from their growth surface using 0.02 % EDTA, centrifuged at 400 g for 6 minutes to pellet the cells. To a pellet of 1x10^6 cells, 300 μl of solution A (trypsin in citrate stabilizing buffer) was added and incubated for 15 minutes at room temperature with continuous agitation. Step 2 involved the addition of 200 μl of solution B (trypsin inhibitor and Ribonuclease A in citrate stabilizing buffer) and incubation for a further 15 minutes. The samples were then centrifuged at 400 g for 6 minutes and stained with 200 μl of solution C (propidium iodide and spermine tetrahydrochloride in citrate stabilizing buffer) for 30 minutes at room temperature. The cell cycle was analysed using an orthocyte benchtop flow cytometer attached to a PC computer and the % of cells in G0/G1, S and G2/M phases of the cell cycle was determined. The G0/G1 peak channel number and the coefficient of variation (C.V.) of this peak were recorded and used to produce histograms. Samples with a C.V. greater than 10 channels were considered to be of poor quality due to inadequate cellular or DNA staining.

2. 3. 20. Flow Cytometric Analysis of Posterior Uveal Melanoma Cell Cycle Kinetics.

After enucleation, the globe was bisected and samples of tumor tissue removed and placed in growth medium. The tumor tissue was minced into fragments of 1 mm^3 and digested using a cocktail containing collagenase type II (0.025 %), pronase E (0.05 %) and dithiothreitol (0.5 μM) (Sigma, Poole, England) for 2 hours at 37°C. The tumor cell suspension was washed twice with growth medium by centrifugation
at 400 g for 6 minutes and following a final wash in ice-cold PBS the suspension was held on ice at 4°C.

Cell suspensions were filtered through a 50 μm mesh to remove cell clumps prior to DNA staining with the intercalating fluorochrome, propidium iodide (PI) (Sigma Chemical Co Ltd, UK). Staining was as follows, 0.2 % Triton X-100 was used to permeabilise cell membranes (100 μl/10⁶ cells) and DNA was stained with 20 μg/ml PI for 15 minutes at room temperature.

Cell suspensions were analysed using a FACS 420 flow cytometer with an accessory Consort 30 computer (Beckton Dickinson [UK] Ltd) using a 488 nm excitation laser wavelength, and measuring the emission fluorescence of PI through a 530 nm band pass filter. A minimum of 10,000 cells were analysed for each sample. The intensity of the fluorescence was considered proportional to their DNA content.

Cell cycle measurements were made using the planimetric method of Chrisman et al. (1974) and Dean (1980) assuming that G₀/G₁, S, and G₂/M phase cells adopt binomial distributions.

The ploidy of the sample was calculated, with normal lymphocytes forming the diploid standard, using the equation:

\[
ploidy\ \text{index} = \frac{G₀/G₁ \text{ peak channel (tumour cells)}}{G₀/G₁ \text{ peak channel (normal cells)}}
\]

Diploid cells therefore have an index of one and tetraploid cells have an index of two in accordance with the convention on nomenclature for DNA cytometry (Hiddeman et al., 1984). The quality of the sample was assessed by the coefficient of variation (CV) of the G₀/G₁ peak.
2. 3. 21. Flow Cytometric Immunostaining.

Following completion of the experimental protocol, the cells were harvested and centrifuged in Dulbecco’s medium containing 10% FCS at 400 g for 6 minutes. The pellet was resuspended in PBS and aliquots of 1x10^6 cells were placed in LP3 tubes, pelleted by centrifugation at 400 g for 6 minutes and stained using specific monoclonal antisera as follows. The cell pellet was incubated with an excess of monoclonal antibody (MoAb) (W6/32, 10 μl; HLA-DR, 5 μl and ICAM-1, 20 μl, as determined by titration assays) at 4°C for 30 minutes then washed with PBS containing 1% FCS (PBS-FCS) The PBS was removed and the cells incubated with 100 μl (1/60 diluted according to titration assays) of fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse F(ab’)2 antibodies at 4°C for 30 mins after thorough mixing. This incubation was terminated by washing with PBS-FCS, and the cells were resuspended and fixed in 1% paraformaldehyde and analysed with an orthocyte benchtop flow cytometer attached to a PC computer (Ortho Diagnostic Systems Ltd). Background fluorescence was assessed by including a control with no primary MoAb added. Mean fluorescence intensity was given as channel number on a linear scale from 1 to 256.

2. 4. NAMES AND ADDRESSES OF SUPPLIERS.

Amersham International Ltd.
UK Sales Office, Lincoln Place, Green End, Aylesbury, Bucks. HP20 2TP.

Becton Dickinson Ltd.
Between Towns Road, Cowley, Oxford OX4 3LY.

Biological Industries Ltd.
56 Telford Road, Cumbernauld, Glasgow G67 2AX.

Blood Transfusion Service.
Langley Road, Sheffield.

Boehringer Mannheim.
Boehringer mannheim, Bender & Co., Vienna, Austria.
Bradsure Biologicals.
65 Park Lane, Sutton bonington, Loughborough, Leicestershire LE12 5NQ.

British Biotechnology Ltd.
Brook House, Waltington Road, Cowley, Oxford OX4 5LY.

Caltag Laboratories Inc.
436 Rozzi Place, South San Francisco, California, 94080, USA.

Costar.
205 Broadway, Cambridge, MA 02139, USA.

DAKO Ltd.
16 Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks. HP31 5RE.

Dynatech Laboratories Ltd.
Caux Road, Billingshurst, Sussex RH14 9SJ.

ECACC.
PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, SP4 OJG.

Fisons Scientific Equipment .
Bishop Meadow Road, Loughborough, Leicestershire LE11 )RG.

Glaxo Institute for Molecular Biology S.A.
Route des Acacias 46, 1211 Geneva 24, Switzerland.

Imperial Cancer Research Fund Laboratories.
P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX.

Laboratory Impex Ltd.
111-113 Waldegrave Road, Teddington, Middlesex TW11 8LL.

Life Technologies Ltd.
P.O. Box 35, Washington Road, Abbotsinch Industrial Estate, Paisley PA3 4EP.

N.I.B.S.C.
National Institute For Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG.
Merck Ltd.
Fourways, Carlyon Road Industrial Estate, Atherstone, Warwickshire CV9 1JH.

Northumbria Biologicals Ltd. (NBL).
South Nelson Industrial Estate, Cramlington, Northumberland NE23 9HL.

Nycomed Ltd.
Nycomed House, 2111 Coventry Road, Sheldon, Birmingham, B26 3EA.

Ortho Diagnostic Systems Ltd.
Enterprise House, Station Road, Loudwater, High Wycombe, Bucks, HP10 9UF.

Seralab Ltd.
Crawley Down, Sussex RH10 4FF.

Sigma Chemical Co. Ltd.
Fancy Road, Poole, Dorset BH17 7NH.

Skatron.
Unit 11, Studlands park Avenue, Newmarket, Suffolk CB8 7DB.

Smith, Kline & Beecham's Laboratories.
Sweedland, PA, USA.

Sterilin Co.
Lampton House, Lampton Rd., Hounslow, Middlesex TW3 4EE.
CHAPTER 3

INTRODUCTION: THE USE OF ANIMALS IN THE DETERMINATION OF THE METASTATIC PHENOTYPE.
3. 1. INTRODUCTION: THE USE OF ANIMALS IN THE DETERMINATION OF THE METASTATIC PHENOTYPE.

Despite significant improvements in early diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths from cancer are due to metastases that are resistant to conventional therapies (Fidler, 1978; Fidler et al., 1978; Fidler, 1984; Fidler and Balch, 1987; Fidler and Hart, 1982; Sugarbaker, 1985). The metastatic foci can be located either in lymph nodes and visceral organs distant to the primary tumor or in various regions of the tumor's primary tissue, producing a clinical state which is difficult to treat.

The isolation of tumor cells with enhanced metastatic potential, in an animal model, from a primary tumor has been possible by selection either *in vivo* or *in vitro*. Following implantation of the cells subcutaneously (s. c.), intra-dermally (i. d.), intra-muscularly (i. m.) or intravenously (i. v.) into mice, cells may be derived from metastatic lesions which have an increased metastatic propensity compared with the original cell inoculum. This procedure has been successfully used to establish a number of tumor cell lines with increased metastatic capacity (Fidler, 1973; Fidler, 1978; Poste, 1982; Talmadge and Fidler, 1982; Raz and Hart, 1980; Brunson and Nicolson, 1978). More recently attention has focussed on the development of spontaneously metastatic tumor models, rather than those which involve i. v. injection of tumor cells to produce "experimental metastasis". This form of metastasis whilst more clinically relevant is less frequently observed. There are however a number of reports of spontaneously metastatic human melanoma cell lines in nude mice (Cornil et al., 1989; Iliopoulos et al., 1989; Fodstad et al., 1988; Ishikawa, et al., 1988).

An alternative approach has been to select cells for the enhanced expression of a phenotype believed to be important in determining the metastasis of a tumor cell. The cells are then tested in an appropriate host to determine their metastatic potential (Hart, 1979; Poste *et al.*, 1980; Sloane and Honn, 1984).

Clinical studies in cancer patients and in experimental rodent tumor models have shown that certain tumors produce metastasis to specific organs independent of
vascular anatomy, rate of blood flow, and number of cells delivered to each organ. The dissemination of radiolabelled melanoma cells in experimental rodent systems demonstrates that tumor cells reach the microvasculature of many organs (Fidler, 1970; Price et al., 1986; Fidler et al., 1977; Hart et al., 1981), however, growth does not occur in all the organs encountered. Extravasation into organ parenchyma and proliferation of the tumor cells occurs only at specific body sites, and the presence of viable tumor cells within a particular organ is not indicative of successful growth and formation of a metastatic lesion (Nicolson, 1988; Horak et al., 1986; Naito et al., 1987; Fidler and Talmadge, 1986).

The attempts to understand the mechanisms that regulate the pattern of site specific metastasis began in 1889, when Paget analysed over 735 autopsy records of women with breast cancer, and suggested that the non random pattern of metastases was not due to chance but, rather, that certain tumor cells (the "seed") had a specific affinity for growth in certain organs (the "soil"). Metastasis resulted when the seed could grow in the soil. Experimental evidence for the "soil and seed" hypothesis was obtained using the B16 murine melanoma cell line (Hart, 1982; Hart and Fidler, 1981). Tumor growth developed in the lungs, and was shown to be present in fragments of pulmonary or ovarian tissue but not in renal tissue. Experiments with organ derived soluble growth factors indicate that soil factors can have profound effects on certain tumor cell subpopulations (Nicolson, 1988).

An appropriate model for studies of human cancer metastasis must fulfill two requirements: the cells must be metastatic, and they must grow in the relevant organ environment. The first report of the successful growth of human tumor xenografts in athymic nude mice (Rygaard and Povlsen, 1969), has been followed by numerous studies which have used this procedure to assess the metastatic properties of human tumors. Unfortunately, many highly malignant tumors fail to form metastases when grafted into nude mice (Fidler and Nicolson, 1987; Sordat et al., 1982), and this has seriously restricted the use of xenografts in studying human metastasis.
The aim of the present study was to assess the tumorigenicity and spontaneous metastatic potential in nude mice of a series of established long term human cutaneous melanoma cell lines and short term cultured uveal melanoma cell lines, or fresh uveal melanoma tissue implants. In addition, tail vein injection of cutaneous melanoma cell lines was used to determine their lung colonising ability, which was used as a measure of "experimental" metastatic potential. Tumors developing following s. c. or i. d. injection were excised, and the animals observed for greater than 12 weeks to allow the development of occult metastases to a detectable size. Animals were killed and subjected to autopsy at times greater than 12 weeks. Tumor growth occurring at secondary body sites following s. c. or i. d. injection were considered to be examples of spontaneous metastasis, portions of the tumor tissue were snap frozen in liquid nitrogen, formalin fixed or recultured, if sufficient quantity of tissue was available. In some cases, mice with s. c. or i. d. tumors were killed and autopsied immediately rather than allowing possible occult metastases to develop.
3. 2. RESULTS.

3. 2. 1. Uveal Melanoma Tumorigenesis in Nude Mice Following Subcutaneous and Intradermal Injection.

Uveal melanoma tissue fragments and short term cell lines were assessed for their ability to grow either subcutaneously or intradermally in nude mice. Following tumor resection the bulk of the tumor was either stored or disaggregated as described above (Materials and Methods) and used for \textit{in vitro} studies. In some cases a portion of the tumor was used for \textit{in vivo} innoculation, and tissue fragments implanted within 2 hours of receiving the tumor.

Cell lines were grown to approximately 70 % confluency, washed with PBS and detached from their growth surface with EDTA. The cell suspension was washed with Hams F12 medium, followed by PBS, and cells were finally resuspended at a concentration of $3.5 \times 10^6$-$1 \times 10^7$ cells/ml; 0.1 ml was typically injected.

Results of uveal melanoma tissue implantation and cell line injection are given in tables 3. 1. and 3. 2. respectively. Tissue fragments from 24 uveal melanomas were implanted, 22 subcutaneously and 2 intradermally. Tumor take was low with 6 out of 24 (25 %) (MEL20, 23, 40, 50, 52 and 62) showing tumor growth. Four uveal melanoma cell lines were injected subcutaneously of which one grew (MEL23), results are shown in table 3. 2. The characteristics of the primary tumors are compared with successful tumor growth in table 3. 3. Tumors which grew in nude mice displayed low 1.9 % and 3.8 % proliferation rates, as measured by flow cytometric analysis, there appeared to be an association with cell type, ploidy and the location of the tumor within the eye prior to removal. Mixed and epithelioid cell tumors accounted for all six tumors which successfully grew, five and one respectively. Tumors having diploid, diploid with an aneuploid subpopulation and diploid with a tetraploid subpopulation accounted for all tumor growth, these represented three, two and one tumors respectively. Analysis of the site of the primary tumor showed a correlation with tumors arising from choroid (3) or involving both the choroid and ciliary body (3). All tumors implanted were initially of a large size (greater than 15 mm diameter
and 5 mm depth) when removed from the eye. Upon autopsy one tumor was found to have spontaneously metastasised to the liver and spleen (MEL52), no pulmonary or skin metastases were detected. The characteristics of this tumor were assessed, it was a diploid tumor having an aneuploid subpopulation, of mixed cell type, and involved both the choroid and ciliary body. The patient is alive with no detectable metastatic disease.
Table 3. 1.
Tumorigenic potential of uveal melanoma tissue implanted subcutaneously in nude mice.

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<tr>
<th>Tumor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor take&lt;sup&gt;b&lt;/sup&gt;/number of mice implanted</th>
<th>Duration of experiment (Months)</th>
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<td>MEL20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3/3</td>
<td>12</td>
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<tr>
<td>MEL21</td>
<td>0/2</td>
<td>11, 14</td>
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<tr>
<td>MEL22</td>
<td>0/1</td>
<td>8</td>
</tr>
<tr>
<td>MEL23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1/2</td>
<td>3</td>
</tr>
<tr>
<td>MEL30</td>
<td>0/3</td>
<td>7</td>
</tr>
<tr>
<td>MEL31</td>
<td>0/1</td>
<td>13</td>
</tr>
<tr>
<td>MEL37</td>
<td>0/1</td>
<td>10</td>
</tr>
<tr>
<td>MEL38</td>
<td>0/1</td>
<td>15</td>
</tr>
<tr>
<td>MEL40</td>
<td>1/1</td>
<td>13</td>
</tr>
<tr>
<td>MEL48</td>
<td>0/4</td>
<td>12</td>
</tr>
<tr>
<td>MEL49</td>
<td>0/2</td>
<td>12</td>
</tr>
<tr>
<td>MEL50</td>
<td>1/2</td>
<td>14</td>
</tr>
<tr>
<td>MEL51</td>
<td>0/1</td>
<td>11</td>
</tr>
<tr>
<td>MEL52&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1/38</td>
<td>14</td>
</tr>
<tr>
<td>MEL53</td>
<td>0/2</td>
<td>9</td>
</tr>
<tr>
<td>MEL54</td>
<td>0/2</td>
<td>8</td>
</tr>
<tr>
<td>MEL56</td>
<td>0/1</td>
<td>12</td>
</tr>
<tr>
<td>MEL57</td>
<td>0/4</td>
<td>10</td>
</tr>
<tr>
<td>MEL59</td>
<td>0/2</td>
<td>9</td>
</tr>
<tr>
<td>MEL61</td>
<td>0/2</td>
<td>12</td>
</tr>
<tr>
<td>MEL62&lt;sup&gt;e, f&lt;/sup&gt;</td>
<td>2/2</td>
<td>9</td>
</tr>
<tr>
<td>MEL63</td>
<td>0/1</td>
<td>8</td>
</tr>
<tr>
<td>MEL64&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0/2</td>
<td>6</td>
</tr>
</tbody>
</table>
a Following tumor resection the bulk of the tumor was either stored or disaggregated as described above (Materials and Methods) and used for in vitro studies. In some cases a portion of the tumor was used for in vivo inoculation, and tissue fragments were passed through a gauge needle to disaggregate them and implanted within 2 hours of receiving the tumor.
b Tumor take was detected as vertical growth of a tumor nodule at the site of implantation.
c This tumor achieved 3 successful in vivo passages.
d This tumor achieved 2 successful in vivo passages.
e This tumor achieved 1 successful in vivo passage.
f Intradermal injections.
g Spontaneous metastases detected in the liver and spleen.
Table 3.2.
Tumorigenic potential of uveal melanoma cell lines injected subcutaneously in nude mice.

<table>
<thead>
<tr>
<th>Tumor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor take&lt;sup&gt;b&lt;/sup&gt;/number of mice injected</th>
<th>Duration of experiment (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL17 P1</td>
<td>0/2</td>
<td>2</td>
</tr>
<tr>
<td>MEL18 P1</td>
<td>0/2</td>
<td>13</td>
</tr>
<tr>
<td>MEL23 P3</td>
<td>1/1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12</td>
</tr>
<tr>
<td>MEL37b P6</td>
<td>0/1</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cell lines were grown to approximately 70% confluency, washed with PBS and detached from their growth surface with EDTA. The cell suspension was washed with Ham's F12 medium, followed by PBS, and cells were finally resuspended at a concentration of 3.5x10<sup>6</sup>-1x10<sup>7</sup> cells/ml; 0.1 ml was typically injected.

<sup>b</sup> Tumor take was considered positive if vertical growth occurred at the site of injection was detected.

<sup>c</sup> This tumor achieved 2 successful in vivo passages.
Table 3.3.
Comparison of tumor tissue displaying vertical growth in nude mice with the characteristics of the primary tumor.

<table>
<thead>
<tr>
<th>Cell Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mixed</th>
<th>Epithelioid</th>
<th>Spindle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5/19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1/3</td>
<td>0/2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Choroid</th>
<th>Choroid and Ciliary body</th>
<th>Ciliary body</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3/13</td>
<td>3/10</td>
<td>0/1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ploidy&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Diploid</th>
<th>Diploid/ Aneuploid</th>
<th>Diploid/ Tetraploid</th>
<th>Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3/15</td>
<td>2/6</td>
<td>1/1</td>
<td>0/0</td>
</tr>
</tbody>
</table>

<sup>a</sup> As determined by the histological criteria of McLean et al. 1983
<sup>b</sup> As determined by histological analysis following enucleation of the eye.
<sup>c</sup> Ploidy values were calculated using the method of Rennie et al. 1989, data was not available for two tumors.
3. 2. 2. Derivation of the A375/NUPR1 Melanoma Cell Line from the A375 Melanoma by Subcutaneous Passage in Nude Mice.

The human melanoma cell line A375/NUPR1 was derived from the parental A375 melanoma cell line following tumor growth in a Balb/c nude mouse and established as a long term cell culture as follows. Subcutaneous injection of $1 \times 10^5$ A375 cells into the right flank of a Balb/c nude mouse resulted in the development of a tumor, which was resected, minced into fragments of $1 \text{ mm}^3$, and disaggregated using a cocktail containing collagenase type II (0.025 %), pronase E (0.05 %) and dithiothreitol (0.5 $\mu$M) for 2 hours at 37°C. The tumor cell suspension was washed twice with growth medium and the suspension seeded onto a 75 cm$^2$ vented tissue culture flask in Dulbecco's medium supplemented with 20 % fetal calf serum and antibiotics at 37°C in a humidified incubator (5 % CO$_2$ and 95 % air). Tumor growth was observed from both explants and single cells. The tumor cell line was confirmed as abnormal by cytogenetic analysis and verified as mycoplasma free using a nucleic acid hybridisation kit. The tumor cell line A375/NUPR1 was shown to be related to A375 by cytogenetic analysis but had an altered karyotype (shown in fig. 3. 1). This subline was used subsequently in $in$ $vitro$ experiments to establish its phenotype in relation to cytokine response and gelatinolytic activity.
Cytogenetic analysis of A375 and A375/NUPR1.

A375

<table>
<thead>
<tr>
<th>Lab. No.</th>
<th>Karyotype</th>
<th>Col. Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>5</td>
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</tr>
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<td>9</td>
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<td>10</td>
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<td>11</td>
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<td>13</td>
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<td>17</td>
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<td>18</td>
<td></td>
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<td>21</td>
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</tr>
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</tr>
<tr>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Karyotypes of A375 and A375/NUPR1 demonstrating that the same ploidy and structural rearrangement are common to both.
Figure 3. 1. continued

Cytogenetic analysis of A375 and A375/NUPR1.

A375/NUPR1

Karyotypes of A375 and A375/NUPR1 demonstrating that the same ploidy and structural rearrangement are common to both.
3.2.3. In Vivo Growth Characteristics of A375 and A375/NUPR1 Melanoma Cell Lines in Nude Mice.

Subconfluent cultures were prepared as described above except that cells were titrated prior to subcutaneous injection such that the final number injected was in the range 1x10^4-1x10^6 in a volume of 0.1 ml of ice cold PBS.

To determine in vivo growth kinetics, tumors were measured weekly at orthogonal angles. The mean tumor diameter and doubling time were calculated as described in materials and methods.

Experiment 1.

To determine the tumor growth rate, 1x10^6 cells were injected subcutaneously into groups of up to 3 Balb/c nude mice and the tumors measured at weekly intervals. Growth was rapid, with mean doubling times of 6.3 ± 3.1 days and 3.9 ± 0 days for A375 and A375/NUPR1 respectively. However, when the number of tumor cells injected was decreased to 5x10^5 the mean doubling times decreased to 7.7 ± 2.7 days and 7.6 ± 2.5 days for A375 and A375/NUPR1 respectively (Table 3.4). An injection of greater than 1x10^5 tumor cells was required for tumor take and, no significant difference in tumor take rate was observed between the two cell lines.

Experiment 2.

The experiment was repeated, however, the number of cells injected was in the range 1x10^5-1x10^6. Tumor doubling times were calculated as previously described and are shown in table 3.4. When 1x10^6 cells were injected subcutaneously into groups of up to 3 Balb/c nude mice the mean doubling times were 3.0 and 3.5 ± 0.5 days for A375 and A375/NUPR1 respectively. However, when lower cell numbers were injected the mean doubling times remained of a similar magnitude, in contrast to the previous experiment, where they increased approximately two fold. Furthermore, tumor take was at a higher frequency with 1x10^5 cells effectively producing a tumor. The duration of the second experiment was 28 days in contrast to 49 days for the first experiment. The reasons for these differences are at present unknown.
Table 3.4.
Growth potentials of A375 and A375/NUPR1 human malignant melanoma cells in nude mice following subcutaneous injection: Experiments 1 and 2.

<table>
<thead>
<tr>
<th>Number of cells injected/cell line</th>
<th>Number of mice with s. c.</th>
<th>Tumor doubling timea (Days)</th>
<th>Number of mice showing spontaneous metastasis/number injectedb</th>
<th>Number of spontaneous metastasesc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10^6 A375</td>
<td>3/3 (1/1)</td>
<td>6.3 ± 3.1 (3.0)</td>
<td>None (None)</td>
<td>Na (Na)</td>
</tr>
<tr>
<td>1x10^6</td>
<td>3/3 (3/3)</td>
<td>3.9 ± 0</td>
<td>None (None)</td>
<td>Na (Na)</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td></td>
<td>(3.5 ± 0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5x10^5 A375</td>
<td>2/3 (2/3)</td>
<td>7.7 ± 2.7</td>
<td>1/3 (None)</td>
<td>2 (L) (Na)</td>
</tr>
<tr>
<td>5x10^5</td>
<td>2/3 (3/3)</td>
<td>7.6 ± 2.5</td>
<td>2/3 (None)</td>
<td>1,1 (L) (Na)</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td></td>
<td>(3.2 ± 0.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5x10^5 A375</td>
<td>0/3 (ND)</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>2.5x10^5</td>
<td>0/3 (ND)</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1x10^5 A375</td>
<td>0/3 (2/3)</td>
<td>Na (3.5 ± 0.1)</td>
<td>Na (None)</td>
<td>Na (Na)</td>
</tr>
<tr>
<td>1x10^5</td>
<td>0/3 (3/3)</td>
<td>Na (3.1 ± 0.3)</td>
<td>Na (None)</td>
<td>Na (Na)</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5x10^4 A375</td>
<td>0/2 (ND)</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>5x10^4</td>
<td>0/2 (ND)</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results of experiment 2 are shown in brackets

a Mean value ± standard deviation.

b Mice were subjected to autopsy at the time of tumor resection, and the extent of spontaneous metastasis was determined.

c The number of spontaneous metastases present in the skin (S), lungs (L) and abdominal organs (A) was determined.

Na Not applicable, ND Not done
3. 2. 4. Intradermal Growth Characteristics of A375 and A375/NUPR1 In Nude Mice.

Tumor cells were prepared as described above except that $1 \times 10^6$ cells were injected intradermally using a fine bore needle. If no tumor take was observed the mice were killed 12 weeks after injection of the tumor cells and gross necropsies were performed to determine if spontaneous metastasis had occurred. No macroscopic tumor growth was detected in any organ. Developing tumors were resected when the tumor measured 0.5-1.0 cm mean diameter and the tumor tissue was then divided into a number of portions for either reinjection, in vitro culture, histology or snap freezing. Results are shown in table 3. 5. for experiments 1 and 2 respectively. The growth rate of melanoma cells injected intradermally was determined as described previously (materials and methods). Growth rates and spontaneous metastasis rates were comparable between the two experiments and although the growth rate for intradermal growth was slightly higher the rate of spontaneous metastasis was comparable to that observed with subcutaneous injection and growth.
Table 3.5.
Growth potentials of A375 and A375/NUPR1 human malignant melanoma cells in nude mice following intradermal injection: Experiments 1 and 2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of mice with tumor growth/number injected</th>
<th>Tumor doubling time</th>
<th>Number of mice showing spontaneous metastasis</th>
<th>Number of metastases&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>6/8</td>
<td>1.9 ± 0.5</td>
<td>2</td>
<td>2, 1 (L)</td>
</tr>
<tr>
<td></td>
<td>(10/11)</td>
<td>(2.1 ± 0.4)</td>
<td>(None)</td>
<td>(Na)</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td>7/8</td>
<td>1.7 ± 0.4</td>
<td>None</td>
<td>Na</td>
</tr>
<tr>
<td></td>
<td>(11/15)</td>
<td>(1.6 ± 0.2)</td>
<td>(None)</td>
<td>(Na)</td>
</tr>
</tbody>
</table>

Results of experiment 2 are shown in brackets.

<sup>a</sup>Spontaneous metastasis was determined 12 weeks after tumor resection.

<sup>b</sup>The number of spontaneous metastases present in the skin (S), lungs (L) and abdominal organs (A) was determined.

Na not applicable.
3.2.5. Intravenous Injection of a Series of Cutaneous Melanoma Cell Lines as a Measure of their Experimental Metastatic Potential in Nude Mice.

Experimental metastasis assay

Subconfluent cultures were detached using 2 mM EDTA, resuspended in Dulbecco's medium containing 10 % FCS, centrifuged at 400 g for 6 minutes and then resuspended in ice-cold PBS. Cells used in these studies were >95 % viable as determined by trypan blue exclusion, and adjusted to a final concentration of $1 \times 10^7$ cells/ml by the addition of ice-cold PBS; mice (4-6 weeks old Balb/c nudes) received $1 \times 10^6$ viable cells in 0.1 ml into the lateral tail vein. Animals were killed 12 weeks post inoculation by cervical dislocation following etherisation and subjected to gross necropsies. Lungs were fixed in neutral buffered formal saline and the extent of lung colonisation determined, the number of surface colonies was generally low and were quantified macroscopically. All cell lines showed a low experimental metastatic potential in nude mice, results are shown in table 3.6. Overall A375/NUPR1 appeared to show a higher incidence of lung colonisation than A375 when injected intravenously.
Table 3.6.
Experimental metastatic potentials of a series of human malignant melanoma cells in nude mice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of mice with lung metastases/number injected</th>
<th>Number of lung metastases(^b)/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>3/28</td>
<td>2, 3, 5</td>
</tr>
<tr>
<td>A375/NUPR1(^a)</td>
<td>15/31</td>
<td>2, 4, 6, 1, 1, 1, 1, 3, 2, 7, 1, 2, 3, 1, 10,</td>
</tr>
<tr>
<td>DX.3(^c)</td>
<td>1/5</td>
<td>6</td>
</tr>
<tr>
<td>DX.3 LT 5.1(^c)</td>
<td>3/9</td>
<td>12, 8, 7</td>
</tr>
<tr>
<td>G361(^c)</td>
<td>0/16</td>
<td>Na</td>
</tr>
</tbody>
</table>

\(^a\) This assay was performed 3 times on the first two occasions the results were 6/8 and 7/8 however on the third assay the result was 2/15, a level similar to A375. The increased metastatic propensity of A375/NUPR1 was not reproducible and may be due to the formation of homotypic tumor emboli or other artifacts which may be involved in the establishment of this assay.

\(^b\) The presence of metastases in the skin, peritoneal cavity and abdominal organs was also determined and found to be negative.

\(^c\) Results of a single experiment.
3. DISCUSSION.

Melanoma metastases represent a major clinical problem, since the therapeutic approaches so far tested have, in the main, failed to improve the prognosis of melanoma patients. Following the initial report of the development of an experimental model for tumor growth in nude mice, several studies have indicated that human melanoma tissue shows a high tumor take rate when xenotransplanted subcutaneously into nude mice (Giovanella et al., 1974; Sharkey et al., 1978). Many highly malignant human tumors do not form metastases when xenotransplanted into nude mice (Fidler, 1986; Fidler et al., 1984; Giovanella and Fogh, 1985; Sordat et al., 1982), however, a few exceptions have been reported (Sharkey et al., 1978; Sordat et al., 1982; Neulat-Duga et al., 1984; Kozlowski et al., 1984). This benign, localised growth of tumor cells is one of the limiting factors to the use of the nude mouse for testing therapeutic modalities on human metastases.

The aims of this study were to assess the tumorigenic and metastatic ability in nude mice of a series of primary uveal melanoma tumor tissues and short-term cultures of human uveal melanoma. Comparisons with other patient parameters were also attempted, an in-depth description of these investigations is given in chapter 5. A series of five cutaneous melanomas were also studied with respect to their experimental metastatic potential in nude mice following tail vein injection. Two cell lines were chosen for further study of both their in vivo and in vitro characteristics (see chapters 4 and 6). Furthermore, attempts were made to establish tumor cell sublines from both spontaneously and experimentally metastatic human cutaneous melanoma lesions in nude mice as cell lines. The growth kinetics and spontaneous metastatic potential of two related human melanomas, A375 and A375/NUPR1, implanted at different sites, subcutaneous and intradermal, in nude mice was also studied.

Initial studies using human uveal melanomas, both fresh tissue and short term cultured cells, showed them to have a low tumorigenic potential when injected subcutaneously into nude mice. Subsequent studies used uveal melanoma tissue
injected intradermally. Of the 24 ocular melanomas tested, five were tumorigenic after subcutaneous injection and one was tumorigenic following intradermal injection, but none displayed growth in nude mice further than three *in vivo* transplantations. Likewise, spontaneous metastases were observed only once following subcutaneous injection and never following intradermal injection. These results are in agreement with the reports from other groups who describe a low incidence of tumor growth and metastasis, observing either no tumor take with fresh tissue or only a benign localised lesion. Reports by Fidler (1990) describing an increased incidence of tumor take and spontaneous metastasis following intradermal injection of tumor cells cannot at this stage be verified, however, it should be noted that one of the two tumors injected intradermally grew as a detectable lesion. Further studies involving an increased number of tumors are needed to confirm these initial observations.

Analysis of the characteristics of the primary uveal melanoma tissue, which successfully grew in nude mice, revealed a number of trends which are interesting. However, due to the low numbers in this study these observations should be treated with caution until they are either verified or found to be false following completion of a more extensive study. Despite this, these preliminary results will be discussed in the context of other investigations. Kerbel *et al.* (1984) reported an association between the metastatic capacity and tetraploidy of a human melanoma cell line. The ploidy values and karyotypes of the uveal melanomas from the present study were also assessed. When compared with the growth and metastasis of this tumor type in mice it was found that three of the six tumors displaying growth in nude mice were diploid, two were diploid with an aneuploid subpopulation and one was diploid with a tetraploid population; the metastatic tumor, was diploid with an aneuploid subpopulation. It cannot be confirmed that the findings of Kerbel *et al.* (1984) apply to uveal melanoma. As will be discussed later (chapter 5), uveal melanoma, has both similarities and differences to cutaneous melanoma.

The tumors which grew when implanted at subcutaneous or intradermal sites in nude mice were MEL 20, 23, 40, 50, 52 and 62. There appears to be no obvious
correlation with patient status and tumor growth upon implantation (see table 3. 7). This lack of correlation between patient status and growth in nude mice was also observed with respect to spontaneous metastasis in nude mice.

The location of the primary tumor within the eye did appear to be related to the ability of the tumor tissue to grow in mice, with tumors originating from primary tumors involving the choroidal or the choroid and ciliary body, no tumors which were solely of ciliary body origin grew or metastasised in nude mice. Furthermore, tumor cell type is used as an indicator of tumor aggression. Two cell types are recognised, spindle and epithelioid, and mixtures of the two cell types are frequently observed (Callender, 1931). Epithelioid cell tumors have the worst prognosis followed by mixed cell tumors and pure spindle cell tumors which have the most favourable prognosis (Callender, 1931). We observed that five of the six tumors growing in mice were of mixed cell composition, the remaining tumor was epithelioid.

Alterations in chromosomes 1, 3, 6, and 7, which have been reported in other primary and metastatic malignant melanoma biopsies or cell lines (Balaban et al., 1984), do not appear to be closely associated with uveal melanoma tumorigenic or metastatic potential in nude mice, although alterations in chromosome 3 were frequently observed (a complete list of tumor karyotypes and a more comprehensive discussion of this area is present in chapter 5).
Table 3.7.
Comparison of patient status and uveal melanoma growth in nude mice with karyotypic abnormalities.

<table>
<thead>
<tr>
<th>Tumors displaying vertical growth(^a)</th>
<th>Karyotype(^b)</th>
<th>Patient status(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Na</td>
<td>Alive</td>
</tr>
<tr>
<td>23</td>
<td>Na</td>
<td>Alive</td>
</tr>
<tr>
<td>40</td>
<td>Na</td>
<td>Alive</td>
</tr>
<tr>
<td>50</td>
<td>Na</td>
<td>Alive</td>
</tr>
<tr>
<td>52</td>
<td>-3 +i8q del 11q t (1.10)</td>
<td>Alive</td>
</tr>
<tr>
<td>62</td>
<td>-3 +8 +i8q</td>
<td>Alive</td>
</tr>
</tbody>
</table>

\(^a\) Determined by macroscopic analysis of the tumor lesion.
\(^b\) Results obtained by collaboration with K. Sisley.
\(^c\) Clinical details kindly provided by I. G. Rennie.
\(^d\) Pathological analysis of lung nodules by Dr M. A. Parsons.
Further studies are needed to establish which genotypic or phenotypic characteristics are associated with the growth of uveal melanomas in nude mice, and the clinical relevance of such findings. Orthotopic implantation, the implantation of tumor tissue or cells directly into the eye is a possible approach, however, due to the severity of this type of experiment this option has not been taken at the present time. Intrasplenic injection of tumor tissue has been reported to produce in vivo growth of melanoma in nude mice (Nicolson, 1987). Since the site of metastasis in 60-70% of patients with uveal melanoma is the liver (Char, 1989), intrasplenic injection of tumor cells may aid in the development of a metastatic model for uveal melanoma. Initial results would suggest that this may be an appropriate site of injection since MEL17 formed metastatic lesions in the liver when injected via an intrasplenic route. However additional experiments are needed to confirm this initial result.

The cutaneous melanoma cell lines used in this study, A375 and A375/NUPR1, when titrated, were found to be capable of subcutaneous tumor formation when the total number of cells injected per mouse was $1 \times 10^5$ although this was higher upon repeat experiments. The incidence of spontaneous metastatic potential for the two cell lines was low, only 3 out of 24 mice showed metastasis; the numbers of metastases were also of a low magnitude typically one or two lesions were detected upon macroscopic analysis. Spontaneous metastasis was not detected in a second experiment.

Intradermal injection of A375 or A375/NUPR1 tumor cells followed by tumor resection and subsequent observation of the mice for longer than 12 weeks showed that although the doubling time for the tumors was reduced to approximately 2 days, when compared to subcutaneous injection (approximately 3.5 days), the incidence of spontaneous metastasis was not significantly different from that observed following subcutaneous injection; again the intradermal growth rates of the parent line (A375) and subline (A375/NUPR1) were not significantly different.

The experimental metastatic potential of these tumor lines, when injected into the lateral tail vein, was low, and the number of metastases detected in individual animals
was minimal, typically only 1-15 exclusively pulmonary located lesions. *In vitro* experiments determined that there were differences in gelatinase expression and its modulation by peptide regulatory factors between the different melanoma cell lines (see chapter 4). The two cell lines, A375 and A375/NUPR1, were analysed further. Both the constitutive expression of MHC class I, II and ICAM-1 was determined as was the response of the cells to the cytokines IL-1 and TNFα (see chapter 6).

There is now considerable evidence that alteration in gene expression by genetic or epigenetic processes can lead to the acquired phenotypic alterations in tumor cells which confer upon a minority of them a selective or competitive growth advantage. Host selective pressures may ensure the preferential survival and outgrowth of these cells, which would then become the progenitors of new and more aggressive clones. Analysis of such a dynamic evolutionary process involving the emergence and decline of successive new clonal subpopulations known as clonal dominance (Kerbel *et al*., 1988) has been attempted through the use of endogenous or radiation induced cytogenetic markers (Hu *et al*., 1987; Talmadge and Fidler, 1982), drug resistance markers (Kerbel *et al*., 1982), by the use of X-linked isoenzyme mosaicism (Ootsuyama *et al*., 1987) or by the use of transferred DNA markers (Kerbel *et al*., 1988). This study showed that primary tumors have a tendency to become rapidly overgrown by the progeny of a small number of clones. In our own study, the cell line A375/NUPR1 was found to display an increased level of cellular homogeneity when compared to the parent tumor, A375, and in one experiment to have an increased growth rate in nude mice when injected subcutaneously. Whilst our study is too limited to draw definite conclusions as to the nature of clonal selection within a primary tumor, it is probable that A375/NUPR1 is an example of a selected subpopulation of the primary tumor which has an increased growth rate, possibly due to altered responsiveness to host microenviromental factors, compared with other clones, and has achieved dominance within the tumor. In *in vitro* experiments A375/NUPR1 was found to react differently when exposed to cytokines, and this
alteration in susceptibility to the cytokines IL-1 and TNFα may be a result of in vivo passage (see chapter 7).

In our hands the A375 melanoma did not metastasise following intravenous injection at the level reported in the literature (Kozlowski et al., 1984). This may however be due to genotypic and/or phenotypic alterations which may have occurred following in vitro passage of this cell line. It should be noted that the in vitro growth medium used in our study differed from that used by Kozlowski et al. (1984). In the present study cells were maintained in Dulbecco's minimal essential medium supplemented with 10% FCS, whereas Kozlowski et al. (1984) maintained their cultures in Eagle's minimal essential medium supplemented with 10% FCS, sodium pyruvate, nonessential amino acids, l-glutamine and 2-fold vitamin solution. This may have introduced a selective growth advantage for a particular subpopulation of tumor cells thereby altering the metastatic potential of the total population.

The ability to extravasate is dependent upon the destruction of some of the major constituents of the basement membrane. The basement membrane is a dense scaffold of type IV and V collagen, laminin, entactin and heparan sulphate proteoglycan. The type IV collagenolytic (gelatinase) activity of these tumors is investigated in chapter four of this thesis. Hendrix et al. (1990a) have shown that the measurement of gelatinase activity or the ability of tumor cells to traverse reconstituted basement membrane may be good predictors of in vivo metastatic properties. In addition there is increasing evidence of an association between intercellular adhesion molecule 1 (ICAM1) expression and melanoma progression (Holzman et al., 1987; Johnson et al., 1988; Johnson et al., 1989; Natali et al., 1990). These studies report that ICAM-1 is expressed at higher levels on successfully metastatic melanoma cells than on the cells in the primary tumor or precancerous lesions, suggesting that ICAM-1 may play a role in the metastatic process. Furthermore, Feldman et al. (1988) and Kushtai et al. (1988) have correlated major histocompatibility antigen expression with metastatic propensity. These aspects are considered in the subsequent studies on uveal and cutaneous melanomas.
CHAPTER 4

INTRODUCTION: GELATINASE EXPRESSION AND METASTASIS.
4. 1. INTRODUCTION: GELATINASE EXPRESSION AND METASTASIS.

Tumor invasion, the process of the removal of the extracellular matrix surrounding the tumor, allowing cell migration and tumor growth, is an initial event in the metastatic cascade. The removal of the extracellular matrix is thought to occur through the action of a variety of degradative enzymes which are produced by either the tumor (Review: Tryggvason et al., 1987) or stimulated host cells (Basset et al., 1990). Tumor invasion may occur by a number of different mechanisms, which are not mutually exclusive, and may involve active locomotion (Strauli and Haemmerli, 1984). Three theories of invasion have been proposed:

1) the mechanical theory (Eaves, 1973)
2) the enzymatic theory (Review: Tryggvason et al., 1987)
3) the migratory theory (Strauli and Haemmerli, 1984)

In addition to invasion of the interstitial matrix, the tumor cell must invade the vascular wall or lymphatic channels during dissemination if it is to successfully metastasise to a distant organ or tissue. The major structural barrier to invasion of these vessels is the basement membrane, a specialised extracellular matrix, produced by epithelial cells, endothelial cells, and many mesenchymal cells, whose functions include molecular sieving, impeding the passage of cells from one tissue compartment to another and cell attachment, growth and differentiation (Review: Farquhar, 1981). Liotta et al. (1986), suggested a three step theory for invasion. The first step involves attachment of tumor cells to basement membrane components, typically laminin and type IV collagen (Kramer et al., 1989; Ramo, et al., 1990), followed by secretion of proteolytic enzymes to cause degradation of the extracellular matrix (Turpeenniemi-Hujanen et al., 1986), and finally tumor cell migration into the area of degradation. A number of studies have reported these steps to be closely linked, for example, attachment to laminin induces increased enzyme expression (Turpeenniemi-Hujanen et al., 1986) which is itself a chemotactic stimulant for the tumor cells (Terranova et al.,
1989), as indeed are the degraded basement membrane components (Terranova et al., 1989).

Animal studies have indicated that that tumor cell invasion and metastasis are not random events, but arise from tumor cell subpopulations expressing a higher metastatic potential (Fidler and Hart, 1982). Many studies have focussed on biochemical and genetic markers of the invasive phenotype, which would allow patients at greater risk of developing metastatic disease to be identified.

A major group of enzymes associated with matrix degradation during tumor invasion are the proteases (Goldfarb and Liotta, 1986), which can be subdivided into exopeptidases (peptidases) and endopeptidases (proteinases). Proteinases which appear to be the more important group, are classified according to their active sites, as serine-, cysteine-, aspartic- and metalloproteinases (McDonald, 1985).

Tumor cells capable of metastasis have been shown to produce proteinases capable of degrading basement membrane type IV collagen (Tryggvason et al., 1987; Turpeenniemi-Hujanen et al., 1986; Garbisa et al., 1987; Nakajima et al., 1987), and the production of these enzymes constitutes an important mechanism by which migrating tumor cells degrade basement membrane components. Gelatinase enzymes are metalloproteinases capable of degrading type IV collagen (Collier et al., 1988; Fessler et al., 1984; Liotta, 1986; Murphy et al., 1989) and there is evidence to suggest that the type IV collagenase is a gelatinase (Review: Woessener, 1991; Collier et al., 1988; Murphy et al., 1985; Seltzer et al., 1981). Two molecular forms of progelatinase have been identified, a 72 kDa species, expressed by connective tissue cells and tumor cells (Collier et al., 1988; Murphy et al., 1989; Seltzer et al., 1981; Yamagata et al., 1988; Yamagata et al., 1989), and an immunologically distinct glycosylated 92 kDa species, mainly expressed by monocytes, macrophages, polymorphonuclear leukocytes (Murphy et al., 1989) and tumor cells (Yamagata et al., 1988; Yamagata et al., 1989). Both molecular species of gelatinase are secreted as latent proenzymes which require modification prior to attaining their active state (Stetler-Stevenson et al., 1989).
The expression of the 92 kDa gelatinase has been shown to be induced by ras oncogene transfection (Ballin et al., 1988) and has been correlated with increasing metastatic potential (Yamagata et al., 1988; Yamagata et al., 1989) in a number of malignant cell types. In addition, 92 kDa gelatinase expression was shown to correlate with the metastatic phenotype in transformed rat embryo cells (Bernhard et al., 1990).

The investigations described in this chapter have involved the study of the modulation of gelatinase by a number of cytokines, growth factors and extracellular matrix components. It is possible that a metastasising tumor cell may encounter these factors during the metastatic process which may subsequently influence the metastatic propensity of the cell.

The cytokines used in this study were the interleukins: IL-1α and β, IL-2, IL-4, and IL-6; transforming growth factor beta 2 (TGFβ2); interferons alpha (IFNα) and gamma (IFNγ); insulin like growth factors 1 (IGF 1) and 2 (IGF 2), epidermal growth factor (EGF), platelet derived growth factor (PDGF) and acidic fibroblast growth factor (aFGF). In addition to these soluble mediators of cell communication a number of extracellular matrix components were also studied, these were laminin, fibronectin, and collagen types I and IV. Later studies involved combinations of factors to determine possible inhibitory or synergistic actions. The combined literature on these factors is extensive and only their reported effects on gelatinase expression shall be discussed in this introduction.

The structures of the genes (Huhtala et al., 1990; 1991) and the cDNA sequences (Collier et al., 1988; Wilhelm et al., 1989) for human 72 kDa gelatinase and 92 kDa gelatinase respectively are known. The 5' regulatory region of the 72 kDa gelatinase gene contains neither a TATA, TPA responsive element (TRE) or CAAT box, but there are however two putative SPI binding sequences (GC rich regions) and one potential AP-2 consensus sequence. In contrast, the 92 kDa gelatinase promoter region contains a TATA-like sequence (TTAAA), no CAAT motif and only one GC box. Two putative TREs are present as is a TGFβ inhibitory element (TIE).
Both the activity of 72 kDa gelatinase (Salo et al., 1985) and the mRNA levels (Salo et al., 1989) have been reported to be increased by phorbol esters in human fibroblasts. TNFα has been reported to stimulate the production of both active gelatinase and collagenase and to cause de novo expression of an 80 kDa gelatinase in carrageen-induced granulation tissue in organ culture. However, granulation tissue contains a number of different cell types including many immunocytes, and the cell type(s) responsible for the production of this enzyme was not given (Nakagawa et al., 1987). A recent study, published during the present investigations, reported that 92 kDa gelatinase was induced and stimulated by TNFα whereas 72 kDa gelatinase expression was unchanged (Okada et al., 1990). In addition this group reported that IL-1α, PDGF, EGF, bFGF and TGFβ had no effect on gelatinase expression. In contrast to this report Wilhelm et al. (1989) reported that EGF and IL-1β were inducers for a 92 kDa gelatinase in SV40-transformed human lung fibroblasts.

Although expression of the 92 kDa gelatinase may be an important indicator of malignancy, it cannot be considered to be the only determinant for metastatic propensity, since upregulation or alteration of a number of different phenotypic traits have been correlated with metastatic cells.
4. 2. RESULTS.

4. 2. 1. Zymographic Analysis of Proteinases Constitutively Expressed by Cutaneous and Uveal Melanomas in Tissue Culture.

To investigate proteinase expression in human melanoma cell lines, zymograms of gelatin and/or casein embedded polyacrylamide gels were used for the qualitative analysis of enzymes (Heussen and Dowdle, 1980). Serum free medium or cell lysates are dissolved in a sample buffer containing SDS and are separated by electrophoresis. The technique of zymography or substrate gel electrophoresis (Heussen and Dowdle, 1980) presents a number of advantages over classical assays for enzymatic activity using either natural or synthetic substrates, but principally its ability to detect different molecular species of enzymes and enzyme-inhibitor complexes. SDS was found to activate both serine proteinases (Granelli-Piperno and Reich, 1978) and proMMP-2 (72 kDa progelatinase) without changing their apparent Mr (Collier et al., 1988) and to dissociate and subsequently activate the proMMP-9 present in proMMP-9-TIMP complexes (Wilhelm et al., 1989), but not to dissociate those formed between active MMP-9 (92 kDa gelatinase) and TIMP (Murphy et al., 1989). Furthermore, it has been shown to reactivate several serine proteinase-inhibitor complexes without dissociating them (Granelli-Piperno and Reich, 1978).

When 24 hour serum free conditioned medium was analysed on gelatin zymograms, proteinases of apparent molecular weights 92 kDa and 72 kDa were detected as clear bands. The cutaneous melanoma cell lines A375, and SK23 expressed only one gelatinolytic activity, which had an apparent molecular weight of 72 kDa whereas the cutaneous melanomas A375/NUPR1, DX3 and LT5.1 in addition constitutively expressed the 92 kDa species. A375/NUPR1 expressed a 92 kDa gelatinolytic activity at a low level, so that whilst this enzyme could easily be detected in 10 fold concentrated conditioned medium, it was rare to detect this species in unconcentrated medium. The same pattern of enzyme activities was found in the Triton X-100 cell-lysates (table 4.1).
Fifteen short term (1-5 passage levels) posterior uveal melanoma cell lines were qualitatively analysed for gelatinolytic and caseinolytic proteinase activity. All fifteen cell lines secreted a gelatinolytic metalloproteinase, with an apparent molecular weight of 72 kDa, into protein free culture media; and nine of these secreted an additional gelatinolytic metalloproteinase with an apparent molecular weight of 92 kDa; neither species had the ability to degrade casein. The results are shown in table 4.2.

Table 4.1.
Identification of gelatinase activities constitutively expressed by a series of human cutaneous melanoma cell lines.

<table>
<thead>
<tr>
<th>Cell linea</th>
<th>Cellularb</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td>72 (92)c</td>
<td>72 (92)</td>
</tr>
<tr>
<td>DX3</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>LT5.1</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>SK23</td>
<td>72</td>
<td>72</td>
</tr>
</tbody>
</table>

a Subconfluent cell cultures were washed twice with PBS for 1 hour then incubated for 24 hours at 37°C in serum free medium. The medium was centrifuged for 5 minutes at 15000 g prior to analysis, or storage at -70°C.
b Cell lysates were prepared in 0.25 M Tris-HCl pH 7.4 containing 200 mM NaCl, 5 mM CaCl₂ and 0.1% Triton X-100.
c 92 kDa gelatinase was expressed at the limit of detection, however it was clearly present in 10 fold concentrated medium.
Table 4.2.
Identification of gelatinase activities constitutively expressed by a series of uveal melanoma cell lines.

<table>
<thead>
<tr>
<th>Cell line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cellular&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Extracellular</th>
</tr>
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<td>MEL14</td>
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<td>MEL22</td>
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<td>72, 92</td>
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<td>MEL30</td>
<td>72, 92</td>
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</tr>
<tr>
<td>MEL35</td>
<td>72, 92</td>
<td>72, 92</td>
</tr>
<tr>
<td>MEL37a</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>MEL37b</td>
<td>72, 92</td>
<td>72, 92</td>
</tr>
<tr>
<td>MEL40</td>
<td>72</td>
<td>72</td>
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<tr>
<td>MEL44</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>MEL47</td>
<td>72, 92</td>
<td>72, 92</td>
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<tr>
<td>MEL50</td>
<td>72, 92</td>
<td>72, 92</td>
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<td>MEL52</td>
<td>72, 92</td>
<td>72, 92</td>
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<td>MEL55</td>
<td>72, 92</td>
<td>72, 92</td>
</tr>
<tr>
<td>MEL56</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>MEL57</td>
<td>72, 92</td>
<td>72, 92</td>
</tr>
</tbody>
</table>

<sup>a</sup>Subconfluent cell cultures were washed twice with PBS for 1 hour at 37°C then incubated for 24 hours at 37°C in serum free medium. The medium was centrifuged for 5 minutes at 15000 g prior to analysis, or storage at -70°C.

<sup>b</sup>Cell lysates were prepared in 0.25 M Tris-HCl pH 7.4 containing 200 mM NaCl, 5 mM CaCl<sub>2</sub> and 0.1 % Triton X-100.
4. 2. 2. Proteinase Inhibitor and Enzyme Activation Studies.

The 72 kDa and 92 kDa enzyme species, present in supernatants or cell lysates, did not appear when the chelating agents EDTA, a general divalent cation chelator, or 1, 10, phenanthroline, a preferential zinc chelator, (both used at 10 mM) were added to the incubation buffer. However PMSF, a general serine proteinase inhibitor, (2 mM), Net-MAL, a general cysteine proteinase inhibitor, (10 mM) and E-64, an inhibitor specific for cathepsins, (10 mM) did not inhibit enzyme activity (Fig. 4. 1). In addition, all the observed gelatinase species were activated by incubation with 1 mM p-APMA suggesting that constitutively expressed species are latent metalloproteinases. Fig. 4. 2 shows the presence of an additional lower molecular weight activation band following treatment of the serum free A375 melanoma cell conditioned growth medium with p-APMA. When casein was used as a substrate, bands of degradation comparable to those seen with gelatin were not apparent (Fig. 4. 3) indicating that the enzyme activities were not due to stromelysins (Chin et al., 1985). These results infer that the gelatinases produced by melanoma cell cultures are latent gelatinolytic metalloproteinases with apparent Mr values similar to those reported for proMMP-2 and proMMP-9 respectively.
Fig 4. 1.

Zymography inhibitor studies.

EDTA (10 mM)

1, 10 Phenanthroline
Serum free conditioned medium from LT 5.1 and A375 cell cultures was analysed by gelatin zymography on 7.5% \( w/v \) polyacrylamide gels, following electrophoresis, the gels were washed and incubation buffer as previously described. Inhibitors specific for the different mechanistic classes of proteinases were included in the incubation buffer: Control gels were incubated in buffer plus the appropriate solvent,
Fig 4.2.
Activation of progelatinase by p-APMA.

A375 melanoma cell conditioned medium containing constitutively expressed progelatinase species was incubated with 1 mM p-APMA for 4 hours at 37°C. The samples were analysed by zymography on 7.5% w/v polyacrylamide minigels containing 1 mg/ml gelatin. Lane 1, A375 conditioned medium; Lane 2, A375 conditioned medium incubated with p-APMA as described above.
Expression of caseinolytic enzymes.

Serum free conditioned medium containing gelatinolytic enzyme species was analysed by casein zymography. Casein zymograms contained 1 mg/ml casein copolymerised in 7.5 % (w/v) polyacrylamide minigels. Lane 1, A375 conditioned medium; Lane 2, A375/NUPR1 conditioned medium; Lane 3, LT5.1 conditioned medium; Lane 4, DX3 conditioned medium; Lane 5, SK23 conditioned medium. Zymography was as previously described.
4. 2. 3. Cellular Location of 72 kDa Progelatinases in A375 and A375/NUPR1 as Determined by Triton X-114 Phase Separation.

Cell lysates were prepared as previously described: typically 1X10^7 cells were lysed on ice in 1 ml of 50 mM Tris-HCl pH 7.4 containing 200 mM NaCl, 5 mM CaCl_2 and 0.1 % Triton X-114. Integral membrane proteins form mixed micelles with nonionic detergents, and it is possible to isolate them from hydrophilic proteins by phase separation above the cloud point of the detergent. Phase separation of integral membrane proteins was performed by the method of Bordier, (1981). As a control ovalbumin, a hydrophilic protein, was phase extracted in parallel with the sample. The resulting aqueous and detergent phases were analysed by gelatin zymography or in the case of the ovalbumin control on an 7.5 % polyacrylamide SDS containing gel which was coomassie brilliant blue R 250 stained. The 72 kDa progelatinase species were found both in the detergent and aqueous phases suggesting that the enzymes exist as both integral and either intracellular or peripheral membrane proteins (Fig. 4. 4a and b).
Cell lysates were prepared using a Triton X-114 containing lysis buffer as previously described and the samples phase separated using the method of Bordier, 1981. Aqueous and detergent phases were analysed by zymography on 7.5 % w/v polyacrylamide minigels containing 1 mg/ml gelatin. Lane 1, A375 aqueous phase; Lane 2, A375 detergent phase; Lane 3, A375/NUPR1 aqueous phase; Lane 4, A375/NUPR1 detergent phase.
As a control to determine if phase separation was occurring when the method of Bordier, 1981 was used a 0.5 mg/ml solution of ovalbumin was prepared using Triton X-114 containing lysis buffer. This solution was phase separated in parallel with cell lysate preparations. The respective detergent and aqueous phases were separated on 7.5% w/v polyacrylamide gels and the proteins stained using coomassie brilliant blue R 250. Lane 1, blank; Lane 2, detergent phase; Lane 3, aqueous phase; Lane 4, aqueous phase; Lane 5, detergent phase.
4. 2. 4. Cytokine Modulation of Progelatinase Expression in Melanomas by IL-1α, IL-2, IL-6, IFNα, IFNγ, TNFα, and TGFβ2.

The cytokines IL-1α and β, IL-6, IFNα, IFNγ and TNFα were used at a final concentration of 1000, 100, and 10 U/ml against nine melanoma cell lines (table 4.3); TGFβ2 was used at a final concentration of 100, 50, 10, 1, and 0.1 ng/ml. When added as single activating agents, none of these cytokines, with the exception of TNFα and IL-1α and β, altered the pattern of progelatinase expression by any of the cell lines used in the study. However, a pronounced and consistent response to TNFα was shown by A375, and A375/NUPR1 melanoma cells (table 4.3). 24 hour exposure to TNFα induced a 92 kDa progelatinase in a dose dependent manner in both of these related cell lines, detectable in both cell lysates and conditioned medium (Fig. 4.5). IL-1α and β induction of the 92 kDa progelatinase was observed with A375/NUPR1 cells but not A375 cells following a 24 hour exposure (table 4.3).
Dose dependent stimulation of the secretion of 92 kDa progelatinase by the A375 human melanoma cultures in response to 24 hour TNFα treatment.

Conditioned medium was collected from subconfluent A375 melanoma cultures stimulated with TNFα, 1000, 100, and 10 U/ml, for 24 hours. An aliquot (20 μl) was electrophoresed on 1 mg/ml gelatin, 7.5 % w/v crosslinked polyacrylamide minislab gels and analysed by zymography (48 hours incubation) as described earlier.
Dose dependent stimulation of the cell associated expression of 92 kDa progelatinase by the A375 human melanoma cultures in response to 24 hour TNFα treatment.

Cell lysates were prepared from subconfluent A375 melanoma cultures stimulated with TNFα, 1000, 100, and 10 U/ml, for 24 hours. An aliquot (20 μl) was electrophoresed on 1 mg/ml gelatin, 7.5 % w/v crosslinked polyacrylamide minislab gels and analysed by zymography (48 hours incubation) as described earlier.
Table 4.3.
Modulation of progelatinase activities by tumor necrosis factor α and interleukin one α and β

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control\textsuperscript{a}</th>
<th>TNFα\textsuperscript{b}</th>
<th>IL-1α/β\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>72</td>
<td>72 92</td>
<td>72</td>
</tr>
<tr>
<td>A375/NUPR1</td>
<td>72 (92)\textsuperscript{d}</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>DX3</td>
<td>72 92</td>
<td>72 92</td>
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<td>LT5.1</td>
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<td>MEL57</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Subconfluent cultures were washed twice for 1 hour at 37°C, then incubated in serum free medium for 24 hours at 37°C.
\textsuperscript{b} TNFα treated cultures were washed as described and exposed to 1000 U/ml of TNFα for 24 hours.
\textsuperscript{c} IL-1α/β treated cultures were washed and incubated as described and exposed to 1000 U/ml of IL-1α for 24 hours.
\textsuperscript{d} 92 kDa progelatinase was expressed at the limit of detection, however, it was clearly present in 10 fold concentrated medium.
4. 2. 5. Neutralisation of the Cytokine Modulation of 92 kDa Progelatinase Expression Using Specific Anti-Cytokine Anti-sera.

Specific neutralising antibodies were used to block the effects of TNFα and IL-1α on 92 kDa progelatinase induction. TNFα (1000 U/ml) and IL-1α (100 U/ml) were incubated with sufficient specific anti-sera to neutralise the effect of the cytokine present in serum free medium for 1 hour at 37°C prior to addition of the serum free medium to cell cultures. The conditioned medium was collected after 24 hours and analysed using gelatin zymography. Controls with and cytokine and anti-sera were performed in parallel. The effect of TNFα on A375 melanoma cell cultures and the effects of TNFα and IL-1α on A375/NUPR1 cell cultures were blocked by the respective anti-sera (shown in fig.4. 6). These results infer that TNFα and IL-1α exert a direct effect upon A375 and A375/NUPR1 melanoma cell cultures.
Specific neutralising antibodies were used to block the effects of TNFα and IL-1α on 92 kDa progelatinase induction. TNFα (1000 U/ml) and IL-1α (100 U/ml) were incubated with specific anti-sera to neutralise the effect of the cytokine present in serum free medium for 1 hour at 37°C prior to addition of the serum free medium to cell cultures. The conditioned medium was collected after 24 hours and analysed using gelatin zymography as previously described. Lane 1, A375/NUPR1 incubated with 1000U/ml TNFα for 24 hours; Lane 2, As lane 1 except the serum free cytokine containing medium was incubated with 110 neutralising units of anti-TNFα antibody for 1 hour prior to addition to the cells; Lane 3, A375/NUPR1 incubated with 100 U/ml IL-1α for 24 hours; Lane 4, As lane 3 except the serum free cytokine containing medium was incubated with 11 neutralising units of anti-TNFα antibody for 1 hour prior to addition to the cells.

Fig. 4. 6.
Neutralisation of TNFα and IL-1 induction of 92 kDa gelatinase expression.
4.2.6. Time Course Studies to Determine the Minimum Time of Exposure of Melanoma Cells to TNFα and IL-1α Necessary to Induce Expression of 92 kDa Progelatinase.

Time course studies, in which A375 cells were incubated with TNFα (1000 U/ml) for various time periods, 1, 4, 8, 16, and 24 hours, showed that the minimum time of exposure required to induce the 92 kDa progelatinase using A375 melanoma cells was 4 hours (Fig. 4.7), and 16 hours for A375/NUPR1 (summarised in table 4.4). Further studies revealed that IL-1α and β (both were used at 100 U/ml) induced the expression of 92 kDa progelatinase in A375/NUPR1 but not A375 cells, the time periods used were 1, 4, 8, 16, and 24 hours, the enzyme activity was first detectable in conditioned medium at the 16 hour time point (results are summarised in table 4.4).

4.2.7. Time Course Studies to Determine the Duration of 92 kDa Progelatinase Expression Following 24 Hour Exposure to TNFα and IL-1α.

Additional time course studies established that, following a 24 hour exposure of A375 cells to TNFα (1000 U/ml), the enzyme was still strongly expressed, in both cell lysates and conditioned medium, 72 hours after removal of exogenous TNFα by washing.

A375/NUPR1 treated with TNFα displayed similar longevity of expression, however, activity was undetectable at 96 hours post TNFα stimulation; in contrast IL-1α/β (100 U/ml) stimulated cells still expressed 92 kDa gelatinase activity at 96 hours post stimulation, results are summarised in table 4.5.
Time course studies using the A375 melanoma to determine minimum exposure times for induction of 92 kDa gelatinase by TNFα.

A375 melanoma cell cultures were incubated with TNFα (1000 U/ml) for the indicated time periods and the medium removed, the cells were washed twice with PBS for 5 minutes and incubated for the reminder of the 24 hour period in serum free medium. The serum free conditioned medium was analysed using gelatin zymography.
Table 4.4.

Modulation of 92 kDa progelatinase by the cytokines IL-1α/β and TNFα.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control¹</th>
<th>TNFα²</th>
<th>IL-1α³</th>
<th>IL-1 β³</th>
<th>Control¹</th>
<th>TNFα²</th>
<th>IL-1α³</th>
<th>IL-1 β³</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A375/NUPR1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
</tr>
<tr>
<td>12</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
</tr>
<tr>
<td>16</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
</tr>
<tr>
<td>24</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
<td>72 (92)</td>
</tr>
</tbody>
</table>

⁠¹ Cells were seeded at a concentration of 4x10⁴ cells/cm², allowed to settle for 24 hours in serum containing medium and washed for 1 hour twice with PBS prior to stimulation with cytokines. The conditioned medium and cell lysates were prepared as previously described.

⁠² Cells were exposed to TNFα at a concentration of 1000 U/ml for the designated time period, washed with PBS twice for 5 minutes and incubated in serum free medium for the duration of the experiment. The post TNFα stimulation incubation medium was assayed for enzyme expression by zymography.

⁠³ Cells were exposed to IL-1 at a concentration of 100 U/ml for the designated time period, washed with PBS twice for 5 minutes and incubated in serum free medium for the duration of the experiment. The post IL-1 stimulation incubation medium was assayed for enzyme expression by zymography.
Table 4.5.

Duration of 92 kDa progelatinase expression following stimulation by the cytokines IL-1α/β and TNFα for 24 hours.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Controla</th>
<th>TNFαb</th>
<th>IL-1αc</th>
<th>IL-1βc</th>
<th>Controla</th>
<th>TNFαb</th>
<th>IL-1αc</th>
<th>IL-1βc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72</td>
<td>72 (92)</td>
<td>72</td>
<td>92</td>
</tr>
<tr>
<td>48</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72</td>
<td>72 (92)</td>
<td>72</td>
<td>92</td>
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<td>72</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
<td>72</td>
<td>72 (92)</td>
<td>72</td>
<td>92</td>
</tr>
<tr>
<td>96</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72 (92)</td>
<td>72</td>
<td>92</td>
</tr>
</tbody>
</table>

A375/NUPR1

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Controla</th>
<th>TNFαb</th>
<th>IL-1αc</th>
<th>IL-1βc</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
</tr>
<tr>
<td>48</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
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<tr>
<td>72</td>
<td>72</td>
<td>72</td>
<td>92</td>
<td>72</td>
</tr>
<tr>
<td>96</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
</tbody>
</table>

a Cells were seeded at a concentration of 4x10^4 cells/cm^2 allowed to settle for 24 hours in serum containing medium and washed for 1 hour twice with PBS prior to stimulation with cytokines. Following completion of the experiment the conditioned medium and cell lysates were prepared as previously described and assayed by zymography.

b Cells were exposed to TNFα at a concentration of 1000 U/ml for 24 hours, washed with PBS twice for 5 minutes and incubated in serum free medium for the duration of the experiment.

c Cells were exposed to IL-1 at a concentration of 100 U/ml for 24 hours, washed with PBS twice for 5 minutes and incubated in serum free medium for the duration of the experiment.
4.2.8. Effects of Simultaneous Co-Stimulation of A375 with Multiple Cytokines.

Investigations into the possible synergistic or inhibitory actions of multiple cytokines were performed to determine if the expression of the 92 kDa progelatinase could be modulated by the simultaneous co-stimulation of A375 with TNFα plus another cytokine. TNFα was used at a constant concentration of 500 U/ml and the cytokines IL-1α, IL-2, IL-4, IL-6, IFNα, and IFNγ were titrated at final concentrations of 1000, 100, and 10 U/ml, TGFβ2 was used at final concentrations of 100, 50, 10, 1, and 0.1 ng/ml. These combinations were found to be without effect except for TNFα and TGFβ2, where TGFβ2 caused an upregulation of the 92 kDa proenzyme expression, detected as an increase in the size of the band. Epidermal growth factor when used at final concentrations of 1000, 100, and 10 U/ml with TNFα was also ineffective in altering progelatinase expression.
4. 2. 9. Synergistic Effect of TNFα and TGFβ2 on both Cutaneous and Posterior Uveal Melanoma Cell Lines.

Studies were undertaken to investigate the possible synergistic action of TNFα and TGFβ2 on progelatinase production. A375 tumor cells were exposed to TNFα at 1000, 100, and 10 U/ml together with TGFβ2 at 100, 50, 10, 1, and 0.1 ng/ml. This caused an apparent increase of 92 kDa progelatinase secretion (Fig. 4. 8) at all concentrations of TGFβ2 used. There was no quantitative or qualitative change in the secretion of the 72 kDa gelatinase by the A375 melanoma cell line as a result of co-stimulation with TNFα with TGFβ2. Similar results were obtained with A375/NUPR1 (table 4. 6). In further studies it was shown that exposure of SK23 and MEL56 to TNFα and TGFβ2 resulted in the detection of a 92 kDa progelatinase both in the cell lysate and conditioned medium (table 4. 6). Neither TNFα nor TGFβ2 alone induced this molecular weight species of progelatinase in SK23 nor MEL56 cells.
Synergistic action of TNFα and TGFβ2 on the secretion of 92 kDa progelatinase by the human melanoma A375.

Subconfluent A375 melanoma cultures were stimulated with TNFα (1000 U/ml) and TGFβ2, at 100, 50, 10, 1, and 0.1 ng/ml either singularly or in combination for 24 hours. The conditioned medium and cell lysates were electrophoresed on 1 mg/ml gelatin, 7.5 % w/v polyacrylamide minigels and analysed by zymography as described previously.
Table 4.6.
Modulation of progelatinase activities by tumor necrosis factor alpha and transforming growth factor beta 2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control\textsuperscript{a}</th>
<th>TNF\textsuperscript{b}</th>
<th>TNF + TGF\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>72</td>
<td>72 92</td>
<td>72 92\textsuperscript{d}</td>
</tr>
<tr>
<td>DX3</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>LT5.1</td>
<td>72 92</td>
<td>72 92</td>
<td>NT</td>
</tr>
<tr>
<td>SK23</td>
<td>72</td>
<td>72</td>
<td>72 92</td>
</tr>
<tr>
<td>MEL47</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>MEL52</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92\textsuperscript{d}</td>
</tr>
<tr>
<td>MEL56</td>
<td>72</td>
<td>72</td>
<td>72 92</td>
</tr>
<tr>
<td>MEL57</td>
<td>72 92</td>
<td>72 92</td>
<td>NT</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Subconfluent cultures were washed twice for 1 hour at 37\textdegree C, then incubated in serum free medium for 24 hours at 37\textdegree C.

\textsuperscript{b} TNF\textalpha treated cultures were washed as described and exposed to 1000 U/ml of TNF\textalpha for 24 hours.

\textsuperscript{c} TNF\textalpha + TGF\textbeta 2 treated cultures were washed and incubated as described but contained 10 ng/ml of TGF\textbeta 2 in addition to 1000 U/ml TNF\textalpha.

\textsuperscript{d} An apparent upregulation of the 92 kDa progelatinase was observed.

NT Not tested
4. 2. 10. Activation of Induced Progelatinase Activities by p-APMA.

Progelatinase-containing conditioned medium was incubated with p-APMA for varying periods of time (1-24 hours) to determine if the cytokine induced enzyme species were active or latent forms of gelatinase. Activation was determined to have occurred if there was a decrease in apparent molecular weight of the enzyme of approximately 6-8 kDa. This was observed following 4 hour incubation of the supernatant with 1 mM p-APMA (Fig. 4. 10), but total loss of the 92 kDa enzyme could not be achieved at any time point studied. The reason for this is at present unknown.
Fig 4.9.

Activation of progelatinase by p-APMA.

Serum free conditioned medium containing constitutively expressed and induced progelatinase species were incubated with 1 mM p-APMA for 4 hours at 37°C. The samples were analysed by zymography on 7.5 % w/v polyacrylamide minigels containing 1 mg/ml gelatin. Lane 1, A375 conditioned medium; lane 2, A375 conditioned medium following incubation with p-APMA; Lane 3, A375 conditioned medium following incubation with TNFα (1000 U/ml) for 24 hours; Lane 4, A375 conditioned medium following incubation with TNFα (1000 U/ml) for 24 hours and subsequently activated with p-APMA for 4 hours.
4. 2. 11. Modulation of Progelatinase Expression in the A375 Melanoma by Growth Factors: IGF 1, IGF 2, ACIDIC FGF, PDGF, and EGF.

The growth factors, Insulin like growth factors 1 and 2, acidic fibroblast growth factor, platelet derived growth factor and epidermal growth factor were added as single activating agents to subconfluent serum free A375 cell cultures for 24 hours. Insulin like growth factors 1 and 2 were used at final concentrations of 1000, 500, 100, 10 and 1 U/ml, epidermal growth factor, acidic fibroblast growth factor and platelet derived growth factor were used at final concentrations of 10,000, 1000, 100 and 10 U/ml. Following exposure to these growth factors in serum free conditions for 24 hours at 37°C no qualitative or quantitative alterations in progelatinase expression were observed (summarised in table 4. 7); similarly no induction of caseinolytic enzymes was observed.
Table 4.7.

Modulation of 92 kDa progelatinase expression in the A375 melanoma by growth factors.

<table>
<thead>
<tr>
<th>Progelatinase activities (kDa)</th>
<th>Control(a)</th>
<th>IGF 1(b)</th>
<th>IGF 2(c)</th>
<th>PDGF(d)</th>
<th>aFGF(e)</th>
<th>EGF(f)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
</tbody>
</table>

\(a\) The appropriate control was used for each growth factor, this was the solution in which the growth factor was reconstituted added to the serum free medium, no modulation of progelatinase expression was observed for any control solution used. Subconfluent melanoma cell cultures (4x10⁴ cells/cm²) were washed twice with PBS for 1 hour and incubated with growth factor containing or control medium for 24 hours at 37°C. The conditioned medium was collected and cell lysates were prepared as previously described.

\(b\) IGF 1 was used at final concentrations of 1000, 500, 100, 10 and 1 U/ml.

\(c\) IGF 2 was used at final concentrations of 1000, 500, 100, 10 and 1 U/ml.

\(d\) PDGF was used at final concentrations of 10,000, 1000, 100 and 10 U/ml.

\(e\) aFGF was used at final concentrations of 10,000, 1000, 100 and 10 U/ml.

\(f\) EGF was used at final concentrations of 10,000, 1000, 100 and 10 U/ml.

The extracellular matrix components collagens type I and IV, fibronectin and laminin were used to coat the growth surface of tissue culture flasks as described previously (materials and methods). Tumor cells were then seeded at a density of 1x10^6 cells/25cm^2 flask and allowed to attach and grow for 24 hours prior to washing with PBS twice for 1 hour at 37°C. The cell cultures were incubated for 24 hours in serum free medium, which was collected and stored as previously described. The conditioned medium was analysed by gelatin and casein zymography. Seeding of the cell lines A375, DX3, LT5.1 and SK23 onto the extracellular matrix components listed was without effect on progelatinase expression in this experimental system (table 4. 8). No caseinolytic enzymes were observed.
Table 4.8.

Modulation of Progelatinase expression by extracellular matrix components.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>Collagen type I</th>
<th>Collagen type IV</th>
<th>Fibronectin</th>
<th>Laminin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>DX3</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>LT5.1</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
<td>72 92</td>
</tr>
<tr>
<td>SK23</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
<td>72</td>
</tr>
</tbody>
</table>

a Subconfluent melanoma cell cultures (4 x 10⁴ cells/cm²) were washed twice with PBS for 1 hour and incubated in serum free medium for 24 hours at 37°C. The conditioned medium was collected and cell lysates were prepared as previously described. Cell counts were performed at the end of the experiment and cell numbers were found to be similar for each matrix component. All cell lines appeared to attach equally well to each matrix component over the 24 hour equilibration period.
b Tissue culture flask growth surfaces treated with the solution in which the extracellular matrix component was dissolved were used as controls.
c Collagen type I was used at final concentrations of 5, 10 and 15 µg/cm².
d Collagen type IV was used at final concentrations of 5, 10 and 15 µg/cm².
e Fibronectin was used at final concentrations of 1, 5 and 10 µg/cm².
f Laminin was used at final concentrations of 1, 2.5 and 5 µg/cm².
4. 2. 13. Modulation of Progelatinase Following Co-Stimulation of A375 Melanoma Cells with both Laminin and Cytokines.

The basement membrane component laminin was used to coat the growth surface of tissue culture flasks at a concentration of 5 μg/cm². Tumor cells were then seeded at a density of 1x10⁶ cells/25 cm² flask in serum free medium and allowed to attach and grow for 48 hours prior to washing with PBS twice for 1 hour at 37°C. The cell cultures were incubated for 24 hours in serum free medium or serum free medium containing either TNFα (1000 U/ml), TGFβ2 (10 ng/ml) or both TNFα and TGFβ2 combined (concentrations as before), which was collected and stored as previously described. The conditioned medium was analysed by gelatin and casein zymography. Seeding of the melanoma cell line A375 onto laminin and subsequent co-stimulation with the listed cytokine combinations failed to affect progelatinase modulation by cytokines in this experimental system (table 4. 9).
Table 4.9.
Modulation of A375 progelatinase expression by co-stimulation with cytokines and laminin.

<table>
<thead>
<tr>
<th>Progelatinase activities (kDa)</th>
<th>No Laminin</th>
<th>Laminin&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TNFα&lt;sup&gt;c&lt;/sup&gt;</td>
<td>TGFβ&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGFβ&lt;sup&gt;e&lt;/sup&gt;</td>
<td>TNFα +</td>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>TNFα&lt;sup&gt;c&lt;/sup&gt;</td>
<td>TGFβ&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>TNFα +</td>
<td>TGFβ&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>TGFβ&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

- 72 72 92 72 72 92 72 72 92

<sup>a</sup> Plates were coated with 5 μg/cm² of laminin, uncoated plates were treated with an identical volume PBS.

<sup>b</sup> Control cultures were incubated in serum free medium as previously described.

<sup>c</sup> TNFα was used at a final concentration of 1000 U/ml.

<sup>d</sup> TGFβ was used at a final concentration of 10 ng/ml.

<sup>e</sup> TNFα and TGFβ co-stimulated cultures were exposed to 1000 U/ml of TNFα and 10 ng/ml TGFβ.
4. 3. DISCUSSION.

An important characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at distant locations in the body. In the initial stages of tumor metastasis, the tumor cells become detached from the primary tumor and migrate into the surrounding tissues. In most cases the invading cells reach lymphatic or blood vessels from which they can spread to secondary organs, extravasate and form metastases (Poste and Fidler, 1980).

The influence of host factors on tumor metastasis due to the modulation of the expression of tumor products is of considerable interest, and studies by Dabbous et al. (1988) have demonstrated that cytokines could stimulate invasion in an in vitro assay.

Akedo et al. (1989) reported that preculture of rat ascites hepatoma cells with rat macrophages enhanced both their in vitro and in vivo invasion, as did TGFβ and activated platelets. This group suggested that the potentiation of invasive ability was mediated by oxygen radicals which caused aggregation of the tumor cells. It is also possible that a contributory factor in the potentiation of invasion is the activation of latent metalloproteiinases by an oxidative mechanism (Review: Van Wart and Birkedal-Hansen, 1990). In addition, activated macrophages are known to produce a number of cytokines including TNFα and TGFβ, it is possible that these cytokines may influence tumor cell behavior. Host cells, including platelets, endothelial cells and the various immunocytes which are known to be associated with tumors are capable of secreting a number of cytokines and growth factors. An aim of this study was to determine which, if any, of these factors were capable of modulating gelatinase expression. Basement membrane and interstitial matrix components were also studied, in order to provide a comprehensive appraisal of agents which may alter proteinase production and influence metastatic potential.

Wilhelm et al. (1989) using SV40 transformed fibroblasts demonstrated that the mRNA for a 92 kDa progelatinase could be induced by exposure of the cells to IL-1, EGF and TPA. In contrast, Turpeenniemi-Hujanen et al. (1986) reported that expression of type IV procollagenase by the A2058 melanoma was down regulated by
EGF. Furthermore, Okada et al. (1990) demonstrated that TNFα is a potent inducer of 92 kDa progelatinase expression in a variety of sarcoma cell lines, although a number of cytokines and growth factors: IL-1α, PDGF, EGF, bFGF and TGFβ failed to influence the progelatinase profile of the cells. In the present study the effect of a number of cytokines, IL-1α, IL-2, IL-6, IFNα, IFNγ, TNFα and TGFβ2 and growth factors, IGF 1 and 2, aFGF, PDGF and EGF on progelatinase expression by melanomas was investigated. In agreement with previous studies (Okada et al., 1990) these factors were found to be without effect except for TNFα. Differences between our studies and those of Wilhelm et al. (1989) may reflect tissue or cell type differences between the cells studied, or differences in the response of cells transformed with other agents e.g. SV40, or responses which are tumor tissue specific.

Initially TNFα was reported to cause haemorrhagic necrosis of murine tumors (Carswell et al., 1975), suggesting that it may be of value in patient therapy. However, a consistent anti-tumor effect in cancer patients could not be achieved (Frei and Spriggs, 1989). The therapeutic action of TNFα in animal models is thought to be due to a combination of biological effects and, in part, due to its cytotoxic and/or cytostatic potential (Sugarman et al., 1985), and its ability to cause activation of anti-tumor immune surveillance mechanisms (Palladino et al., 1987). Recently TNFα has been reported to have multiple effects on both tumor and host cell behaviour, including the stimulation of angiogenesis (Frater-Shroder et al., 1987), osteoclastic bone resorption (Bertolini et al., 1986), tumor cell invasion and metastasis of human ovarian carcinoma xenografts (Malik et al., 1989), enhanced experimental metastasis in mice (Malik and Balkwill, 1991), and most recently in the induction of 92 kDa gelatinase in osteosarcoma and fibrosarcoma cells (Okada et al., 1990). TNFα may therefore play an important role in modulating the invasive properties of tumor cells, and the results of the present study are consistent with this hypothesis.

Reports by Yamagata et al. (1988; 1989) have correlated expression of the 92 kDa gelatinase with increased metastatic potential in a number of tumor types, and our
results using the A375 melanoma show that exposure to TNFα can induce the expression of this molecular weight species of enzyme. A recent study by Overall et al. (1989), reported that progelatinase production by fibroblasts was stimulated by TGFβ, although the results of the present study show that human melanoma cells are unaffected by the addition of TGFβ2. TGFβ2 was found to synergise with TNFα to increase progelatinase detection in A375 and to induce the expression of the 92 kDa progelatinase in SK23 and MEL56. These results suggest that some melanoma cells either do not require TGFβ as a co-stimulant to regulate enzyme production, or that they may constitutively release TGFβ2, thereby requiring only exogenous TNFα to induce gelatinase gene expression. Analysis of TGFβ2 production by the tumor cell lines used in this study would be beneficial in determining if this is a possible mechanism. Whether tumor cells in vivo may be affected by TNFα secreted from infiltrating mononuclear cells is not yet known, although cell-cell communication (between tumor and host cells) is likely to influence tumor cell behaviour. The A375 melanoma has been shown to be capable of inducing TNFα secretion from a monocyte cell line in vitro (Sabbatini et al., 1990), suggesting the production of this cytokine is regulated by tumor cell signals, and this in turn could serve to increase the invasive and metastatic capacity of cells, through the induction and/or increase in gelatinase activity.

In this study a subpopulation of the A375 melanoma (A375/NUPR1), selected by in vivo passage in nude mice, was sensitive to the action of IL-1α and β whereas the total (parental) tumor population shows no detectable induction of the 92 kDa gelatinase. TNFα and IL-1α/β treatment of A375/NUPR1 cells both result in the detection of the 92 kDa progelatinase species, however the time of detection is markedly different. It is possible that IL-1 may act indirectly by inducing TNFα expression by the tumor cells, resulting in autocrine stimulation of progelatinase expression. Further studies are needed to investigate this possibility. A discussion of the respective gelatinase promoter structures and their possible role in explaining these observations is present in the general discussion chapter of this thesis.
Phase separation studies revealed the presence of a membrane associated 72 kDa progelatinase species in both A375 and A375/NUPR1, in addition a hydrophilic species was also detected. Zucker et al. (1985a, 1987a, 1987b, 1990) reported the presence of membrane associated gelatinases, and elucidating the importance of membrane associated enzymes versus secreted enzymes is of intense interest to enzymologists. Sas et al. (1986) reported that degradation of extracellular matrix fibronectin, laminin and type IV collagen occurred only beneath or along the path of the tumor cell migration, indicating that degradation was due to cell surface associated rather than secreted proteinases. In addition, Chen and Chen, (1987) have reported that degradation of fibronectin by rous sarcoma virus-transformed fibroblasts occurs only at the sites of cell-substrate contact and a 150 kDa metalloproteinase was extracted from the cell membrane of transformed but not normal cells. However, melanoma cells have been reported to secrete high levels of gelatinase relative to enzyme concentrations in the plasma membranes (Zucker et al., 1990). Whilst it is recognised that there are advantages to the membrane localisation of proteinases, namely protection from proteinase inhibitors and co-localisation of the enzyme and its substrate, membrane localisation may represent merely a transient situation prior to the shedding of membrane vesicles (Zucker et al., 1987c). It is not known if the extracellular activities observed in this study are soluble or located within vesicles.

It is interesting to note that both cutaneous and posterior uveal melanomas express the 72 kDa and 92 kDa species of progelatinases, and the activities are modulated by the same cytokines. However, the expected sites of metastasis for these types of melanoma are different. Whilst progelatinase expression is associated with the metastatic phenotype it may not determine the secondary site for metastasis. This may be dependent upon cell-cell, cell-substratum interaction via adhesion molecules, or due to the specific organ microenvironment.

Gelatinase, in addition to its role in basement membrane invasion is thought to have a synergistic relationship with interstitial collagenase in the degradation of type I
collagen. Enhanced matrix degradation could be a contributing factor for increasing the \textit{in vivo} growth rate of tumors.

Turpeenniemi-Hujanen \textit{et al.} (1986), demonstrated that laminin could increase the expression of type IV collagenase from malignant cells and Emonard \textit{et al.} (1990) confirmed the effect of laminin on type IV collagenase expression using trophoblasts. In our studies, laminin was found to be without effect on melanoma cells, however, other melanoma cell lines have been reported to be responsive to laminin: the A2058 melanoma, B16 F10 melanoma and the fibrosarcoma cell line HT 29. Laminin is known to have a number of cell binding sites (Review: Humphries, 1990) and it is possible that the human melanoma cells used in this study may not express the receptor associated with an increase in type IV collagenase expression.

The level of active enzyme is dependent upon enzyme: inhibitor ratios, whilst this qualitative study has identified progelatinase expression patterns further studies to quantitate metalloproteinases and their inhibitors would be informative.
CHAPTER 5

INTRODUCTION: POSTERIOR UVEAL MELANOMA: PROGNOSTIC INDICATORS
5. 1. INTRODUCTION: POSTERIOR UVEAL MELANOMA: PROGNOSTIC INDICATORS.

Uveal melanoma is a rare cancer (Deves et al., 1987), but it holds considerable interest because of the rapid and unexplained rise in incidence of the related but more common melanomas of the skin (Cutler and Young, 1975). They represent approximately 80% of all non-cutaneous melanomas (Scotto et al., 1976). The etiology of uveal melanoma is at present uncertain, possible causes including viral infection (Albert, 1979), chemical agents (Albert et al., 1980) and malignant transformation of benign nevi of the choroid. Although uncommon in the context of general neoplasia, uveal melanomas of the ciliary body and choroid, are the most common primary adult intraocular malignancy, they frequently metastasize, and approximately half of the patients die from their disease within 15 years of enucleation (Egan et al., 1988). Metastatic disease is responsible for virtually all tumor-related deaths. However, not all tumors have the same metastatic potential; some tumors have little or no metastatic capabilities whereas others metastasise readily. Once metastases occur the prognosis is extremely poor, with a median survival time of six months (Seddon et al., 1983), and despite recent advances in treatments, including many directed at preserving the eye, the overall prognosis has remained unaltered (Zimmerman and McLean, 1984). Traditional prognostic indicators rely heavily on the assessment of the morphological and histological appearances of the tumor; the tumor location and size are of prognostic significance. Generally "posterior" uveal melanomas located anterior to the equator have a less favorable prognosis than ones located posteriorally (Shammas and Blodi, 1977). In addition, large tumors (tumor diameter greater than 10 mm) have a less favorable prognosis than small tumors (Shammas and Blodi, 1977).

Histologically, uveal melanomas may be classified as: spindle cell, epithelioid cell or mixed cell tumors. Pure spindle cell tumors generally have a favourable prognosis with a five year survival rate of approximately 80%, whereas mixed and epithelioid tumors fare less favourably, with pure epithelioid cell tumors having a five year
survival of only 40% (Paul et al., 1962). Calculation of the inverse standard deviation of the nucleolar area has been assessed as an indicator of survival (John et al., 1977).

In addition to metastatic potential, the relative growth rates of the primary tumors may also vary (Augsburger et al., 1984; Friberg et al., 1983). Flow cytometry enables the rapid analysis of single cell suspensions by virtue of the light scatter properties of cells, which may or may not be stained with DNA or protein specific fluorochromes. It has been shown to be a reliable quantitative method of determining the DNA content of tumor cells (Freidlander et al., 1984); and from such analyses, details of the cell cycle kinetics, the percentage of cells undergoing division (those cells in G2/M and S phase of the cell cycle relative to those in G0/G1) and ploidy (total DNA content) of the tumor cell population can be obtained. The presence of non-diploid (aneuploid) cells in some human tumors may infer a poor prognosis (Freidlander et al., 1984).

Prior to our studies, flow cytometric studies of uveal melanomas have been limited to analysis of histological material recovered from paraffin embedded specimens (Meecham and Char, 1986; Shapiro et al., 1986). Meechan and Char (1986) retrospectively correlated ploidy and clinical outcome, and found that hyperploidy correlated with poor prognosis. Due to the reported technical difficulties encountered when using paraffin embedded material for flow cytometry (Lawry et al., 1987), namely formal saline fixation induced artefacts, including reduced fluorescent intensity and high levels of cellular debris which make histogram interpretation difficult (Shapiro et al., 1986), we have used flow cytometry in a prospective study to determine the cellular DNA content in fresh tumor tissue obtained from 49 uveal melanomas. Initial studies were published (Rennie et al., 1989) and these together with the results of further studies are included in this chapter. In addition to providing a ploidy index for the tumor tissue, this technique permits us to obtain an index of cell proliferation by studying the percentage of cells in each phase of the cell cycle. The results and their relationship to various tumor parameters including, anatomical location, tumor size and histological cell type are discussed. For many biological studies of tumor cell characteristics, it is necessary to establish in vitro cell cultures.
Thus, a lack of an economically acceptable culture system which permits the routine in vitro growth of uveal melanomas from biopsy specimens has hindered the study of the basic properties of this tumor type.

The ability to grow human uveal melanoma in vitro enables the investigation of a number of diverse phenotypic properties which may be involved in both tumorigenesis and metastasis. The first reported attempt to grow uveal melanoma cells in vitro was that of Kirby (1929). A number of studies demonstrated that uveal melanoma cells can survive in culture for periods of several months if passage of the cells is not attempted (Pomerat et al., 1956; Barishak et al., 1960). The culture medium shown to be optimal for growth was a modification of Hams F12.

Albert et al. (1984) described a long term in vitro culture system for these tumors which relied upon tumor cell migration from explants which were seeded onto a fibroblast feeder layer, in addition they used 12-O-tetradecanoyl-phorbol-13-acetate (PMA) to inhibit the growth of fibroblasts and keratinocytes. This group determined that for long term culture of uveal melanoma the following factors were important:

1) the use of Hams F12 basal medium.
2) the use of the human fibroblast cell line MRC-5 as a feeder layer.
3) addition of epidermal growth factor, and cholera toxin to the medium.
4) a combination of fetal bovine serum and horse serum.
5) a high concentration of glucose which seemed to increase the longevity of the cultures.

Albert et al., (1984) used a high concentration of epidermal growth factor (10 μg/ml) in their culture system, but in the present study the routine use of this concentration of EGF was found to be too expensive; cholera toxin and PMA were omitted from the growth medium as were fibroblast feeder layers, where a high risk of fibroblast overgrowth could occur. The use of different basal medium, and supplements was studied. In addition a variety of extracellular matrix components and tissue culture plastics were used as growth substrata.
Tissue cultures were identified as being abnormal by cytogenetic analysis (Sisley et al., 1990), which has allowed examination of a number of different phenotypic and genotypic parameters associated with this tumor type with the aim of developing new prognostic indicators for this tumor type. Using the described tissue culture procedure it has been possible to grow successfully 80 % of tumors for periods of up to 3 months, and for several in vitro passages. Successful establishment of tumor cell lines was dependent upon the quantity of tumor material available as was the duration of the cell line in tissue culture.

In spite of their importance in the context of ocular neoplasia, little information is available on the cytogenetics of uveal melanomas. Rey et al. (1985) and Griffin et al. (1988) reported on single cases, however more recent studies have reported non-random abnormalities of chromosomes 3, 6, and 8 (Horsman et al., 1990; Prescher et al., 1990; Sisley et al., 1990), most of the published work on melanocytic tumors being confined to cutaneous melanomas (Pathak et al., 1983; Balaban et al., 1984; Cowan et al., 1986; De Lucca et al., 1988).

The overall ability of uveal melanoma to produce metastases is high, but it is recognised that metastatic potential varies from tumor to tumor, some having little or no metastatic potential, whereas others readily metastasise, and in part, these differences are reflected by the histological appearances of the tumor (Paul et al., 1962). Tumor invasion and metastasis are complex interrelated multistage events which involve detachment and migration of tumor cells from the primary tumor mass into the surrounding tissues followed by spread to distant sites via the vasculature or lymphoid system. A major barrier to intravasation is the vascular basement membrane, a similar membrane is found around the lymphoid channels, this membrane is synthesised and lined by a layer of endothelial cells. If tumor metastasis is to occur, the tumor cell must attach to either the endothelial cells or to denuded basement membrane should this option be available, possibly as a result of fenestrations or damage to the endothelial cell layer. In the first situation the endothelial cells must be
stimulated to retract allowing the tumor cells access to the basement membrane, once this has occurred basement membrane degradation may proceed. Alternatively tumor cells may stimulate endothelial cells to alter the balance between synthesis and degradation of the basement membrane.

Intra-ocular tumors, because of the lack of lymphatics, metastasise to distant sites exclusively via the blood stream, which implies that tumor cells must be capable of traversing blood vessel walls, including the basement membrane, and it is likely that tumor or host derived proteinases are major participants in this process. Expression of a number of different enzymes by tumor cells has been found to correlate with tumor invasion and/or metastasis: (a) metalloproteinases including interstitial collagenase (Liotta et al., 1982; Wooley, 1984), stromelysin (Matrisian et al., 1986) and gelatinase/type IV collagenase (Liotta et al., 1982); (b) serine proteinases including plasminogen activator (Quax et al., 1991), elastase (Kao et al., 1982) and mast cell chymase (Chiu and Lagunoff, 1972); (c) cysteine proteinases including cathepsins B, H and L (Sloane and Honn, 1984; Murnane et al., 1991), and (d) glycosidases (Bernacki et al., 1985) including hyaluronidases (Fiszer-Szaforz and Gullino, 1970) and heparanases (Nakajima et al., 1984). In the reports cited above it should be noted that each tumor type has a specific enzyme profile. One of the aims of this study was to identify the enzyme profile associated with uveal melanomas which enables some tumors to metastasise and not others.

Evidence has accumulated suggesting that a proteinase cascade involving serine and/or cysteine proteinase activation of one or more metalloproteinases, is pivotal to the breaching of basement membranes; in part due to the ability of these enzymes to degrade type IV collagen (Nakajima et al., 1987; Fessler et al., 1984; Liotta et al., 1982).

The metalloproteinase family of enzymes includes interstitial collagenase (Goldberg et al., 1986), 72 kDa gelatinase (type IV collagenase) (Collier et al., 1988), 92 kDa gelatinase (Wilhelm et al., 1989), stromelysin-1 (Wilhelm et al., 1987), stromelysin-2 (Muller et al., 1988), stromelysin-3 (Basset et al., 1990), and Pump-1
The metalloproteinase enzymes are secreted as zymogens which require modification to their structure prior to attaining enzymic activity (Stetler-Stevenson et al., 1989). Several studies have implicated these enzymes, and in particular stromelysin and type IV collagenase, in basement membrane degradation at the tumor cell-host interface; this process represents one of the key events in the invasive process (Nakajima et al., 1987; Fessler et al., 1984; Liotta et al., 1982). Yamagata et al. (1988; 1989), used zymography to compare the properties of two gelatinases (60 and 95 kDa forms), secreted in the conditioned media of murine fibroblasts, macrophages and various tumor cell lines, and demonstrated that cell lines with the greatest metastatic potential secreted the highest amount of the 95 kDa gelatinase (Yamagata et al., 1988; 1989). Furthermore, the present study has shown that in both cutaneous and posterior uveal melanoma cell lines, gelatinase production is influenced by the presence of certain cytokines and growth factors, particularly TNFα and TGFβ (Cottam et al., 1991).

In this chapter, the expression of metalloproteinase enzymes in short term (1-5 passages) cultures of human uveal melanomas is reported, and the expression of enzyme subtypes by these tumors are assessed in relation to other prognostic features of these tumors.

Tumor cell invasion of the vascular basement membrane and the extracellular matrix is thought to occur by the synergistic action of a number of proteinases (Reich et al., 1988; Mignatti et al., 1986). In addition to gelatinase, posterior uveal melanomas were found to express tissue-type plasminogen activator (tPA). Plasminogen activator expression and its relationship with tumor malignancy and other prognostic indicators was investigated. High levels of expression of tPA have been associated with cutaneous melanomas (Wilson et al., 1980) but the expression of plasminogen activators by posterior uveal melanomas has received little attention. In this study the expression of plasminogen activators in a series of short term posterior uveal melanoma cell cultures was established and compared to the level of expression.
of tPA in a series of established cutaneous melanoma cell lines. A recent report by Tsuboi and Rifkin (1990) suggested that a high level of expression of plasminogen activator may inhibit invasion rather than increase invasion of a denuded amniotic membrane due to the weakening of cell substratum contacts. However tPA has a number of functions which may augment metastasis rather than inhibit the process. tPA has a platelet binding site (Vaughan et al., 1989) which may, upon the formation of heterotypic platelet-melanoma cell emboli, facilitate the localisation of this enzyme in a similar way to the cell surface UK receptor. tPA is also reported to have a role in both platelet activation (Ohlstein et al., 1987; Niewiarowski et al., 1973) and platelet inhibition (Niewiarowski et al., 1973) and to cause platelet disaggregation (Loscalzo et al., 1987; Niewiarowski et al., 1973); this variety of actions appears to be the result of the concentration of plasmin produced as well as the duration of exposure to a given plasmin concentration (Loscalzo et al., 1987; Niewiarowski et al., 1973). The secretion of a number of platelet associated growth factors is linked with these events (Poggi et al., 1988). Thus platelets may contribute to successful haematogenous spread by (1) forming a protective barrier between the tumor cells and the immune system following platelet aggregation, (2) causing release of platelet associated growth factors and (3) expression of platelet adhesion molecules which may enable attachment to basement membrane or endothelial cells (Albelda and Buck, 1990). In addition tPA is involved in stimulating endothelial cell migration (Inyang et al., 1990) and in fibrinolysis (Astrup et al., 1947), both of these actions may be important in allowing tumor cells access to the basement membrane. Subsequent basement membrane degradation may occur due to the action of tPA alone or by the synergistic action of tPA and gelatinases. Uveal melanomas, in addition to haematogenous spread, may invade locally through the sclera, which is composed of a mixture of proteoglycans, associated with fiber forming collagens. Whilst neither PA nor gelatinase can directly degrade the interstitial collagen triple helix, gelatinase is thought to have a synergistic action with interstitial collagenase in the degradation of these collagen types and plasmin is known to degrade the telopeptide link regions between collagen fibers. This
suggests an important interrelationship between gelatinase and PA, which could potentiate tumor cell invasion.
5. 2. RESULTS.

5. 2. 1. DNA Ploidy and Rate of Proliferation of Posterior Uveal Melanoma Cells.

Based on the finding of this study, one publication has arisen and another is under review, a summary of the findings is given below.

5. 2. 1. 1. Patient Details

Forty nine tumors were analysed by flow cytometry, of the 49 patients, there were 27 males (55 %) and 22 females (45 %) with a mean age of 61.5 years (range 21-90 years). The mean period of follow up was 20.7 months (range 0-47 months). During this time four patients died, two had proven metastatic disease, one had suspected metastatic disease and for the other patient no details were available. Mean survival times for these patients were 16.2 ± 10.9 months (range 8 to 32 months).

5. 2. 1. 2. Pathological Details of the Tumors.

Thirty one patients had choroidal melanomas (63 %), four patients (8 %) had ciliary body tumours and fourteen patients (29 %) had tumors which involved both the choroid and ciliary body. Forty two tumors were classified as large (86 %) and seven were medium (14 %).

Two tumors were classified as spindle (4 %), 34 as mixed (69 %) and 11 as epithelioid (22 %), no data was available on 2 tumors. Lymphocyte infiltration was described as low.

5. 2. 1. 3. Flow Cytometry.

Flow cytometric analysis of 49 posterior uveal melanoma tumors showed that 47 (96 %) had a diploid cell population, of these 2 (4 %) had a tetraploid subpopulation and 12 (24 %) had an aneuploid subpopulation, 3 (6 %) tumors were purely aneuploid (representative histograms are shown in figure 5. 1).

The percentages of cells in G2/M and S phases in the 32 pure diploid tumors were summed to provide an index of cell proliferation, the percentage of cells in G2/M/S was 7.3 ± 4.1 % (range 1.9 to 18.2 %).
The flow cytometric results for pure diploid tumors were then compared with histological cell type. The proportion of cells in G2/M/S for the 22 mixed cell tumors was 7.5 ± 4.6 % and for the 6 epithelioid cell tumors it was 7.5 ± 3.6 %, the 2 diploid spindle cell tumors had a value of 4.5 ± 1.2 %, cell type data was not available for two diploid tumors (results are summarised in table 5.1).

Whilst proliferation rates could not be determined for non-diploid tumors comparisons with the cell type classification were performed. Of the 2 diploid tumors which contained a tetraploid subpopulation 1 was mixed, data was not available on the other. Of the 12 diploid tumors with aneuploid subpopulations 3 were epithelioid, 8 were mixed and 1 was spindle. 2 of the aneuploid tumors were mixed cell and 1 was epithelioid. There appeared to be a link between epithelioid cell type of the tumor and aneuploidy.

There appeared to be a difference in the percentage of cells in G2/M/S between medium (5.8 ± 1.6 %) and large (7.8 ± 4.7 %) tumors. In agreement with our earlier report we found that there was no statistically significant difference between tumor location in the eye and the percentage of cells in G2/M/S. The proportion of cells in G2/M/S was 6.8 ± 6.4 % for ciliary body tumors, 7.0 ± 4.2 % for choroidal tumors and 7.9 ± 4.3 % for tumors involving both the ciliary body and choroid. The three patients who died of proven metastatic disease had mean G2/M/S phase values of 11.5 ± 1.6 % (range 9.6 to 14.7 %). The coefficient of variation (CV) of the G0/G1 peaks, which is a measure of sample quality was low, 7.5 ± 2.3 % (range 3.3 to 13.5 %).
Table 5.1.
Comparison of cell type with respect to mean percentage cells in the G$_2$/M/S phases of the cell cycle.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>N</th>
<th>Mean$^b$</th>
<th>Std. Dev</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spindle</td>
<td>2</td>
<td>4.5</td>
<td>1.2</td>
<td>3.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Mixed</td>
<td>22</td>
<td>7.5</td>
<td>4.6</td>
<td>1.9</td>
<td>16.8</td>
</tr>
<tr>
<td>Epithelioid</td>
<td>6</td>
<td>7.5</td>
<td>3.6</td>
<td>3.0</td>
<td>11.3</td>
</tr>
</tbody>
</table>

$^a$ Determined using the cell type classification system of McLean et al. 1983.

$^b$ Determined by flow cytometric analysis of digested fresh uveal melanoma tissue (Rennie et al., 1989).
Fig. 5.1.

A series of representative FACS analyses.

Uveal melanoma samples were analysed using flow cytometry as previously described and their ploidy values were determined in comparison to a lymphocyte control sample which represented a diploid population of cells.

MEL22, Diploid uveal melanoma.

MEL35, Aneuploid uveal melanoma.

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**DNA Content**

**Control**

G₀/G₁ peak channel 51.2
= 91.2 %, CV = 2.7

**MEL22**

G₀/G₁ peak channel 50
= 81.0 %, CV = 4.4

**MEL35**

G₀/G₁ peak channel 50
= 90.6 %, CV = 6.8
MEL52. Diploid and aneuploid populations within the tumor.

Control

MEL52

G₀/G₁ peak channel 51.2
= 91.2 %, CV = 2.7

G₀/G₁ (dip) peak channel 51.2
= 96.6 %, CV = 8.0

G₀/G₁ (aneu) peak channel 61.0
= 88.0 %, CV = 7.6

MEL23. Diploid and tetraploid populations within the tumor.

Control

MEL23

G₀/G₁ peak channel 50
= 90.6 %, CV = 5.2

G₀/G₁ (dip) peak channel 46
= 67.3 %, CV = 6.8

G₀/G₁ (tet) peak channel 91
= 23.2 %, CV = 75.1
5. 2. 2. The Development of a Short Term Culture System for Posterior Uveal Melanoma.

Preliminary investigations to determine the most efficient tumor digestion protocol resulted in the following procedure: mincing of the tumor tissue with scalpels until the tissue fragments were less than 1mm³ and the suspension of them in a digestion cocktail containing collagenase type II (0.025 %), pronase E (0.05 %) and dithiothreitol (0.5 μM) in PBS; the digested tumor tissue was seeded onto a number of different growth surfaces in a variety of growth media. The optimum combination of factors was determined using different tumors. Less comprehensive studies were performed using individual tumors to 'fine tune' the protocol but these usually had a negligible effect upon successful melanoma cell culture establishment and longevity of the culture in vitro and shall not be discussed. As a consequence of the diversity of tumor cell phenotype the tumor was routinely suspended in 10 ml of growth medium and seeded onto two 25 cm² tissue culture flasks, if the tumor was exceptionally large or small this would be increased or decreased accordingly. Whilst this study has not fully elucidated the optimum growth conditions for uveal melanoma cell culture it has provided a reliable culture system enabling investigation of the in vitro characteristics of this tumor type. Photographs of uveal melanoma cells in culture are shown in figure 5. 2.

Study One.

Determination of optimal growth medium for cell attachment and growth.

Freshly digested uveal melanoma cells were suspended at a concentration of 1 x 10⁶ cells/ml in seven different growth media and seeded onto tissue culture plastic using a volume to growth area ratio of 1 ml of cell suspension/5cm² of growth surface (5 x 10⁶ cells/25 cm² tissue culture flask). The cells were allowed to attach for 4 days at which point the medium was replaced with fresh growth medium. Growth medium was subsequently changed every 4 days until the end of the experiment. The different growth media were compared for their ability to allow successful attachment, growth
and their ability to support continued cellular growth over extended periods of time. The optimum growth medium was found to have a Hams F12 base supplemented with 15 % FCS, 5 % Donor horse serum, glucose (2 g/l) and epidermal growth factor (2 ng/ml) (results are summarised in table 5.2).
Table 5.2.

Determination of optimal growth medium for cell attachment and growth.

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>Attachment(^a)</th>
<th>Growth(^b)</th>
<th>Longevity of the Culture(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 + 10 % FCS</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RPMI 1640 + 20 % FCS</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulbecco's + 10 %</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulbecco's + 20 %</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hams F12 + 10 % FCS</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hams F12 + 20 % FCS</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Oc. Mel Media(^d)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

\(^a\) Cell cultures were assessed by eye and the percentage of attached cells to the percentage of floating cells determined and results recorded as follows: + low (0-30 % attachment), ++ medium (30-60 % attachment), +++ high (60-100 % attachment)

\(^b\) Indicates the comparative time required to reach confluence (90 % of the growth area covered by the cell monolayer) and results are represented as follows: - no growth, + low proliferation (the culture reaches confluence after 1 month), ++ average proliferation (the culture reaches confluence after 2-3 weeks), +++ high proliferation rate (the culture reaches confluence after 1-2 weeks).

\(^c\) Indicates the comparative number of in vitro passages for the culture to senescence (no detectable increase in cellular confluency for 2-3 weeks) and is represented as follows: - no initial growth, + low growth potential (reaches passage 1-2), ++ medium growth potential (reaches passage 3), +++ high growth potential (reaches passage 4-5).

\(^d\) Composition: Hams F12 basal medium supplemented with 15 % FCS, 5 % Donor horse serum, glucose (2 g/l) and epidermal growth factor (2 ng/ml).
Study Two.
Determination of optimal growth substratum for cell attachment and growth.

Freshly digested uveal melanoma cells were suspended at a concentration of $1 \times 10^6$ cells/ml in ocular melanoma growth media and seeded onto six different growth surfaces using a volume to growth area ratio of 1 ml of cell suspension/5 cm$^2$ of growth surface ($5 \times 10^6$ cells/25 cm$^2$ tissue culture flask). The cells were allowed to attach for 4 days at which point the medium was replaced with fresh growth medium. Growth medium was subsequently changed every 4 days until the end of the experiment. The different growth surfaces were compared for their ability to allow successful attachment, growth and their ability to support continued cellular growth over extended periods of time. The optimum growth substrata were found to be collagens type I and IV and Costar vented tissue culture flasks the reason for the difference between the two different costar tissue culture flasks is unknown but may be due to improved circulation of CO$_2$ (results are summarised in table 5.3). For simplicity Costar vented tissue culture flasks were routinely used.
Table 5.3.

Determination of optimal growth substratum for cell attachment and growth.

<table>
<thead>
<tr>
<th>Growth surface</th>
<th>Attachment$^a$</th>
<th>Growth$^b$</th>
<th>Longevity in Culture$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Laminin</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Costar tissue</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>culture flasks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Costar vented</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>tissue culture flasks</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Cell cultures were assessed by eye and the percentage of attached cells to the percentage of floating cells determined and results recorded as follows: + low (0-30% attachment), ++ medium (30-60% attachment), +++ high (60-100% attachment)

$^b$ Indicates the comparative time required to reach confluence (90% of the growth area covered by the cell monolayer) and results are represented as follows: - no growth, + low proliferation (the culture reaches confluency after 1 month), ++ average proliferation (the culture reaches confluency after 2-3 weeks), +++ high proliferation rate (the culture reaches confluency after 1-2 weeks).

$^c$ Indicates the comparative number of in vitro passages for the culture to senescence (no detectable increase in cellular confluency for 2-3 weeks) and is represented as follows: - no initial growth, + low growth potential (reaches passage 1-2), ++ medium growth potential (reaches passage 3), +++ high growth potential (reaches passage 4-5).
5. 2. 3. Cytogenetic Analysis of Short Term Posterior Uveal Melanoma Cultures.

In a collaborative study (with Miss K. Sisley), who analysed short term cell cultures for cytogenetic abnormalities, the karyotypes of sixteen posterior uveal melanomas were analysed. Cytogenetic analysis of lymphocyte preparations showed that all patients had a normal constitutional chromosome complement. The results of the analysis are summarised in table 5.4 and representative karyotypes are shown in figure 5.3. Based on the cytogenetic findings of this study, one publication has arisen and another has been accepted for publication, a summary of genetic characteristics is given below.

No cytogenetically detectable abnormalities were detected in two cases (MEL17 and MEL61). 3 patients showed the loss of a sex chromosome, the Y chromosome (cases MEL22, MEL35 and MEL60). Nine tumors (MEL11, MEL22, MEL30, MEL35, MEL37, MEL53, MEL47, MEL59, and MEL63) showed structural anomalies of chromosome 6, which took the form of a deletion of the long arm, or trisomy of the short arm. Seven ciliary body tumors (MEL10, MEL30, MEL35, MEL37, MEL52, MEL57 and MEL63) had monosomy of chromosome 3 with one or two copies of i (8q). In MEL52 and MEL62 there was evidence of clonal evolution with some cells demonstrating increased dosage of the long arm of chromosome 8. One ciliary body tumor (MEL53) had monosomy of chromosome 3 but no associated i(8q), and one choroidal melanoma (MEL59) had trisomy 8 but no corresponding loss of chromosome 3. Three tumors (MEL52, MEL47, and MEL62) had deletions of the long arm of chromosome 11. Other abnormalities involved chromosome 1 (MEL11, MEL22 and MEL52), two of these were deletions of the short arm (MEL11 and MEL22) and chromosome 13 was abnormal in MEL53 and MEL59. Trisomy 7 was observed in MEL22 and MEL35 and monosomy 16 in MEL30 and MEL35. In MEL22 two derivative chromosomes 22 were present with material possibly of Y origin, but it was not possible to perform C-banding to confirm this. A marker chromosome was present in MEL30, but could not be identified as a derivative of the
missing chromosome 13. Two clones were evident in MEL60 and MEL52 demonstrated several sub clones.

Table 5. 4.

Karyotypes of a series of short term cultured uveal melanoma cell lines: major changes.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chromosomal alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL 10</td>
<td>Normal/ +6p</td>
</tr>
<tr>
<td>MEL 11</td>
<td>-3/+ i8q</td>
</tr>
<tr>
<td>MEL 22</td>
<td>+7/ deleted chromosome 1 der 6q</td>
</tr>
<tr>
<td>MEL 27</td>
<td>Normal</td>
</tr>
<tr>
<td>MEL 30</td>
<td>-3/+ i8q (6.9)</td>
</tr>
<tr>
<td>MEL 35</td>
<td>-3/+i8q i6p</td>
</tr>
<tr>
<td>MEL 37</td>
<td>Normal/-3 +i8q</td>
</tr>
<tr>
<td>MEL 47</td>
<td>der 6. 19. 22.</td>
</tr>
<tr>
<td>MEL 52</td>
<td>-3/+i8q del 11q t(1.10)</td>
</tr>
<tr>
<td>MEL 53</td>
<td>-3. der 6. (9./17.)</td>
</tr>
<tr>
<td>MEL 57</td>
<td>Normal/ -3 +8q</td>
</tr>
<tr>
<td>MEL 59</td>
<td>t(6. 13) -13, 22</td>
</tr>
<tr>
<td>MEL 60</td>
<td>Normal/-y</td>
</tr>
<tr>
<td>MEL 62</td>
<td>-3 +8, +i8q</td>
</tr>
<tr>
<td>MEL 63</td>
<td>der (6)</td>
</tr>
</tbody>
</table>

The following tumors had apparently normal karyotypes: MEL17, MEL36, and MEL61.

a Chromosomal analysis obtained in collaboration with K. Sisley.
Fig. 5.3.

Representative uveal melanoma karyotypes.

The karyotype of MEL11.

The karyotype of MEL22.

Karyotypes of MEL11 and MEL22 showing the loss of chromosome 3 and an additional isochromosome 8q in MEL11. In contrast MEL22 shows no obvious detectable cytogenetic alterations.
5. 2. 4. Progelatinase Expression by Posterior Uveal Melanomas In Short Term Culture.

A series of posterior uveal melanomas have been studied to establish the pattern of gelatinase production (described in chapter 4) and this has been compared with other established prognostic indicators to assess its value in the prediction of both local invasion and metastasis. Histologically, eleven (78.6 %) tumors were of mixed cell type and three were found to be composed of purely epithelioid cells (21.4 %). Evidence of overt extrascleral spread was noted in five specimens (36 %) (see table 5. 5. for patient details). By zymography all melanoma cell lines released an enzyme of approximately 72 kDa molecular weight, and 10 cell lines secreted an additional 92 kDa progelatinase (summarised in table 4. 2). All enzyme activities detected could be activated with p-APMA, suggesting that the enzymes were latent metalloproteinases (examples of p-APMA activation are shown in Figs. 4. 2. and 4. 9). Enzyme activity in supernatants from all tumor cells could be completely inhibited by the inclusion of EDTA or 1, 10 phenanthroline in the incubation buffer, but PMSF, Net-MAL and E-64 had no effect on enzyme activity, thus suggesting that substrate degradation was not due to serine or cysteine proteinase activities (examples shown in fig. 4. 1). No caseinolytic activities could be detected in any of the supernatants inferring that the observed metalloproteinase activities were not due to stromelysins (examples shown in fig. 4. 3). This study is contained in chapter 4.

There was no statistically significant difference (p=<0.05) between the mean ages of patients secreting both molecular weight species of metalloproteinase (mean age 63.8 ± 9.2 years) and for those expressing only the 72 kDa progelatinase (mean age 68.4 ± 14.2 years). Although the numbers are too small to permit a reliable statistical comparison, there does not appear to be any correlation between either location of the primary tumor, cell type or development of metastatic disease with progelatinase enzyme production. However, it is of interest to note that all four tumors with overt extrascleral spread secreted both 72 kDa and 92 kDa progelatinases.
Table 5.5.

Patients details.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Tumor location</th>
<th>Histological cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL14a</td>
<td>M</td>
<td>52</td>
<td>Choroid</td>
<td>Mixed</td>
</tr>
<tr>
<td>MEL17</td>
<td>M</td>
<td>64</td>
<td>Choroid</td>
<td>Mixed</td>
</tr>
<tr>
<td>MEL22</td>
<td>M</td>
<td>79</td>
<td>Ciliary Body + Choroid</td>
<td>Mixed&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL30a</td>
<td>M</td>
<td>80</td>
<td>Ciliary Body + Choroid</td>
<td>Mixed&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL35</td>
<td>M</td>
<td>66</td>
<td>Choroid</td>
<td>Epithelioid</td>
</tr>
<tr>
<td>MEL37a</td>
<td>F</td>
<td>90</td>
<td>Ciliary Body</td>
<td>Mixed&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL40b</td>
<td>F</td>
<td>48</td>
<td>Choroid</td>
<td>Mixed</td>
</tr>
<tr>
<td>MEL44</td>
<td>M</td>
<td>76</td>
<td>Ciliary Body + Choroid</td>
<td>Mixed</td>
</tr>
<tr>
<td>MEL47</td>
<td>M</td>
<td>67</td>
<td>Choroid</td>
<td>Mixed</td>
</tr>
<tr>
<td>MEL50</td>
<td>F</td>
<td>48</td>
<td>Ciliary body + Choroid</td>
<td>Mixed&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL52</td>
<td>F</td>
<td>72</td>
<td>Ciliary body + Choroid</td>
<td>Mixed</td>
</tr>
<tr>
<td>MEL55</td>
<td>M</td>
<td>72</td>
<td>Ciliary body + Choroid</td>
<td>Epithelioid&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL56b</td>
<td>M</td>
<td>58</td>
<td>Ciliary Body + Choroid</td>
<td>Epithelioid</td>
</tr>
<tr>
<td>MEL57</td>
<td>F</td>
<td>63</td>
<td>Ciliary Body + Choroid</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

M = male  F = female

<sup>a</sup> Deceased.

<sup>b</sup> Local resection, the eye was not removed surgery involved only removal of the tumor and the tissue contained within the resection margins which were typically 3-5 mm from the tumors edge.

<sup>c</sup> Evidence of extrascleral spread.
5. 2. 5. Plasminogen Activator Expression by Posterior Uveal Melanomas In Short Term Culture.

Attempts have been made to correlate PA activity with patient status for the uveal melanomas, and a series of cutaneous melanoma cell lines have been included for comparison. Histologically, eight (80%) tumors were of mixed cell type and two were found to be composed of pure epithelioid cells (20%). Evidence of overt extrascleral spread was noted in four specimens (44%), evidence of extraocular spread was also noted in three specimens (see table 5. 5). By zymography all melanoma cell lines released an enzyme of approximately 70 kDa molecular weight, and most cell lines expressed an enzyme species or enzyme inhibitor complex with a molecular weight of approximately 110 kDa. MEL37b and MEL50 expressed an additional enzyme species with a molecular weight of 50-60 kDa; these activities had the ability to degrade casein only if plasminogen was co-polymerised in the gel (Fig. 5. 5). Zymographic analysis of conditioned medium derived from cutaneous melanoma cell cultures displayed similar patterns of plasminogen-dependent degradation with bands of lysis of apparent molecular weights of 70 and 110 kDa respectively. There were no lower molecular weight species of plasminogen activator, as observed in MEL37b and MEL50, and no plasminogen-independent lysis of casein was detected.

Using a chromogenic assay specific for plasminogen activators, the cell lines were found to express only fibrinogen fragment-dependent plasminogen activator activity, which is most probably tissue-type plasminogen activator (tPA). However, the activities detected in uveal melanoma cell supernatants were 10 fold lower than those detected in cutaneous melanoma cell supernatants, values were expressed as the mean ± the standard deviation; 33.3 ± 25.5 mIU/ml (range 0-85 mIU/ml) and 469 ± 245 mIU/ml (range 256-737 mIU/ml) for the uveal melanomas and cutaneous melanomas respectively.
The patterns of plasminogen activator expression were compared with the morphological and histological characteristics of the primary tumors as summarised in table 5.6. The tPA activity secreted by uveal melanoma cell cultures did not correlate with tumor location, cell type or ploidy values. However, there was a correlation between tumors which secreted the highest levels of tPA and extrascleral spread; these tumors also co-expressed the 92 kDa progelatinase species.
Table 5.6.
Comparison of the morphological and histological characteristics of the primary tumor with expression of tissue plasminogen activator.

<table>
<thead>
<tr>
<th>Patient</th>
<th>tPA activity&lt;sup&gt;a&lt;/sup&gt; (mIU/ml)</th>
<th>ploidy&lt;sup&gt;b&lt;/sup&gt;</th>
<th>extrascleral spread</th>
<th>92 kDa progelatinase expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEL30</td>
<td>46 ± 1.6</td>
<td>Diploid</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MEL35</td>
<td>19 ± 2.3</td>
<td>Aneuploid</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>MEL37a</td>
<td>19 ± 1.0</td>
<td>Diploid</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>MEL37b</td>
<td>65 ± 14</td>
<td>Diploid</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MEL40</td>
<td>26 ± 2.0</td>
<td>Diploid</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>MEL44</td>
<td>20 ± 0.6</td>
<td>Diploid</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>MEL47</td>
<td>0 ± 0.4</td>
<td>Diploid</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>MEL50</td>
<td>86 ± 6.4</td>
<td>Diploid/</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MEL52</td>
<td>20 ± 5.0</td>
<td>Diploid/</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>MEL55</td>
<td>32 ± 9.4</td>
<td>Diploid/</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A375</td>
<td>737 ± 25</td>
<td>Na</td>
<td>Na</td>
<td>No</td>
</tr>
<tr>
<td>LT5.1</td>
<td>256 ± 85</td>
<td>Na</td>
<td>Na</td>
<td>Yes</td>
</tr>
<tr>
<td>SK23</td>
<td>416 ± 57</td>
<td>Na</td>
<td>Na</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup>These values are the mean of two separate triplicate determinations using the chromogenic assay of Leprince <i>et al.</i> 1989.

<sup>b</sup>As determined by Facs analysis of the digested tissue.
The conditioned medium was analysed by zymography on 7.5 % w/v polyacrylamide gels containing 1 mg/ml casein and 60 µg/ml plasminogen, control gels containing casein without plasminogen were run in parallel. Lane 1, MEL30; Lane 2, MEL50; Lane 3, MEL37b; Lane 4, MEL40; Lane 5, MEL 47.
5. 3. DISCUSSION.

Not all tumors give rise to populations of cells endowed with the ability to metastasise and successfully establish tumors in secondary organs. Genotypic and phenotypic properties are important in allowing tumor cells to traverse basement membranes and endothelial cell barriers, to survive the hostile enviroments of the blood (escaping host immune defence systems) and to colonise and grow at distant sites of the body (Fidler, 1990). Although certain clinical and histological criteria are associated with tumor progression, there are as yet no definitive markers of the invasive phenotype.

A number of studies of cutaneous melanoma (Barlogie et al., 1980; Hansson et al., 1982; Sondergaard et al., 1983) have shown an increased incidence of aneuploidy amongst patients who died early of metastatic disease; the incidence of aneuploidy in cutaneous melanoma has been reported to be high (70-89 %). Two previous studies (Meecham and Char, 1986; Shapiro et al., 1986) involving the estimation of DNA ploidy in uveal melanomas reported that the incidence of aneuploidy was 36 % and 77 % respectively. In contrast we observed a much lower level of aneuploidy (15.8 %) (Rennie et al., 1989), and have extended our initial study to show that with increased numbers the incidence of aneuploidy is 6 %; 12 tumours (24 %) were shown to have both diploid and and aneuploid cell populations. This latter observation may indicate that these tumours are undergoing clonal expansion of an aneuploid subpopulation, which is linked with increasing malignancy (Shapiro et al., 1986). If these tumours are combined with the purely aneuploid tumours the incidence of aneuploidy is 30 % which is comparable to that observed by Meecham and Char (1986). At this time it cannot be confirmed that hyperploidy is linked to poor patient prognosis, since the only patients to have died of metastatic disease had diploid tumours.

In the present study four patients with solely diploid cell profiles have died, two with proven metastatic disease and one with probable metastatic disease. When the rate of cell turnover within the diploid group was determined by summation of the total number of cells counted in G2/M and S phases it was found that the patients with
metastatic disease all had values greater than 9.5 % for their G2/M/S phase populations. This value should be interpreted with caution since the tumor will contain a number of non-tumor cell types, including vascular endothelial and stromal cells, which may be undergoing cellular division. However since this cell division may be stimulated by tumor derived growth factors and related to tumor-induced angiogenesis, it is unlikely to significantly influence the results. In addition to the fibrovascular population of the tumour it is probable that immunocytes will be present within the tumour, as has been reported by Hansson et al. (1982), however little or no infiltrate was observed in the tumors described in this study.

We have previously found the percentage of cells in G2/M/S phases to be significantly lower than the 30 % reported by Shapiro et al. (1986), in the present study this ranged between 1.9 % and 18.2 % (7.3 ± 4.1 %). Shapiro et al. (1986) used paraffin fixed material whereas fresh material was analysed here. The results of this enlarged study are in agreement with our previous observations that spindle cell neoplasms have a lower cell turn over rate than epithelioid and mixed cell tumours, 4.5 ± 1.2 , 7.5 ± 3.6 and 7.5 ± 4.6 respectively (table 5. 1). In contrast to the earlier observations there appears to be a difference between the rate of cell turn over and tumor size, medium and large tumours having values of 5.8 ± 1.6 % and 7.8 ± 4.7 % respectively. Obviously these values represent the rate of cellular turnover at the time of enucleation and it has been reported that choroidal melanomas are capable of rapid growth following periods of quiescence (Char et al., 1983).

In the light of this larger study of ploidy and proliferation rates in posterior uveal melanomas, the initial observations (Rennie et al., 1989) can be confirmed; however, two new trends have become apparent, that of an apparently higher rate of cellular turnover in large tumours as compared to medium sized tumours and the correlation between high diploid tumour cell turnover rates and metastatic disease. Whilst this study is biased towards large and medium sized tumours due to current patient management it has relevance to uveal melanoma metastasis since it is these large tumours which pose the greatest threat to patients.
There are few investigations of the chromosomal changes associated with posterior uveal melanoma, mainly due to the rarity of this tumor type. Cytogenetic studies by our laboratory have confirmed the non-random abnormalities described by Griffin et al. (1988) and Rey et al. (1985) involving chromosomes 3, 6, and 8. However our study has analysed 16 tumors, compared with only a single tumor in the studies by Griffin et al. (1988) and Rey et al. (1985). Griffin et al. (1988) observed a single abnormality, trisomy of 6p, in one uveal melanoma and Rey et al. (1985) found structural anomalies of chromosomes 6 and 8, in the form of i (6p) and +8p which was virtually trisomic for 8q, in a uveal melanoma metastatic to the brain. Recently Prescher et al. (1990) also demonstrated a non-random association of chromosomes 3 and 8 in a series of fourteen tumors. Associated abnormalities between chromosomes 3 and 8 have been observed in both renal cell carcinoma and lung adenocarcinoma (Drabkin et al., 1985; Miura et al., 1990), and involvement of c-myc and erb-A proto-oncogenes have been suggested (Drabkin et al., 1985; Wong et al., 1986; Rabbitts et al., 1989). Tucker (1985) demonstrated an increased risk in adult males with uveal melanoma for the development of lung cancer. Hence it is possible that as abnormalities of erb-A and c-myc are shared by cancers such as renal, lung and posterior uveal melanoma. Whilst both cutaneous and uveal melanomas are melanocytic tumors, abnormalities of chromosomes 3 and 8 are rarely observed in cutaneous neoplasms (Limon et al., 1988) but appear to be common in uveal melanoma, thus indicating that uveal melanomas are genotypically distinct from their supposed cutaneous counterparts. In addition, these abnormalities appear to be characteristic of tumors arising in the ciliary body. Only one choroidal tumor had trisomy of chromosome 8 and no corresponding abnormality of chromosome 3. Monosomy of chromosome 3 and i (8q) may be uniquely associated with a subset of uveal melanomas.

Another common abnormality of both cutaneous and uveal melanoma are rearrangements of chromosome 6, resulting in either deletions of the long arm or trisomies of the short (Cowan et al., 1986; Limon et al., 1988; Prescher et al., 1990;
Sisley et al., 1990). It has been suggested that abnormalities of chromosome 6 are linked with tumor progression, rather than genesis (Limon et al., 1988), and a recent study (Trent et al., 1990) demonstrated that introduction of chromosome 6 into human melanoma cell lines could reverse the the malignant phenotype. Thus structural changes of chromosome 6 may be an integral part of melanocytic transformation.

Our studies are in agreement with those of Prescher (1990) who found structural abnormalities of chromosomes 1, 11 and 13 in both ciliary body and choroidal tumors. We found rearrangements of chromosome 1 in 3 cases; this is a common feature of many solid tumors (Mitelman, 1988) which may reflect tumor progression (Limon et al., 1988; Dracopoli et al., 1989). In addition to these previously reported abnormalities, we have detected abnormalities in chromosomes 11 and 13. Three cases, 10, 12 and 15, showed deletions of the long arm of chromosome 11. No breakpoint was common to all, but two tumors did have a breakpoint in the region 11q23, which is in agreement with a tumor from Prescher's (1990) series of fourteen uveal melanomas which also had a breakpoint at 11q23. Several proto-oncogenes have been identified on the long arm of chromosome 11, including ets-lat 11q23-24 and hst, bcl-1, int-2 and sea at 11q13. Both deletions of 11q23 in this study were found in tumors with choroidal involvement, and may therefore may be a characteristic feature associated with these tumors. The deletion of 11q13 in case 15 was not found in all cells examined and may therefore reflect tumor progression. Two cases, 11 and 14, had abnormalities of chromosome 13. Monosomy of chromosome 13 was found in case 11, while in contrast case 14 displayed a translocation between chromosome 6 and 13. Prescher (1990) observed both monosomy and structural rearrangements of tumors in his series of uveal melanomas. In addition to the presence of various oncogenes on chromosome 11, the genes for both interstitial collagenase and stromelysin are present on the long arm of chromosome 11 (Spurr et al., 1988). Whilst the gene for 72 kDa gelatinase is located on 16q21 (Huhtala et al., 1990), the location of the gene for 92 kDa is not known. It is interesting to note that we found no evidence for the expression of interstitial collagenase or stromelysin in the uveal
melanomas studied. There are currently few reports of abnormalities of chromosome 13 in cutaneous melanomas, and this chromosomal abnormality may be a distinguishing feature of uveal melanomas.

One important aspect of tumor cell metastasis is the ability of cells to migrate across basement membranes, a mechanism associated with the production of enzymes expressing gelatinolytic activity (Liotta et al., 1982; Fessler et al., 1984; Nakajima et al., 1987). In an attempt to further understand the association between such enzymes and tumor cell invasion, a series of uveal melanomas, propagated in vitro for 1 to 5 passages, have been assessed for their ability to produce differing molecular weight species of enzymes with gelatinolytic activity. Approximately fifty percent of patients undergoing surgical removal of their ocular melanomas will succumb to metastatic disease, commonly to the liver, within a fifteen year period. Thus any property of the tumor which may predict metastasis would be valuable in determining the prognosis, and assist in patient management.

This study identified two molecular weight forms of gelatinolytic enzymes, (72 kDa and 92 kDa) produced by posterior uveal melanoma cells upon short term culture. Both these enzyme species were able to degrade gelatin, but not casein. Stromelysin is known to degrade casein under the assay conditions used in the present investigation (Wilhelm et al., 1987), but no caseinolysis was seen, and it is therefore unlikely that the active enzymes released from uveal melanoma cell cultures are stromelysins. There are however, several proteinases capable of degrading basement membrane and intercellular matrix components, including the plasminogen activators (Mackay et al., 1990). One possibility is that the synergistic action of these two proteinase groups may contribute to the invasive properties of the tumor.

Although, it is of academic interest to identify the production of a 92 kDa gelatinase in posterior uveal melanomas and speculate on its potential role in tumor invasion, the relatively small sample size and short follow up periods prevent any firm conclusions being drawn as to its clinical significance. A long term longitudinal study of the pattern of proteinase secretion and its clinical correlate is currently in progress.
The acquisition by a tumor cell of an invasive malignant phenotype is in part due to the increased expression, or altered control of a number of proteases (Dano et al., 1985). Plasminogen activator expression has been shown to be involved in cellular invasion (Mignatti et al., 1986; 1989; Yagel et al., 1988; 1989). Varani et al. (1987) found high levels of PA activity in the culture medium of malignant cells including malignant cutaneous melanoma cell lines. To the best of our knowledge the type and level of plasminogen activators expressed by uveal melanomas in short term culture has not been reported, and the present study demonstrates that posterior uveal melanoma cells secrete tissue-type plasminogen activator (tPA) into their culture medium at a level which is low (approximately 10 fold lower) in relation to a series of cutaneous melanoma cell cultures. In addition, two other species of plasminogen activator were observed; a 110 kDa PA and a 50-60 kDa PA. The 110 kDa species may represent the tPA: inhibitor complex reported by Dano et al. (1985) and the molecular weight of the 50-60 kDa species corresponds to that of urokinase-type plasminogen activator, although no fibrinogen-independent plasminogen activation was detected in these samples. This suggests that either no biologically active urokinase-type plasminogen activator was present, possibly as a result of complex formation with plasminogen activator inhibitors, although such enzyme: inhibitor complexes could not be detected as discrete bands by zymography or that the sensitivity of this assay is lower than that of zymography. Recent work by Quax et al. (1991) has identified expression of both tPA and UK by invasive cutaneous melanoma cell lines, and correlated this co-expression with the incidence of spontaneous metastasis in nude mice.

Uveal melanomas, with the exception of one tumor which did not express PA activity, expressed tissue plasminogen activator at levels which varied between 19 and 86 mIU/ml whereas the cutaneous melanomas studied expressed tPA at levels between 256 and 737 mIU/ml. Whilst differences in tissue culture growth conditions or cell proliferation rate could account for these differences; the uveal melanoma cell cultures proliferated at a slower rate than the cutaneous melanomas used in this study. It is also
possible that the level of active enzyme is lower as a result of increased plasminogen activator inhibitor production by uveal melanomas. Relatively high tPA activities in uveal melanomas was found to correlate with extrascleral invasion in four tumors (MEL30, 37, 50 and 55) from patients with either clinical or histological evidence of extrascleral spread at the time of surgery. Two of these patients (MEL30 and 37) subsequently died from metastatic disease, whilst the remaining two are alive and have no detectable metastatic disease at present. The expression of both 72 and 92 kDa progelatinases by these cell lines was also studied, and it was noted that the four cell lines which expressed the highest tPA activities all expressed both 72 and 92 kDa progelatinases. Although nine out of fifteen tumors expressed both species of gelatinase, only the four expressing tPA activity had invaded the sclera. It is therefore tempting to suggest that it is possible that the synergistic actions of these two enzyme groups is important in scleral invasion. The sclera, which is composed of a mixture of interstitial collagens and sulphate-containing proteoglycans, may be degraded by the combined action of metalloproteinases and plasmin.

The role of tissue-type plasminogen activator in metastasis is complex. Plasmin, the product of the action of plasminogen activators on plasminogen, has been shown to activate endothelial cell migration (Inyang et al., 1990), to degrade type IV collagen (Mackay et al., 1990), and to activate latent metalloproteinases (Werb et al., 1977). In addition to these actions, which can be correlated directly with vascular and connective tissue invasion, the ability to modulate platelet function with respect to the de novo expression of platelet related growth factors may be important for tumor metastasis. In chapter 2 the modulation of both uveal and cutaneous melanoma gelatinase expression by a series of cytokines was studied and uveal melanomas were found to respond in a similar way to cutaneous melanomas when insulted with TNFα and TGFβ2 either singularly or in combination. The site specific metastasis, which is characteristic of uveal melanoma is, if this fact is taken in isolation, independent of degradative enzyme expression. However the expression of a particular profile of enzymes may be a prerequisite for tumor cell invasion, either through the sclera or through the vascular
basement membrane, it is only after escape from the primary tumor site has been accomplished that the phenotypic determinants for liver metastasis would become important, assuming that such factors are different from those involved in tumor cell escape.
CHAPTER 6

INTRODUCTION: MELANOMA CELL PHENOTYPIC PARAMETERS ASSOCIATED WITH THE METASTATIC PHENOTYPE AND THEIR REPORTED MODULATION BY SELECTED CYTOKINES
6. 1. INTRODUCTION: MELANOMA CELL PHENOTYPIC PARAMETERS ASSOCIATED WITH THE METASTATIC PHENOTYPE AND THEIR REPORTED MODULATION BY SELECTED CYTOKINES.

The in vivo growth characteristics of A375 and A375/NUPR1 in nude mice and the expression of gelatinase and modulation of gelatinase expression in vitro by cytokines have been studied in chapters 3 and 4 respectively. The complexity of the metastatic cascade necessitates that a successfully metastatic tumor cell must express a wide range of genetic, biochemical, immunological and biological traits, such as altered morphology, growth properties, cell surface components and receptors, a resistance to therapeutic agents, and the ability to invade and metastasize. The expression of some of these phenotypic traits are investigated in this section, together with the modulatory effect of cytokines which were found to induce the expression of 92 kDa gelatinase in A375 and A375/NUPR1.

Melanoma cells share a number of antigenic determinants with lymphoid cells, amongst these are the Thy antigen (Hersey et al., 1983), HLA class II antigens (Winchester et al., 1978; Wilson et al., 1979) ICAM-1 (Johnson et al., 1989) and the common acute lymphblastic leukemia antigen (CALLA) or CD10 (Carel et al., 1983).

The term cytokine was first used by Dr S. Cohen in 1974 to describe any soluble substance produced by lymphoid and/or non-lymphoid cells that exercises specific effects in its target cells. This definition has been modified to "a class of inducible, water soluble, heterogeneous proteinaceous mediators of animal origin of molecular weight greater than 5000 that exercise specific, receptor mediated effects in target cells and/or in the mediator-producing cells themselves. Such effects are typically the normal physiological control of

(a) mitogenes:s which is required for cell proliferation and thus tissue development and repair, and
(b) cell function which is required for the maintenance of homeostatic and defence mechanisms, and the integrated control of different physiological systems (Meager, 1990).

Whilst many types of animal cell do not normally divide, cells are often stimulated to proliferate during normal physiological processes such as tissue repair, angiogenesis and morphogenesis. Malignant cells are characterised by their accelerated and apparently uncontrolled growth rates, and for convenience these can be subdivided into four distinct stages in the cell division cycle G1, S, G2, and M. The first stage, G1 is of 8-10 hours duration and involves cellular preparation for S phase, this typically involves synthesis of RNA and protein. S phase involves a period of DNA synthesis, RNA and protein synthesis also continue during this phase. The third stage, G2, is characterised by a cessation of DNA synthesis but RNA and protein synthesis continue for a further 3-4 hours. During the final stage, M phase, mitosis and cell division occur during which little or no DNA, RNA or protein synthesis takes place. Cells may either enter the cell cycle or become growth arrested, which is associated with entry into G0 (O'Keefe and Pledger, 1983).

Initially, TNFα was reported to cause haemorrhagic necrosis of murine tumors (Carswell et al., 1975), suggesting its potential in therapy, but a consistent anti-tumor effect in cancer patients could not be achieved (Frei and Spriggs, 1989). The anti-tumor activity of TNFα has been demonstrated both in vivo (Carswell et al., 1975; Helson et al., 1979; Watanabe et al., 1985a; Niitsu et al., 1988a) and in vitro (Watanabe et al., 1985b; Watanabe et al., 1988; Sugarman et al., 1985), and its action in animal models is thought to be due, in part, to its cytotoxic and/or cytostatic potential (Sugarman et al., 1985), together with the ability to cause activation of anti-tumor immune surveillance mechanisms (Palladino et al., 1987). Recently TNFα has been reported to have multiple effects on both tumor and host cell behaviour, including the stimulation of angiogenesis (Frater-Shroder et al., 1987), osteoclastic bone resorption (Bertolini et al., 1986), tumor cell invasion and metastasis of human ovarian carcinoma xenografts (Malik et al., 1989). TNFα has been reported to
modulate the expression of the cell surface molecules HLA class I and II which are involved in the generation and regulation of the immune response and cell-cell adhesion molecules such as intercellular adhesion molecule 1 (Maio et al., 1989), and to have an antiproliferative effect on human melanoma cells (Morinaga et al., 1989). The ability of TNFα to lyse mouse fibroblasts during M phase is possibly due to its' ability to delay cell progression through G2 to M (Darzynkiewicz et al., 1984). Also it has been shown that A375 is capable of inducing TNFα expression in monocytic cells following release of tumor derived GM-CSF (Sabatini et al., 1990). Also, TNFα is not equally cytotoxic to all tumor cells, and certain tumor cell types are resistant to its effect (Sugarman et al., 1985; Shepard and Lewis, 1988). A suggested mechanism is that endogenous TNFα production acts as a protective protein against the action of exogenous TNFα (Spriggs et al., 1987; Niitsu et al., 1988). Alternatively IL-6 (Defilippi et al., 1987), HER2/erb-B2 (Hudziak et al., 1988) and the Mr 14,700 protein of adenovirus E3 region (Gooding et al., 1988) have been reported to act as protective factors.

Recently expression of the TNFα gene has been reported to correlate with reduced tumorigenicity and reduced invasion in vivo (Vanhaesebroeck et al., 1991). In contrast, Lollini et al. 1990 reported that murine B16 melanoma cells when treated in vitro prior to use in an experimental metastasis assay displayed enhanced lung colonisation in C57BL/6 mice. In related studies using ovarian cancer, Malik et al. (1989) showed that TNFα therapy promoted both peritoneal invasion and solid tumor formation of ovarian ascites in xenograft models. Later studies determined that cell expressing TNFα, following transfection with the TNFα gene, exhibited enhanced invasion and metastasis in nude mice Malik et al. 1990.

Cellular exposure to IL-1 in vitro shows similarities to the effects of TNFα on tumor cells (Dinarello, 1989; Endo et al., 1988) and has been reported to arrest A375 melanoma cells in the G0-G1 stage of the cell cycle (Morinaga et al., 1990). IL-6 has been reported both to contribute to the antiproliferative effect of IL-1 by acting as an autocrine factor (Morinaga et al., 1989) and also to have a strong inhibitory effect.
itself (Heinrich et al., 1990; Kohase et al., 1986). In vivo intravenous administration of IL-1 prior to cell injection resulted in increased lung colony formation of the A375 melanoma (Giavazzi et al., 1990) and daily intraperitoneal treatment increased the incidence of spontaneous lung and liver metastases following footpad tumor inoculation of B16-BL6 melanoma cells (Bani et al., 1991). However this effect was only observed with cell lines which had previously been shown to be metastatic in nude mice and did not endow non-metastatic cell lines with the ability to metastasise. Activation of endothelial cells by cytokines has been shown to augment metastasis (Belloni and Tressler, 1989; Nicolson, 1988; Barbera-Guillem et al., 1989) possibly as a result of the de novo expression of adhesion molecules involved in the binding of malignant cells (Dejana et al., 1988; Lauri et al., 1990; Rice and Bevilacqua, 1989); this could result in site specific metastasis due to organ specific endothelial cell phenotypes.

Modulation of HLA class I expression by tumor cells may influence T-cell or natural killer cell recognition of tumor cells (Elliott et al., 1989). An increase in class I MHC expression can be associated with a loss of susceptibility of some tumor cell lines to NK-mediated lysis. In addition class I MHC molecules have a role in intercellular adhesion (Bartlett and Edidin, 1978), contact inhibition of epithelial cell movement (Curtis and Rooney, 1979), and the control of endothelial cell clustering in vitro (Birkby et al., 1988). Class II MHC antigens play a role in the processes of cell proliferation and differentiation, the majority of the evidence for this being derived from studies on B cells (Cambier and Lehmann, 1989; Forsgren et al., 1984). Cross-linking class II molecules expressed on B cells has been shown to trigger elevation of intracellular calcium and cAMP, as well as nuclear translocation of cytosolic protein kinase C (Cambier et al., 1987).

The majority (approximately 70 %) of melanomas in long term culture express HLA class II antigens (Herlyn et al., 1980; Pellegrino et al., 1981; Houghton et al., 1982), which appears to be unrelated to their establishment from either primary tumors or metastases or to their growth characteristics in nude mice (Reynier et al.,
1984; Welch et al., 1991). However, Feldman et al. (1988) and Kushtai et al. (1988) have shown that expression of class I and II histocompatibility antigens correlates with the metastatic propensity of murine tumors.

Intercellular adhesion molecule 1 (ICAM-1) is frequently found on cells of the melanocytic lineage, transformed cells showing a higher incidence of expression (Johnson et al., 1989; Natali et al., 1990). The expression of ICAM-1 in primary lesions from stage 1 melanoma patients has been shown to correlate with tumor thickness (Johnson et al., 1989; Natali et al., 1990), which is itself a prognostic indicator. The frequency of expression is higher in metastases than in primary lesions, indicating a possible selective process. Alternatively, this phenomena may be due to the effect of immunocyte derived cytokines (Matsui et al., 1987; Pober et al., 1986; Dustin et al., 1986; Rothlein et al., 1986; Dustin et al., 1988; Maio et al., 1988). The high frequency of ICAM-1 expression in metastases is interesting, since ICAM-1 has been shown to be a ligand for lymphocyte function antigen-1 (LFA-1) in T cell mediated cytotoxicity (Makgoba et al., 1988) although LFA-1, LFA-3 and ICAM-1 were reported not to be the major adhesion ligands for LAK cell mediated lysis on a series of target tumor cell lines none of which were melanoma cell lines (Quillet-Mary et al., 1991).
6. 2. RESULTS.

6. 2. 1. Modulation of the Proliferation Rates and Cell Cycle Kinetics of A375 and A375/NUPR1 by TNFα and IL-1α.

The effects of TNFα (1000 U/ml) and IL-1α (100 U/ml) on cell proliferation, when used both as single agents or in combination for 24 hours, was investigated using ³H thymidine uptake. The results shown in Figures 6. 1 and 6. 2 represent the mean value ± the SD of 3 experiments. Both TNFα and IL-1α were able to decrease ³H thymidine uptake; this decrease was statistically significant (p<0.05, Student's t-test). This effect was more pronounced with IL-1α and the magnitude of this effect could be further increased by co-stimulation with TNFα. The effect of TNFα and IL-1α on cell cycle kinetics, was investigated following staining of cellular DNA with propidium iodide and subsequent analysis by flow cytometry.

Figures 6. 3 and 6. 4 show changes in the cell cycle distribution which occurred in treated and untreated A375 and A375/NUPR1 cells. The percentage of cells in S/G2/M appeared to decrease in both A375 and A375/NUPR1 cell lines in the presence of both cytokines either individually or in combination.
Fig. 6. 1.

$^3$H thymidine incorporation by the A375 melanoma, used as a measure of cellular proliferation: modulation by TNF$\alpha$ and IL-1 $\alpha$.

![Graph](image-url)

Melanoma cells were seeded onto 96 well plates at a concentration of $4 \times 10^4$ cells/well, and allowed to equilibrate for 24 hours at $37^\circ$C in Dulbecco's medium containing 10% FCS in an atmosphere of 5% CO$_2$ in air. Following two 1 hour washes with PBS at $37^\circ$C, the cells were incubated in serum free medium containing cytokines (the concentrations used were: TNF$\alpha$ at 1000 U/ml; IL-1$\alpha$ at 100 U/ml), either used alone or in combination, for 24 hours in the conditions described previously. Cells were pulsed with $^3$H thymidine, harvested and assayed as previously described in materials and methods. Values are the mean value ± the standard deviation of 3 experiments.

* Statistically significant decrease ($p<0.05$, Student's t-test).
Fig. 6. 2.

3H thymidine incorporation by the A375/NUPR1 melanoma, used as a measure of cellular proliferation: modulation by TNFα and IL-1α.

Melanoma cells were seeded onto 96 well plates at a concentration of $4 \times 10^4$ cells/well, and allowed to equilibrate for 24 hours at 37°C in Dulbecco's medium containing 10% FCS in an atmosphere of 5% CO₂ in air. Following two 1 hour washes with PBS at 37°C, the cells were incubated in serum free medium containing cytokines (the concentrations used were: TNFα at 1000 U/ml; IL-1α at 100 U/ml), either used alone or in combination, for 24 hours in the conditions described previously. Cells were pulsed with 3H thymidine, harvested and assayed as previously described in materials and methods. Values are the mean value ± the standard deviation of 3 experiments.

* Statistically significant decrease (p<0.05, Student's t-test).
Cell cycle distribution of A375 cell cultures: modulation by TNFα and IL-1α.

The cell cycle kinetics of the A375 melanoma was determined using a DNA analysis kit (Becton Dickinson Ltd., Oxford, England); the kit was used by following the manufacturers instructions. Alterations in cell cycle kinetics due to cytokine treatment was studied as described in materials and methods. The cell cycle was analysed using an orthocyte benchtop flow cytometer attached to a PC computer and the % of cells in Go/G1, S and G2/M phases of the cell cycle was determined. The Go/G1 peak channel number and the coefficient of variation (CV) of this peak were recorded and used to produce histograms. Samples with a CV greater than 10 channels were considered to be of poor quality due to inadequate cellular or DNA staining and not used.
Cell cycle distribution of A375/NUPR1 cell cultures: modulation by TNFα and IL-1α.

The cell cycle kinetics of the A375/NUPR1 melanoma was determined using a DNA analysis kit (Becton Dickinson Ltd., Oxford, England); the kit was used by following the manufacturer's instructions. Alterations in cell cycle kinetics due to cytokine treatment was studied as described in materials and methods. The cell cycle was analysed using an orthocyte benchtop flow cytometer attached to a PC computer and the % of cells in G0/G1, S and G2/M phases of the cell cycle was determined. The G0/G1 peak channel number and the coefficient of variation (CV) of this peak were recorded and used to produce histograms. Samples with a CV greater than 10 channels were considered to be of poor quality due to inadequate cellular or DNA staining and not used.

The expression and modulation of class I and II major histocompatibility antigens by cytokines in melanoma-related human melanocytes was investigated. Both cell lines showed similar levels of expression of class I and II antigens and TNFα, which was enhanced following 24 hour exposure to the cytokines either as single agents or in combination (S and 6.6).
6. 2. 2. The Influence of TNFα and IL-1α on The Major Histocompatibility Class I and II Antigens and Intercellular Adhesion Molecule-1 (ICAM-1) Expression by A375 and A375/NUPR1.

The expression and modulation of class I and II major histocompatibility antigens by cytokines in two related human cutaneous melanoma cell lines, A375 and A375/NUPR1 was investigated in vitro. Both cell lines showed similar levels of expression of class I and II MHC, which was unaltered following 24 hour exposure to the cytokines IL-1α and TNFα either as single agents or in combination (figures 6.5 and 6.6).

The expression and modulation of ICAM-1 by cytokines in A375 and A375/NUPR1 was investigated in vitro. Both cell lines showed similar levels of ICAM-1. IL-1α and TNFα was shown to increase ICAM-1 expression, TNFα having a greater effect. Co-incubation of the cell lines with both cytokines failed to increase expression of ICAM-1 to levels significantly higher than those achieved with TNFα alone. The magnitude of the TNFα induced increase in ICAM-1 expression was 5 fold and 7 fold for A375 and A375/NUPR1 respectively. IL-1α caused 2.5 fold and 5.4 fold increases for A375 and A375/NUPR1 respectively; co-incubation causing 5 fold and 7.5 fold increases for A375 and A375/NUPR1 respectively (figure 6.7).

This study was performed in collaboration with Mr M. Mesri.
Expression of MHC class I on the related melanoma cell lines A375 and A375/NUPR1 and its modulation by TNFα and IL-1α.

Melanoma cells were seeded onto 25 cm² tissue culture flasks at a concentration of 1 x 10⁶ cells/flask, and allowed to equilibrate for 24 hours at 37°C in Dulbecco's medium containing 10 % FCS in an atmosphere of 5 % CO₂ in air. Following two 1 hour washes with PBS at 37°C, the cells were incubated in serum free medium containing cytokines (the concentrations used were: TNFα at 1000 U/ml; IL-1α at 100 U/ml), either used alone or in combination, for 24 hours in the conditions described previously. Following completion of the experimental protocol, the cells were harvested and stained with an excess of monoclonal antibody (MoAb) (W6/32, 10 µl as determined by titration assays) as described in materials and methods. The cells were resuspended and fixed in 1 % paraformaldehyde and analysed with an orthocyte benchtop flow cytometer attached to a PC computer (Ortho Diagnostic Systems Ltd). Background fluorescence was assessed by including a control with no primary MoAb added. Mean fluorescence intensity was given as channel number on a linear scale from 1 to 2048. Results are the mean of 3 experiments ± the SD.
Expression of MHC class II on the related melanoma cell lines A375 and A375/NUPR1 and its modulation by TNF\(\alpha\) and IL-1\(\alpha\).

Melanoma cells were seeded onto 25 cm\(^2\) tissue culture flasks at a concentration of 1 x 10\(^6\) cells/flask, and allowed to equilibrate for 24 hours at 37\(^{\circ}\)C in Dulbecco's medium containing 10\% FCS in an atmosphere of 5\% CO\(_2\) in air. Following two 1 hour washes with PBS at 37\(^{\circ}\)C, the cells were incubated in serum free medium containing cytokines (the concentrations used were: TNF\(\alpha\) at 1000 U/ml; IL-1\(\alpha\) at 100 U/ml), either used alone or in combination, for 24 hours in the conditions described previously. Following completion of the experimental protocol, the cells were harvested and stained with an excess of monoclonal antibody (MoAb) (HLA-DR, 5 \(\mu\)l as determined by titration assays) as described in materials and methods. The cells were resuspended and fixed in 1\% paraformaldehyde and analysed with an orthocyte benchtop flow cytometer attached to a PC computer (Ortho Diagnostic Systems Ltd). Background fluorescence was assessed by including a control with no primary MoAb added. Mean fluorescence intensity was given as channel number on a linear scale from 1 to 2048. Results are the mean of 3 experiments \(\pm\) the SD.
Melanoma cells were seeded onto 25 cm² tissue culture flasks at a concentration of 1 x 10⁶ cells/flask, and allowed to equilibrate for 24 hours at 37°C in Dulbecco's medium containing 10% FCS in an atmosphere of 5% CO₂ in air. Following two 1 hour washes with PBS at 37°C, the cells were incubated in serum free medium containing cytokines (the concentrations used were: TNFα at 1000 U/ml; IL-1α at 100 U/ml), either used alone or in combination, for 24 hours in the conditions described previously. Following completion of the experimental protocol, the cells were harvested and stained with an excess of monoclonal antibody (MoAb) (ICAM-1, 20 μl, as determined by titration assays) as described in materials and methods. The cells were resuspended and fixed in 1% paraformaldehyde and analysed with an orthocyte benchtop flow cytometer attached to a PC computer (Ortho Diagnostic Systems Ltd). Background fluorescence was assessed by including a control with no primary MoAb added. Mean fluorescence intensity was given as channel number on a linear scale from 1 to 4096. Results are the mean of 3 experiments ± the SD.
6. 2. 3. The Effect of Pretreatment With TNFα on the Experimental Metastatic Potential of A375.

A375 cells pretreated by 24 hour incubation with TNFα were used in an experimental metastasis assay to determine the effect of TNFα on lung colonisation. The assay was performed as previously described, and animals were examined for lung colonies 12 weeks after injection. Control mice received A375 cells which had been placed in serum free conditions for 24 hours. TNFα was not found to increase experimental metastasis when this protocol was used; the results are shown in table 6.1.

Table 6.1.
The effect of preincubation of A375 tumor cells with TNFα prior to determination of experimental metastatic potential.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>number of mice with lung metastases/number injected</th>
<th>Number of lung metastases/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>0/16</td>
<td>Na</td>
</tr>
<tr>
<td>A375+TNFα</td>
<td>1/16</td>
<td>1 (L)</td>
</tr>
</tbody>
</table>

Subconfluent cultures were washed twice for 1 hour at 37°C, then incubated in serum free medium for 24 hours at 37°C. TNFα treated cultures were washed as described and exposed to 1000 U/ml of TNFα for 24 hours.

Mice were examined at autopsy for the macroscopic presence of lung (L), liver (Li), and peritoneal (P) metastases. Na Not applicable.
6. 3. DISCUSSION.

The effects of TNFα on a number of A375 melanoma cell phenotypic characteristics reported to be associated with the metastatic phenotype were studied and related to in vivo experimental metastatic potential in nude mice. The aims of this study were (1) to determine if pretreatment of A375 melanoma cells with TNFα prior to intravenous injection could augment experimental metastatic propensity in nude mice; (2) to establish which, if any, of the phenotypic characteristics studied were modulated by TNFα and/or IL-1α treatment. In this context, the differences in the expression, by A375 and A375/NUPR1, of HLA class I and II molecules and ICAM-1 in vitro and their modulation by exposure to TNFα and IL-1α were investigated. The effects of exposure to TNFα and IL-1α for 24 hours on A375 and A375/NUPR1 melanoma cell proliferation rate and cell cycle kinetics in vitro.

There is increasing evidence of an association between intercellular adhesion molecule 1 (ICAM-1) expression and melanoma progression (Holzman et al., 1987; Johnson et al., 1988; Johnson et al., 1989; Natali et al., 1990). These studies report that ICAM-1 is expressed at higher levels on cells which have successfully completed the metastatic cascade of events than on the cells in the primary tumor or precancerous lesions, suggesting that ICAM-1 may play a role in the metastatic process. In addition Feldman et al. (1988) and Kushtai et al. (1988) have correlated expression of major histocompatibility antigens with metastatic propensity; however in contrast, Rodolfo et al. (1988) found that HLA class I and II antigens did not correlate with metastatic ability in nude mice.

Exposure of both cell lines to TNFα and IL-1α alone caused a decrease in proliferation rate to occur, IL-1α having the more pronounced effect. Co-incubation with both cytokines produced an increase in the magnitude of the growth inhibition relative to the effects of a single cytokine. This decrease in proliferation rate was linked with an decrease in the number of cells present in S/G2 and M phases of the cell cycle.
In addition, this study has shown that A375 and A375/NUPR1 cells constitutively express similar levels of MHC class I and II and ICAM-1 in vitro. Class I and II MHC expression on both cell lines was unaltered following 24 hour exposure to the cytokines IL-1α and TNFα either as single agents or in combination. However, both IL-1α and TNFα increased ICAM-1 expression, TNFα having a greater effect than IL-1α. Co-incubation of the cell lines with both cytokines failed to increase expression of ICAM-1 to levels significantly higher than those achieved with TNFα alone. The magnitude of the TNFα induced increase in ICAM-1 expression was 5 fold and 7 fold, 2.5 fold and 5.4 fold for IL-1α. Co-incubation caused 5 fold and 7.5 fold increases for A375 and A375/NUPR1 respectively. It is interesting to note that HLA class I expression was unaltered by treatment with TNFα and/or IL-1α and therefore unaffected by the changes they induced in cell cycle kinetics and proliferation rate. In contrast ICAM-1 expression was increased by these cytokines and expression was found to be inversely related to the rate of proliferation; an increase in the number of cells in the S/G2 and M phases of the cell cycle perhaps being indicative of increased ICAM-1 expression in these phases of the cell cycle. This study has illustrated the complex changes in melanoma cell phenotype which may occur following exposure to TNFα and/or IL-1α. In vivo this may occur through contact with or close proximity to tumor infiltrating lymphocytes, tumor associated monocytes and/or other mesenchymal, endothelial or epithelial host cells. Growth inhibition and cytolysis of tumor cells is of obvious importance in cancer therapy but the associated changes in tumor cell phenotype, possibly creating a phenotype associated with successfully metastatic cells should not be overlooked.

Intravenous injection of TNFα treated A375 melanoma cells did not increase experimental metastatic potential. The inhibitory effects of TNFα and IL-1α on cellular proliferation rate may be responsible for the lack of an increase in experimental metastatic potential since this decrease in proliferation rate may preceed cytolysis, which although undetected in vitro may occur after arrest in the nude mouse. Alternatively the reduced rate of proliferation may inhibit growth at the secondary site.
It is possible that an increase in ICAM-1 expression may be irrelevant to increasing metastatic propensity, since it is a possibility that receptor:ligand interactions will not function due to the species barrier involved, or the immunodeficient status of the nude mouse, which enables successful xenografting to occur, may reduce the number of lymphocyte: tumor cell interactions and hence possible lymphocyte aided metastasis will be reduced. Alternatively other tumor cell: host cell interactions may be altered following exposure to cytokines resulting in tumor cells unable to interact effectively with host cells i.e. adherence to vascular endothelium. Recent reports (Mortarini et al., 1991) have indicated that the integrin receptors VLA 1, 2 and 6 were weakly expressed on primary tumors compared to metastases. The expression of these molecules and their modulation by cytokines was not studied in our investigation, hence, we have no information regarding their modulation by TNFα or IL-1α or their possible role in experimental metastasis in our model.

However, it is interesting to speculate about the relative importance of adhesion molecule expression, MHC expression and rate of proliferation, to successful completion of the metastatic cascade. One could hypothesise that following intravenous injection of tumor cells, the first obstacle to overcome would be the hosts immune response, then attachment followed by degradation of the basement membrane and subsequent invasion into the interstitial stroma. The ability to proliferate and form a detectable lesion may then be the critical factor in the establishment of a metastasis. This scenario is in no way complete. We do not know the relative abilities of the two cell lines to withstand the mechanical trauma of the vasculature; if this is modulated by cytokines; whether in vitro ICAM-1 and gelatinase levels of expression are important in this model or the duration of the growth inhibition caused by TNFα.
CHAPTER 7

GENERAL DISCUSSION
CHAPTER 7
GENERAL DISCUSSION

Tumor metastasis is an extremely complex process involving many aspects of cell biology. Markers for metastasis have involved the study of ploidy values, genetic alterations, at both the molecular and cytogenetic levels, and the identification of phenotypic traits and alterations in cellular behavior possibly responsible for organ specific metastasis. The data contained in this thesis has been obtained by the use of a number of diverse and complimentary investigations of in vivo tumor growth characteristics, metastatic potential and also the study of specific molecules thought to be involved in invasion in vitro. It is pertinent to consider the implications of these findings for tumor metastasis.

This thesis has addressed a number of potentially important questions relating to the nature of uveal melanoma and its similarities and/or differences to cutaneous melanoma. These have involved comparative studies in vivo (chapter 3) and in vitro, with particular emphasis on the expression and modulation by cytokines, of proteinases thought to be involved in invasion (chapters 4 and 5). A number of other phenotypic characteristics considered to be involved in determining the metastatic propensity of tumor cells by TNFα and IL-1 were also studied (chapter 6). The influence of pretreatment of A375 melanoma cells with TNFα on experimental metastatic potential in nude mice was investigated.

Uveal melanoma displays a characteristic pattern of metastasis, showing involvement of the liver in 97% of cases (Jensen, 1963), and metastasis to regional lymph nodes is virtually unknown (Zimmerman 1980b), whereas cutaneous melanoma displays a different pattern of metastatic spread which frequently shows lymph node involvement (Nicolson, 1982). The reason for this disparity is unclear and it is unlikely that there is a single determining factor, although such a possibility cannot be excluded. Rather it may be the case that a number of contributory factors are involved. Using the seed-soil hypothesis (Paget, 1889), it is tempting to speculate that
there may be factors present in the liver that promote one or more of the following events: attachment, invasion into the organ parenchyma and growth and possibly inhibition of the development of lymph node metastases.

*In vivo* studies of uveal melanoma growth and metastasis were performed in congenitally athymic nude mice and initial observations concluded that tumor growth was a rare event (21%), and with the tumors that grew, the growth was slow (taking up to 12 weeks to form visible tumors) in comparison to the established cutaneous melanoma cell lines. This observation is in contrast to that of Phillips *et al.* (1989), who reported that ocular melanomas failed to grow in either nude or SCID mice. Ocular melanoma growth was infrequent and slow, and such tumors did not metastasise to the liver, this being the most frequent site of metastasis for uveal melanoma in patients (Jensen *et al.*, 1963). Therefore, this system cannot be considered a model for uveal melanoma metastasis. Further studies could involve the use of SCID mice, and possibly intra-ocular implantation of melanoma tissue or cell lines (Harning and Szalay, 1987), or intra-splenic injection of dissociated tumor samples, which has proved a successful route for colon carcinoma (Kozlowski *et al.*, 1984). Intra-splenic injection has been used previously to select for melanoma subpopulations with increased liver colonisation potential (Sargent *et al.*, 1988; Ladanyi *et al.*, 1990) and this increase in liver colonisation has been shown to be due to growth stimulation of the tumor cells in the presence of hepatocytes (Sargent *et al.*, 1988). The applicability of animal studies to human cancer metastasis is controversial since the introduction of human cells into a mouse immediately introduces an aspect of heterogeneity into any subsequent observation, in addition tumor growth in these models is frequently rapid which is in contrast to the growth of many human neoplasms in patients.

It was interesting to note that the doubling time for cutaneous melanomas following intradermal implantation was lower than for subcutaneous implantation, the reason for this is at present unknown, but seeding of the tumor cells onto the basement membrane may be responsible, possibly because of the ability of the
basement membrane to stimulate and polarise cellular growth (Review: Farquhar, 1981). However, a number of different explanations could be given to account for this observation such as, a reduction in the resistance that the growing tumor may encounter due to this site or a more generally suitable microenvironment for growth. Whilst the level of lung colonisation observed, following tail vein injection of a single cell suspension of either A375 or A375/NUPR1, showed that the experimental metastatic potential of A375/NUPR1 was higher than that of A375, the incidence of spontaneous metastasis displayed by these cell lines was approximately equal and of a low magnitude.

In attempts to explain this increase in experimental metastatic potential of A375/NUPR1 in nude mice, a number of parameters, thought to be involved in metastasis, were studied in vitro, these included, progelatinase expression (Yamagata et al., 1989; 1990), MHC class I and II (Van Muijen et al., 1991), ICAM-1 expression (Johnson et al., 1989) and the relative rates of proliferation for the two cell lines. These were generally found to be of similar orders of magnitude; with the exception that A375/NUPR1 which constitutively expressed the 92 kDa progelatinase in vitro whereas the parent cell line did not; however, no data concerning the expression of this proteinase in vivo is available at present. It is unlikely that the increase in experimental metastatic potential observed for A375/NUPR1 is solely due to the de novo expression of the 92 kDa progelatinase, and may be due to a number of, as yet undetermined, parameters. Further studies presented here examined the modulation of these factors by cytokines, and established that the two cell lines responded differently to TNFα and IL-1. The effect of IL-1 on A375/NUPR1 melanoma cell cultures was more pronounced than its effect on A375 cell cultures. MMP-9 (92 kDa gelatinase) was upregulated by IL-1 in A375/NUPR1 cells but not in A375 cells. Similarly, the magnitude of ICAM-1 modulation was greater in A375/NUPR1 than in A375 cells, suggesting that a sub-population may have been selected following in vivo passage of the parental cells. The different response to IL-1
is possibly due to altered signal transduction pathways, although the precise mechanism has still to be established.

A large component of the study involved the identification of phenotypic characteristics common to uveal and cutaneous melanomas. These focussed on proteinase expression by these cell types, particularly MMP-9 and MMP-2 (92 and 72 kDa progelatinases respectively), and tissue plasminogen activator. Not only did both types of melanoma express the same enzyme profiles, but they were also susceptible to modulation by the same agents. In situ hybridisation for mRNA and immunolocalisation of proteinase and proteinase inhibitor molecules may determine whether their expression in situ is similarly controlled.

The fact that melanoma cell cultures constitutively express progelatinase and secrete high amounts in vitro is interesting. The enzyme was found in a latent state, requiring loss of an amino-terminal peptide for activity. However, the mechanisms involved in the activation of the metalloproteinases are exquisitely complex, and much research is directed towards elucidating the details of this process (Review: Van Wart and Birkedal-Hansen, 1990). The mechanism of activation of the metalloproteinases in vivo is generally accepted to be undefined, however, in vitro there appear to be differences in the control of activation between the different metalloproteinase groups. MMP-2 and MMP-9 (72 and 92 kDa progelatinases) are enzymes which display characteristics not yet observed in other proteinases to date. The 72 and 92 kDa gelatinases have been found to co-purify with TIMP-2 and TIMP-1 respectively, as stable proenzyme-inhibitor complexes with the inhibitor bound at a site other than the active site (Goldberg et al., 1989; Wilhelm et al., 1989). It is not known if these enzymes-inhibitor complexes are secreted as separate molecules which associate following secretion, or if the enzyme-inhibitor complex is assembled intracellularly and then subsequently secreted. This situation is in contrast to the majority of known enzymes which are capable of binding inhibitors only at their active sites when active. The 72 kDa gelatinase, once it has formed a complex with TIMP-2, has a reduced susceptibility to auto-activation (Howard et al., 1991) and this stabilisation may be
important *in vivo*. Removal of TIMP from its complex with gelatinase results in auto-activation and the production of a number of unstable but highly active enzyme fragments (Howard *et al.*, 1991). Furthermore, once activated, they may be inhibited by TIMP. Interestingly this unusual complex formation of TIMP-1 with the MMP-9 and TIMP-2 with MMP-2 has not been observed with the other matrixins, although TIMP-2 has been shown to preferentially inhibit type II matrixins (Howard *et al.*, 1991). This dual control of the enzyme (activational repression and inhibition) by TIMP suggests an important role for these enzymes *in vivo*.

The study of *in situ* patterns of enzyme and inhibitor expression by different tumor types may prove to be useful in understanding invasive tumors, and it is interesting that this study found that melanoma, which is considered to be a highly metastatic tumor, constitutively expresses only the type II matrixins (gelatinases). The expression of type III matrixins (stromelysins) by melanomas has not been reported, and expression of type I matrixins (interstitial collagenases) although reported by one group (Smyth Templeton *et al.*, 1990) awaits confirmation. Neither type I or type III matrixins were detected in the present study using gelatin and casein zymography. It is interesting to speculate about this pattern of enzyme expression and its association with melanoma: of the three matrixin families, the type II matrixins are the only enzymes which are thought to be capable of binding to their substrates prior to activation or as an enzyme-inhibitor complex. This most likely occurs because of the presence of a fibronectin-like collagen binding domain present in only the 72 kDa and 92 kDa gelatinases (Goldberg *et al.*, 1989). In addition an α2 (V) collagen-like domain is present in the 92 kDa gelatinase, the function of which is unknown, but which may be involved in the binding of this enzyme to type V collagen. In this study it was observed that high levels of gelatinase were secreted into the culture medium; should this occur *in vivo* and the enzyme be capable of binding to its substrate in an *in vivo* milieu, then subsequent degradation of associated TIMP-1 by elastase (Okada *et al.*, 1988) may result in an autoactivated enzyme associated with its substrate in a way that may prevent inhibition by TIMP, due to the close proximity of the enzyme.
and its substrate. This combination of factors: high levels of enzyme secretion; the ability of the enzyme to bind to substrate prior to activation to form an enzyme-inhibitor complex and subsequent activation by removal of the stabilising inhibitor by proteolysis, suggests a scenario where enzyme/enzyme-inhibitor complexes attached to its appropriate substrate awaits activation by the appropriate stimulus. This stimulus may be the arrival of cells possibly by macrophages or neutrophils, secreting elastase (to degrade TIMP-1) or other proteinases capable of degrading TIMP-2 at this site, which may facilitate the rapid invasion commonly associated with melanoma.

The *in vitro* experimental data presented in this thesis determined that certain cytokines, TNFα, TGFβ and IL-1 α/β, are capable of altering melanoma cell phenotypic characteristics in such a way as to enhance the metastatic ability of these cells; however, other characteristics such as proliferation rate are decreased. When TNFα treated cells were used in an experimental metastasis assay in nude mice, their metastatic ability remained unaltered, suggesting that the factors which were upregulated were not rate limiting for successful experimental metastasis. The role of TNFα and IL-1 in tumor metastasis is an area of current interest. Lollini *et al.* (1990) reported that murine B16 melanoma cells when treated *in vitro* prior to use in an experimental metastasis assay displayed enhanced lung colonisation in C57BL/6 mice, and Bani *et al.* (1991) studied the effect of intravenous injection of IL-1 in nude mice on the experimental metastatic potential of the A375 melanoma, which was found to be increased. In related studies using ovarian cancer, Malik *et al.* (1989) showed that TNFα therapy promoted both peritoneal invasion and solid tumor formation of ovarian ascites in xenograft models. Later studies determined that cells expressing TNFα, following transfection with the TNFα gene, exhibited enhanced invasion and metastasis in nude mice (Malik *et al.* 1990). The study of Lollini *et al.* (1990) involved the use of murine melanoma cells in a murine system, and using a subline resistant to H-2 induction they provided evidence that the increase in experimental metastatic potential was due to the increase in H-2 expression associated with TNFα treatment. This study determined that *in vitro* treatment of A375 melanoma cells with TNFα did
not alter MHC expression or experimental metastatic potential in nude mice, therefore confirming the study of Lollini et al. (1990) but in a human xenograft model. The study by Bani et al. (1991) involved analysis of the role of IL-1 in the augmentation of experimental metastasis due to increased endothelial cell-tumor cell interactions caused by endothelial cell activation by IL-1. A more comprehensive study by Burrows et al. (1991) investigated the effect of melanoma cell secretion of IL-1 on melanoma-endothelial cell adhesive interactions, but did not investigate modulation of experimental metastatic potential. These two studies do not address the question of the effect of TNFα treatment on experimental metastasis but rather that of the effect of a cytokine with a similar spectrum of activities to TNFα (IL-1) on endothelial cells and their role in metastasis. Studies by Malik et al. (1989; 1990) provide evidence for the role of TNFα in the invasion and metastasis of ovarian cancer cells in nude mice.

The differences observed in gelatinase modulation by the cytokines TNFα, IL-1 and TGFβ may possibly be explained by examining the differing promoter sequences of the two gelatinase genes. TNFα and IL-1 have been shown to induce expression of c-fos and c-jun the components of the transcription factor AP-1 (Lin and Vilcek, 1987; Conca et al., 1989). AP-1 is capable of stimulating gene expression following binding to TRE sequences in their promoter elements (Angel et al., 1987). The 92 kDa gelatinase promoter contains 2 TRE motifs but the 72 kDa gelatinase lacks such a sequence (Huhtala et al., 1990; 1991). Furthermore, the 92 kDa gelatinase promoter contains a TGFβ inhibitory element (TIE), the 72 kDa gelatinase again does not contain this motif (Huhtala et al., 1990; 1991).

In the interstitial collagenase promoter there is a TPA oncogene responsive unit (TORU), composed of a TRE motif and a PEA3 binding sequence which act together to regulate the level of expression of the enzyme. If only the AP-1 binding motif is occupied, basal expression of the enzyme results, but if both sites are filled, maximal transcription occurs (Gutman and Wasylyk, 1990). It is possible that the AP-1 binding sites and the TIE motif are acting in such a way as to regulate 92 kDa gelatinase gene transcription. Alternatively, rather than a direct effect of TGFβ on
protein/DNA binding, TGFβ may stimulate the de novo production of a factor which is capable of binding at a site in the promoter other than the TIE motif. Interestingly, the 92 kDa gelatinase promoter contains an Spi-1 binding motif (5' GAGGAA 3') (Goebl, 1990) at -536 to -542 position 5' to the transcriptional start site (personal observation), which is one nucleic acid away from the 5' TRE sequence; it is possible that these two sequences may act together to regulate enzyme expression. The putative oncogene Spi-1 has been reported to produce the PU-1 transcription factor which may act as a lymphoid-specific enhancer, which has been detected in macrophage and B-cell but not in T-lymphocyte cell lines (review: Goebl, 1990). In addition, it is a member of the ets transcription factor family and may be able to bind to PU boxes in a similar way to that of the oncogene c-ets-1 binding to PEA3 sites (Goebl, 1990).

Synergy between TNFα and TGFβ has previously been observed in the induction of monocytic differentiation of the human promyelocytic leukemia cell line HL-60 (De Benedetti et al., 1990); furthermore, the induction of a 94 kDa gelatinase has been reported to occur during HL-60 differentiation (Davis and Martin, 1990); the role of Spi-1 in this event has not been determined.

Since the 72 kDa gelatinase lacks TRE sequences, it is not surprising that it is refractory to the action of AP-1 inducing cytokines. However, the constitutive expression of the 72 kDa gelatinase by all melanoma cell cultures studied to date may indicate that melanomas, as a tumor type, either have a tumor specific transcription factor stimulating gelatinase expression, or have lost a constitutively expressed inhibitory factor. Further studies are needed to elucidate the exact mechanism of transcriptional regulation of the different gelatinases.

Whilst all melanoma cell cultures studied expressed the 72 kDa progelatinase, only a proportion of them expressed the 92 kDa progelatinase. A recent report by Burrows et al. (1991) showed that high levels of ICAM-1 expression on melanomas was due to endogenous IL-1 production by the tumor cells, and a number of the cell lines used in the present study were also used in the study of Burrows et al. (1991). It will be interesting to determine if expression of the 92 kDa gelatinase by DX3 (a cell line
shown by Burrows et al. (1991) to secrete IL-1 and in this study to express 92 kDa progelatinase) can be inhibited by anti-IL-1 antibodies. Cell lines shown not to secrete IL-1 (Burrows et al., 1991) were A375 and SK23, however, both A375 and SK23 failed to express 92 kDa progelatinase at a detectable level. This study has not investigated the expression of cytokines by melanoma cell cultures, but further studies may address the expression of cytokines by melanoma cells and the modulation of tumor derived cytokine expression by the addition of exogenous cytokines. The production of cytokines such as TNFα and IL-1 may profoundly affect the ability of a melanoma cells to metastasise, due to both autocrine effects and the influence of these cytokines on host cells such as endothelial cells.

The studies in this thesis attempting to identify potential new prognostic indicators for uveal melanoma were:

(i) analysis of the role of ploidy in metastatic spread.
(ii) cytogenetic analysis of uveal melanoma tissue.
(iii) studies involving the expression of proteinases such as gelatinases and plasminogen activators by uveal melanomas.

Analysis of tumor cell karyotypes has shown that cells exhibit increasingly altered karyotypes and ploidy values, as they progress from benign to malignant neoplasms (Yunis, 1983; Nowell, 1989). Therefore, as the tumor progresses it should exhibit an increased rate of genetic mutation, since cellular homeostatic mechanisms are likely to become altered or inactivated. Experimental evidence for this was provided by Cifone and Fidler (1982) who showed that highly metastatic cells were both phenotypically and genotypically more unstable than non-metastatic cells. In contrast, this study found that the majority of the tumors studied were diploid, and it was in this group of tumors that deaths due to metastasis occurred rather than in the aneuploid or tetraploid groups of tumors. Fidler and Talmadge (1986) demonstrated that a single tumor cell has the ability to form a metastatic lesion, and whilst the majority of tumor cells within a tumor were diploid, it is possible that a minor sub-population of tumor cells may be present and have a higher metastatic potential and associated alterations in ploidy.
values. It is these tumor cells that may escape from the primary tumor mass and form the metastases. Unfortunately metastatic deposits were not available for analysis, so this hypothesis could not be proved. It is possible that this subclone, whilst it is adapted for invasion and metastasis, may not achieve dominance within the tumor due to growth inhibitory interactions (Kerbel et al., 1984).

The observation that the uveal melanoma patients who died of metastatic disease all had tumors which had high numbers of cells, which were undergoing cell division, has important implications for invasion. Theories of how cells invade have included mechanical explanations (Eaves, 1973), which suggest that invasion may be accomplished solely by the build up of pressure due to tumor growth, which will eventually allow the tumor to force its way through the extracellular matrix and into the newly available tissue space. However, later theories included cyto-migration as an additional factor (Strauli and Haemerli, 1984), and finally active degradation of extracellular matrix (Tryggvason et al., 1987). It is reasonable to accept that such a combination of theories could explain tumor invasion. A high proliferation rate may be a basic requirement for tumor invasion and as such it may be responsible for certain forms of tumor invasion, especially when the tumor does not express degradative enzymes. However, if degradative enzymes are expressed by such tumors they may increase the rate of invasion. Furthermore, the products of matrix degradation have been shown to be chemotactic for tumor cells (Terranova et al., 1989), and as such may augment tumor invasion into the surrounding stroma by providing a strong chemotactic signal for the tumor cells to migrate to the periphery of the tumor, and into the surrounding stroma. The role of degradative enzymes in tumor invasion may be more complex than at first thought, due to the effects of both the enzymes and their inhibitors on host cell behavior (Inyang and Tobelem, 1990; Takigawa et al., 1990).

Many of these studies presented here were possible due to the development of a short term in vitro culture system for uveal melanoma. Whilst a protocol for cell growth had been previously described by Albert et al. (1984), the media requirements were expensive and not feasible for the present study. Whilst the culture system used
failed to maintain cells *in vitro* for extended periods of time, it is possible to grow cell cultures for periods of 2-3 months and up to five *in vitro* passages. This provides sufficient uveal melanoma cells to perform selected experiments. Cytogenetic analysis of uveal melanoma cell cultures was performed at a passage level equal to or later than the cells used in other experiments, so verifying the cells as abnormal (Sisley *et al.*, 1990; Sisley *et al.*, 1992 in press). A major problem in the continuing culture of uveal melanomas was the poor attachment of the cells following sub-culture. Although tumor cells may attach directly to plastic, such binding is generally considered to be non-physiological and cannot be disrupted by the agents commonly used in cell culture. Rather cells are considered to attach to matrix factors present in the serum or to factors produced by themselves (Review: Yamada, 1983). If we speculate that uveal melanomas lack expression or display reduced levels of expression of the receptors capable of binding to these matrix components, this may, in part, explain their site specific metastasis as a negative selection event. Whilst it is possible that liver associated extracellular matrix components or cell types express adhesion receptors which allow specific attachment of uveal melanoma cells at this site, it is also possible that because the liver does not have a basement membrane layer (Martinez-Hernandez, 1984) it does not impede the extravasation of uveal melanoma cells as other sites may. In contrast to other organs the liver has a loosely organised basement membrane-like matrix which is found in the space of Disse and allows the hepatocyte direct contact with the blood plasma. This irregular organisation of extracellular matrix contains collagen type III (Geerts *et al.*, 1986) and fibronectin (Martinez-Hernandez, 1984) in addition to classic basement membrane components such as collagen type IV (Martinez-Hernandez, 1984), laminin (Maher *et al.*, 1988) and heparin sulphate proteoglycan (Friedman *et al.*, 1985) Recently a novel heparan sulphate proteoglycan has been reported to be present exclusively in liver extracellular matrix (Soroka and Farquhar, 1991). It is interesting that type III collagen which is not normally present in basement membranes has been detected in Bruch's membrane and the space of Disse (Geerts *et al.*, 1988). Adhesion to this collagen type may be responsible for
arrest in the liver, since it is possible that the tumor cells may express receptors for this collagen type due to their previous close proximity in the eye to Bruch's membrane and the sclera (Zimmerman, 1985). However, at present little is known about the adhesion molecules expressed by uveal melanomas. Further studies would investigate this possibility, possibly using cryostat sections of the liver, and other organs to determine if specific adhesion occurs. Subsequent studies may involve the identification of cell-type specific or matrix specific binding. Alternatively, it would be informative to study in vitro adhesion molecule expression on uveal melanomas using specific anti-sera to adhesion molecules (such studies are currently underway in collaboration with Dr. I. R. Hart at the Imperial Cancer Research Fund, London).

Complementary studies aiming to identify growth factors and cytokines capable of modulating uveal melanoma cell growth may be undertaken using tritiated thymidine incorporation and flow cytometry, as a measure of cell proliferation. Identification of factors capable of increasing the growth rate of uveal melanomas in vitro may help in determining factors important in vivo, especially if these factors were present at higher concentrations in the liver (Sargent et al., 1988). This would offer a possible explanation for the site specific metastasis associated with this tumor type, that of growth potentiating factors present in the liver.

Increasing genetic abnormality is associated with a tumor's progression from a benign to malignant neoplasm. Since few cytogenetic abnormalities were detected in uveal melanoma cells, they would be expected to have a low metastatic propensity. Only three patients out of forty nine patients have died of metastatic disease to date, and it has not been possible to correlate cytogenetic analysis with the development of metastatic disease. Whilst both cutaneous and uveal melanomas are melanocytic tumors, abnormalities of chromosomes 3 and 8 are rarely observed in cutaneous neoplasms (Limon et al., 1988; Grammatico et al., 1990) but appear to be common in uveal melanoma (Sisley et al., 1990; 1992 in press), thus indicating that uveal melanomas are genotypically distinct from their supposed cutaneous counterparts. In addition, these abnormalities appear to be a characteristic of tumors arising in the
ciliary body. Only one choroidal tumor had trisomy of chromosome 8 and no corresponding abnormality of chromosome 3 (Sisley et al., 1990; 1992 in press). Monosomy of chromosome 3 and i (8q) may be uniquely associated with a subset of uveal melanomas. Whilst there are a number of phenotypic similarities between cutaneous and uveal melanomas there are also significant genetic changes observed in uveal melanoma that have so far not been detected in cutaneous melanoma. These specific changes may, in some way, be linked with the characteristic pattern of metastasis observed in uveal melanoma.

In common with cutaneous melanomas, uveal melanomas express tissue plasminogen activator as the major plasminogen activator species detected. Interestingly, a plasminogen activator with an apparent molecular weight similar to that described for urokinase type plasminogen activator was also expressed by cell cultures established from highly aggressive tumors. Quax et al. (1991) reported that expression of tissue and urokinase plasminogen activator correlated with spontaneously metastasis in nude mice. The in vitro levels of active enzyme were 10 fold lower than those secreted by the series of cutaneous melanomas studied. Whilst this observation may be due to increased expression of PA by the cutaneous melanomas, equally the increased expression of PA inhibitors by uveal melanomas may be responsible. Although a plasminogen activator with a molecular weight comparable to urokinase was detected using casein/plasminogen zymography, no activity was detectable in the conditioned medium (chapter 6). Urokinase is thought to be more involved in tissue remodelling than tissue plasminogen activator, and as such has a receptor allowing the enzyme to remain active following binding to the cell. It is possible that if cell lysates were assayed urokinase activity may well be detectable.

These studies have shown that aggressive uveal melanomas express a number of degradative enzyme species which may act synergistically to degrade the components of the sclera and in so doing potentiate metastasis (Cottam et al., 1992 in press).

Further studies to determine the in situ expression at the site of scleral invasion would be informative, and provide evidence for the production of these enzymes in

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the patient. Therapeutic manipulation of the enzymes or the inhibitors may prove beneficial in the treatment of invasive neoplasms, however, localisation of the modulators at the site of the tumor may prove difficult. A systemic reduction of proteinases or an increase in the expression of proteinase inhibitors would be disruptive to a number of physiological processes, for example, systemic decreases in tissue-type plasminogen activator may adversely affect fibrinolysis and wound healing and a reduction in the expression of gelatinase may affect wound healing, ovulation, uterine and breast involution (Mullins and Rohrlich, 1983). The role of these enzymes in physiology should be investigated extensively before therapeutic modulation is attempted.

The major findings of the work in this thesis can be summarised as follows:

Although uveal and cutaneous melanoma display different cytogenetic changes, in vitro, cell cultures of both melanomas express similar patterns of degradative enzymes, the 72 and 92 kDa gelatinases and tissue plasminogen activator. Expression of the 92 kDa gelatinase was found to be modulated in both melanoma types by the cytokine TNFα and TGFβ was found to synergise with TNFα in the induction of the 92 kDa gelatinase in some melanoma cell cultures. The uveal melanomas which expressed the highest levels of tissue plasminogen activator activity expressed the 92 kDa gelatinase and were found to have invaded the sclera, suggesting a synergistic action between these enzyme families in the process of invasion.

The related cutaneous melanoma cell lines A375 and A375/NUPR1 showed different levels of experimental metastatic potential, but expressed similar levels of a number of melanoma associated markers, MHC class I and II and ICAM-1, and had similar proliferation rates in vitro and doubling times in vivo. ICAM-1 expression was increased by TNFα and IL-1 but MHC expression was unaltered. Differences in the magnitude of modulation were observed between the two cell lines, A375/NUPR1 showing a greater response than A375. The proliferation of both cell lines was decreased following stimulation with both TNFα and IL-1. In vitro pretreatment of
A375 cell cultures with TNFα prior to tail vein injection did not alter the extent of lung colonisation.

Further analysis of the metastatic process aiming to reveal the underlying molecular mechanisms of metastasis will be likely to involve the study of matrix metalloproteinases and plasminogen activators. No doubt, the roles of these molecules and their inhibitors in this process will be extended to include their actions upon tumor and host cell behavior and the effects upon cell differentiation caused by alterations in the extracellular matrix induced by their action.
List of Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A,</td>
<td>abdominal organs</td>
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<tr>
<td>Ad-2 E1A,</td>
<td>adenovirus 2 E1A</td>
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<td>AP-1,</td>
<td>activator protein-1</td>
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<td>BSA,</td>
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<td>cAMP,</td>
<td>cyclic adenosine monophosphate</td>
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<td>chloramphenicol acetyl transferase</td>
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<td>CV',</td>
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<td>del,</td>
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<td>der,</td>
<td>derived chromosome</td>
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<td>DNA,</td>
<td>deoxyribonucleic Acid</td>
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<td>DTNB,</td>
<td>5,5'-Dithiobis-(2-nitrobenzoic Acid)</td>
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<td>FACS,</td>
<td>fluorescence activated cell sorting</td>
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<td>IMP,</td>
<td>inhibitor of metalloproteinases</td>
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<td>IU,</td>
<td>international units</td>
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<tr>
<td>i. v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>KCL,</td>
<td>pottasium Chloride</td>
</tr>
<tr>
<td>kDa,</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L,</td>
<td>lungs</td>
</tr>
<tr>
<td>LAK,</td>
<td>lymphokine activated killer</td>
</tr>
<tr>
<td>lb/sq in,</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>LFA-1,</td>
<td>lymphocyte function antigen-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>VLA</td>
<td>very late activation antigen</td>
</tr>
<tr>
<td>v-mos</td>
<td>viral mos oncogene</td>
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<tr>
<td>v-raf</td>
<td>viral raf oncogene</td>
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<tr>
<td>v-src</td>
<td>viral src oncogene</td>
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<tr>
<td>v-myc</td>
<td>viral myc oncogene</td>
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