THE ROLE OF REACTIVE OXYGEN SPECIES IN WALKER 256 TUMOUR CELL AND PLATELET INTERACTIONS WITH THE VESSEL WALL

By

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VESSEL WALL INJURY AND REACTIVE OXYGEN SPECIES IN METASTASIS

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ABSTRACT

Cancer cells frequently disseminate via the bloodstream where the endothelium and underlying basement membrane act as a barrier between the circulating tumour cells and the extravascular tissue. Previous studies have shown that metastasis was promoted following endothelial cell injury. Although several of the agents used in these studies may have caused endothelial cell injury by the generation of reactive oxygen species, a role for such species in the promotion metastasis has only been postulated thus far. Therefore, we have performed experiments to show that rat Walker 256 cells (W256 cells), and human platelets generate reactive oxygen species and that these are capable of perturbing human umbilical-vein endothelial cells. We also present data which suggests that W256 cells secrete a latent metalloproteinase which can be activated by reactive oxygen species.

W256 cells were shown to generate oxygen-derived free radicals when activated with the chemotactic peptide N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP). fMLP-stimulated W256 cells, suspended to a concentration of 5 X 10^6 cells/ml, produced luminol chemiluminescence equivalent

to that generated by 6.5 X 10^{-3} units/ml xanthine oxidase. The activation of 5 X 10^{6} W256 cells by 10^{-6}M fMLP, resulted in the extinction of scopoletin fluorescence corresponding to the generation of 18 ± 1 nmoles of hydrogen peroxide/hr. Direct evidence for the generation of reactive oxygen species by W256 cells was obtained morphologically. In the presence of $CeCl_3$, approximately 82% of the fMLP-stimulated W256 cells demonstrated cerium perhydroxide deposits either on their cell surface or intracellularly. The addition of 2000 units/ml of catalase reduced this to 38% ± 6.

We also examined human platelets for their ability to generate reactive oxygen species since these are often found at sites of tumour cell arrest in vivo. While others had inferred that human platelets generate reactive oxygen species, we have obtained direct morphological evidence confirming that this can occur. Platelet suspensions activated with 2.5 units/ml thrombin or 1 X 10^{-6} M phorbol myristate acetate produced luminol chemiluminescence. This was inhibited by the addition of catalase (2000 units/ml), superoxide dismutase (450 units/ml) or mannitol (1 mM). When activated platelets were added to endothelial monolayers, cerium perhydroxide deposits were found between adherent platelet aggregates and the endothelial cells. The formation of cerium perhydroxide precipitates was inhibited by mannitol.

Preliminary studies showed that in the presence of

the reactive oxygen species - generating system xanthine-xanthine oxidase, the release of ^{3}H-2-deoxyglucose from prelabeled endothelial cell monolayers was a sensitive index of endothelial cell perturbation. Incubating fMLP-activated W256 cells with ^{3}H-2-deoxyglucose-labeled endothelial cell monolayers, increased specific isotope release by 27% ± 5. Catalase (1000 units/ml) inhibited tumour cell-induced release of ^{3}H-2-deoxyglucose by 84% whereas superoxide dismutase (1 mg/ml) had no effect. Tumour cell contact with the endothelium was required in order to observe isotope release. Cell-free supernatants from fMLP-activated W256 cells did not cause ^{3}H-2-deoxyglucose release. Pretreatment of W256 cells with 1 μM cytochalasin B reduced the release of isotope although chemiluminescence was increased three-fold. Electron microscopy revealed that fewer cytochalasin B-treated W256 cells were attached to the endothelial cell monolayer than in untreated controls.

Since the previous experiments suggested that tumour cell contact with the endothelium was required in order to observe isotope release, we asked if the release of ^{3}H-2-deoxyglucose was dependent upon the adhesion of W256 cells to the endothelium. Four hour pretreatment of endothelial cell monolayers with 10 ng/ml rIL-1 resulted in a two-fold increase in the expression of vitronectin receptor, an 80% increase in the adhesion of PMA-activated W256 cells and a

42% increase in the release of isotope. The release of ^{3}H-2-deoxyglucose from labelled endothelial cell monolayers correlated directly with tumour cell adhesion ($r = 0.98$, $p < 0.005$). A polyclonal antiserum to the vitronectin receptor inhibited adhesion of activated W256 cells to rIL-1-pretreated endothelium by 39% and inhibited tumour cell-dependent isotope release by 66%. We suggest that W256 cell adhesion to endothelial cell monolayers is partially regulated by vitronectin receptor expression and that endothelial cell perturbation by reactive oxygen species is dependent on tumour cell adhesion.

We have also obtained evidence suggesting that W256 cells degrade subendothelial cell matrices by a process involving both the generation of hydrogen peroxide and the secretion of a metalloproteinase. As an assay for basement membrane degradation, ^{3}H-proline-labelled subendothelial matrices were exposed to 5×10^{6} fMLP-activated W256 cells. The addition of 2000 units/ml catalase completely inhibited the release of ^{3}H-proline. ^{3}H-proline release was also inhibited by the metalloproteinase inhibitors 1,10-phenanthroline or EDTA but not by α1-antitrypsin or α2-macroglobulin. Cell-free supernatants obtained from activated W256 cells were still able to promote isotope release. Electrophoresis of the cell-free supernatants in gelatin-containing SDS-PAGE gels revealed a major band of gelatinolytic activity at 94 kD. The 94 kD band may

represent the activity of a normally latent gelatinase since incubation of 0.001M 4-aminophenylmercuric acetate (APMA; a known activator of latent metalloproteinases) with the supernatants of PMA-activated W256 cells resulted the loss of gelatinolytic activity at 94 kD band and the appearance of five new bands of lower molecular weight (Mw 86 kD, 79 kD, 74 kD, 70 kD, 66 kD). Two of these lower molecular weight bands (Mw 86 kD and 66 kD) were also found in the absence of APMA, following 10-fold concentration of the cell-free supernatants. When the cell- free supernatants of PMA-activated W256 cells (concentrated 10 fold) were incubated with increasing concentrations of hydrogen peroxide (0.12% to 0.24%), the band at 66 kD demonstrated enhanced gelatinolytic activity. We suggest that the W256 cells can secrete a latent metalloproteinase of molecular weight 94 kD which may be activated, with a loss in molecular weight, by hydrogen peroxide or APMA.

In summary, we provide evidence which supports the novel concept that some tumour cells may mediate vessel wall injury by generating reactive oxygen species and suggest that this may promote the metastasis of these cells <u>in vivo</u>. Such a hypothesis has not been postulated previously.

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APMA	4-aminophenylmercuric acetate
BCNU	1,3-bis(chloroethyl)-1-nitrosourea
BSA	bovine serum albumin
BSO	buthionine sulfoximine
CDNB	1-chloro-2,4 dinitrobenzyne
CoF	cobra venom factor
COL	collagen
C5a	complememt component 5a
DEM	diethylmaleate
EC	endothelial cell
EDTA,	ethylenediaminetetraacetic acid
ELAM-1	endothelial leukocyte adhesion molecule-1
FITC	fluorescein isothiocyanate
fMLP,	N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine
Fn	fibronectin
FnR	fibronectin receptor
GSH	reduced glutathione
GRGDSP	Glycine-Arginine-Glycine-Aspartic acid-Serine-Proline
GRGESP	Glycine-Arginine-Glycine-Glutamic acid-Serine-Proline
HBSS	Hanks' balanced salt solution
HHLGGAKQAGDV	Histidine-Histidine-Leucine-Glycine-Glycine-Alanine-Lysine- Glutamine-Alanine-Glycine-Aspartic acid-Valine

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	intercellular adhesion molecule - 1
IFN-	interferon gamma
IGF	insulin like growth factor
INCAM-110	inducible cell adhesion molecule - 110
^{125}IUdr	^{125}Iiododeoxyuridine
i.v.	intravenous
LFA-1	lymphocyte - function - associated antigen
LM	laminin
LPS	lipopolysaccharide
MEM	modified minimal essential medium
Mw	molecular weight
NRS	normal rabbit serum
PBS	phosphate buffered saline
PDGF	platelet - derived growth faccor
PMA	phorbol 12-myristate, 13-acetate
PRCGVPDV	Proline-Arginine-Cysteine-Glycine-Valine-Proline-Aspartic acid-Valine
RGD	Arginine - Glycine - Aspartic acid
rIL-1α,	recombinant interleukin - 1alpha
RPMI 1640	Roswell Park Memorial Institute medium 1640
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	superoxide dismutase

TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases
TNFα	tumour necrosis factor alpha
u-PA	urokinase-plasminogen activator
VAAHELGHSLGLSHS	Valine-Alanine-Alanine-Histidine-Glutamic acid-Leucine-Glycine-Histidine-Serine-Leucine-Glycine-Leucine-Serine-Histidine-Serine
VCAM-1	vascular cell adhesion molecule - 1
VLA	very late activation antigen
Vn	vitronectin
VnR	vitronectin receptor
VWF	von Willebrand factor

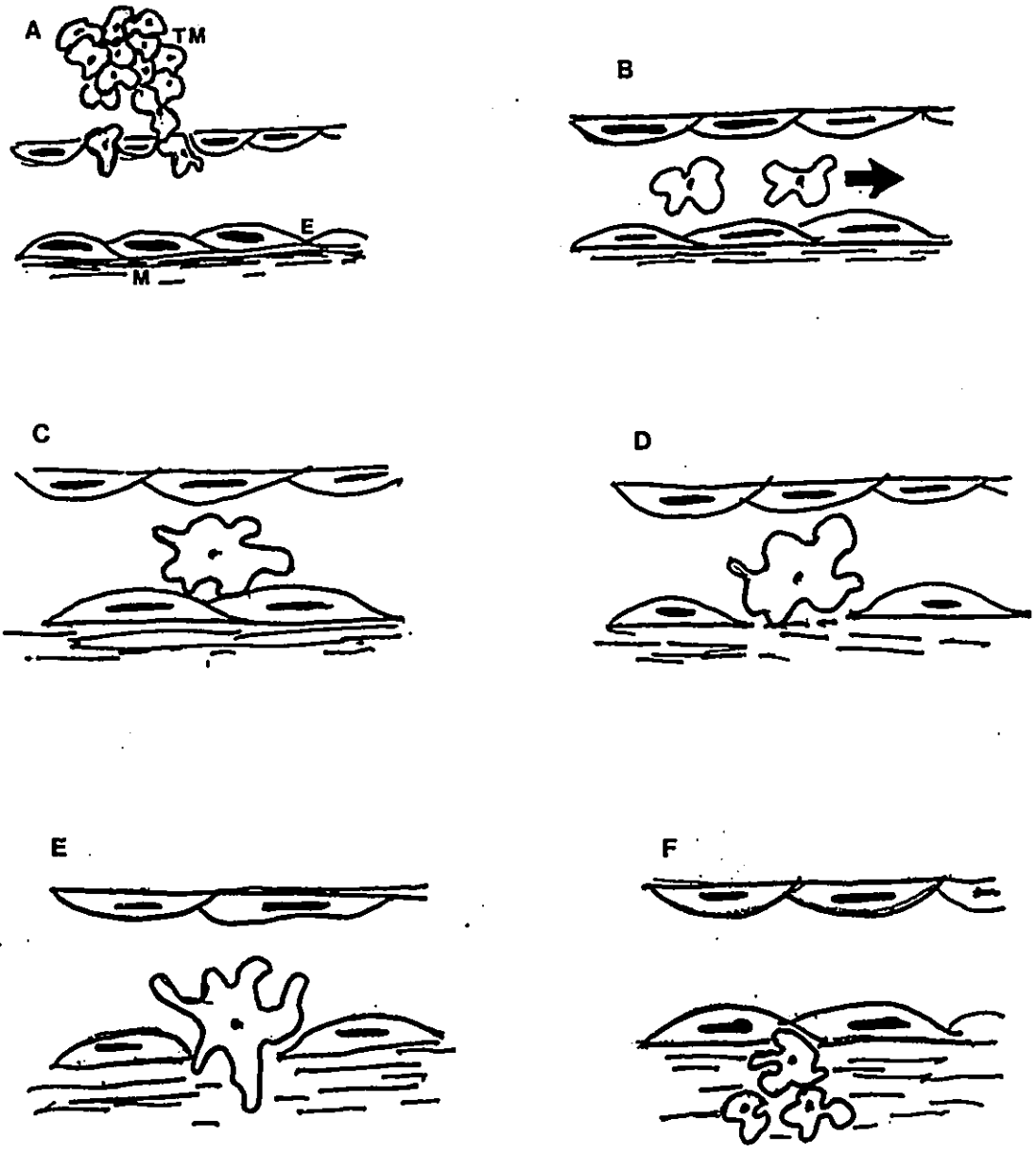
1 INTRODUCTION

1.1 General model of tumour metastasis

1.1.1 Mechanisms of tumour cell metastasis

Metastasis is the process by which cancer cells form secondary growths in organs at sites distant from the primary tumour. While this process is often lethal it is also largely an inefficient process. The metastatic potential of a given tumour cell is dependent upon its ability to perform a series of complex interactions with host cells (Figure 1). To disseminate from the primary tumour, cancer cells must: a) enter the bloodstream or lymphatics either by active migration of single cells or by detaching as the tumour mass (TM) invades; b) survive passage through the circulatory system; c) arrest within a microvascular bed; d) destroy the capillary wall at sites of arrest; e) pass across both the endothelium (E) and its underlying extracellular matrix (M); and, f) proliferate at the secondary site. These secondary lesions can subsequently metastasize themselves. This sequence of events has been named the metastatic cascade (Jones et al.,1971; Crissman et

Figure 1. The Metastatic Cascade



al., 1985; Crissman et al., 1988).

Several properties of the tumour cell appear to be essential in order for it to cross the barrier formed by endothelium and basement membrane. These include: the expression of adhesive moieties on the tumour cell surface; the production of proteolytic enzymes; tumour cell motility, and tumour growth.

1.1.2 Tumour cell adhesion to extracellular matrices and endothelium

Tumour cells are thought to adhere to components of both the interstitial stroma and basement membrane during invasion and adhere to the endothelium when leaving the bloodstream. In vitro, tumour cells bind more strongly to the basement membrane than to endothelial cell monolayers, (Kramer et al.,1980; Vlodavsky et al., 1982). In vivo, they preferentially adhere to sites of denuded basement membrane. Many adhesive moieties involved in tumour cell adhesion to the basement membrane or interstitial stroma have been identified. For example, those mediating adhesion to types I, II, III and IV collagen, laminin, fibronectin, and vitronectin, belong to the integrin family of adhesion molecules. In addition, two members of this family, a fibronectin receptor (FnR) and a vitronectin receptor (VnR), appear to also mediate tumour cell attachment to the

endothelium. These molecules are described in detail in sections 1.3 and 1.4.

The adhesive moieties, present on both the tumour cell and the endothelium, appear to be important in determining the pattern of tumour cell-dissemination and colonization in vivo. Many blood-borne tumour cells metastasize to the first organ they encounter. This is consistent with the view that tumour cells physically arrest within the microvasculature on the basis of anatomical considerations alone. However, some cancer cells appear to metastasize to specific organs regardless of anatomical considerations. For example, carcinomas of the prostate frequently metastasize to bone and not to lung as would be expected (Nicolson, 1988). This non-random pattern of metastasis was first explained by the "seed and soil" hypothesis of Paget (Paget, 1889). He postulated that gross tumour development was a consequence of tumour cells (the seeds) finding a fertile environment (the soil) in which to proliferate.

While site-specific metastasis may depend in part on events which occur subsequent to the arrest of tumour cells, such as tumour growth, the attachment of tumour cells to organ-specific endothelium determines which organs are colonized. Alby and Auerbach (1984) demonstrated that capillary endothelial cells derived from different organs, differ in their adhesivity for tumour cells. Thus, they

demonstrated that tumour cells which metastasize preferentially to a specific organ in vivo are more adhesive to endothelial cells isolated from that organ (Nicolson, 1982; Alby and Auerbach, 1984; Auerbach et al.,1987). For example, teratoma cells which metastasized to the ovary following intraperitoneal injection, adhered preferentially to ovary-derived endothelium, while glioma cells derived from brain metastasis adhered preferentially to brain microvascular cells. In addition, hepatoma cell lines derived from liver metastasis adhered preferentially to capillary endothelium derived from liver (Alby and Auerbach, 1984; Auerbach et al.,1987). These studies indicated differences between the capillary endothelial cells derived from different organs and that site-specific metastasis may be the consequence of endothelial-derived adhesion molecules, which mediate attachment of circulating tumour cells within specific microvascular beds.

1.1.3 Tumour cell degradation of extracellular matrices

Tumour cell invasion is the process by which tumour cells migrate into the extracellular space, occupied by host cells or extracellular matrix. Invasion can be viewed as occuring in three steps: a) tumour cells adhere to the extracellular matrix; b) there is local degradation of the matrix; c) tumour cells migrate into the region of matrix

modified by degradation (Liotta et al.,1980). Tumour cell-displacement or degradation of the extracellular matrix is of particular importance during both intravasation and extravasation. Morphological studies indicate tumour cells extravasate (or intravasate) in one of two ways (Baserga and Saffiotti, 1955; Crissman et al., 1985). Some tumour cells, once arrested, proliferate within the lumen of the capillary eventually leading to vessel wall damage and subsequent extravasation, (Baserga and Saffiotti, 1955). This process requires only mechanical forces and suggests the tumour cells have displaced the extracellular matrix which lies in their path (Eaves, 1973). A second model of extravasation involves the direct migration of tumour cells through the capillary wall with little or no proliferation (Wood, 1958; Ludatscher et al., 1967; Kinjo, 1978). In this case, proteolytic degradation of the extracellular matrix appears to be the primary mechanism by which tumour cells are able to invade surrounding tissues.

Correlations have been established between tumour cell invasion, their metastatic potential and their ability to degrade type IV collagen (Liotta et al.,1980; Turpeenniemi-Hujanen et al.,1985; Mignatti et al.,1986; Nakajima et al.,1987). Nakajima et al. (1987), examined a series of rat 13762NF mammary adenocarcinoma cell clones of various metastatic potentials for their ability to degrade subendothelial cell matrices and purified type IV collagen.

Metastatic potentials were simultaneously determined based on spontaneous lung metastasis after subcutaneous implantation or experimental lung metastasis after intravascular injection of the tumour cells. Highly metastatic 13762NF cells solubilized isolated subendothelial matrices and purified type IV collagen at higher rates than poorly-metastatic cells (Nakajima et al.,1987).

There is now considerable evidence implicating the metalloproteinase family of degradative enzymes in the processes of invasion and metastasis (Mignatti et al.,1986; Reich et al., 1988). These enzymes degrade type IV collagen as well as the interstitial collagens, type I, II and III, and are specifically inhibited by a variety of compounds. These include EDTA, 1,10-phenanthroline, tissue inhibitor of metalloproteinases (TIMP), and SC-44463. The migration of tumour cells across polycarbonate filters coated with matrigel or human amnion is inhibited by addition of these metalloproteinase inhibitors (Mignatti et al.,1986; Reich et al.,1988). In addition, in models of experimental metastasis, the formation of metastatic lung - lesions is reduced greatly when tumour cells are co-injected with the metalloproteinase inhibitor, SC-44463 (Reich et al.,1988). The family of metalloproteinase enzymes are described in detail in Section 1.5.

1.1.4 Tumour cell motility

Tumour cells must migrate into the region of matrix modified by degradation in order to enter or leave the circulation. The migration of tumour cells, in vitro, can be directed by synthetic peptides, cytokines, complement components, and components of extracellular matrices (Orr et al.,1978; McCarthy et al.,1983; McCarthy and Furcht,1984; Rayner et al,1985; Orr et al.,1985). Components of extracellular matrices can direct tumour cell migration by both chemotactic and haptotactic mechanisms. Chemotactic migration involves directed migration in response to a soluble stimuli whereas haptotactic migration is the directed migration of cells in response to a substratum-bound attractant.

The extracellular matrix components, collagen, fibronectin, and laminin are chemotactic for a wide variety of tumour cells (McCarthy et al.,1983; McCarthy and Furcht, 1984). The migration of B16 mouse melanoma cells was increased by 100-fold in response to a gradient of laminin, and was inhibited by the addition of laminin antiserum (McCarthy et al.,1983; McCarthy and Furcht, 1984). The cells migrated in response to laminin by both haptotaxic and chemotaxic mechanisms since substrate-bound laminin increased tumour cell migration even in the absence of soluble laminin (McCarthy et al.,1983; McCarthy and Furcht,

1984). Tumour cell migration was further enhanced by soluble laminin (McCarthy et al.,1983; McCarthy and Furcht, 1984). The chemotactic and haptotactic properties of collagen, fibronectin and laminin could be important in directing the mobility of tumour cells across basement membranes in the absence of other factors.

Chemoattractants can influence the metastatic behavior of cancer cells in vivo. For example, the fifth component of complement (C5), can be cleaved by leukocyte-derived elastase or cathepsin G and become chemotactic for cancer cells (Orr et al.,1978). C5-dependent chemoattractant activity can be detected in experimentally-induced inflammatory exudates (Orr et al.,1982; Orr et al.,1985). When rats were given intraperitoneal injections of glycogen to induce an acute inflammatory response, prior to the intravenous injection of tumour cells, a significant increase in the number of mesenteric metastases was observed (Orr et al.,1982). Similarily, a significantly greater number of metastases developed in the lungs of animals in which pulmonary inflammation was induced by the intra-tracheal injection of carbon (Orr et al.,1985). These experiments demonstrate that chemoattractants, released during inflammation, can influence the metastasis of circulating tumour cells.

1.1.4.1 Tumour Growth

Tumour cells and/or host cells synthesize a number of factors which can directly stimulate tumour cell proliferation and/or promote tumour growth by increasing tumour vascularization. These factors induce three responses in microvascular endothelial cells which are important in tumour vascularization: a) an increase in endothelial cell protease production which allows the endothelial cells to penetrate surrounding tissue; b) endothelial cell migration towards the source of the angiogenic factor(s); and, c) an increase in the rate of endothelial cell proliferation (Zetter 1988).

A number of different molecules have been shown to be both mitogenic for tumour cells as well as angiogenic, either by virtue of stimulating vascularization *in vivo* and/or by stimulating endothelial cell proliferation *in vitro* (see Table 1). For example, transforming growth factor-α (TGF-α) is angiogenic *in vivo* and can stimulate both tumour cell and endothelial cell proliferation *in vitro* (Schreiber et al.,1986; Imanishi et al.,1989; Rodeck et al.,1990). Transforming growth factor-α shares extensive homology with epidermal growth factor and both factors bind the same receptor (Marguardt et al.,1984). A second growth factor, platelet-derived growth factor (PDGF) is produced by

Table 1. Growth factors produced by tumour cells

Factor	Tumour cell proliferation	Endothelial cell proliferation	Angiogenic	References
TGF-α	+	+	+	Schreiber et al.,1986 Imanishi et al.,1989 Rodeck et al.,1990
PDGF	+	+	+	Ishikawa et al.,1989 Usuki et al.,1989 Maxwell et al,1990
bFGF	+	+	+	Zetter, 1988
IGF-I & II	+	none	none	El-Badry et al.,1989 Cullen et al.,1990 Ohmura et al.,1990
TGF-β	-	-	+	Barnard et al.,1990

various tumour cell lines including human squamous carcinoma and anaplastic thyroid carcinoma cells (Usuki et al.,1989; Ishikawa et al.,1989). This growth factor was first isolated from the α granules of platelets (Usuki et al.,1989). Many tumour cells, while capable of producing PDGF, lack cell surface receptors for it (Bronzert et al.,1987). However, some tumours of the brain, can produce both PDGF and PDGF receptors (Maxwell et al,1990). PDGF can act on host cells to promote angiogenesis in vivo and is chemotactic for bovine aortic endothelial cells in vitro (Ishikawa et al.,1989).

Some tumour cell chemotactic factors do not affect endothelial cells and are not angiogenic in vivo (Table 1). For example, the insulin-like growth factors, IGF-I and II, exert autocrine effects on human colon carcinoma, pancreatic carcinoma and neuroblastoma cells lines but do not affect endothelial cells either in vitro or in vivo (El-Badry et al.,1989; Cullen et al.,1990; Ohmura et al.,1990). Other factors, such as transforming growth factor-β (TGF-β), are produced by a wide variety of tumour cells and inhibit tumour growth (Barnard et al.,1990). TGF-β inhibits endothelial cell proliferation in vitro but promotes angiogenesis in vivo, possibily due to its chemotactic recruitment of monocytes which then release angiogenic factors (Frater-Schroder et al.,1986; Wahl et al.,1987).

1.1.5 Properties of the Walker 256 tumour cell

W256 cells were first isolated from a spontaneous tumor in the mammary gland of a pregnant albino rat (Stewart et al.,1959). Since its isolation in 1928, the W256 cell line has been studied extensively in experimental models of cancer metastasis (Stewart et al.,1959; Jones et al.,1971). Based on its morphology the W256 cell has been classified as a carcinosarcoma (Stewart et al.,1959). Recently however, cells from a line derived by chlorambucil treatment of the W256 cell line were shown to express MAC-1 on their cell surfaces (Simpkins et al.,1991). Since, MAC-1 is exclusively expressed by neutrophils as well as monocytes and further examination revealed an absence of most myeloid markers, the authors concluded that their cell line contained cells of monocytoid origin (Simpkins et al.,1991). Whether or not the W256 cell line used in these studies is a carcinosarcoma or is of monocytoid origin therefore, has yet to be determined.

When observed in experimental models of metastasis, the W256 cell, like many other tumor cell lines, follows a defined sequence events (Jones et al.,1971). Within minutes of being injected intravenously, individual W256 cells were observed, arrested within pulmonary capillaries. Platelets were often found in close association with these cells. By nine hours, the W256 cells crossed the endothelium and were

in contact with the underlying basement membrane. Forty-eight hours later, the W256 cells were clearly perivascular and had breached both the endothelium and the basement membrane (Jones et al.,1971).

In vitro, the W256 cell can be "activated" by either the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) (Rayner et al.,1985) or phorbol esters (Clarke and Varani, 1984). Similar to leukocytes, W256 cells respond to these ligands by chemotaxis, increased adhesiveness and cell swelling (Wass et al.,1980; Varani and Fantone, 1982; Rayner et al.,1985). In addition leukocytes respond by secreting proteolytic enzymes (Smith et al.,1980) and by generating oxygen-derived free radicals (Root et al.,1975; Briggs et al.,1975). Previous in vitro studies from our laboratory have suggested that W256 cells can also generate reactive oxygen species following their activation by the chemotactic peptide fMLP (Leroyer et al.,1987). Evidence for the generation of reactive oxygen species included: a) the inhibition of luminol-promoted chemiluminescence by the addition of catalase, superoxide dismutase, and mannitol; and b) a dose-dependent reduction of acetylated cytochrome c by fMLP-activated W256 cells. These observations serve as the basis for the current study.

1.2 Reactive oxygen species and cancer cell metastasis

1.2.1 Reactive oxygen species

A free radical is any atom or molecule that contains one or more unpaired electrons. A normal chemical bond consists of a pair of electrons, opposite in spin, which share a single molecular orbital. Thus, free radicals are highly reactive since their unpaired electron is stabilized when a new bond with another atom is formed. If two radicals react, both radicals are eliminated. If a radical reacts with a non-radical, another free radical is produced. These characteristics enable free radicals to participate in chain reactions, which are terminated only if one free radical reacts with another.

The superoxide anion is an oxygen molecule containing one extra unpaired electron, and, is therefore, a free radical species. Superoxide anion can be protonated to form highly reactive perhydroxyl radicals at acid pH (pKa = 4.8) (Badwey and Karnovsky, 1980; Weiss and LoBuglio, 1982). The reactivity of superoxide anion is dictated, in part, by its rapid dismutation (see Figure 2). In aqueous solutions at neutral pH, superoxide anion either spontaneously dismutates, forming hydrogen peroxide or functions primarily as a reductant (Koppenol, 1976; Badwey and Karnovsky, 1980;

FIGURE 2. Proposed mechanisms of reactive oxygen species formation (from Fantone and Ward, 1985).

Spontaneous dismutation of O_2-

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Haber-Weiss reaction

$$O_2^- + H_2O_2 \longrightarrow O_2 + HO^- + HO\cdot$$

Modified Haber-Weiss reaction (Fenton reaction)

$$O_2^- + M^{n+1} \longrightarrow M^n + O_2$$

$$M^n + H_2O_2 \longrightarrow M^{n+1} + HO^- + HO\cdot$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + HO^- + HO\cdot$$

Myeloperoxidase-hydrogen peroxide-chloride system

$$H_2O_2 + Cl^- \longrightarrow OCl^- + H_2O$$

Weiss and LoBuglio, 1982). Superoxide anion can donate electrons to a variety of compounds including cytochrome c and nitroblue tetrazolium. Both have been used to quantitate superoxide anion production by neutrophils (Lichtenstein, 1987). Superoxide anion can also directly reduce hydrogen peroxide to form the potent hydroxyl-radical (OH·) (Koppenol, 1976; Badwey and Karnovsky, 1980; Weiss and LoBuglio, 1982; Fantone and Ward, 1985). This reaction, the Haber-Weiss reaction, also generates HO- and molecular oxygen (see Figure 2). In a modified version of this reaction O_2- first reduces a metal ion such as Fe^{3+}, which then reacts with hydrogen peroxide to form OH- and OH· (Figure 2). This reaction, also called the Fenton reaction, dominates over the classical Haber-Weiss reaction under physiological conditions (Koppenol, 1976; Badwey and Karnovsky, 1980; Weiss and LoBuglio, 1982).

1.2.2 Endothelial damage by reactive oxygen species

Vascular injury due to the production of reactive oxygen species, has been implicated in the pathology of a number of diverse processes including acute inflammation, ischemia-reperfusion injury, oxygen and bleomycin toxicity. The source of reactive oxygen species generation in many of

these processes may be the leukocyte. Phagocytes (neutrophils, monocytes, macrophages and eosinophils) generate reactive oxygen species following their activation by various stimuli such as bacteria, opsonized zymosan, immune complexes, chemotactic factors such as C5a or bacterial - derived formylated oligopeptides (eg. fMLP) and phorbol myristate acetate (PMA). Following exposure to such agents, neutrophils utilize NADPH and molecular oxygen to produce superoxide anions ($O_2^{\cdot -}$) (Cross et al.,1985). This reaction is catalysed by a "NADPH oxidase", composed of cytochrome b-245 and a flavoprotein dehydrogenase (Segal et al.,1981; Gabig and Lefker,1984). Purified cytochrome b-245 is a heterodimer composed of a 76-92kD β and a 23kD α subunit (Parkos et al.,1987). Its midpoint potential at -245 mV is the lowest of any mammalian cytochrome b which accounts for its ability to reduce directly oxygen to superoxide anion (Gabig and Lefker, 1984).

Neutrophils and monocytes from a variety of species also contain myeloperoxidase, a heme-enzyme in their lysosomal granules (Dahlgren and Stendahl, 1983). Phagocytes release this enzyme into their phagocytic vacuoles or into the external milieu upon activation. Myeloperoxidase catalyses the formation of hypohalous acids in the presence of hydrogen peroxide and halides. The iodide derivatives are the most reactive of the hypohalous acids on a molar basis, however, in most biological systems, hypochlorous acid is

the predominant species formed (Winterbcurn et al.,1985).

Hydrogen peroxide is formed by phagocytes following the spontaneous dismutation of superoxide anion. Studies have shown that hydrogen peroxide is an effective oxidant, capable of injuring endothelial cells. Neutrophils can damage endothelial cell monolayers by generating hydrogen peroxide (Sacks et al.,1978; Weiss et al.,1981). In vitro endothelial cell injury can be quantitated by monitoring isotope release from cultures of ^{51}Chromate or ^{3}H-2-deoxyglucose - labeled human umbilical vein-endothelial cells (Sacks et al.,1978; Andreoli et al., 1985). In such experiments, damage was found to be dependent upon stimulation of the neutrophils with PMA or C5a and could be inhibited by the addition of catalase (Sacks et al.,1978; Weiss et al.,1981; Andreoli et al.,1985). Hydrogen peroxide, and not superoxide anion or HOCl, (generated from hydrogen peroxide and Cl_2 by myleoperoxidase) was the damaging agent since superoxide dismutase, nonspecific inhibitors of myleoperoxidase (azide or cyanide), and scavengers of HOCl (isoleucine, valine and serine) all failed to inhibit neutrophil-mediated injury (Sacks et al.,1978; Weiss et al.,1981). Further, myeloperoxidase-deficient neutrophils were still able to promote the release of isotope (Weiss et al.,1981). Similarily, the OH· scavengers, mannitol and ethanol, also failed to inhibit neutrophil-mediated injury (Weiss et al.,1981). Together, these results suggest that

neutrophil-dependent endothelial cell injury is mediated by the generation of hydrogen peroxide. Hydrogen peroxide can also damage endothelial monolayers in cell-free model systems. The addition of glucose-glucose oxidase or xanthine-xanthine oxidase hydrogen peroxide-generating systems to prelabelled endothelial cell monolayers promotes the release of ^{51}chromate (Sacks et al.,1978; Weiss et al.,1981). Damage, in this case, was found to be inhibited by the addition of catalase but not by superoxide dismutase (Sacks et al.,1978; Weiss et al.,1981).

In direct contrast to the above studies, others have been unable to demonstrate that activated neutrophils can damage endothelial cell monolayers by the generation of reactive oxygen species (Harlan et al.,1981; Harlan et al.,1985). Differences in the levels of endogenous antioxidant enzymes, from one endothelial cell strain to the next, has been postulated to account for this discrepancy. Harlan et al. (1984), examined the role of the glutathione redox cycle as an antioxidant defense mechanism in cultured bovine and human endothelial cells. They demonstrated that some strains of endothelial cells were resistant to lysis by PMA-activated neutrophils or glucose-glucose oxidase generated-hydrogen peroxide (Harlan et al.,1984). However, when these endothelial cells were treated with drugs which lower the levels of reduced glutathione before being exposed to hydrogen peroxide - generating systems, chromate release

was observed (Harlan et al.,1984; Suttorp et al.,1985). The inhibitors of glutathione synthesis used in these studies included 1,3-bis(chloroethyl)-1- nitrosourea (BCNU), which inhibits regeneration of reduced glutathione, buthionine sulfoximine (BSO), an inhibitor of reduced glutathione synthesis and diethylmaleate (DEM) or 1-chloro-2,4 dinitrobenzyne (CDNB) which catalyse the breakdown of reduced glutathione (Figure 3),(Harlan et al.,1984; Suttorp et al.,1985). Treatment of endothelial monolayers with BSO (1 mM) increased the specific release of ^{51}Chromate from undetectable levels to 80% and 15% in the presence of glucose oxidase and neutrophils respectively (Harlan et al.,1984; Suttorp et al.,1985). Endogenous catalase does not appear to play a role in protecting endothelial cells from oxidant injury. Catalase catalyses the reaction:

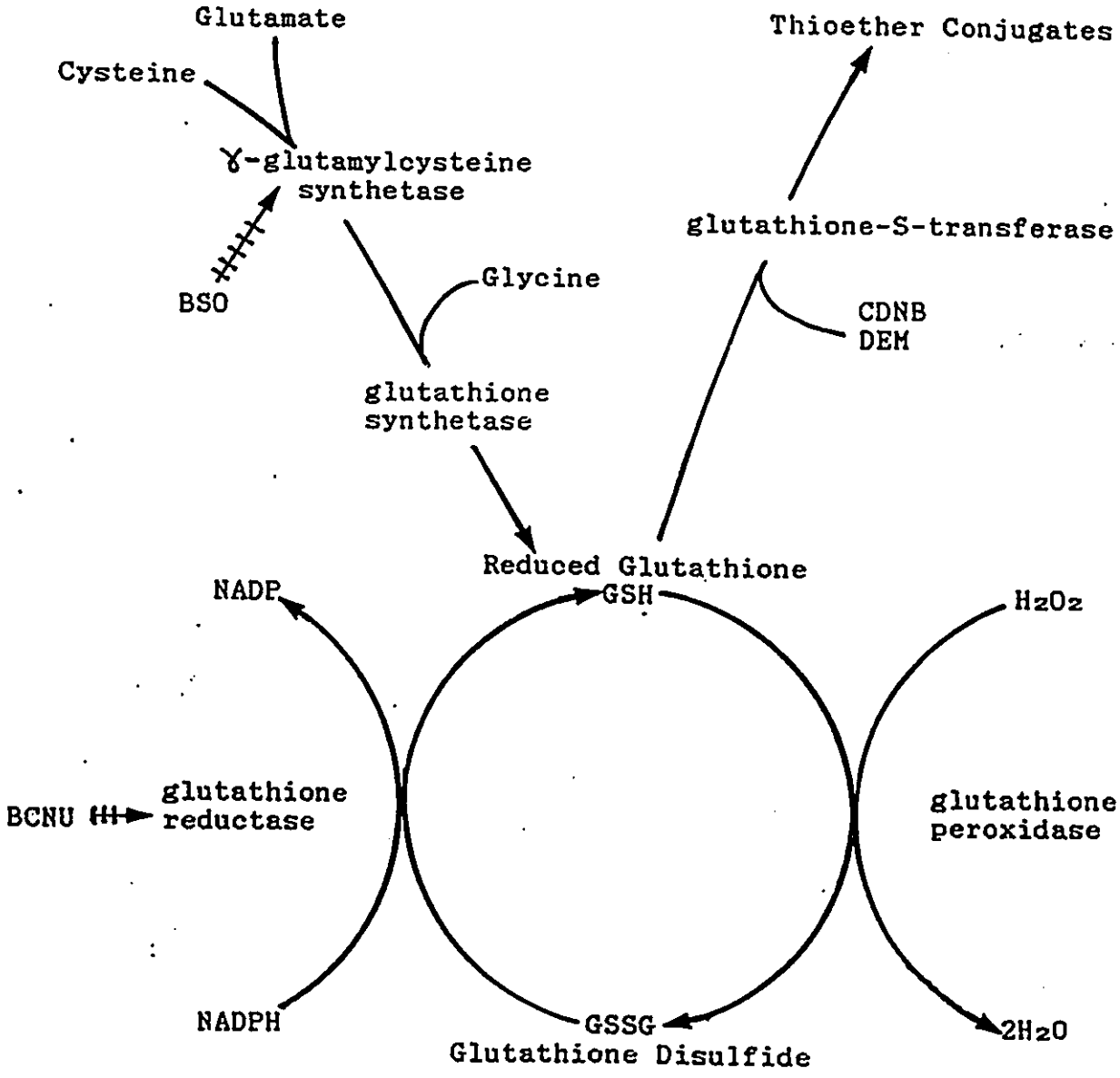
$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

and is inhibited by 3-amino-1,2,4-triazole (Suttorp et al.,1985). Suttorp et al. (1985) inhibited endogenous catalase in human umbilical cord endothelial cells by 96% with 50 mM 3-amino-1,2,4-triazole and found that neither glucose oxidase, nor neutrophil-mediated endothelial cell injury, was increased.

1.2.3 Reactive oxygen species and the extracellular matrix:

There is little evidence that free radicals can

Figure 3. The glutathione redox cycle (from Suttrop et al.,1986)


Glutamate
Cysteine
γ-glutamylcysteine synthetase
BSO
Glycine
glutathione synthetase
Thioether Conjugates
glutathione-S-transferase
CDNB
DEM
Reduced Glutathione
GSH
NADP
NADPH
BCNU
glutathione reductase
H2O2
glutathione peroxidase
2H2O
GSSG
Glutathione Disulfide

degrade basement membranes directly. However, it has been suggested that free radicals may enhance proteolytic damage to basement membranes by activating latent proteolytic enzymes (Weiss et al.,1985; Peppin and Weiss, 1986 and Shah et al.,1987) or inactivating proteinase inhibitors (Weiss and Regiani, 1984; Weiss et al.,1986; Strandberg et al.,1991).

1.2.3.1 Oxidative autoactivation of neutrophil metalloproteinases

Neutrophils activated with phorbol myristic acetate or serum-opsonized zymosan particles, secrete an interstitial collagenase capable of digesting type I collagen fibrils. This enzyme is distinct from tumour cell or fibroblast-derived interstitial collagenases (Weiss et al.,1985). Activated neutrophils also secrete a 92 kD type IV collagenase capable of degrading denatured collagens, human glomerular basement membrane, and purified type IV and V collagen (Hibbs etal.,1985; Peppin and Weiss, 1986; Shah et al.,1987). These enzymes were shown to be secreted in a latent form which was then postulated to be activated by the simultaneous generation of oxygen radicals (Weiss et al.,1985; Peppin and Weiss, 1986 and Shah et al.,1987). In the presence of catalase, activated neutrophils secrete, but do not activate these metalloproteinases (Weiss et al.,1985;

Peppin and Weiss, 1986). Under these conditions, full activity is restored by the addition of APMA to the cell free supernatants of activated neutrophils (Weiss et al.,1985; Peppin and Weiss, 1986). In the absence of catalase, approximately 50% of the secreted interstitial collagenase and 15% of the secreted 92 kD gelatinase are activated by reactive oxygen species (Weiss et al.,1985; Peppin and Weiss, 1986). Neutrophils, stimulated in the presence of the myeloperoxidase inhibitor, azide, or the HOCl scavenger, methionine, also secrete inactive enzymes (Weiss et al.,1985; Peppin and Weiss, 1986 and Shah et al.,1987). Enzyme activity is restored by the addition of HOCl directly to the cell-free supernatants of neutrophils activated in the presence of azide (Peppin and Weiss, 1986).

1.2.3.2 Inactivation of proteinase inhibitors

Both the proteinase inhibitors, human α-1-proteinase and plasminogen activator inhibitor type 1, can be oxidatively inactivated by reactive oxygen species. Alpha-1-proteinase inhibitor is found in plasma at high concentrations and can inactivate the serine proteinase, elastase, by forming 1:1 molar complexes with it (Johnson and Travis, 1979). Plasminogen activator type-1, similarily inhibits urokinase-type or tissue-type plasminogen activator activity. While tissue-type plasminogen activators are

considered to be of importance in fibrinolysis, the urokinase-type plasminogen activators are considered important in local tumour growth, invasion, and metastasis (Mignatti et al.,1986; Reich et al.,1988). Elevated elastase activity has been implicated in both inflammation and lung emphesema.

Oxygen radicals, produced by activated human polymorphonuclear leukocytes or human alveolar macrophages, can partially suppress the elastase-inhibiting capacity of whole human serum and inactivate purified human α1-proteinase inhibitor (Carp and Janoff, 1979; Carp and Janoff, 1980; Matheson et al.,1981; Weiss and Regiani, 1984; Weiss et al.,1986). While addition of hydrogen peroxide alone does not inhibit α1-proteinase inhibitor, its activity is diminished in cell free systems consisting of purified myeloperoxidase, a glucose oxidase-hydrogen peroxide generating system and Cl-, indicating that hypochlorous acid is the agent responsible for inactivating α1-proteinase inhibitor (Carp and Janoff, 1979; Carp and Janoff, 1980; Weiss and Regiani, 1984; Weiss et al.,1986). Oxidatively inactivated α1-proteinase inhibitor is unable to bind free elastase (Johnson and Travis, 1979; Clark et al.,1981; Zaslow et al.,1985; Banda et al.,1987). The loss of elastase binding activity, in the presence of the myeloperoxidase system is very rapid, being reduced more than 50% within 10 seconds (Clark et al.,1981). Similarily, plasminogen

activator type-1 activity is inhibited following oxidation with either hydrogen peroxide, N-chlorosuccinimide or chloramine T (Strandberg et al.,1991).

The oxidative inactivation of α1-proteinase inhibitor is mediated by the specific oxidation of a methionine residue located at the P1 position of the reactive centre (Johnson and Travis, 1979). Amino acid analysis of oxidized α1-proteinase inhibitor has shown that 2 of the 8 methionyl residues are oxidized to their sulfoxide derivatives by myeloperoxidase or N-chlorosuccinimide (Johnson and Travis, 1979). The 2 methionines are 8 residues apart and one of these (Methionine-358) is located in the reactive site (Johnson and Travis, 1979; Matheson et al.,1981; Banda et al.,1987). The oxidation of these 2 methionines, caused by treatment with increasing concentrations of N-chlorosuccinimide, correlates with a loss of α1-proteinase-inhibitory activity (Johnson and Travis, 1979). Methionine, at a concentration of 0.1 mM completely inhibits, by direct competition, the inactivation of α1-Proteinase inhibitor by both the purified myeloperoxidase system and activated neutrophils (Weiss and Regiani, 1984; Clark et al.,1981). In contrast, addition of oxidized methionine has no effect (Clark et al.,1981).

Plasminogen activator inhibitor also contains a methionine residue within its reactive centre at the P_1 position (Strandberg et al.,1991). Both α-1-proteinase

inhibitor and plasminogen activator inhibitor type 1 can be genetically engineered to replace the P_1 methionine with a nonoxidizable valine residue and still remain active. However, while genetically engineered α-1-proteinase, with valine in its active site, is less susceptable to oxidative inactivation by chlorosuccinimide, plasminogen activator inhibitor is not (Strandberg et al.,1991). Oxidation of PAI-1 appears to induce a conformational change in plasminogen activator inhibitor type 1, independent of the reactive centre P1 methionine residue, which results in a loss of activity (Strandberg et al.,1991).

1.2.4 Reactive oxygen species and the inhibition of metastasis

In vivo experiments have demonstrated that less than 1% of tumour cells survive in the circulation to proliferate into secondary lesions. A number of factors contribute to the death of cancer cells. Circulating tumour cells experience mechanical trauma often leading to cell death as they pass through the microvasculature (Weiss et al.,1990). In addition, cancer cells interact with host cells, such as neutrophils, which are capable of causing cancer cell death by the generation of free radicals. Pretreatment of mice with superoxide dismutase substantially increased the retention of intravenously-injected radiolabelled Lewis lung

carcinoma cells in the lung, liver, kidney and spleen of these mice (Glaves 1986). There was also an increase in the number of pulmonary nodules developing in the lungs of superoxide dismutase-treated mice (Glaves 1986).

Hydrogen peroxide generated by PMA-activated macrophages or neutrophils can also cause the lysis of tumour cells (Nathan et al.,1979ab). Co-incubation of ^{51}chromium-prelabelled tumour cells with activated neutrophils, resulted in complete release of isotope within 4.5 hours. Tumour cell lysis was completely blocked by the addition of catalase. While the release of isotope was not affected in the presence of superoxide dismutase, release was inhibited by the addition of ferricytochrome c (Nathan et al.,1979ab). These results suggest that hydrogen peroxide arising from the spontaneous dismutation of superoxide anion, and not superoxide anion itself, is the major oxygen metabolite responsible for tumour cell lysis. In addition to catalase, the myeloperoxidase inhibitors, azide and cyanide, also inhibit neutrophil-mediated lysis of tumour cells (Clark and Klebanoff, 1979; Clark and Szot, 1981). Further, neutrophils, isolated from a patient with a hereditary deficiency in myeloperoxidase, demonstrated a marked reduction in their cytotoxicity towards tumour cells (Clark and Szot, 1981). Cytotoxicity was restored upon the addition of purified myeloperoxidase. Tumour cell lysis was dependent on the presence of either chloride, iodide, or bromide as

well as hydrogen peroxide suggesting the actual damaging agent is a hypohalous acid (Clark and Szot, 1981). Both macrophages and endothelial cells can cause tumour cell lysis by the generation of the reactive oxygen species, nitric oxide (Hibbs et al.,1988; Stuehr and Nathan, 1989; Li et al.,1991). Nitric oxide is also an endothelial-derived relaxation factor for smooth muscle cells and an inhibitor of platelet aggregation (Palmer et al.,1987; Ignarro et al.,1987; Ignarro, 1989).

Tumour cells can protect themselves against lysis by hydrogen peroxide by the glutathione (GSH) redox cycle (Figure 3). The enzyme glutathione peroxidase catalysis the reaction:

Reduced Glutathione + H_2O_2 ⟶ Glutathine Disulphide + $2H_2O$.

Tumour cells with depleted glutathione levels are more sensitive to hydrogen peroxide generated by glucose oxidase or PMA-activated phagocytes (Nathan et al.,1980; Arrick et al.,1982; O'Donnell-Tormey et al.,1985). Depletion of intracellular glutathione can be accomplished by: inhibiting glutathione reductase with 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU); inhibiting γ-glutamyl cysteine synthetase with buthionine sulfoximine (Figure 3); or, reducing glutathione peroxidase levels by passaging the

tumour cells in selenium-deficient mice. All of these treatments enhance tumour sensitivity to hydrogen peroxide generating systems (Nathan et al.,1980; Arrick et al.,1982; O'Donnell-Tormey et al.,1985). In contrast, inhibition of endogenous catalase with aminotriazole has no effect on the sensitivity of tumour cells to peroxide-mediated lysis (Nathan et al.,1980).

1.2.5 Reactive oxygen species and the promotion of metastasis by vessel wall injury

The endothelium acts as a barrier between circulating tumour cells and the extravascular tissue. Endothelial cell injury, postulated to be a result of free radical generation by environmental factors or host cells, can promote the metastasis of intravascular tumour cells (Adamson et al.,1986; Orr et al.,1986; Adamson et al.,1987; Orr and Warner, 1987; Orr and Warner, 1990). These data, described below, and the possibility that activated W256 cells produce reactive oxygen species suggest the hypothesis that reactive oxygen species released from tumour cells might induce vessel wall injury, thus promoting their own metastasis. This is the working hypothesis of this thesis.

In vivo, neutrophils activated by the intravenous injection of cobra venom factor (CoF), can promote pulmonary endothelial cell injury, as determined by the increased

vasopermeability and morphology (Orr and Warner, 1987; Orr and Warner, 1990). In both rats (Orr and Warner, 1987) and mice (Orr and Warner, 1990) CoF injection leads to complement activation and neutropenia with sequestration of neutrophils in the lung. When ^{125}IUdr-labeled cancer cells were injected intravenously during periods of maximal endothelial cell injury, there was a 3-fold increase in retention of these cells in the lung 24 hours later (Orr and Warner, 1987; Orr and Warner, 1990). After 14 days, there was a 3 to 20 fold increase in the number of metastatic tumours in CoF-treated animals. Support for the involvement of reactive oxygen species included evidence that, in both rats and mice, tumour cell localization was reduced 70-80% by pretreatment of the animals with catalase or anti-neutrophil antiserum (Orr and Warner, 1987).

In mice, endothelial injury induced by bleomycin (120 mg/kg) or by exposure to 90% oxygen for 2-4 days, also significantly increased the metastasis of circulating cancer cells (Adamson et al.,1986; Orr et al.,1986; Adamson et al.,1987). Bleomycin is a glycopeptide-derived antibiotic used as a chemotherapeutic agent in many clinical treatment protocols. The drug binds to DNA as an iron-bleomycin complex and can react with molecular oxygen to produce reactive oxygen species, including superoxide anion and hydroxyl radical (Pepin and Langer, 1985). Exposure to high concentrations of oxygen is associated with the

sequestration of neutrophils in the lungs of exposed animals during periods of maximum injury. When mice are given a single intravenous injection of bleomycin (120 mg/kg) or exposed to an atmosphere of 90% oxygen for a period of 2-4 days, increased vascular permeability is easily detectable (Adamson et al.,1986; Orr et al.,1986; Adamson et al.,1987). When radiolabeled cancer cells were injected into mice during this period, a significant increase in tumour cell retention at 24 hrs can be observed. After 14 days, the number of metastatic tumours in bleomycin-treated mice or mice exposed to 90% oxygen were greater than in untreated controls (Adamson et al.,1986; Orr et al.,1986; Adamson et al.,1987).

1.3 Tumour cell adhesion to endothelium

1.3.1 Identification of the adhesive moieties expressed by endothelial cells

Previous studies attempted to determine if endothelial cells express specific surface proteins which can bind tumour cells. Detergent solubilized endothelial cell-derived plasma membrane fractions were electrophoresed on polyacrylamide gels, transfered to nitrocellulose and the ability of radiolabeled or biotinylated tumour cells to bind to specific components determined. RAW117-H10 tumour cells, which colonize liver, were shown to bind specifically to components of murine hepatic sinusoidal endothelial cells immobilized on nitrocellulose at Mw 48 kD, 32 kD, 30 kD, 25 kD, and 20 kD (Nicolson 1988). The binding of this tumour cell to extracts obtained from different endothelial cell sources was found to correlate with its organ preference *in vivo*. RAW117-H10 cells were shown to bind to a lesser extent to the same components obtained from the electrophoresed extracts of lung endothelial cells and not to bind to extracts of bovine aortic endothelial cells (Nicolson 1988). While such studies have been instrumental in demonstrating

that endothelial cell-derived adhesive moieties exist for tumour cells, they have met with limited success in further characterizing or identifying such molecules.

Recent studies have concentrated on identifying and characterizing those adhesive moieties whose expression is inducible by cytokine treatment of the endothelium (Dejana et al.,1988; Rice et al.,1988; Lauri et al.,1989 and Lauri et al.,1990). Treatment of endothelial cell monolayers with cytokines increases the adhesion not only of cancer cells (ie malignant melanomas and carcinoma cell lines) but also leukocytes and lymphocytes. This response is mediated by the induced expression of a defined set of adhesive moieties on the endothelium. Induction is time-dependent and requires protein synthesis (Dejana et al.,1988; Rice et al.,1988; Lauri et al.,1989). Moieties involved in tumour cell/endothelial cell adhesion include INCAM-110 (or VCAM-1), ICAM-1, and the vitronectin receptor, $\alpha V\beta 3$, as well as ELAM-1 (see Table 2).

The metastatic potential of tumour cells can be increased by the pretreatment of animals with cytokines. The retention of tumour cells within the lung, measured 24 hrs after intravenous injection is a measure of tumour cell adhesion/entrapment within lung capillaries. When nude mice were pretreated for 4 hr with rIL-1α, there was a 5 to 6-fold increase in tumour cell retention when compared to

Table 2. Tumour cell - endothelial cell adhesion molecules

Receptor	Distribution	Ligand	Function	References
ELAM-1	IL-1,LPS,lymphotoxin or TNF-activated endothelium	?	Colon carcinoma cell-EC adhesion HL60-EC adhesion PMN-EC adhesion	Bevilacqua et al.,1985 Bevilacqua et al.,1987 Bevilacqua et al.,1989
INCAM-110	IL-1 or TNFα-activated endothelium Lymphoid dendritic cells Tissue macrophages Reactive mesothelial cells	$\alpha_4\beta_1$	Melanoma-EC adhesion Lymphocyte-EC adhesion Monocyte-EC adhesion	Rice and Bevilacqua, 1989 Rice et al.,1991
Vitronectin Receptor $\alpha_v\beta_3$	IL-1 or LPS-activated endothelium	?	A549 carcinoma and W256 carcino sarcoma cell-EC adhesion	Shaughnessy et al.,1991 Lafrenie et al., In Press
LFA-1	C5a,fMLP -activated Leukocytes Monocytes Lymphoma cells PHA-activated Lymphocytes	ICAM-1	Lymphoma/ Leukocyte/ Lymphocyte-EC adhesion	Pohlman et al.,1986 Marlin and Springer, 1987 Dustin and Springer,1988 Oppenheimer-Marks et al.,199C
ICAM-1	IL-1,TNF,rIFNr or LPS-activated endothelium Thymic epithelial cells Fibroblasts Tissue macrophages Germinal centre dendritic cells in tonsils, lymph nodes and Peyers patches	LFA-1	Lymphoma/ Leukocyte-EC adhesion	Dustin et al.,1986 Dustin and Springer, 1988

untreated controls (Lauri et al.,1990). Intravenous administration of human recombinant IL-1β, 1 hr before tail vein injection of melanoma or carcinoma cells increased lung colony formation (experimental metastasis) in nude mice and syngeneic recipients (Bani et al.,1991). IL-1β treatment did not induce lung colony formation by cell lines which are non-metastatic to lung such as a human rectal carcinoma or a murinereticulum sarcoma (Bani et al.,1991). Spontaneous metastasis was studied in C57BL/6 mice bearing a solid B16-BL6 melanoma tumour (metastatic to lung) in their footpad or a subcutaneous M5076 reticulum cell sarcoma (metastatic to liver) (Bani et al.,1991). Daily intraperitoneal injections with IL-1β increased metastasis of both tumours to their respective organs without increasing the rate of growth of the primary tumour (Bani et al.,1991).

1.3.1.1 Endothelial Leukocyte Adhesion Molecule 1 (ELAM-1)

ELAM-1 has been implicated in the binding of both carcinoma cells and neutrophils to IL-1-, TNFβ- and bacterial lipopolysaccaride (LPS)-pretreated endothelium. The observed increases in tumour cell or neutrophil adhesion were dependent on the duration of endothelial cell pretreatment (peaks at 4-6 hrs) and could be blocked by protein synthesis inhibitors (Bevilacqua et al.,1985;

Pohlman et al.,1986; Rice and Bevilacqua, 1989). Monoclonal antibodies to ELAM-1 (H4/18,H18/7) have been reported to block the adhesion of human neutrophils (>50%), HL-60 cells (>60%) and the human colon carcinoma cell line HT-29 to cytokine-stimulated endothelium (Bevilacqua et al.,1987; Rice and Bevilacqua, 1989). The adhesion of the human colon carcinoma cell line HT-29 was not blocked by addition of monoclonal antibodies recognizing INCAM-110 (Rice and Bevilacqua, 1989). INCAM-110 is described below. The ELAM-1 monoclonal antibodies, H4/18 and H18/7, immunoprecipitate a polypeptide (major species, Mw 115 kD; minor species, Mw 97 kD, reduced) (Bevilacqua et al.,1987). Sequence analysis of ELAM-1 shows it is a member of the vascular selectins, a family of proteins which includes the lymphocyte homing receptors, MEL-14 and granule membrane protein-140 (GMP-140). The primary sequence of ELAM-1 demonstrates an amino-terminal lectin-like domain, followed by a epidermal growth factor (EGF) domain and six tandem repetitive motifs (about 60 amino acids each), related to those found in complement regulatory proteins (Bevilacqua et al.,1989). A similar structure is also found in the MEL-14 lymphocyte cell surface homing receptor, and GMP-140 (see above).

ELAM-1 is not expressed by the vascular endothelium of normal tissues, but has been detected in venules affected with certain inflammatory conditions, including appendicitis and acute granulomatous lymphadenitis (Rice et al.,1991).

ELAM-1 is transiently expressed (peak 4 hrs, decline by 24 to 48 hrs) suggesting a role in the acute inflammatory process (Bevilacqua et al.,1987).

1.3.1.2 Vitronectin receptor (αVβ3)

The integrins are a large and diverse family of adhesion molecules, each member consisting of an α subunit noncovalently linked to a β subunit. To date 12 different α chains and 7 different β chains have been identified (Table 3, page 68). Particular integrin receptors are usually classified according to the identity of the β subunits in the dimer.

The β_3 group of integrins includes the vitronectin receptor $\alpha_V\beta_3$ and gpIIb/IIIa. Both receptors have promiscous ligand specificities recognizing at least six different molecules (see table 3). The GPIIIa cDNA sequence encodes a protein of 222 amino acids, which is identical in sequence to the β_3 subunit of the vitronectin receptor (Rosa et al., 1988; Ginsberg et al., 1987, Fitzgerald et al., 1987). The vitronectin receptor is found on very many different cell types (see Table 3). gpIIb/IIIa however is found primarily on platelets although it is present at very low levels on cultured HEL and endothelial cells (Plow 1991). An important characteristic of $\alpha_V\beta_3$ is its high affinity for RGD containing peptides (Pytela et al., 1985; Cheresh and

Harper, 1987; Cheresh 1987; Pytela et al., 1986). This integrin has been isolated from tumour cell extracts by affinity chromatography on GRGDSPK - Sepharose columns (Cheresh and Harper, 1987; Cheresh 1987).

Lauri et al. (1990) and Lafrenie et al. (1991) have reported inhibition of human A549 lung carcinoma and M6 melanoma cell-adhesion to IL-1α pretreated endothelial cell monolayers, following the addition of GRGDSP peptides. The integrin $\alpha_V\beta_3$, a vitronectin receptor (VnR), was subsequently shown to mediate the adhesion of these cells (Lafrenie et al., in press). Cell-cell adhesions mediated by ELAM-1, ICAM-1 or INCAM-110 are not affected by the GRGDS peptide (see below). Adhesion was partially inhibited in the presence of a polyclonal antiserum to the human VnR, $\alpha_V\beta_3$, or a VnR monoclonal, LM609. The latter monoclonal specifically recognizes the integrin $\alpha_V\beta_3$. Immunofluorescence flow cytometry, antibody binding and western blotting all demonstrated that, $\alpha_V\beta_3$ was expressed by the endothelium and not by the A549 carcinoma or M6 melanoma cells (Lafrenie et al., in press).

1.3.1.3 INCAM-110/VCAM

Inducible cell adhesion molecule 110 (INCAM-110), is a 110-kd glycoprotein which mediates adhesion of monocytes, lymphocytes and melanoma cells to IL-1 or tumour necrosis

factor-α - activated endothelium (Rice and Bevilacqua, 1989; Rice et al.,1991). Originally, this adhesive moiety was identified by a monoclonal antibody, E1/6, which blocked adhesion of melanoma cells to activated endothelium (Rice and Bevilacqua, 1989). Cytokine pretreatment of endothelial cell monolayers increased the surface expression of INCAM-110 by 10 to 15 fold, which was sustained for at least 48 hrs (Rice and Bevilacqua, 1989). Little or no INCAM-110 was detected on the endothelium of normal human tissues (Rice et al.,1991). However, during episodes of chronic inflammation such as sarcoidosis, elevated INCAM-110 expression was observed on post capillary venules (Rice et al.,1991). A role for endothelial INCAM-110 in the pathophysiology of both acute and chronic inflammatory reactions has been suggested (Rice et al.,1991).

Recently, the anti-INCAM-110 antibody, E1/6, was found to recognize COS cells transfected with a cDNA clone encoding a portion of another adhesive moiety, VCAM-1 (Rice et al.,1991 - unpublished). This suggests that INCAM-110 and VCAM-1 are the same molecules (Rice et al.,1991). Further, both INCAM-110 and VCAM-1 are expressed by endothelial cells 2 hrs following IL-1 or TNF-α pretreatment and are maintained for at least 72 hrs (Osborn et al.,1989). Sequence analysis shows that VCAM-1 belongs to the immunoglobulin gene superfamily and has 26% identity with ICAM-1 (see below) (Osborn et al.,1989). The counter

receptor for VCAM-1 is the integrin $\alpha_4\beta_1$ (VLA-4) (Elices et al.,1990).

1.3.1.4 Intercellular adhesion molecule-1 (ICAM-1)

Purified intercellular adhesion molecule 1 (ICAM-1), isolated from endothelial cells migrates as a single band of 90 kD on SDS-PAGE (Marlin and Springer, 1987; Dustin and Springer, 1988). ICAM-1 is a member of the immunoglobulin gene superfamily. The proposed amino acid sequence of ICAM-1 specifies an integral membrane protein with an extracellular domain of 453 residues. This domain contains five regions of significant homology with members of the immunoglobulin supergene family (Staunton et al.,1988). Unlike other integrin ligands, ICAM-1 does not contain an RGD sequence (Staunton et al.,1988). ICAM-1 is detectable on advanced human melanomas in situ but is not found on benign melanocytes or early melanomas (Johnson et al.,1989). The frequency of ICAM-1 expression on malignant melanomas in situ increased with increasing tumour thickness, an index of tumour progression (Johnson et al.,1989). An increase in the vertical thickness of these tumours also correlated to an increased risk of metastasis. The acquisition of ICAM-1 expression during tumour progression is postulated to contribute to the development of subsequent metastasis of melanoma (Johnson et al.,1989). In addition, because

increased expression of ICAM-1 in activated endothelium in vitro is more sustained than ELAM-1, it has been suggested that ICAM-1 mediates chronic inflammatory processes.

1.3.2 "Counter Receptors" in tumour cell - endothelial cell adhesion

Tumour cells also express specific surface proteins which can promote their adhesion to the endothelium. To date, two of these "counter receptors" have been identified. Both belong to the integrin family of cell surface receptors (see section 1.4.2 below). The first is $\alpha_4\beta_1$ (VLA-4), which is the counter receptor for VCAM. It is found on B and T lymphocytes, monocytes, lymphocytic leukemias but not on platelets, PMNs, or HUVECs (Wayner et al.,1989). $\alpha 4\beta 1$ may also be expressed by melanoma cells since VCAM-1 is INCAM-110. The second counter receptor is LFA-1, which associates with ICAM-1 (Marlin and Springer, 1987; Dustin and Springer, 1988). LFA-1 is expressed on monocytes, granulocytes, peripheral blood lymphocytes, and lymphoma cells (Sanchez-Madrid et al.,1983; Haskard et al.,1986; Cavender et al., 1987; Dustin and Springer, 1988; Roossien et al.,1989; Oppenheimer-Marks et al.,1990).

1.3.2.1 VLA-4; $\alpha_4\beta_1$

VLA-4 ($\alpha_4\beta_1$) is a member of the integrin family of adhesion molecules. The integrins are a large and diverse family of adhesion molecules consisting of an α subunit noncovalently linked to a β subunit (see below). Most of the integrins, particularily those possessing the β1 chain, mediate adhesion of cells to basement membrane components, such as collagen, laminin and fibronectin. In contrast, although VLA-4 ($\alpha_4\beta_1$) mediates adhesion to fibronectin, it also mediates cell-cell adhesion by binding to VCAM (Elices et al.,1990). There are distinct binding sites within the $\alpha4\beta1$ molecule for VCAM and fibronectin (Elices et al.,1990). Soluble 38 kD trypsin fragments of fibronectin block attachment of α_4-transfected K-562 cells to fibronectin but are unable to inhibit the adhesion of transfected K-562 cells to VCAM-1 transfected COS cells or TNF-α-activated endothelial cells (Elices et al.,1990). However, the adhesion of transfected K-562 to fibronectin or to VCAM-1 transfected COS cells is completely blocked by the addition of anti-VLA-4 monoclonal antibodies (Elices et al.,1990).

1.3.2.2 LFA-1

The human lymphocyte function-associated antigen-1

(LFA-1) is one of three defined β_2 integrin adhesion molecules which are involved in cell-cell adhesion rather than cell-matrix adhesion (see section 1.4.2). These include LFA-1, Mac-1 and p150,95. LFA-1 is a heterodimeric adhesion molecule consisting of α and β subunits. LFA-1, the complement receptor-associated Mac-1 (OKM1) and p150,95 all share a common β subunit (95kD) noncovalently associated with an unique α subunit (177, 165, 150 kD respectively)(Sanchez-Madrid et al.,1983; Kishimoto et al.,1987; Larson et al.,1989). LFA-1 is the counter receptor for ICAM-1. ICAM-1 supports the adhesion of both T and B lymphocytes when reconstituted into liposomes or bound to plastic (Marlin and Springer, 1987; Dustin and Springer, 1988). Only LFA-1 positive cells bind to reconstituted ICAM-1; LFA-1-deficient cells do not (Marlin and Springer, 1987; Dustin and Springer, 1988). Lymphocyte adhesion to purified ICAM-1 reconstituted into liposomes or bound to plastic can be completely blocked by the addition of monoclonal antibodies to either LFA-1 or ICAM-1 (Marlin and Springer, 1987; Dustin and Springer, 1988). Adhesion of lymphocytes to cultured endothelium, however, can only be partially inhibited by monoclonal antibodies to LFA-1 or ICAM-1.

LFA-1 expression is increased by treating neutrophils with fMLP, PMA, C5a, leukotriene B4 or TNF (Tonnesen et al.,1984; Harlan et al.,1985; Bevilacqua et al.,1987; Tonnesen et al.,1989). In adhesion assays, the

addition of fMLP, C5a, leukotriene B4, or TNF enhances neutrophil adherence to untreated cultured human microvascular cells or human umbilical vein endothelial cells (Tonnesen et al.,1984; Gamble et al.,1985; Pohlman et al.,1986; Tonnesen et al.,1989). The increase in adherence occurs rapidly (within 15 minutes) and is independent of protein synthesis (Tonnesen et al.,1984; Gamble et al.,1985; Tonnesen et al.,1989). Monoclonal antibodies to the $\beta 2$ subunit of LFA-1 (ie TS1/18), can completely inhibit C5a- or fMLP-induced increases in neutrophil adhesion (Tonnesen et al.,1989). TNF-α increases neutrophil adhesion as a result of affects on both the neutrophils and endothelial cells (Gamble et al.,1985; Pohlman et al.,1986).

The importance of LFA-1 in neutrophil function is illustrated by patients with the hereditary condition, Leukocyte Adhesion Deficiency (LAD). These patients are characterized by recurrent bacterial infections without pus formation. Neutrophils isolated from these patients do not express MAC-1, LFA-1 or p150,95 on their cell surface since they lack a functional β_2 subunit (Ruoslahti 1991). Unlike normal neutrophils, the adhesion of these neutrophils to endothelium is not increased by fMLP, PMA, calcium ionophore A23187 or C5a treatment (Harlan et al.,1985; Tonneson et al.,1989). Similarily, compared to normal neutrophils, neutrophils isolated from LAD patients are markedly reduced in their ability to adhere to LPS, IL-1 and TNF-pretreated

endothelial cells (5-11% neutrophils adhered verses 43%-54%) (Pohlman et al.,1986). The ability of neutrophils isolated from these patients, to migrate across endothelial cell monolayers in response to fMLP is also reduced. Treatment of normal neutrophils with an LFA-1 monoclonal antibody inhibits their ability to migrate across endothelial cell monolayers in response to fMLP and can also inhibit neutrophil adhesion to LPS, IL-1, or TNFα-pretreated endothelial cells by 60-70% (Harlan et al.,1985, Pohlman et al.,1986).

Mutant lymphoma cell lines which show impaired synthesis of either α or β precursor of LFA-1 have a reduced capacity to invade cultures of hepatocytes or fibroblast monolayers *in vitro* and also demonstrate greatly reduced metastatic capacity *in vivo* compared to the LFA positive parent (Roossien et al.,1989).

1.4 Tumour cell adhesion to sub-endothelial matrices

Tumour cells adhere rapidly to subendothelial extracellular matrices (Kramer et al., 1980). Extracellular matrices are a complex mixture of laminin, collagen type IV, heparin sulfate, enactin, nidogen and accumulated serum proteins such as fibronectin, vitronectin and thrombospondin. Tumour cells express a number of adhesion molecules for most of these components. Many of these adhesive moieties, for example those which mediate adhesion to type IV collagen, laminin, fibronectin, vitronectin and thrombospondin, belong to the integrin family of adhesion molecules. A non-integrin adhesion molecule which mediates tumour cell attachment to laminin has also been identified.

1.4.1 Non-integrin laminin receptors

The ability of tumour cells to bind laminin correlates with their metastatic potential (Malinoff et al., 1984). Metastatic potential is increased when poorly metastatic cell lines are preincubated with laminin prior to intravenous injection or is decreased when highly metastatic tumour cells are preincubated with anti-laminin antisera

(Malinoff et al., 1984; Terranova et al., 1982). When bound to its receptor(s), laminin can mediate cell binding to type IV collagen and can therefore increase the metastatic potential of tumour cells in vivo (Terranova et al., 1982, Terranova et al., 1983). A receptor which binds laminin has been isolated from a variety of tumour cells, including murine melanoma cells, murine fibrosarcoma cells and human breast carcinoma cells (Malinoff and Wicha, 1983; Rao et al., 1983; Terranova et al., 1983). This receptor is also expressed by macrophages and endothelial cells and has been demonstrated in fixed sections of adult human tissue (Hand et al., 1985). The isolated receptor has a molecular weight of between 67 and 69 kD and binds to laminin with high affinity (k_d = 2 nM) (Malinoff and Wicha, 1983; Rao et al., 1983).

1.4.2 Integrin expression by tumour cells

Integrin expression by tumour cells can mediate their adhesion to extracellular matrix. The $\beta 1$ group of integrins includes receptors for type IV collagen ($\alpha_1\beta_1$,$\alpha_2\beta_1$), laminin ($\alpha_6\beta_1$) and fibronectin ($\alpha_4\beta_1$,$\alpha_5\beta_1$) (Pytela et al., 1985; Pytela et al., 1987; Ramos et al., 1990; Kramer and Marks, 1989). This group of integrins is also known as the VLA's (very late antigens) since they are expressed by T cells as a "late" event in T-cell activation.

Table 3. Components of extracellular matrices and their receptors

Receptor Subunits	Distribution	Molecular Weight kD	Ligands	References
α5β1	MG-63 osteosarcoma cells	140/140	FN	(Pytela et al.,1985; Pytela et al.,1987)
α4β1	Lymphocytes, monocytes lymphocytic leukemias		FN	(Wayner et al.,1989)
αVβn	Neuroblastoma cells	135/115	FN, type I COL	(Dedhar and Gray, 1990)
αIIbβ3	platelets, HEL cells		VN,FB,VWF FN	(Haverstick et al.,1985; Pytela et al.,1986; Poncz et al.,1987)
αVβ3	MG-63 osteosarcoma cells, melanoma cells endothelial cells, Platelets	130/105	VN,FB,VWF	(Pytela et al.,1985; Cheresh and Harper, 1987; Cheresh and Spiro, 1987; Cheresh, 1987; Lam et al., 1989)
αVβ1	Embryonal kidney cells	150/120	VN	(Bodary and McLean, 1990)
αVβ5(βX)	Carcinomas,placenta		VN	(Smith et al.,1990)
αVβS	MG-63 cells, fibroblasts endothelial cell		VN	(Freed et al.,1989)
α1β1	Melanoma cells	200/110	type I and IV COL	(Kramer and Marks, 1989)
α2β1	Melanoma cells platelets	150/110	type I, II, III and IV COL	(Kramer and Marks,1989; Staatz et al.,1989)
α6β1	Melanoma cells macrophages	140/120	LM	(Ramos et al.,1990; Shaw et al.,1990)

Abbreviations used FN, fibronectin; COL, collagen; VN, vitronectin; FB, fibrinogen; VWF, von Willebrand factor; TSP, thrombospondin; LM, Laminin

These receptors all share an identical $\beta 1$ subunit (110 kD), which is noncovalently linked to one of seven unique α chains (140 - 200 kD) (Takada et al., 1987). Many integrins belonging to this group, for example $\alpha_5\beta_1$, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, were first isolated from tumour cells by affinity chromatography of cell extracts on Sepharose covalently bound to the extracellular matrix component of interest (Pytela et al., 1985; Pytela et al., 1987; Ramos et al., 1990; Kramer and Marks, 1989). Macrophages also express $\alpha 6\beta 1$, however their adhesion to laminin requires that the macrophage be treated with either PMA or interferon gamma (Shaw et al., 1990). Such treatment results in the phosphorylation of the α_6 subunit (but not the β_1 subunit) and promotes the association of $\alpha_6\beta_1$ with the cytoskeleton (Shaw et al., 1990). Adhesion via all of the VLAs requires calcium but only adhesion by the fibronectin receptor ($\alpha_5\beta_1$) is also inhibited by Arg-Gly-Asp peptides (Kramer and Marks, 1989). Antibodies to the integrin β_1 subunit are able to inhibit melanoma cell adhesion to purified collagen type 1 or collagen type IV while peptides containing the RGD sequence do not (Kramer and Marks, 1989). VLA-4 ($\alpha_4\beta_1$) mediates not only cell adhesion to fibronectin but is also involved in cell-cell adhesion by binding to V-CAM (see above). VLA-4 mediates adhesion to fibronectin and to V-CAM through two distinct sites (Wayner et al.,1989).

Specific components of extracellular matrix are recognized by more than one receptor. For example, five different receptors for vitronectin, in addition to $\alpha_V\beta_3$ have been described. They are $\alpha_v\beta_s$ and $\alpha_v\beta_3$ expressed by MG63 cells, platelet glycoprotein GPIIb-IIIa, $\alpha_V\beta_5$ isolated from human placenta and the $\alpha_v\beta_1$ which is expressed by human embryonal kidney cell lines (Bodery and McLean, 1990). The $\alpha_v\beta_5$ VnR binds both vitronectin and fibronectin while the $\alpha_v\beta_s$ VnR binds vitronectin as well as additional ligands (Freed et al., 1989). The novel integrins $\alpha_V\beta_s$, $\alpha_V\beta_5$, $\alpha_V\beta_1$ were isolated by monoclonal antibodies (ie LM142) or polyclonal antibodies which recognize the α_V subunit of the classical vitronectin receptor $\alpha_V\beta_3$ (Freed et al., 1989; Smith et al., 1990; Bodary and McLean, 1990). Other integrins have been isolated by immunoprecipitation with a monoclonal antibody which recognizes one of the known β subunits. The integrins, $\alpha_v\beta_n$ and $\alpha_V\beta_s$, have been isolated on GRGDSPK-Sepharose (Freed et al., 1989). Similar to the β_1 subunit of $\alpha_6\beta_1$, the βs subunit of $\alpha_V\beta$s is phosphorylated when MG63 cells are treated with PMA (Freed et al., 1989). When isolated integrins are reconstituted into liposomes their ligand specificity can be determined by assessing the binding of these liposomes to the various components of extracellular matrix (Dedhar and Gray, 1990, Staatz et al.,1989).

On colon carcinoma cells and other epithelial cells

the α_6 subunit is associated with a novel β subunit β_4 (Hemler et al., 1989). The function of $\alpha_6\beta_4$ is unknown (Hemler et al., 1989).

1.4.3 Structure of integrins

Most of the integrin-receptor α and β subunits share some essential structural features. They have large extracellular domains, transmembrane domains and short cytoplasmic domains (Pytela, 1988; Poncz et al., 1987; Suzuki et al., 1986; Larson et al., 1989). The β_4 subunit, however, has both a large cytoplasmic domain and a large extracellular domain.

The amino acid sequences for a number of α and β subunits have been deduced from their respective complementary DNAs (Pytela, 1988; Poncz et al., 1987; Suzuki et al., 1986; Larson et al., 1989). The deduced amino acid sequences show little sequence homology between the α and β chain families. The different α chains however, share 20-46% amino acid sequence homology (Table 4). The five VLA heterodimers were immunopurified from human placenta (VLA-1,VLA-3,VLA-5), platelets (VLA-2) and Molt-4 cells (VLA-4) using a series of monoclonal antibody-Sepharose immunoaffinity columns. N-terminal amino acid sequence for each of the five VLA α subunits showed that they share 42% homology within the first 14 amino acids (Takada et

TABLE 4. Percent homology between integrin α subunits

	α5	αL	αM	αX	αIIb	αV
α5 (FnR)	100%	28%	21%	---	27-36%	46%
αL (LFA-1)	28%	100%	36%	37%	30%	26%
αM (MAC-1)	21%	36%	100%	---	21%	21%
αX (P150/95)	---	37%	---	100%	---	---
αIIb(gpIIb/IIIa)	27-36%	30%	21%	---	100%	34-40%
αV (VnR)	46%	26%	21%	---	34-40%	100%

ref Poncz et al., 1987; Kishimoto et al., 1987; Larson et al., 1989 Pytela, 1988

al.,1987). Many of the α chains (ie α_V, α_{IIb}, α_5) are proteolytically cleaved to generate two fragments, an extracellular heavy chain and a transmembrane light chain, which remain joined by a disulfide bond (Poncz et al., 1987; Suzuki et al., 1986, Lam et al., 1989). Other α subunits (ie.α_M, α_L) however are not proteolytically processed (Pytela, 1988; Larson et al., 1989). The extracellular domain of all α subunits contains a repeated sequence of 4-7 amino acids (DXDXDGXXD) which are homologous to the calcium binding sequences of calmodulin and troponin C (Pytela, 1988; Poncz et al., 1987; Suzuki et al., 1986; Larson et al., 1989). Near the N terminus of α_L, α_X and α_M, there is an insertion of 200 amino acids not found in α_V, gpIIb or α_5 (Pytela, 1988; Larson et al., 1989). This "I" domain has been found to have 20-30% sequence identity with similar domains found in human von Willebrand factor, chicken cartilage matrix protein and complement factor B which are associated with collagen binding (Pytela, 1988; Larson et al., 1989).

The β subunits of integrins also share considerable sequence homology. The β_1 subunit (FnR) amino acid sequence is 47% and 44% homologous to the β2 (LFA-1) and β3 (GPIIb-IIIa) subunits respectively (Poncz et al., 1987; Law et al., 1987). These shared sequences contain a high concentration of cysteine (7.6%) with 24 of the 57 residues being localized within 3 repeating units each with 8 residues (Law

et al., 1987).

1.4.4 Recognition Sites:

1.4.4.1 RGD recognition sequences

Some of the integrin receptors ($\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_m\beta_2$, $\alpha_v\beta_3$, $\alpha_v\beta_5$) appear to recognize the tripeptide sequence RGD (arginine-glycine-aspartic acid) contained within their respective ligands. This sequence is found in many proteins of the extracellular matrix including fibronectin, fibrinogen, thrombospondin, laminin, vitronectin and collagen type I (Pierschbacher and Ruoslahti, 1984; Hayman et al., 1985; Santoro and Lawing, 1987). Cell binding to some of these components of extracellular matrix can be inhibited by synthetic peptides which contain for the RGD sequence and these cells can also bind immobilized RGD peptide (Pierschbacher and Ruoslahti, 1984; Hayman et al., 1985). Conservative substitutions, for example, lysine for arginine, alanine for glycine or glutamic acid for aspartic acid, in this sequence results in a loss of cell-attachment inhibitory activity (Pierschbacher and Ruoslahti, 1984).

RGD-containing peptides have been shown to inhibit both the penetration of human tumour cells through amniotic basement membrane (Gehlsen et al., 1988) as well as the formation of metastatic lesions in models of experimental

metastasis (Humphries et al., 1988; Saiki et al., 1989). Co-injection of tumour cells and GRGDS peptide inhibits pulmonary colony formation by 90% and has been shown to extend the survival time for mice injected intravenously with B16-F10 cells (8/8 vs 0/8 mice alive at 150 days) (Humphries et al., 1988). It was concluded that the GRGDS peptide inhibited metastasis by blocking tumour cell-basement membrane adhesion.

1.4.4.2 HHLGGAKQAGDV recognition sequences

Fibrinogen contains a second recognition sequence, distinct from the RGD sequence, near the carboxyl terminus of its gamma chain corresponding to residues 400-411. This site contains the sequence HHLGGAKQAGDV. Both gpIIb-IIIa and the vitronectin receptor $\alpha_V\beta_3$ recognize the RGD sequence contained within fibrinogen, but only gpIIb/IIIa is able to recognize fibrinogen via the HHLGGAKQAGDV sequence (Smith et al., 1990). Peptides containing the HHLGGAKQAGDV sequence inhibit binding via gpIIb/IIa to both fibrinogen and immobilized RGD peptides. Binding of $\alpha_V\beta_3$ to these substrates, however, is unaffected by the HHLGGAKQAGDV containing peptide (Smith et al., 1990). Conversely, monoclonal antibodies which recognizes the fibrinogen RGD sequence at residues 572-574 inhibit fibrinogen binding to

$\alpha_V\beta_3$ but not to gpIIb/IIIa (Smith et al., 1990). Further, RGD-containing peptides are 150-fold more effective at blocking fibrinogen binding to integrin $\alpha_V\beta_3$ than to gpIIb/IIIa (Smith et al., 1990).

Photoaffinity crosslinking experiments with RGD containing peptides have shown the RGD binding site involves amino acids 109-171 in gpIIIa and 61-203 in the VnR β_3 subunit (D'Souza et al., 1988; Smith and Cheresh 1988). This region is highly conserved in the β subunits of different integrins (ie 76% identity of sequence) (D'Souza et al., 1988). Photoactivatable radiolabelled derivatives of the sequences GRGDSC and HHLGGAKQAGDV were shown to compete for the same binding sites on gpIIb/IIIa since each peptide was capable of inhibiting the labelling of gpIIb/IIIa by the other suggesting that the two binding sites overlap (Santoro and Lawing, 1987).

1.5 Tumour cell degradation of sub-endothelial matrices

There is now considerable evidence implicating the matrix metalloproteinase family of degradative enzymes in the processes of invasion and metastasis (Mignatti et al.,1986; Reich et al., 1988). Specific inhibitors of metalloproteinases can inhibit the migration of tumour cells across polycarbonate filters coated with matrigel or human amnion (Mignatti et al.,1986; Reich et al.,1988). In addition, in models of experimental metastasis, the formation of metastatic lung lesions is greatly reduced when tumour cells are co-injected with specific metalloproteinase inhibitors (Reich et al.,1988). Two members of the metalloproteinase family, the 72 kD and the 92 kD type IV collagenase preferentially degrade basement membrane type IV collagen and are constitutively secreted by a wide variety of tumour cells (Collier et al.,1988; Stetler-Stevenson et al.,1989; Wilhelm et al.,1989).

1.5.1 The matrix metalloproteinase family

The mammalian metalloproteinases can digest all of the major macromolecules of tissue matrices. Although originally characterized from connective tissue cells (ie.

fibroblasts), metalloproteinases are often found at elevated levels in tumour cells. Members of this enzyme family share a number of characteristics: a) they degrade at least one type of collagen; b) they are initially secreted in a latent form; c) activation requires autoproteolysis and generates an active form of lower molecular weight; d) they are inhibited by chelators of divalent cations such as EDTA; and, e) they share close sequence and structural homologies (Wilhelm et al.,1989).

Members of the metalloproteinase family can be distinguished from one another by their substrate specificities and molecular weights (Table 5). Basement membrane type IV collagen is preferentially degraded by two members of the metalloproteinase family, with different molecular weights, the 72 kD and 92 kD type IV collagenases. Both the 72 kD and 92 kD type IV collagenases can degrade type IV collagen and denatured collagens of type I, II, III, IV and V but are unable to degrade interstitial collagens (Fessler et al.,1984; Collier et al.,1988; Stetler-Stevenson et al.,1989; Wilhelm et al.,1989). The interstitial collagens Type I, II and III are degraded by two additional metalloproteinases, an interstitial collagenase first isolated from fibroblasts and what appears to be a distinct interstitial collagenase isolated from granulocytes (Hastey

Table 5.

METALLOPROTEINASE FAMILY OF DEGRADATIVE ENZYMES

Metalloproteinase	MW Before Activation	MW After Activation	Substrate Specificity	References
Interstitial Collagenase	57,52kD	47,42kD	type I,II,III collagen	Chin et al.,1985 Goldberg et al.,1986
Neutrophil Collagenase	75kD	57kD	type I,II,III collagen	Hasty et al.,1990
Stromelysin 1	60,57kD	45kD	proteoglycan, laminin, fibronectin, type IV collagen, type I,II,III, IV,V gelatins	Chin et al.,1985 Wilhelm et al.,1987 Nicholson et al.,1989
Stromelysin 2	----	47kD	type I,II,III gelatins, type IV collagen, fibronectin	Nicholson et al.,1989
72 kd type IV Collagenase	72-70kD	66-62kD	Gelatin, type IV,V collagen	Fessler et al.,1984 Collier et al.,1988 Stetler-Stevenson, 1989
92 kd Type IV Collagenase	92 kd	84kD	Gelatin,type IV, V collagen	Wilhelm et al.,1989
Pump 1	----	21,19kD	Fibronectin, casin and gelatins	Quantin et al.,1989

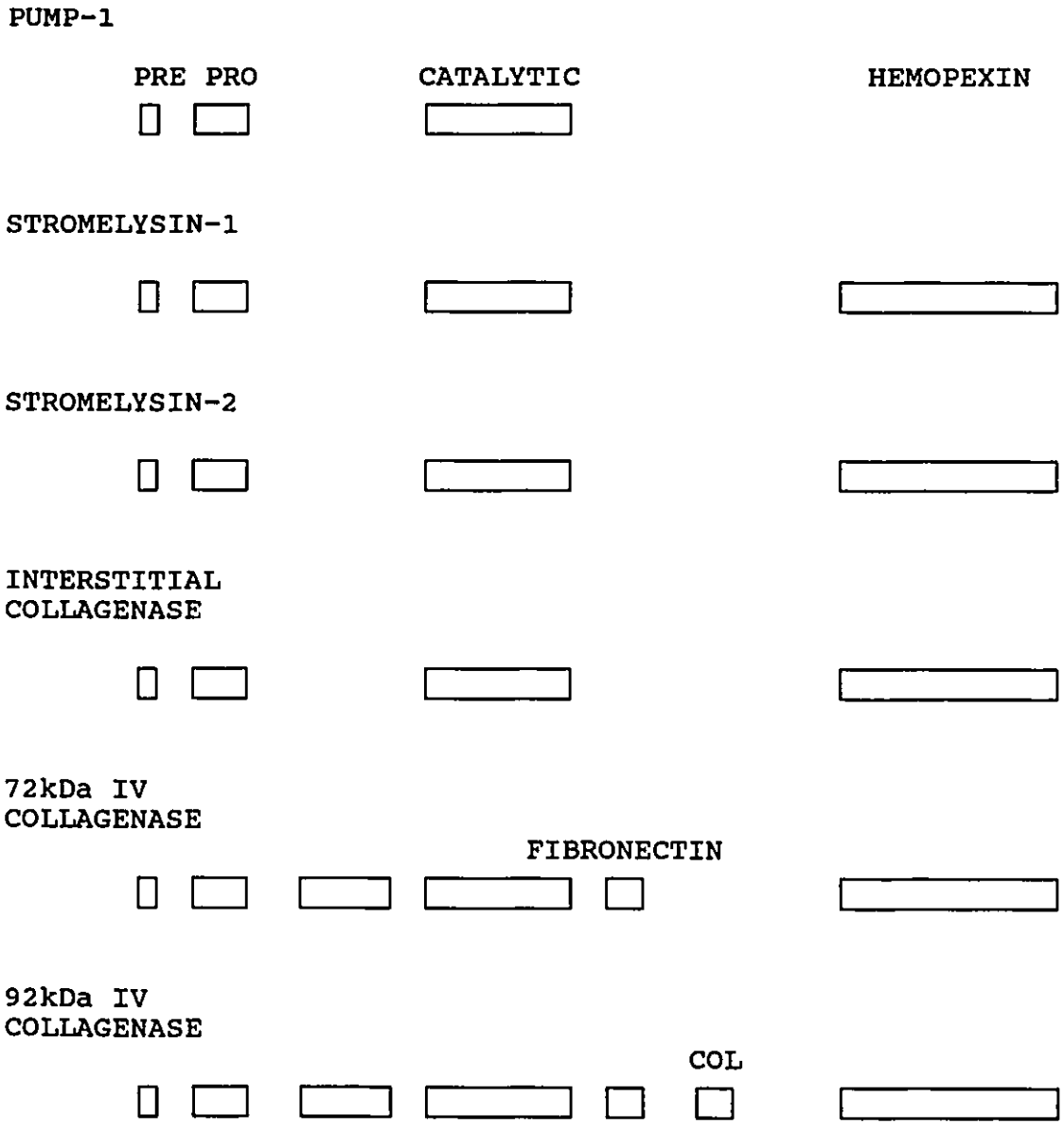
et al.,1990). The skin fibroblast interstitial collagenase and the neutrophil collagenase differ in substrate preference and immunological cross reactivity (Hasty et al.,1990). Degradation of intact collagen by these collagenases generates fragments with an approximate mass ratio of 3:1 (Liotta et al.,1979; Fessler et al.,1984; Nicholson et al.,1989; Hasty et al.,1990). Stromelysin 1 and 2, the remaining members of the metalloproteinase family, preferentially degrade proteoglycans but can also degrade denatured collagen of types I, II and III (Wilhelm et al.,1987; Nicholson et al.,1989). In addition, these enzymes can degrade type IV collagen within its nonhelical domains (Wilhelm et al.,1987; Nicholson et al.,1989).

1.5.2 Structure of metalloproteinases

A close structural relationship exists among the interstitial collagenases, the type IV collagenases and the stromelysins (Figure 4). A comparison of the predicted amino acid sequences of the cloned metalloproteinases reveals that several distinct domains are conserved in all members of this family. The first domain is a 17-19 amino acid long signal peptide, responsible for targeting the enzymes for secretion (Goldberg etal.,1986; Wilhelm et al.,1987; Muller

Figure 4. The structure of metalloproteinases. Several distinct domains are conserved in all members of the metalloproteinase family. These include: an 18 amino acid long signal sequence, called the "pre" domain; a domain 80-84 amino acids long, termed the "pro" domain, which is cleaved off during the activation process; a catalytic domain, which contains the conserved amino acid sequence VAAHELGHSLGLSHS; and a hemopexin-like domain, found in all metalloproteinases except pump-1 (from Wilhelm et al.,1989).

Figure 4.


PUMP-1
PRE PRO
CATALYTIC
HEMOPEXIN
STROMELYSIN-1
STROMELYSIN-2
INTERSTITIAL
COLLAGENASE
72kDa IV
COLLAGENASE
FIBRONECTIN
92kDa IV
COLLAGENASE
COL

et al.,1988; Hastey et al.,1990). The second domain, termed the "pro" - domain, is cleaved off during the activation process (Goldberg et al.,1986; Wilhelm et al.,1987; Muller et al.,1988; Stetler-Stevenson et al.,1989; Hastey et al.,1990). The pro-domain is 80-84 amino acids long and contains the conserved sequence PRCGVPDV at its carboxyl end (Muller et al.,1988; Stetler-Stevenson et al.,1989).The activated 92 kD type IV collagenase, unlike the other metalloproteinases contains the conserved PRCGVPDV sequence near its N-terminus (Wilhelm et al.,1989). The function of this sequence will be discussed below. Metalloproteinases contain at least two additional domains of homology within the active form of these enzymes; the catalytic domain and a hemopexin-like domain (Muller et al.,1988; Wilhelm et al.,1989). The catalytic domains of all of the metalloproteinases contain the conserved amino acid sequence VAAHELGHSLGLSHS (Muller et al.,1988; Wilhelm et al.,1989; Hasty et al., 1990). The histidine residues in this sequence are believed to bind a Zn^{2+} atom associated with the active site of these enzymes (Muller et al.,1988). The hemopexin-like domain found in all metalloproteinases, except pump-1, (Muller et al.,1988; Quantin et al.,1989) has no known function and does not appear to be required for 4-aminophenylmercuric acetate-dependent activation or catalytic activity (Sanchez-Lopez et al.,1988).

The 72 kD and 92 kD type IV collagenases contain an additional gelatin-binding domain allowing their purification by affinity chromatography on gelatin-coated columns (Collier et al.,1988; Wilhelm et al.,1989). The gelatin-binding domain is 175 residues long and is organized into three 58-residue, head to tail, repeats (which are homologous to the type II motif of the collagen binding domain in fibronectin) (Collier et al.,1988; Wilhelm et al.,1989). In gelatinase, the third 58 residue repeat is positioned directly in front of the conserved Zn^{2+}-binding domain (residues 368-385). The 92 kD collagenase has an additional unique 54 amino acid long proline-rich domain homologous to the α_2 chain of type V collagen (30-55% homology) (Wilhelm et al.,1989).

1.5.3 Activation of metalloproteinases; 4-aminophenyl mercuric acetate (APMA)

Members of the metalloproteinase family exist in both latent and active forms. All metalloproteinases contain two regions which are highly conserved and appear to be involved in the activation process. The first, the VAAHELGHSLGLSHS sequence is found in the "catalytic" domain of the protein and is believed to bind a Zn^{2+} atom within the active site. The second conserved sequence, PRCGVPDV, is contained within

the "pro" region of these proteins and is removed along with the entire "pro" region following activation. It is thought that the latent metalloproteinases have their "pro" region folded back onto the active site such that the cysteine residue within the conserved sequence PRCGVPDV forms a complex with the Zn^{2+} ion. During activation this cysteine-Zn^{2+} interaction must be disrupted and the Zn^{2+} freed in order for degradative activity to be expressed.

Experimental support for this model of metalloproteinase activation have been obtained with COS cells transfected with transin (the rat homologue of stromelysin) cDNA (Sanchez-Lopez et al.,1988). The coding for either the VAAHELGHSLGLSHS or the PRCGVPDV sequence were modified. COS cells transfected with wild type transin cDNA, secrete transin in an inactive pro-form which requires pretreatment with the organomercurial, APMA, before caseinolytic activity can be detected (Sanchez-Lopez et al.,1988). In transin, the sequence VAAHELGHSLGLSHS is found between residues 213-227 (Sanchez-Lopez et al.,1988). COS cells transfected with a transin cDNA varient where histidine residue 216 was replaced with leucine and/or histidine 226 with serine produced no caseinolytic activity, even in the presence of APMA (Sanchez-Lopez et al.,1988). COS cells transfected with a transin varient with mutations in the PRCGVPDV region (amino acids 88-96), secreted caseinolytic activity even without prior APMA treatment

(Sanchez-Lopez et al.,1988). Since mutations in the PRCGVPDV sequence do not allow the cysteine-Zn^{2+} interaction to occur, only activated forms of the metallo-proteinase can be isolated. APMA is believed to interact with the Cysteine residue in the conserved sequence PRCGVPDV, causing a conformational change in the molecule, disrupting the cysteine-Zn^{2+} interaction and allowing proteolytic activity to be expressed.

Following activation, all members of the metalloproteinase family cleave off their "pro" domain by intramolecular autoproteolysis, thus remaining permanently activated (Stetler-Stevenson et al.,1989). Fusion proteins constructed between protein A and the complete sequence for either the interstitial collagenase, stromelysin 2 or rat transin molecule can be purified on IgG-Sepharose via their protein A portion (Sanchez-Lopez et al.,1988; Nicholson et al.,1989). In the absence of contaminating proteins, caseinolytic activity can be eluted from the IgG sepharose columns by APMA treatment (Sanchez-Lopez et al.,1988). Thus these metalloproteinases can cleave off their N-terminal end by autoproteolysis. This activity can be inhibited by the addition of EDTA, 1,10, phenanthroline or Tissue inhibitor of metalloproteinases (TIMP) (Sanchez-Lopez et al.,1988).

1.5.3.1 Activation of metalloproteinases following plasmin generation

Plasminogen activators are highly substrate specific enzymes which convert the inactive zymogen, plasminogen, to plasmin. While tissue-type plasminogen activators (t-PA) are considered important in fibrinolysis, the urokinase-type plasminogen activator (u-PA) is considered to be important to local tumour growth, invasion and metastasis (Gaylis et al.,1989). U-PA activity is elevated in several malignant tumours including carcinomas of the colon, prostate, lung, stomach, and breast (Gaylis et al.,1989). Plasmin is a proteolytic enzyme with broad substrate specificity, capable of degrading both laminin and fibronectin. It also appears to be capable of activating members of the metalloproteinase family.

Indirect evidence for the activation of a metalloproteinase by plasmin comes from studies involving invasion of human tumour cells through polycarbonate filters coated with matrigel or human amnion basement membrane (Mignatti et al.,1986; Reich et al.,1988). Tumour cell invasion through these matrices was inhibited by specific antibodies to u-PA (Mignatti et al.,1986; Reich et al.,1988). In addition, plasmin inhibitors, such as aminocaproic acid, benzamidine, soybean trypsin inhibitor and human α_2-antiplasmin also inhibit tumour cell invasion

(Mignatti et al.,1986; Reich et al.,1988). Inhibition of tumour cell invasion can be reversed by the addition of mersalyl (1 mM), an organic mercurial compound which can directly activate metalloproteinases, suggesting that plasmin normally activates these metalloproteinases (Mignatti et al.,1986; Reich et al.,1988).

The activation of metalloproteinases by plasmin has been demonstrated directly. Cultured human skin fibroblasts constitutively secrete interstitial collagenases and stromelysin as proenzymes (He et al.,1989). When these dermal fibroblasts are co-cultivated with epidermal keratinocytes, in the presence of plasminogen, only activated interstitial collagenase and stromelysin are secreted (He et al.,1989). Activation occurs through a urokinase-dependent pathway, since it is blocked by anti-u-PA antibodies. Incubation with plasmin alone, activates purified procollagenase and prostromelysin as determined by a decrease in molecular weight and a 5-8 fold increase in the specific activity (He et al.,1989). Similarily, incubation with trypsin also activates both procollagenase and prostromelysin (He et al.,1989).

1.5.3.2 Activation of metalloproteinases by other metallo-proteinases

Once activated, the metalloproteinases are capable

of acting on each other to further increase their degradative ability. Plasmin- or trypsin-activated collagenase has a molecular weight of 42 kD (He et al.,1989). Collagenase can be further activated by stromelysin, resulting in an additional 2-kD loss in molecular mass and a further 5-8 fold increase in specific activity (He et al.,1989). The decrease in molecular weight is the result of a 15 amino acid residue loss from the carboxyl end of the enzyme. The amino-terminal sequence of the 40 kD form is identical to the 42 kD form (He et al.,1989). Active pump-1 can also further activate collagenase (activity was increased 5 fold above that with APMA alone) (Quantin et al.,1989). Incubation of procollagenase with stromelysin 2 or transin in the absence of APMA, increased collagenase digestion of collagen type I, 8.5 fold and 12.5 fold respectively (Nicholson et al.,1989).

1.5.4 Tissue Inhibitors Of Metalloproteinases (TIMP) Levels Correlate To Metastatic Potential

The secretion and activation of metalloproteinases is not sufficient to ensure extracellular matrix degradation, since tumour cells and host cells (ie. fibroblasts or endothelial cells) also secrete metalloproteinase inhibitors (Stricklin and Welgus, 1983; Welgus and Stricklin, 1983; Hicks et al.,1984; Herron et

al.,1986). These proteins are called "tissue inhibitors of metalloproteinases" or "TIMPs".

Two members of the TIMP family have been isolated, purified and cloned (Stricklin and Welgus, 1983; Welgus and Stricklin, 1983; Docherty et al.,1985; Stetler-Stevenson et al.,1989). TIMP-1 is a 184 amino acid long glycoprotein with an approximate molecular weight of 29 kD (Stricklin and Welgus, 1983). TIMP-2 is a non-glycosylated, 192 amino acid protein with a molecular weight of 21 kD (Goldberg et al.,1989; Stetler-Stevenson et al.,1989). The amino acid sequence of TIMP-2 shows 41% amino acid identity and overall 66% sequence homology to TIMP-1, including the conservation of 12 cysteine residues, and 3 out of 4 tryptophan residues (Docherty et al.,1985; Goldberg et al.,1989; Stetler-Stevenson et al.,1989). TIMP-1 and TIMP-2 differ in regard to which metalloproteinases they can bind and therefore inactivate. TIMP-1 binds only the activated forms of the interstitial collagenase, stromelysin and the 92 kD type IV collagenase (Stricklin and Welgus, 1983; Welgus and Stricklin, 1983). TIMP-2 does not bind these members of the metalloproteinase family but does bind to both the activated and latent forms of the 72 kD type IV collagenase (Goldberg et al.,1989; Stetler-Stevenson et al.,1989). When bound to the latent form of the 72 kD type IV collagenase TIMP-2 does not prevent activation by organomercurials (Goldberg et al.,1989).

Both natural and recombinant TIMP-1 has been shown to inhibit tumour cell invasion and prevent the formation of metastasatic colonies in vivo (Schultz et al.,1988; Khokha et al.,1989). Khokha et al. (1989) have demonstrated that mouse 3T3 cells transfected with plasmid constructs encoding antisence TIMP-1 mRNA become highly invasive in the human amnion invasion assay, and highly tumourigenic in athymic mice (Khokha et al.,1989). These transfected cell lines are down-modulated for TIMP messenger RNA levels and secrete less TIMP into their culture medium. Tail vein injection of nude mice with control 3T3 cells does not produce metastasis, however injection of these mice with TIMP-down-modulated 3T3 cells resulted in numerous lung metastasis. These results have been interpreted to mean that TIMP production can suppress the metastatic potential of tumour cells (Khokha et al.,1989).

1.6 Perspective

Cancer cells frequently disseminate via the bloodstream where the endothelium and underlying basement membrane act as a barrier between the circulating tumour cells and the extravascular tissue. Previous studies have shown that metastasis was promoted following endothelial cell injury. Although several of the agents used in these studies may have caused endothelial cell injury by the generation of reactive oxygen species, a role for such species in the promotion of metastasis has only been postulated thus far. Here we explore the hypothesis that some tumour cells (or host cells) may compromise vessel wall integrity at sites of tumour cell arrest, by generating reactive oxygen species. Specifically, we asked if;

a) W256 cells and/or platelets could produce reactive oxygen species.

b) reactive oxygen species produced by either the free radical generating system xanthine - xanthine oxidase or W256 cells could affect endothelial cell function.

c) the adhesion of W256 cells to endothelial cell monolayers

could affect reactive oxygen species mediated perturbation of the endothelium.

d) W256 cells could degrade subendothelial cell matrices by a mechanism which involves both the secretion of a metalloproteinase and the generation of reactive oxygen species.

2 MATERIALS AND METHODS

2.1 Materials:

The following materials were purchased from the Sigma Chemical Company, St. Louis MO: bovine serum albumin; catalase, from bovine liver; cytochalasin B, from Helminthosporium dematioideum; N-formyl-L-Methionyl-L-Leucyl -L-Phenylalanine (fMLP); luminol (5-amino-2,3-dihydro-1,4-phthalazinedione); phorbol 12-myristate, 13-acetate; superoxide dismutase (SOD) from bovine liver. All media were obtained from GIBCO, Burlington, Ontario. Both the chemotactic peptide, fMLP and the phorbol ester, phorbol 12-myristate, 13-acetate (PMA), were dissolved in absolute ethanol to appropriate stock concentrations. Luminol was similarly prepared using dimethyl sulphoxide as the solvent. All other agents were dissolved in appropriate aqueous buffers.

2-deoxy-D-[1-^{3}H]glucose was obtained from Amersham Corporation, Oakville, Ontario at a concentration of 1.0 mCi/ml, (specific activity 17.0 Ci/mmole), while ^{125}I-deoxyuridine (2200 Ci/mmol) was obtained from Dupont/New England Nuclear, Mississaga, Ontario. Two rabbit anti-human

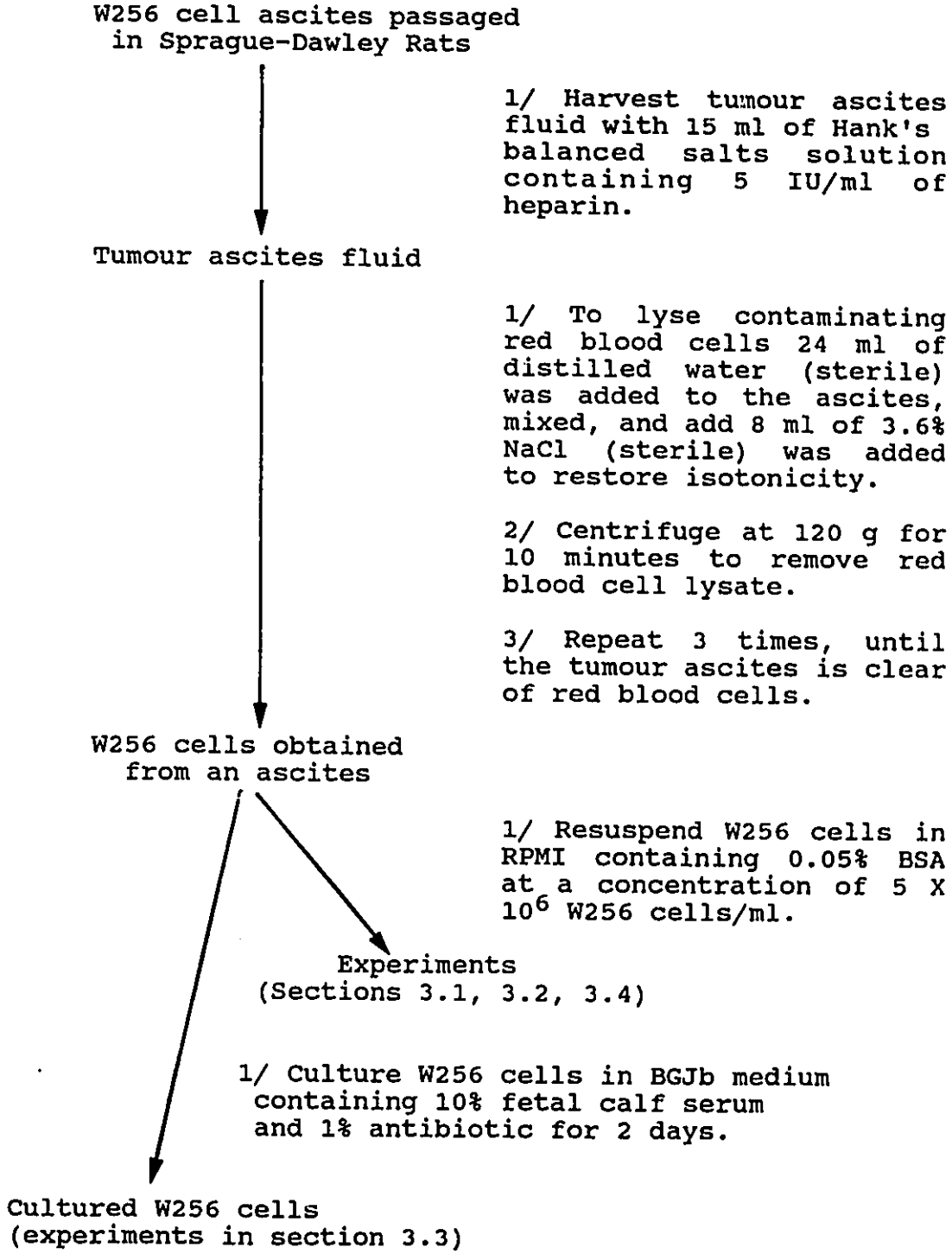
vitronectin receptor (VnR) polyclonal antisera were used in these studies. One of these was a gift from Dr. D.A. Cheresh, Scripps Clinic, La Jolla, California and has been characterized previously (Cheresh and Harper,1987). The other was purchased from Telios Biochemicals ,San Diego, California and has been solid phase absorbed with normal human plasma proteins as well as fibronectin receptor. This antiserum recognized immunoreactive material at 160 kD and 90 kD in immunoblots of extracted endothelial surface proteins. Rabbit anti-human fibronectin receptor (FnR) polyclonal antiserum was also obtained from Telios Biochemicals, San Diego, California. Recombinant human interleukin-1α (rIL-1α) was a gift of Dr. P. Lomedico, Hoffman-La Roche, Nutley, New Jersey.

2.2 Cell lines:

2.2.1 Walker 256 (W256) carcinosarcoma cell line

The W256 cell line was maintained *in vivo* as an ascites tumour within pathogen-free, adult (200 g) male Sprague-Dawley rats (Orr et al.,1978). For experiments, tumour ascites fluids were harvested in heparinized Hanks' balanced salts solution (5 IU heparin/ml) and the contaminating erythrocytes removed by 2-3 cycles of osmotic lysis (Figure 5). Cell suspensions prepared by this method

Figure 5. Isolation of Walker 256 tumour cells



contained 98 ± 1 % tumour cells identifiable on Wright stained cytospin preparations. Mean tumour cell viability was 95% after 6 hours, as estimated by the trypan blue exclusion assay and intact cell morphology. For most experiments, the harvested W256 cells were resuspended at a cell density of 5 X 10^6 cells/ml in RPMI 1640 containing 0.05% bovine serum albumin. Alternatively, the W256 cells were first cultured (seeding density, 2.5 X 10^5 cells/ml) in BGJ^b medium with 10% fetal calf serum and 100 U/ml penicillin, 100μg/ml streptomycin, for 2 days prior to the experiment.

2.2.2 Endothelial Cell Culture

Human umbilical cord endothelial cells were obtained by collagenase treatment and maintained in culture by a method modified from Jaffe et.al. (1973). Briefly the cells were harvested by collagenase treatment and maintained in medium 199 containing 20% heat-inactivated human serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 100 μg/ml pituitary-derived endothelial growth factor. The cells were either passaged once in T25 flasks and then seeded onto fibronectin-coated plastic discs in 24-well tissue culture dishes, or seeded into the multiwell dishes following the collagenase treatment. The cells were identified as being endothelial in origin by their cobblestone morphology as

well as by their expression of von Willibrand factor (see Figure 6). All cells used in experiments were of first or second passage.

Bovine pulmonary endothelial cells were obtained from the American Type Culture Collection (CCL 209) at passage fifteen. These cells were maintained in medium 199 containing 20% heat-inactivated fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin and 1 % glutamine. During passage, the small contaminating smooth muscle cell population was reduced by selection of the endothelial cells after 0.1% trypsin treatment on the advice of the American Type Culture Collection. These cells were also identified as being endothelial in origin by their cobblestone morphology as well as by their expression of von Willibrand Factor (Wagner and Marder, 1983).

2.3 Preparation of washed human platelets

Suspensions of washed human platelets were prepared according to the method of Mustard et. al. (1972). Briefly, venous blood was obtained from healthy donors and mixed with ACD anticoagulant (0.067 M citric acid/trisodium citrate containing dextrose pH 4.5; 1 part anticoagulant to 6 parts blood). See Figure 7. The blood was then centrifuged at 37°C, for 15 minutes, at 190 x g to obtain platelet-rich plasma. The platelet-rich plasma was removed and centrifuged

Figure 6. The expression of von Willibrand Factor by human umbilical vein endothelial cells. W256 cells were pelleted by centrifugation on glass slides and fixed with acetone for 15 minutes at 20°C. The slides were then briefly incubated in 20% swine serum and then incubated with rabbit anti-human von Willebrand Factor antiserum (Dakopatts, of Denmark) at a dilution of 1:100 for 30 minutes. Following the incubation with anti-human von Willebrand Factor antisera, the slides were treated for an additional 30 minutes with swine anti-rabbit peroxidase conjugate at a dilution of 1:25. Von Willebrand Factor was visualized on the endothelial cell surface by light microscopy following diaminobenzidine tetrahydrochloride staining. Magnified 400xs.

Figure 6.

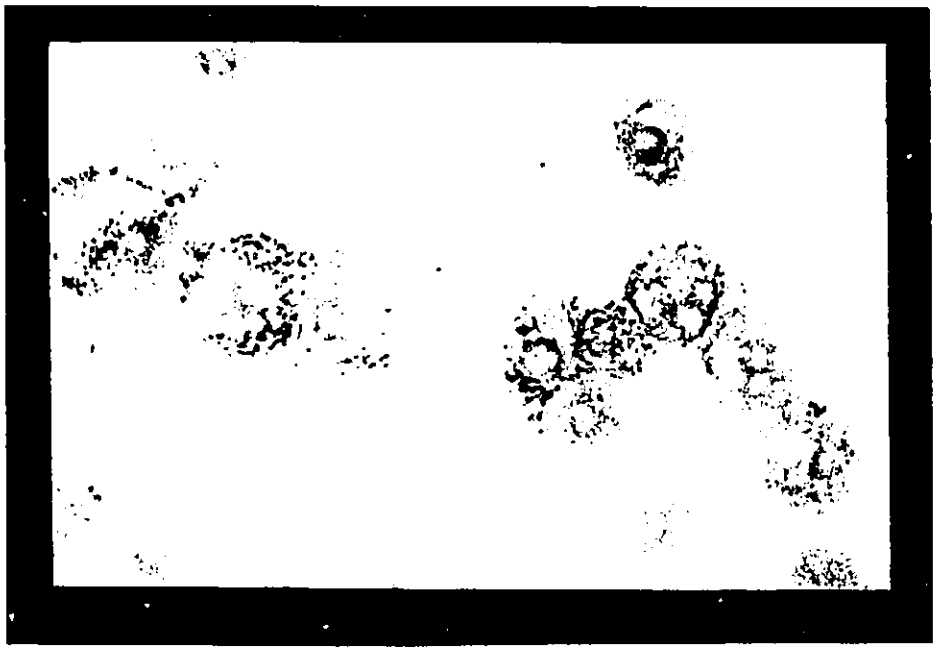
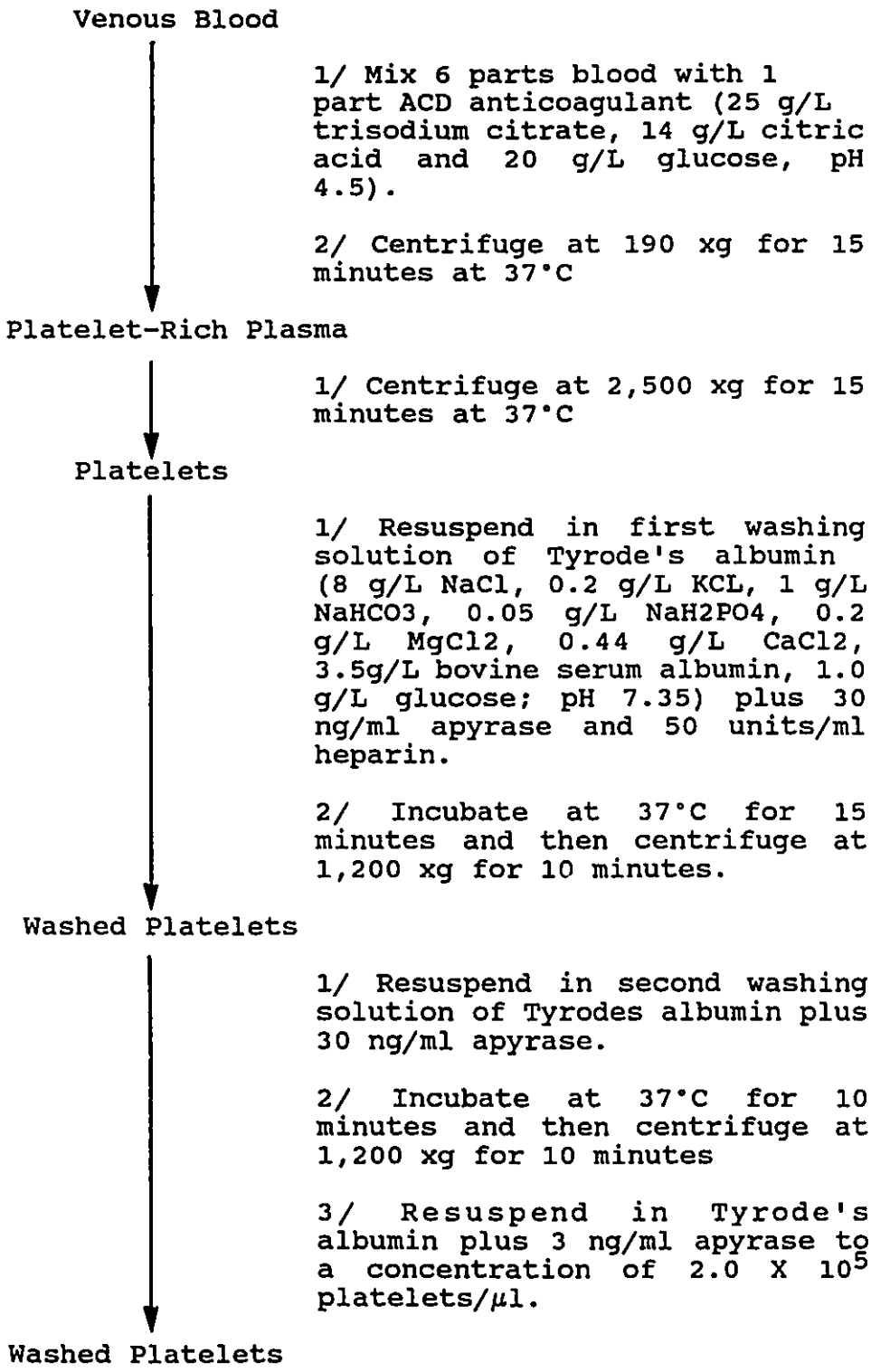


Figure 7. Preparation of washed human platelets



at 2,500 x g for 15 minutes at 37°C. Following removal of the platelet-poor plasma layer, the platelets were resuspended in the first washing solution of Tyrode's albumin containing 30 ng/ml apyrase (at a concentration of 30 ng/ml, apyrase will hydrolyze 500 nmoles of ATP or ADP in 9 minutes) and 50 units/ml heparin (see Figure 7). The platelets were incubated in this solution at 37°C for 15 minutes and then centrifuged at 1,200 x g for 10 minutes. The supernatant was then discarded and the platelets resuspended in a second washing solution of Tyrode's albumin containing 30 ng/ml apyrase. Following an additional 10 minutes incubation, at 37°C, the platelets were centrifuged at 1,200 xg for 10 minutes. The platelets were then resuspended in Tyrode's albumin containing apyrase (30 ng/10 ml of suspension) ata final concentration of 2 X 10^5 platelets/μl.

2.4 Techniques for measuring reactive oxygen species:

2.4.1 Chemiluminescence apparatus and assay

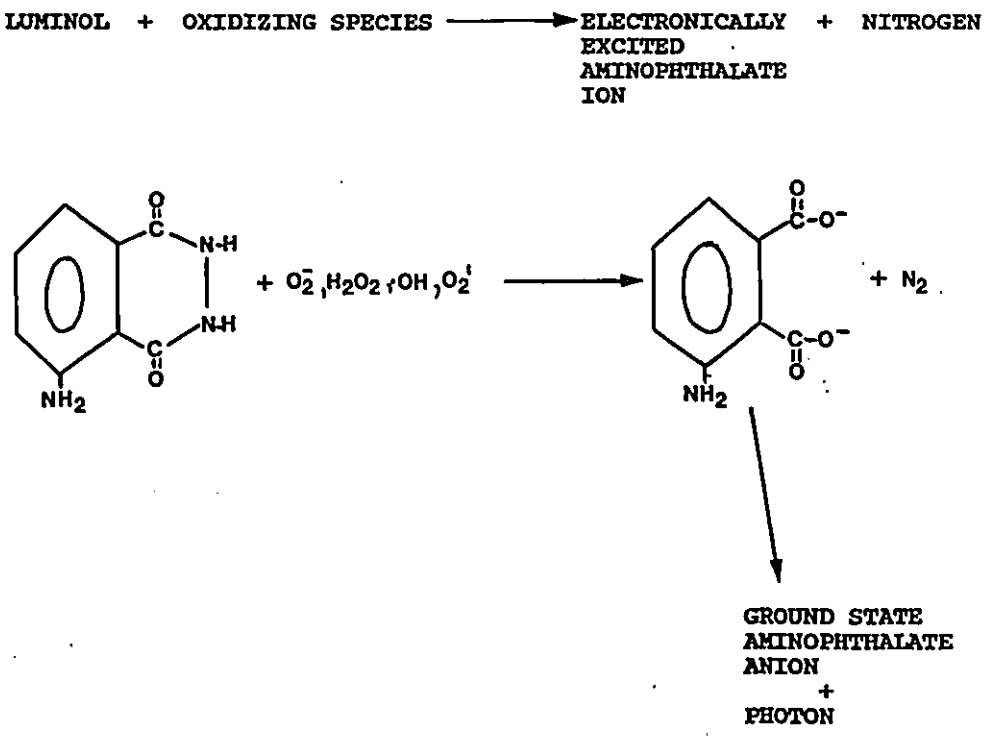
Details of the construction of the chemiluminescence apparatus have been published previously (Leroyer et al.,1987). Briefly, it consists of an EMI 9588A red-sensitive photomultiplier tube which is mounted vertically and monitored by a model C10 photon counter

(Thorn EMI-Gencom instruments). Above the photomultiplier tube there is a light-tight aluminum chamber into which a 6 well culture plate (Linbro plastics #FB6) can be placed. The lids of the culture dishes are replaced with a specially designed cover which includes six miniature stirring motors driving teflon paddles at 17 rpm (to ensure agitation), and holes through which air or reagents can be added. During experiments the temperature in this chamber was maintained at 37^{o} C.

Prior to each experiment, all materials were kept in the dark for a period of at least 1 hour to ensure low background levels of chemiluminescence. Two ml of tumour cell suspension (5 X 10^{6} cells/ml) were added to each culture well in the photometer and allowed to equilibrate for 4 minutes. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was then added to obtain a final concentration of 5 X 10^{-5}M luminol (see Figure 8). This was incubated for another 3 minutes before adding the chemotactic peptide fMLP, to activate the W256 cells. Light emission was recorded over a period of 8-10 minutes. Tracings were evaluated by measuring the peak chemiluminescence (counts per minute) or by calculating the area under the chemiluminescence light curve over eight minutes continuous recording (integral chemiluminescence) (Leroyer et al.,1987). To determine the latter we used a Microplan II Image Analysis System (Laboratory Computer

Figure 8. The chemiluminescence of luminol.

The oxidation of luminol results in the generation of chemiluminescence. The oxidizing species are generated as a result of cellular activation. From Trush et. al., 1978.


LUMINOL + OXIDIZING SPECIES
ELECTRONICALLY EXCITED AMINOPHTHALATE ION
+ NITROGEN
+ O_2^-, H_2O_2, OH, O_2^1
+ N_2
GROUND STATE AMINOPHTHALATE ANION
+
PHOTON

Systems Inc., Cambridge, Massachusetts).

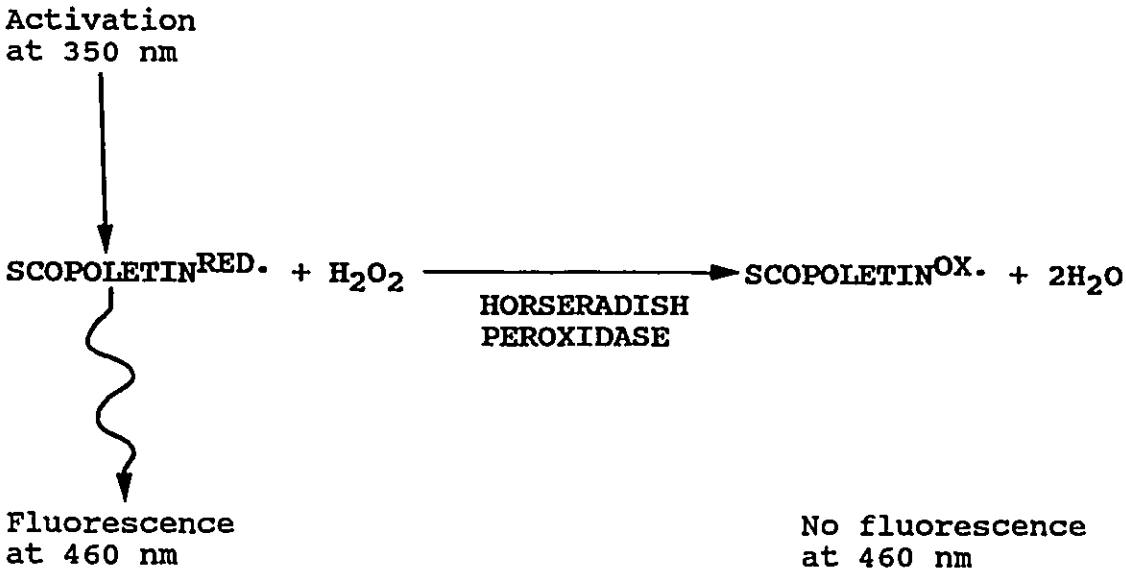
2.4.2 Scopoletin fluorescence assay for hydrogen peroxide production:

The loss of scopoletin fluorescence in the presence of W256 cells was monitored on a Perkin - Elmer fluorescence spectrophotometer (model MPF-44). Briefly, W256 cells were suspended (in 1 ml cuvettes) at a concentration of 5 X 10^6 cells/ml in phosphate buffered saline, pH 7.4, containing 4.8 mM KCl, 0.54 mM $CaCl_3$, and 1.2 mM $MgCl_2$. Excitation was set at 350 nm and emission fluorescence monitored at 460 nm (Root et al.,1975; De la Harpe and Nathan 1985). Fluorescence was recorded continuously following the addition of 10 nmoles scopoletin, 22 nM horseradish peroxidase and 10^{-6} M fMLP respectively (Root et al.,1975; De la Harpe and Nathan 1985) (see Figure 9). The system was standardized by the addition of known amounts of hydrogen peroxide to reagents in the absence of cells. The amount of hydrogen peroxide produced by 5 X 10^6 W256 cells in one hour was calculated as follows:

$$\frac{A\ \Delta F \times 1.0 \text{ nmoles } H_2O_2}{B\ \Delta F} \times 60$$

Where A ΔF represents the loss in scopoletin fluorescence,

Figure 9. Hydrogen peroxide oxidation of reduced scopoletin. Scopoletin is a fluorescent compound which when activated by light at 350 nm, emits light at 460 nm. In the presence of horseradish peroxidase, the fluorescence of scopoletin is diminished in direct proportion to the concentration of hydrogen peroxide found in solution. Figure 9 is from Root et. al., 1975.


Activation
at 350 nm
SCOPOLETIN^RED. + H2O2
HORSERADISH
PEROXIDASE
SCOPOLETIN^OX. + 2H2O
Fluorescence
at 460 nm
No fluorescence
at 460 nm

occurring in 1 minute in the presence of 5 x 10^6 W256 cells and B ΔF represents the loss of fluorescence caused by the addition of 1.0 nmoles of H2O2 in the absence of cells.

2.4.3 Electron microscopic localization of hydrogen peroxide production

To determine directly if W256 cells were capable of generating reactive oxygen species following cellular activation the W256 cells were exposed to fMLP in the presence of $CeCl_3$. $CeCl_3$ reacts with hydrogen peroxide to form the electron dense cerium perhydroxide (Ce-[OH]2 OOH) (Briggs et al., 1975; Vissers et al.,1985). W256 cells were suspended at a concentration of 5 X 10^6 cells/ml in 0.1 M Tris-maleate buffer pH 7.5, containing 7% sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/ml glucose. Following a 2 minute incubation at 37°C in the presence or absence of 10^{-6}M fMLP, the cells were pelleted, resuspended in buffer containing 1 mM $CeCl_3$ and incubated at 37°C for a further 60 minutes. During this step, some cells were incubated in the presence of 2000 units/ml catalase. The samples were then fixed at room temperature by the addition of an equal volume of 4% formaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Further sample preparation was performed by the electron microscopy unit of McMaster University. Briefly, primary fixation was continued for one hour in 2%

buffered glutaraldehyde at room temperature. The cells were then washed for 20 minutes in 0.1M cacodylate buffer, pH 6.0 and postfixed for 30 minutes, at 4°C, in 0.2 M sodium cacodylate buffer containing 1% OsO_4, pH 7.4. Following postfixation, the cells were rinsed in water, and dehydrated through graded ethanol (50%, 70%, 95%, 100%). The samples were then infiltrated for 30 minutes, with a mixture of propylene oxide and Spurr's resin and then transferred into fresh 100% Spurr's resin for 18 hours at 70°C. The embedded samples were then sectioned for transmission electron microscopy and stained with uranyl acetate and lead citrate. During transmission electron microscopy, W256 cells were photographed at random. The number of $CeCl_3$-derived precipitates associated with each W256 cell subsequently determined by visual analysis of the photographs and expressed as a mean ± standard error. The surface area of each W256 cell was determined by analysing the photographs on a Microplan II Image Analysis System (Laboratory Computer Systems Inc., Cambridge, Massachusetts).

2.5 Endothelial Response assays:

2.5.1 The ^{3}H-2-deoxyglucose release assay

Previous studies have utilized the release of ^{3}H-2-deoxyglucose from cultured endothelial cell monolayers as an

index of endothelial cell damage (Andreoli et al.,1985; Andreoli et al.,1986). Since isotope release in these studies was promoted by both activated neutrophils and the reactive oxygen generating system xanthine-xanthine oxidase, we utilized this method to determine if activated W256 cells could also promote the release of ^{3}H-2-deoxyglucose from prelabeled endothelial cell monolayers. Confluent monolayers were labeled overnight by the addition of 1 μCi/well 2-deoxy-[1-^{3}H]-glucose-6-phosphate (Andreoli et al.,1985). Eighteen to 24 hrs later, the media was removed and the endothelial cell monolayers were washed 4 times with 1 ml of RPMI containing 0.5 mg/ml bovine serum albumin (BSA). In some cultures, 1 mM BSO was added to the cells the night prior to the assay and then included at this same concentration in all washes as well as in the assay medium in order to deplete endogenous glutathione levels (Harlan et al.,1984; Suttorp et al.,1985). The assay for endothelial cell damage was initiated by adding various concentrations of xanthine oxidase in 0.5 ml RPMI with 0.5 mg/ml BSA and 0.2 mM xanthine. Alternatively, varying numbers of W256 cells were added in a total volume of 0.5 ml RPMI plus 0.5 mg/ml BSA. In some experiments, the tumour cells were activated by adding 1 X 10^{-6} M fMLP. Following 90 minutes incubation at 37^{o}C in a humidified environment containing 5% CO_2, the medium was removed from each well. The endothelial cell monolayer was washed once with 0.5 ml RPMI

containing 0.5 mg/ml BSA and this was added to the medium. Any cells present in these supernatant fluids were removed by centrifugation for 3 minutes at 200 g. The discs on which the endothelial monolayers had been grown were then removed from the culture dishes, and placed into 0.2 ml of Beckman tissue solubilizer. The radioactivity retained in the monolayer and in the medium was then determined using a Beckman LS 1801 scintillation counter. The release of the ^{3}H-2-deoxyglucose into the medium was calculated as a percentage of the total radioactivity in each well. Considerable variation was found in the release of ^{3}H-2-deoxyglucose between different control endothelial cell preparations. Percent specific release was therefore determined by the formula :

$$A-B/B \times 100$$

where A equals the percent release found in experimental conditions and B equals the percent release found in controls, (medium without xanthine oxidase or tumour cells). Percent release was calculated as a mean of four to six replicates. Percent specific release is expressed as a mean ± standard error.

2.5.2 ^{51}chromate release:

The release of ^{51}chromate from prelabeled endothelial cell monolayers has been used extensively to

examine both neutrophil and xanthine oxidase - mediated endothelial cell injury (Harlan et al.,1984; Suttorp et al.,1985; Andreoli et al.,1985; Andreoli et al.,1986). We performed the ^{51}chromate release assay in a similiar manner to the ^{3}H-2-deoxyglucose assay except that the cells were labeled overnight with 1 μCi/well of Na ^{51}chromate.

2.5.3 Lactate dehydrogenase release:

Lactate dehydrogenase activity was determined using a Kodak Ektachem Analyzer (model 700XR) on cell - free supernatants and endothelial cell monolayers (following sonication) which were exposed to increasing xanthine oxidase concentrations in the presence of 5 mg/ml soyabean trypsin inhibitor and 0.2 mM xanthine. Briefly, lactate dehydrogenase activity was quantitated by recording the decrease in optical density at 340 nm (NADH) in the presence of potassium phosphate buffer containing pyruvic acid impregnated on film.

2.5.4 Quantification of endothelial cell retraction:

Endothelial cell monolayers were exposed to increasing xanthine oxidase concentrations in the presence of 5 mg/ml soyabean trypsin inhibitor and 0.2 mM xanthine and then fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.5 at 37°C. The cells were then

postfixed in 1% OsO_4 in 0.1M sodium cacodylate, at 4°C, for 30 minutes, rinsed in water, and dehydrated through a graded alcohol series (50%, 70%, 90%, 95%). They were then critical-point dried from absolute ethanol and coated with 20 nm of gold in a sputter coater. The samples were viewed on a Phillips 501B scanning electron microscope and photographed at random, at a constant magnification. To determine the extent of endothelial cell retraction, the area between individual endothelial cells was traced from the photographs using a Microplan II Image Analysis System (Laboratory Computer Systems Inc., Cambridge, Massachusetts).

2.6 Walker 256 carcinosarcoma cell adhesion assay:

W256 cells obtained from an ascites fluid were cultured in BGJ_b medium with 10% fetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin, for 2 days before the experiment. The cells were radioactively labeled by the addition of 0.5 μCi/ml of ^{125}I-iododeoxyuridine to the culture medium on day 1 (Lauri et al.,1990; Lafrenie et al.,1990).

Endothelial cells were cultured to confluency, as verified by scanning electron microscopy of selected cultures, on fibronectin-coated plastic discs contained in 24 well tissue culture dishes. Some endothelial cells had

been pretreated for 4 hours with 10 ng/ml of rIL-1α at 37°C to induce expression of adhesion sites (Lauri et al.,1990; Lafrenie et al.,1990). Following IL-1 stimulation, the media were removed and 0.5 ml of ^{125}I-iododeoxyuridine-labeled W256 cells, suspended at a concentration of 5 X 10^6 cells/ml was added. In some experiments, the tumour cells were then activated by adding 1 X 10^{-6} M PMA. Alternatively, the W256 cells were stimulated with 10^{-6} M PMA and washed twice prior to their being added to the endothelial cell monolayers. The labeled W256 cells were incubated for 1.5 hours at 37°C with the endothelial monolayers. Loosely adherent W256 cells were removed from the endothelial cell monolayer by washing the plastic disc on which the endothelium had been grown in unsupplemented M199. Each disc was then counted in a Packard gamma-counter and the number of adherent cells determined (Lauri et al.,1990; Lafrenie et al.,1990).

2.7 Techniques for measuring vitronectin receptor expression:

2.7.1 Immunofluorescence flow cytometry

Cultured W256 cells were isolated by centrifugation, washed twice at 4°C in Hanks balanced saline solution (HBSS) containing 10 mM HEPES pH 7.3, 0.001 % NaN_3, 2 % BSA, 2 % goat serum (binding buffer), (Dustin et al.,1986), and

resuspended in binding buffer at a cell concentration of 10^6 cells/ml. In some experiments, the W256 cells were first activated with 10^{-6} M PMA for 90 minutes prior to isolation. Endothelial cell monolayers were washed twice at 4°C in HBSS containing 0.1% EDTA, 0.001% NaN_3 and were then incubated with the same for 15 minutes at 4°C to release the cells from the substratum. The detached cells were then washed twice at 4°C in HBSS containing 2 mM $CaCl_2$, 2 mM $MnCl_2$, 0.001% NaN_3 and resuspended in binding buffer at a cell concentration of 1 x 10^6 cells/ml. In some experiments, the endothelial cell monolayers were pretreated with 10 ng/ml rIL-1α for 4 hours prior to being isolated.

The cell supensions of W256 or endothelial cells were then incubated at 4°C for 30 minutes with either rabbit anti-human vitronectin receptor antiserum at a titre of 1:50 or normal rabbit serum at the same titre. Following this incubation, the cells were washed 3 times and resuspended in 1 ml of binding buffer at a concentration of 10^6 cells/ml. Fluorescene isothiocyanate-goat anti-rabbit IgG conjugate was then added at a concentration of 0.5 μg/ml. Following a 30 minute incubation at 4°C the cells were washed and resuspended in 1 ml of binding buffer. The samples were either analysed immediately or fixed with 1% paraformaldehyde and analyzed within 4 days (Dustin et al.,1986).

The fluorescent profiles for the final cell

preparation were determined on a EPICS^{TM} profile analyzer (coulter corporation). The fluorochrome was excited at 15 mW of 488 nm light using a argon laser. Light emission was detected by a photomultiplier tube using a band pass filter (530/30 nm). A population of 20,000 cells gated to exclude dead and aggregated cells was analyzed over a log scale of fluorescent intensity.

2.7.2 Antibody binding studies

Endothelial cell monolayers were grown to confluency in 96-well tissue culture plates. The monolayers were then incubated with 10 ng/ml rIL-1α or an equal volume of medium for periods of time ranging from 0 to 24 hours. The monolayers were then washed twice with a 100 μl of binding buffer at 4°C and incubated for 30 minutes, at 4°C, with 100 μl of binding buffer containing either rabbit anti-VnR polyclonal antiserum at a titre of 1:8000 or normal rabbit serum at an equivalent titre. Following this incubation, the monolayers were washed twice with binding buffer and incubated a further 30 minutes with ^{125}I-goat anti-rabbit $F(ab)_2$ fragments (1 mCi/ml). The monolayers were then washed 3 times with binding buffer, solubilized with 50 μl of 2% SDS in HBSS and counted on a Packard gamma-counter. All experiments were performed in quadruplicate.

2.8 Proline release:

To obtain subendothelial matrices for study, confluent cultures of bovine pulmonary artery endothelial cells, grown on gelatin-coated discs, were incubated for 4-5 days in proline-free Dulbecco's minimal essential media with 20 % fetal bovine serum, supplemented with 1 % glutamine, 50 μg/ml ascorbic acid, and 5 μCi/ml ^{3}H-proline. Radiolabelled subendothelial matrices were obtained after lysis of the endothelium with 0.5 % Triton-X-100, 5 mM EDTA in phosphate buffered saline (PBS) and washing of the adherent matrix three times with PBS (Nakajima et al.,1987). The assay was initiated by the addition of 5 X 10^{6} W256 cells. In some cases, W256 cells were activated by adding 10^{-6} M fMLP. Following 120 min incubation at 37^{o}C in a humidified environment containing 5% CO_2, the medium was removed from each well. The matrices were washed once with 0.5 ml RPMI 1640 containing 0.5 mg/ml BSA and this was added to the medium. The discs on which the endothelial monolayers had been grown were then removed from the culture dishes, and placed into 0.2 ml of Beckman tissue solubilizer. The radioactivity retained in the monolayer and in the medium was then determined using a Beckman LS 1801 scintillation counter. The release of the ^{3}H-proline into the medium was calculated as a percentage of the total radioactivity in each well.

2.9 Gelatin Zymography:

Members of the metalloproteinase family of degradative enzymes can be distinguished on the basis of molecular weight following electrophoresis on SDS polyacryamide gels containing gelatin (Chin et al.,1985; Collier et al.,1988; Stetler-Stevenson et al., 1989). Metalloproteinases are secreted in a latent form. Following activation, these enzymes autoproteolytically cleave themselves to a lower molecular weight - active form (Sanchez-Lopez et al.,1988). Both the latent and lower molecular weight active - forms can be visualized by gelatin zymography following displacement of the SDS with Triton X-100 (Chin et al.,1985; Collier et al.,1988).

To obtain W256 cell-free supernatants for analysis by gelatin zymography, 5 X 10^6 W256 cells were suspended in 1 ml of RPMI 1640. In some experiments, the tumour cells were activated by the addition of 1 X 10^{-6} M PMA. Following a 20 minute incubation at 37°C, the W256 cells were pelleted by centrifugation and the cell-free supernatants collected. The cell-free supernatants were incubated with 1.0 mM APMA for 45 minutes at 37°C (Stetler-Stevenson et al., 1989). Alternatively, the cell free supernatants were first concentrated 10 - fold by Amicon filtration (molecular weight exclusion of 30 kD), pooled and 0.25 ml of the

concentrated cell free supernatant (protein concentration 1mg/ml) was treated with 0.12% hydrogen peroxide at 37°C. Following a 45 minute incubation, aliquots of the cell free supernatants were mixed with sample buffer and applied directly, to 9% polyacrylamide gels containing 0.3% gelatin and electrophoresed overnight at 4°C without prior reduction or heating. After removal of SDS from the gel by incubation in 2.5% Triton-X-100 for 30 minutes, the gels were incubated at 37°C for 48 hours in 50 mM Tris-HCl pH 7.5, containing 5 mM $CaCl_2$, 50 mM NaCl, and the detergent 1% Brij-35 (Chin et al.,1985; Collier et al.,1988). Gels were stained for 30 minutes in 30% isopropanol/ 10% acetic acid containing 0.5% (w/v) coomassie blue R-250 and then destained in distilled water.

3 RESULTS

3.1 Reactive oxygen species generation by Walker 256 tumour cells and human platelets

Here we present evidence suggesting that the W256 cell as well as platelets can produce reactive oxygen species. The latter observation is of interest, since platelets are often found at sites of tumour cell arrest *in vivo* (Jones et al.,1971; Crissman et al.,1988).

3.1.1 Walker 256 tumour cells produce reactive oxygen species following chemotactic stimulation

Previous *in vitro* studies had suggested that the Walker 256 carcinosarcoma (W256) cell line could generate reactive oxygen species following activation by the chemotactic peptide N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP) or phorbol 12-myristate, 13-acetate (PMA) (Leroyer et al.,1987). Evidence that the activated W256 cells had generated reactive oxygen species included: 1) the inhibition of luminol-promoted chemiluminescence by the addition of catalase, superoxide dismutase, or mannitol; and 2) a dose dependent reduction of acetylated cytochrome *c*

in the presence of activated Walker 256 cells (Leroyer et al.,1987)

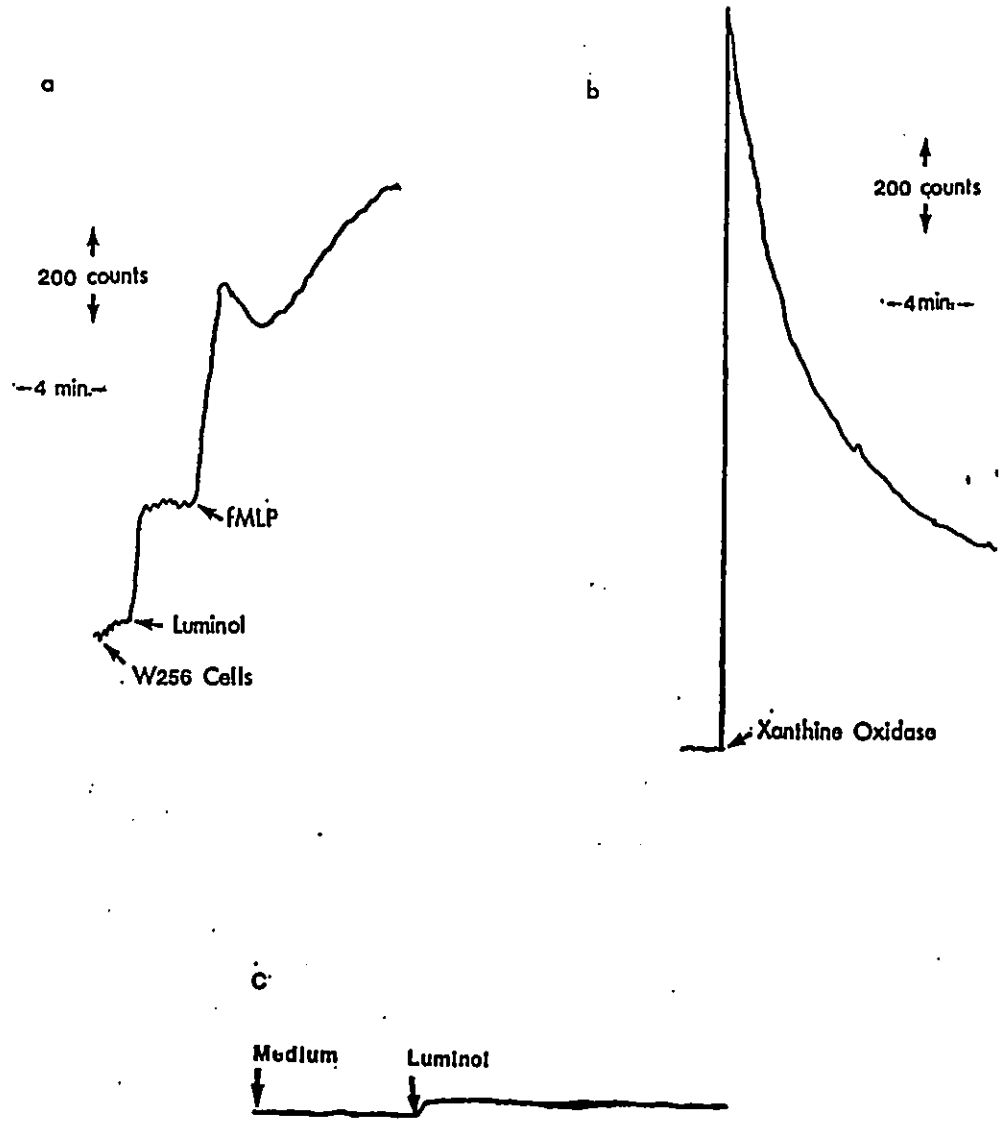
In this study we confirmed that the W256 cell was capable of generating luminol-dependent chemiluminescence, and the amount of hydrogen peroxide produced by these cells was quantitated by the scopoletin fluorescence assay. We also obtained direct morphological evidence that activated W256 cells were generating hydrogen peroxide. In these experiments, $CeCl_3$ was added to W256 ascites preparations following fMLP activation, and the tumour cells were examined by electron microscopy for the formation of electron dense precipitates on their cell surface. The details of these observations are outlined below.

3.1.1.1 Chemiluminescence by Walker 256 tumour cells following chemotactic stimulation:

Figure 10 illustrates a chemiluminescence trace, obtained when 5 X 10^6 W256 cells were incubated with 10^{-6}M fMLP (graph a) or when 6.5 X 10^{-3} units of xanthine oxidase were incubated with 0.2 mM xanthine (graph b). Background production of low level chemiluminescence was observed in studies of unstimulated tumour cells whereas chemiluminescence was not observed in the presence of xanthine

Figure 10: Chemiluminescence produced by W256 cells or xanthine oxidase. (a) 5 X 10^6 W256 cells suspended in RPMI containing 0.05% BSA were stimulated with 1 X 10^{-6} M fMLP. (b) 0.2 mM xanthine was incubated with 6.5 X 10^{-3} units/ml xanthine oxidase in RPMI containing 0.05% BSA. 5 X 10^{-5} M luminol was present in both experiments.

FIGURE 10


a
200 counts
4 min
fMLP
Luminol
W256 Cells
b
200 counts
4min
Xanthine Oxidase
c
Medium
Luminol

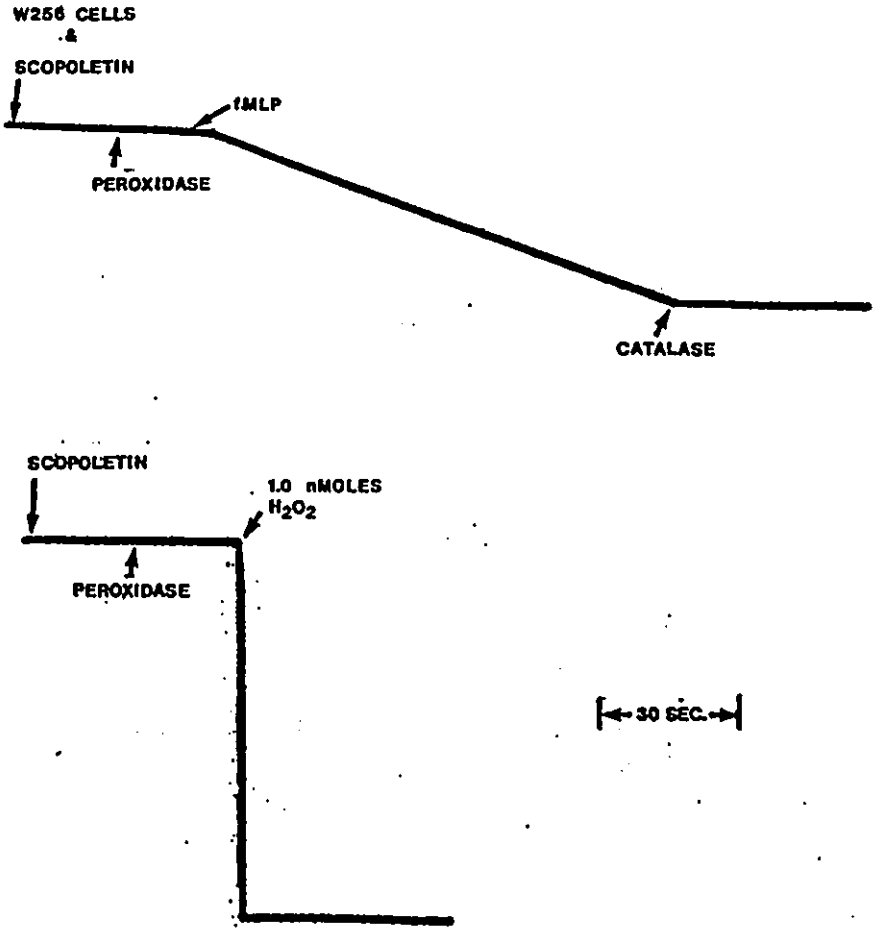
oxidase alone. In contrast to xanthine oxidase chemiluminescence which decreased rapidly after the initial burst of chemiluminescence, W256 cell chemiluminescence was sustained for greater than one hour. Xanthine oxidase chemiluminescence declined to one third its initial peak value within 10 minutes (Figure 10).

3.1.1.2 The loss of scopoletin fluorescence following chemotactic activation of Walker 256 tumour cells:

Scopoletin is a fluorescent compound which, when activated by light at 350 nm, emits light at 460 nm (Root et al.,1975). In the presence of horseradish peroxidase the fluorescence of scopoletin is diminished in direct proportion to the concentration of hydrogen peroxide found in solution (Root et al.,1975; De la Harpe and Nathan, 1985). Scopoletin fluorescence has been previously employed to quantitate the generation of hydrogen peroxide by neutrophils (Root et al.,1975) and macrophages (Nathan and Root, 1977; De la Harpe and Nathan,1985). Unlike increases in luminol-promoted chemiluminescence, the loss of scopoletin fluorescence is quantitative. We found that the fluorescence of scopoletin was decreased when 5 X 10^6 W256 cells were activated with 10^{-6} M fMLP in the presence of 10 units of horseradish peroxidase (Figure 11). Under these

Figure 11. The effect of W256 cells on scopoletin fluorescence. Scopoletin fluorescence was monitored on a Perkin - Elmer Fluorescence Spectrophotometer (model MPF-44). W256 cells, were suspended at a concentration of 5 X 10^6 cells/ml in phosphate buffered saline pH 7.4, containing 4.8 mM KCl, 0.54 mM $CaCl_3$, and 1.2 mM $MgCl_2$ and added to 1 ml cuvettes. The activating light was set at 350 nm and emission fluorescence measured at 460 nm (Root et al.,1975; De la Harpe and Nathan,1985). Fluorescence was recorded continuously following the addition of 10 nmoles scopoletin, 22 nM horseradish peroxidase and 10^{-6} M fMLP respectively (Root et al.,1975; De la Harpe and Nathan, 1985) (Panel A). The system was standardized in the absence of cells by the addition of known amounts of hydrogen peroxide (Panel B).

Figure 11.


W256 CELLS
&
SCOPOLETIN
fMLP
PEROXIDASE
CATALASE
SCOPOLETIN
1.0 nMOLES
H_2O_2
PEROXIDASE
30 SEC.

conditions, fMLP-activated W256 cells were shown to generate 18 nmoles of H_2O_2/hr/5 X 10^6 cells. The loss of fluorescence was inhibited in a dose-dependent manner by the addition of catalase (Table 6). Maximum inhibition was observed at a concentration of 2820 units/ml of catalase.

3.1.1.3 The formation of cerium chloride precipitates by Walker 256 tumour cells following chemotactic stimulation:

Since the W256 ascites typically contains contaminating neutrophils at the level of 1 to 2%, we were concerned that the neutrophils might be responsible for the observed luminol-promoted chemiluminescence in our assays. Although we had not found a correlation between the magnitude of the chemiluminescence response and the number of neutrophils in a given W256 cell preparation, this was indirect evidence (Leroyer et al.,1987). We therefore sought more direct evidence that W256 cells are capable of producing free radicals. This was obtained by morphology. When $CeCl_3$ was added to W256 ascites preparations following fMLP activation, electron dense precipitates were observed both intracellularly and on the tumour cell surface (Figure 12). $CeCl_3$ reacts with hydrogen peroxide to form the electron dense cerium perhydroxide (Ce-$[OH]_2$ OOH) (Briggs et

Table 6. Effect of catalase on the tumour cell-dependent loss of scopoletin fluorescence.

Catalase Concentration (Units/ml)	nMOLES H_2O_2 Generated /hr/5 X 10^6 Cells	
	Activated W256 Cells	Unactivated W256 Cells
0	18.0 ± 1.0	≤ 2.0 ± 0.7[a]
940	10.2 ± 0.5[a]	—
1880	4.2 ± 0.3[a]	—
2820	2.0 ± 0.5[a]	—

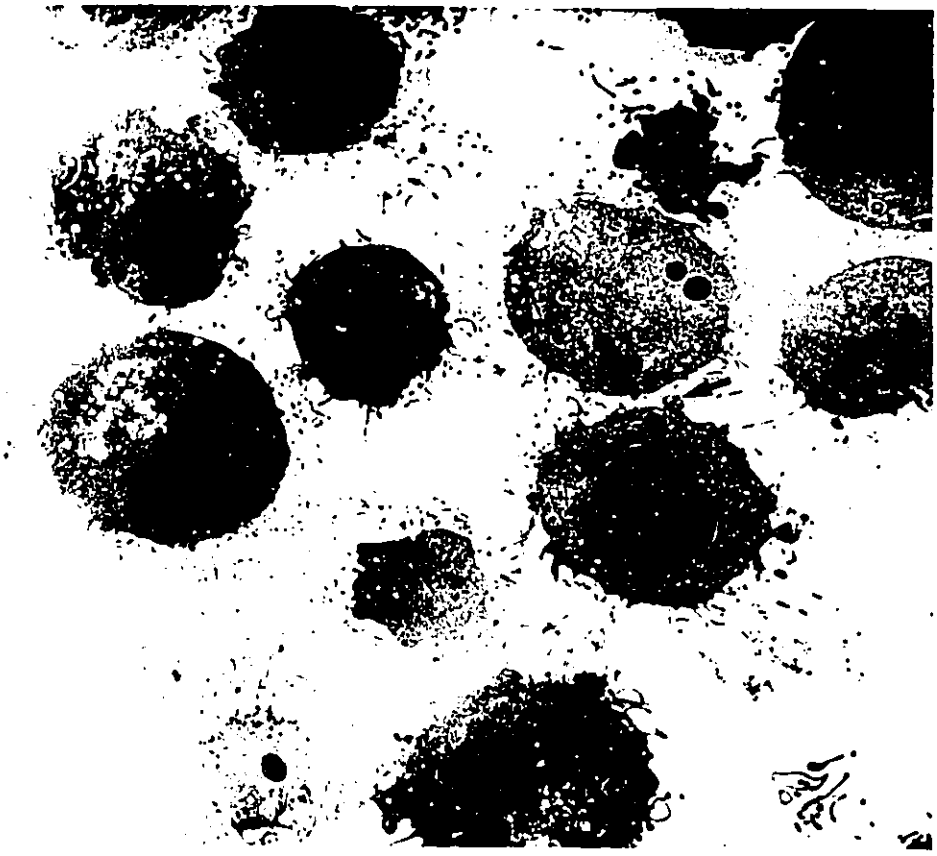
W256 cells, were suspended to a concentration of 5 X 10^6 cells/ml in phosphate buffered saline pH 7.4, containing 4.8 mM KCl, 0.54 mM $CaCl_3$, and 1.2 mM $MgCl_2$. Fluorescence was recorded continuously following the addition of 10 nmoles scopoletin, 22nM horseradish peroxidase, 10^{-6} M fMLP and increasing concentrations of catalase.

[a] $p < 0.05$ when compared to H_2O_2 generated by activated W256 cells in the absence of catalase.

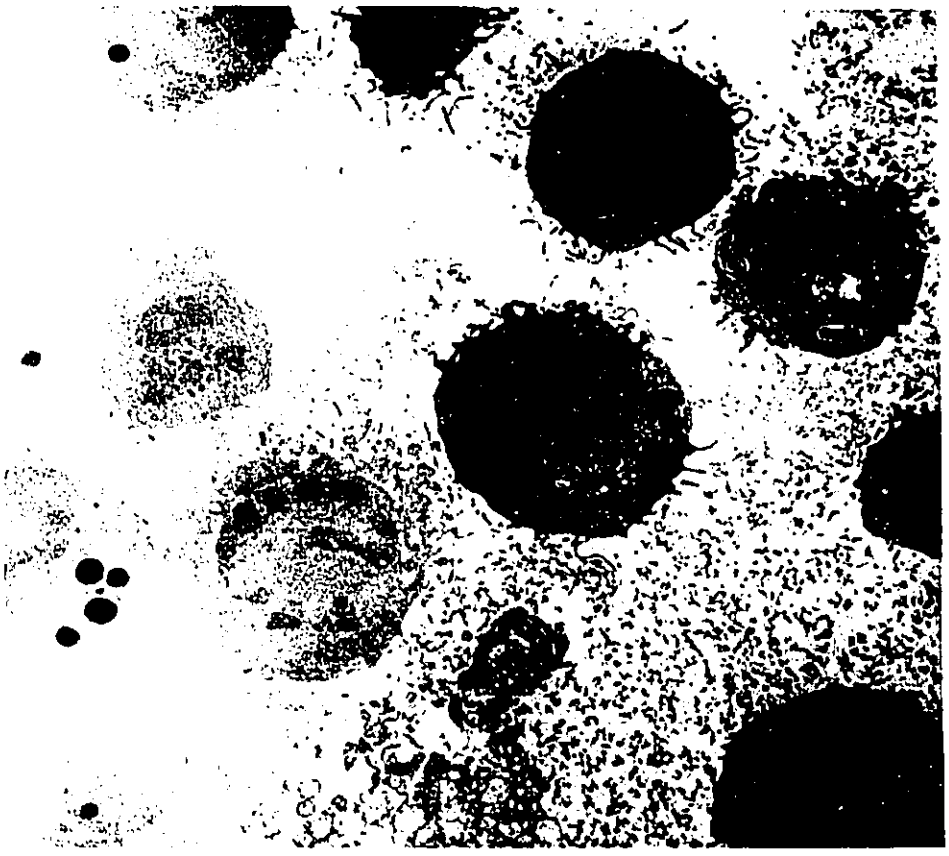
Figure 12. The formation of $CeCl_3$-derived precipitates (arrow) by fMLP-activated (Panel A) and unactivated W256 cells (Panel B). W256 cells were suspended at a concentration of 5 X 10^6 cells/ml in 0.1 M Tris-maleate buffer pH 7.5, containing 7% sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/ml glucose. Following a 2 minute incubation at 37°C in the presence or absence of 10^{-6}M fMLP, the cells were pelleted, resuspended in the above buffer containing 1 mM $CeCl_3$ and incubated at 37°C for a further 60 minutes. The samples were then fixed at room temperature by the addition of an equal volume of 4% formaldehyde and 5% glutaraldehyde in cacodylate buffer and prepared for transmission electron microscopy. [x 2500].

Figure 12

A)



B)



al,1975; Vissers et al.,1985). Figure 12 illustrates that the majority of $CeCl_3$-derived precipitates were found associated with tumour cell pseudopodia or with the cell surface when the W256 cells were activated with 10^{-6}M fMLP. Some deposits were also located intracellularly and appeared to be associated with vesicles. Unactivated W256 cells appeared to have the majority of their precipitate located intracellularly within vesicles.

In the presence of $CeCl_3$, 82 ± 4% of fMLP activated W256 cells demonstrated electron dense deposits on their cell surface and intracellularly (Table 7). Upon the addition of 2000 units/ml of catalase this was reduced to 38 ± 6%. In the absence of 10^{-6} M fMLP only 5.5 ± .3% of the cell population were positive for CeCl3-derived precipitates. Catalase addition did not reduce this value. Activated W256 cells also demonstrated the greatest amount of precipitate on their cell surface. Following the addition of 10^{-6}M fMLP, the W256 cells demonstrated a 28 fold increase in number of CeCl3-derived precipitates per unit surface area. In the presence of 2000 U/ml catalase this was reduced by 50 %. In the presence of catalase or in the absence of fMLP the majority of $CeCl_3$-derived precipitates were found intracellularly.

Table 7. Localization of $CeCl_3$-derived precipitates on Walker 256 tumour cells.

Conditions	% Positive Cells	# Of CeCl3 Precipitates /Unit Surface Area	Ratio Of $CeCl_3$ Precipitates On The Cell Surface Vs Inside The Cell
Activated W256 cells	82.0 ± 4.0	14.1 ± 2.3	10.1 ± 1.8
Activated W256 cells plus catalase	38.0 ± 6.0[a]	6.7 ± 1.7[a]	0.7 ± 0.2[a]
Unactivated W256 cells	5.5 ± 0.3[a]	0.7 ± 0.9[a]	0.3 ± 0.1[a]
Unactivated W256 cells plus catalase	16.7 ± 8.7[a]	1.0 ± 0.4[a]	0.3 ± 0.2[a]

W256 cells were suspended to a concentration of 5 X 10^6 cells/ml in 0.1 M Tris-maleate buffer pH 7.5, containing 7% sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/ml glucose. Following a 2 minute incubation at 37°C in the presence or absence of 10^{-6}M fMLP, the cells were pelleted, resuspended in the above buffer containing 1 mM $CeCl_3$ and incubated at 37°C for a further 60 minutes. Catalase was included in some assays at a concentration of 2000 units/ml.

[a] $p < 0.05$ when compared to the number of $CeCl_3$-derived precipitates associated with the W256 cells in the presence of fMLP.

3.1.2 Platelets generate reactive oxygen species following stimulation with thrombin

A considerable body of evidence exists suggesting an involvement of platelets in metastasis. Briefly, cancer cell emboli are often found surrounded by platelets at sites of vessel wall attachment (Jones et al.,1971; Crissman et al.,1988). In addition, the metastatic potential of some cancer cell lines has been correlated with their ability to activate platelets, ie., induction of thrombocytopenia *in vivo* and/or platelet aggregation *in vitro* (Pearlstein et al., 1984; Fasco et al.,1988). Activated platelets are thought to enhance metastasis by facilitating the attachment of tumour cells to blood vessel walls, by inducing vasopermeability, by sequestering cancer cells from host immune surveillance and by generating platelet-derived growth factors i.e., PDGF and TGF-β (Menter et al.,1987). In addition, since human platelets have been suggested to generate reactive oxygen species, *in vitro* (Marcus et al.,1977; Larsen et al.,1989), one could postulate that platelets, localized at sites of cancer cell attachment, promote metastasis by damaging the endothelium. However, like the W256 cell suspensions, preparations of human platelets often contain host leukocytes and it was therefore not clear in previous experiments, whether the platelets or the contaminating leukocyte population were producing the

reactive oxygen species. Thus we also used the $CeCl_3$ technique to determine if activated platelets could generate reactive oxygen species.

3.1.2.1 Chemiluminescence responses:

To confirm that preparations of human platelets were capable of producing reactive oxygen species, we incubated human platelets (2 X 10^5 platelets/μl) with either 2.5 units/ml of thrombin or 1 X 10^{-6} M PMA. A chemiluminescence response was observed within 1 minute after the addition of thrombin or phorbol ester to freshly isolated platelet suspensions incubated in the presence of 5 X 10^{-5} M luminol (Figure 13). The chemiluminescence burst peaked within 2 minutes and was sustained for an additional 2-4 minutes after which it began to decay (Figure 13). Baseline values for chemiluminescence were not reached within a 20-30 minute period.

The chemiluminescence response of activated human platelets was dependent upon both the concentration of thrombin or phorbol ester used to activate the platelets as well as the platelet concentration. Dose response curves were obtained for thrombin and for PMA demonstrating half-maximal responses at concentrations of 2.5 X 10^{-2} units/ml of thrombin and 5 X 10^{-9} M phorbol myristate acetate (Figure 14a and b). Chemiluminescence was observed to increase with

Figure 13. Chemiluminescence response of human platelets.

Washed human platelets were suspended in Tyrode's albumin (8 g/L NaCl, 0.2 g/L KCL, 1 g/L $NaHCO_3$, 0.05 g/L NaH_2PO_4, 0.2 g/L MgCl2, 0.44 g/L CaCl2, 3.5g/L BSA, 1.0 g/L glucose; pH 7.35) at a final concentration of 2.0 X 10^5 platelets/μl. Chemiluminescence was observed in the presence of 5 X 10^{-5}M luminol (L), when 2.5 units/ml of thrombin (T) was added to a 2 ml suspension of the human platelets.

Figure 13.

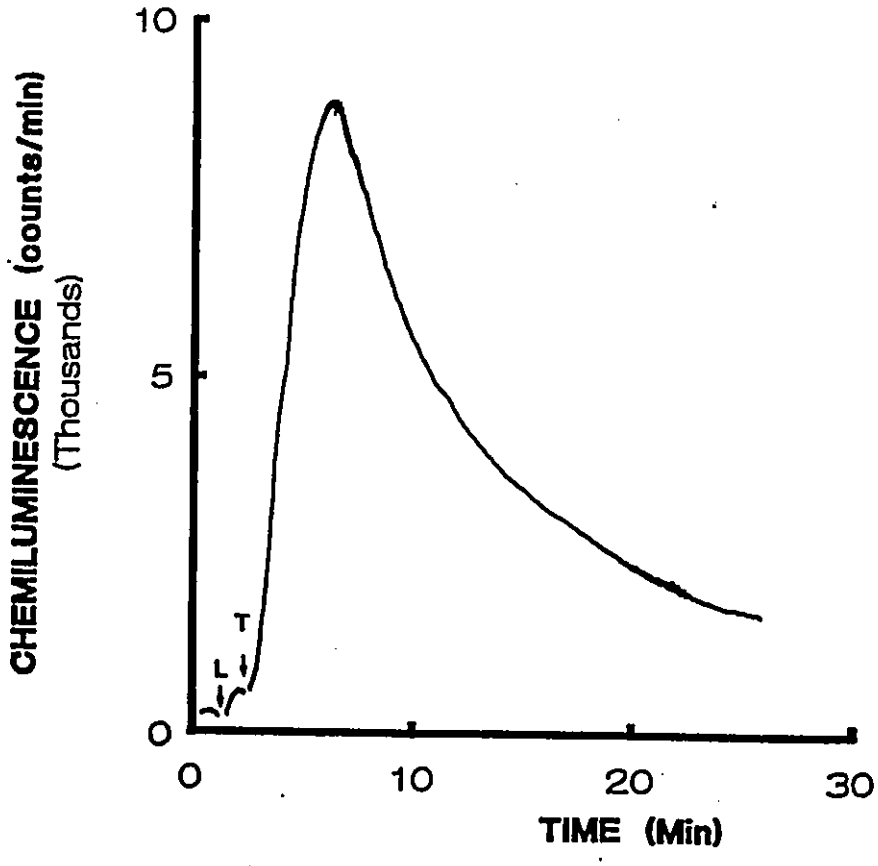
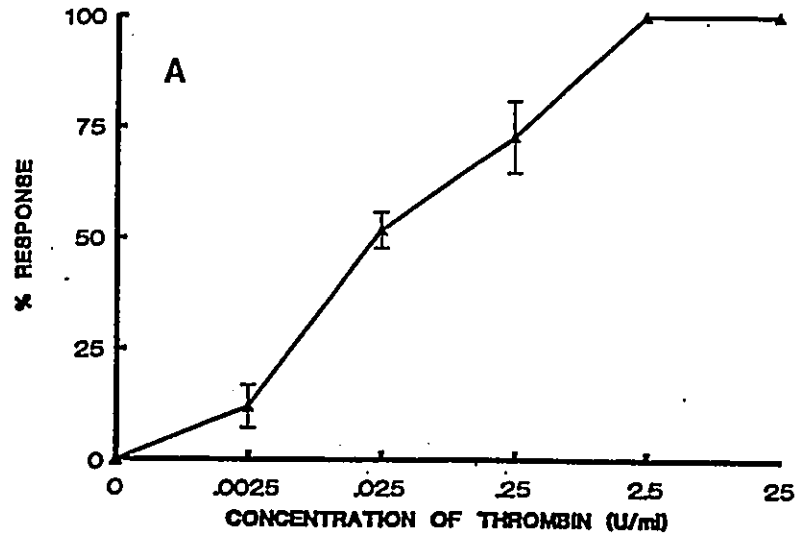
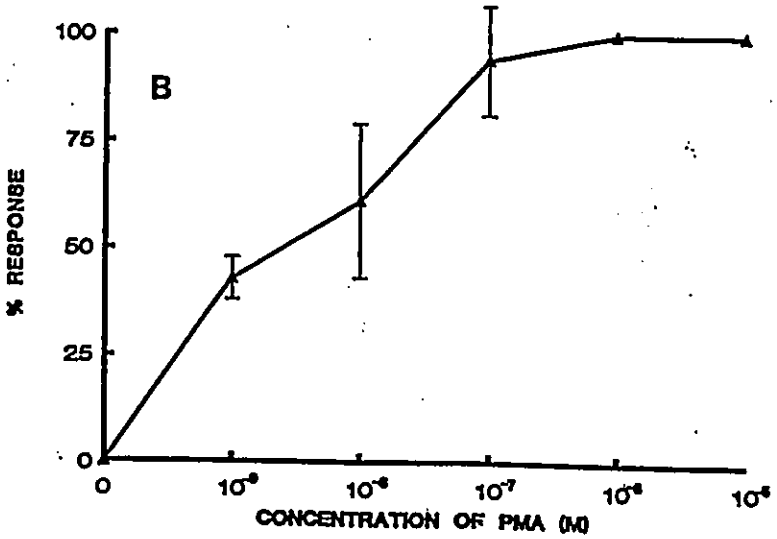

CHEMILUMINESCENCE (counts/min)
(Thousands)
10
5
0
L
T
0
10
20
30
TIME (Min)

Figure 14. Effect of thrombin (Panel A) and phorbol myristate acetate concentration (Panel B) on the chemiluminescence response of human platelets. Responses were obtained from 2 ml volumes of Tyrode's albumin containing 2 X 10^5 platelets/μl and 5 X 10^{-5} M luminol.

Figure 14a and b





increasing platelet concentrations (Figure 15).

These responses were inhibited by the free radical scavengers catalase, mannitol, and superoxide dismutase (Table 8), which suggested that the platelet chemiluminescence response may have been due to the production of oxygen-derived free radicals. The greatest inhibitory effects were obtained with mannitol and superoxide dismutase, which inferred that production of O_2^- and OH· contributed significantly to the response.

3.1.2.2 Morphological evidence for free radical production by platelets:

To confirm that free radicals could be produced by the platelets, we employed the $CeCl_3$ precipitation technique (Briggs et al.,1975; Vissers et al., 1985). In the presence of endothelial cell monolayers, unstimulated platelets were found attached to the monolayer as isolated platelets or in small aggregates (usually 2 -5 platelets) and only occasional platelets were associated with cerium precipitates. The number of precipitate grains associated with each platelet was small (Figure 16, Table 9). In contrast, thrombin stimulation resulted in the formation of large platelet aggregates which were adherent to the

Figure 15. The effect of platelet concentration on the magnitude of the chemiluminescence response. Responses were obtained following stimulation with 2.5 units/ml of thrombin in the presence of 5×10^{-5} M luminol. Data are expressed as a mean ± standard error.

Figure 15

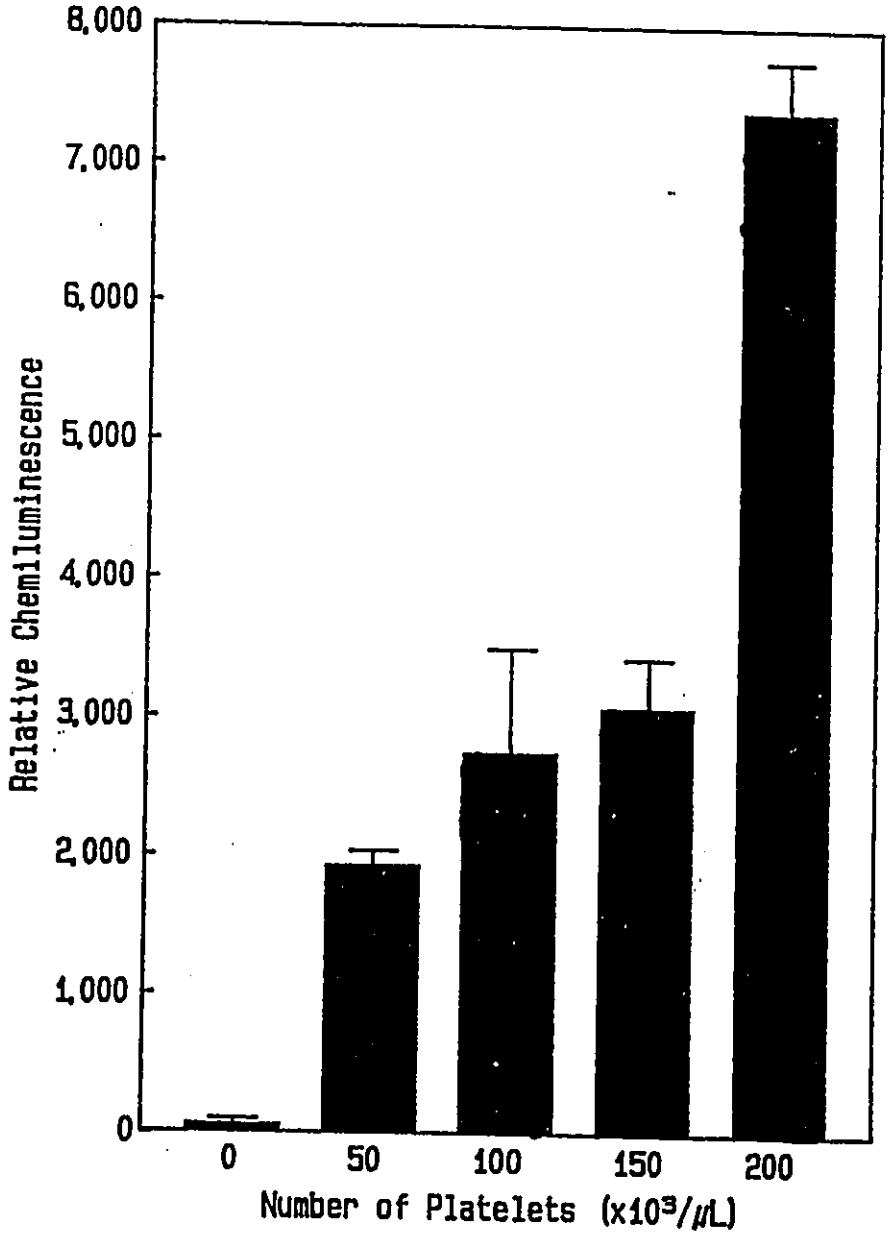

8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
Relative Chemiluminescence
0
50
100
150
200
Number of Platelets (x10³/μL)

TABLE 8: Inhibition of platelet chemiluminescence by free radical scavengers.

Inhibitor	Percent Inhibition of Baseline Response	
	Experiment A	Experiment B
No inhibitor	0 ± 3	0 ± 2
Catalase (2000 u/ml)	13 ± 6	5 ± 1
Mannitol (1 mM)	48 ± 6[a]	32 ± 1[a]
SOD (1mg/ml)	---	47 ± 2[a]

Responses were obtained from 2 ml volumes of Tyrode's albumin containing 2 X 10^5 platelets/μl and 5 X 10^{-5} M luminol. The scavengers catalase (2000 units/ml), Mannitol (1 mM) and superoxide dismutase (1 mg/ml) were added before the addition of 2.5 units/ml of thrombin. Data is expressed as a mean ± standard error.

[a] $P < 0.05$ when compared to the positive control.

Table 9. Quantification of $CeCl_3$-derived precipitates associated with control and stimulated platelets

Treatment	% Positive Platelets[1]	Grains/Platelet
Control	7 ± 7	1.9 ± 1.9
Thrombin	93 ± 3[a]	10.4 ± 2.5[a]
Thrombin + Mannitol	37 ± 11[b]	2.9 ± 0.7[b]

Platelets were suspended at a concentration of 2 X 10^5 platelets/μl in 1ml of Tyrodes albumin and stimulated with 2.5 units/ml of thrombin. Mannitol was used in these experiments at a concentration of 1mM.

[1] a platelet was considered positive if one or more grains were found associated with the surface.

[a] Values significantly greater than negative control ($p<0.05$).

[b] Values significantly less than after thrombin stimulation alone ($p<0.05$).

Figure 16. Platelet aggregation and the formation of $CeCl_3$-derived precipitates by thrombin-activated (Panel A) and unactivated platelets (Panel B). Washed human platelets, suspended in Tyrode's albumin (8 g/l NaCl, 0.2 g/l KCL, 1 g/l $NaHCO_3$, 0.05 g/l NaH_2PO_4, 0.2 g/l $MgCl_2$, 0.44 g/l $CaCl_2$, 3.5g/l bovine serum albumin, 1.0 g/l glucose; pH 7.35) at a final concentration of 2.0 X 10^5 platelets/μl (total volume 0.5ml), were added to monolayers of human umbilical vein endothelial cells. In some experiments the platelets were first activated by the addition of 2.5 units/ml of thrombin. 0.5 ml of 1mM $CeCl_3$ in 0.1 M Tris-maleate buffer pH 7.5 was then added and the platelets/endothelial cells incubated at 37°C for 60 minutes. The samples were then fixed at room temperature by the addition of an equal volume of 4% formaldehyde and 5% glutaraldehyde in cacodylate buffer pH 7.4 and prepared for transmission electron microscopy as described in the Materials and Methods section. [x 5000].

endothelial monolayer and most of the platelets within these aggregates had large numbers of $CeCl_3$-derived grains associated with their surfaces (Figure 16). In the presence of mannitol, the number of detectable $CeCl_3$-derived grains was reduced (Table 9).

3.2 The effect of reactive oxygen species on endothelial cell monolayers

Microvascular walls consisting of endothelium and basement membrane, act as a barrier to both the intravasation and extravasation of metastatic cancer cells. Using several different models of lung injury it was demonstrated that damage to the pulmonary endothelium significantly increased the metastasis of intravenously injected tumour cells (Adamson et al.,1986; Adamson et al.,1987; Orr and Warner, 1987). In these models, injury to the endothelium was postulated to involve the generation of reactive oxygen species however, the possibility that the generation of reactive oxygen species may promote metastasis has not been tested previously. We were therefore interested in determining whether reactive oxygen species generated by the xanthine-xanthine oxidase system or the W256 cell could injure endothelial cell monolayers *in vitro*.

3.2.1 Assays of the endothelial cell response to xanthine-xanthine oxidase

In order to determine if W256 cells could injure endothelial cells, we first determined the effect of exposing endothelial cell monolayers to the reactive oxygen

generating system xanthine - xanthine oxidase. Several different assays were employed to monitor the response of endothelial cells to xanthine oxidase. These included scanning electron microscopy, the release of either ^{3}H-2-deoxyglucose or ^{51}chromate from prelabeled endothelial cell monolayers and LDH release.

Luminol-promoted chemiluminescence was employed to determine if the generation of reactive oxygen species increased with increasing xanthine oxidase concentration. In the presence of 0.2 mmole xanthine, chemiluminescence was dependent upon the presence of xanthine oxidase in concentrations ranging from 6.5×10^{-3} units to 52×10^{-3} units/ml (Table 10). Chemiluminescence increased in a progressive manner with increasing xanthine oxidase concentrations. Chemiluminescence began to decrease at concentrations of xanthine oxidase $\leq 104 \times 10^{-3}$ units. Comparatively, in the presence of fMLP, 5×10^{6} tumour cells/ml produced a level of luminol-promoted chemiluminescence approximately equivalent to that generated by 6.5×10^{-3} units/ml xanthine oxidase. Xanthine oxidase concentrations ranging from 3.0×10^{-3} to 48×10^{-3} units were therefore choosen to assess the response of human umbilical cord endothelial cells to xanthine-xanthine oxidase exposure.

Scanning electron microscopy revealed substantial retraction of endothelial cell monolayers following exposure

Table 10. Relative levels of xanthine oxidase-dependent luminol-promoted chemiluminescence

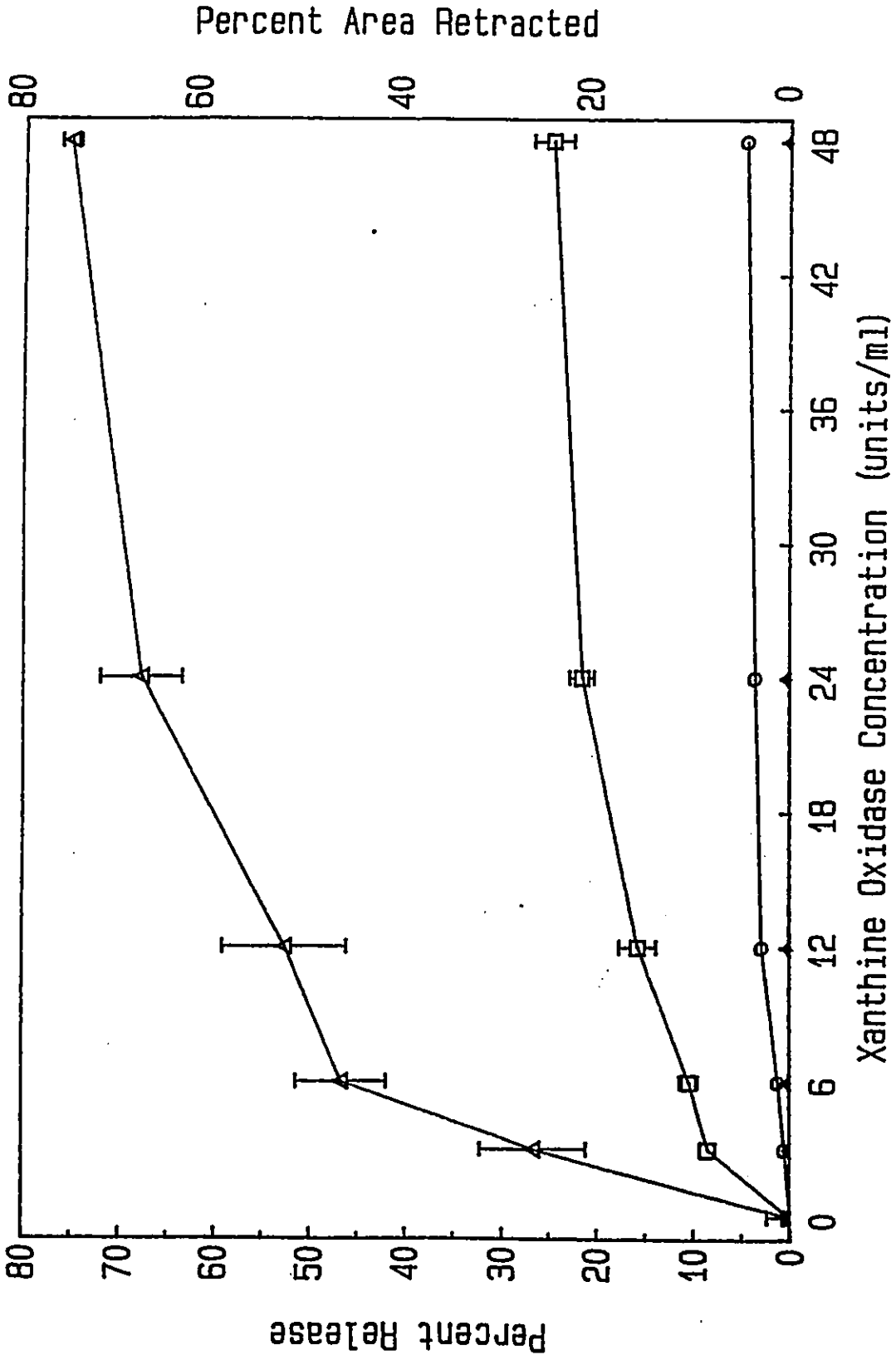
Xanthine Oxidase Concentration (units/ml)	Fluorescent Counts ($\times 10^{-2}$/2 minutes) Exp. 1	Exp. 2
6.5×10^{-3}	59.9 ± 12.8	42.5 ± 9.4
13.0×10^{-3}	130.8 ± 14.9	89.3 ± 9.7
26.0×10^{-3}	145.6 ± 4.9	98.1 ± 5.1
52.0×10^{-3}	224.4 ± 12.4	156.7 ± 2.8
104.0×10^{-3}	186.8 ± 1.5	127.8 ± 6.2

Chemiluminescence was determined by incubating 0.2 mM xanthine with increasing concentrations of xanthine oxidase in RPMI 1640 containing 0.05% bovine serum albumin. 5×10^{-5} M luminol was present in the experiments.

to xanthine-xanthine oxidase at concentrations ranging from 3.0 X 10^{-3} to 48 X 10^{-3} units/ml. Significant retraction of the endothelium was observed following exposure to as little as 3.0 X 10^{-3} units/ml xanthine oxidase (Figure 17). Endothelial retraction rose in a progressive manner with increasing concentrations of xanthine oxidase and correlated directly with xanthine oxidase generated chemiluminescence ($r = 0.97$).

To further confirm that reactive oxygen species could affect endothelial cells, human endothelial cell monolayers prelabeled with ^{3}H-2-deoxyglucose were exposed to xanthine oxidase. Significant release of ^{3}H-2-deoxyglucose from human umbilical cord endothelial cells was observed at concentrations of xanthine oxidase between 3.0 X 10^{-3} and 48 X 10^{-3} units (Figure 17). In contrast, the ^{51}chromate release assay did not detect significant ($p > 0.01$) release of isotope at concentrations of xanthine oxidase less than 12 X 10^{-3} units/ml. No significant release of lactate dehydrogenase occured at even the highest concentrations of xanthine oxidase (Figure 17). Increasing the xanthine oxidase concentration to 104 X 10^{-3} units had no further effect on ^{3}H-2-deoxyglucose release. Specific ^{3}H-2-deoxyglucose release reached levels of approximately 50% at these concentrations of xanthine oxidase. ^{3}H-2-deoxyglucose release correlated directly with xanthine oxidase-generated chemiluminescence ($r = 0.91$).

Figure 17: A comparison of endothelial cell retraction (Δ), ^{3}H-2-deoxyglucose release (□), ^{51}chromate release (○), and lactate dehydrogenase release (▲), following a 90 minute incubation of endothelial cell monolayers with increasing concentrations of xanthine-xanthine oxidase. The endothelial cell monolayers were cultured overnight with 1 mM buthionine sulfoximine. The assay medium contained xanthine (0.2 mM), soybean trypsin inhibitor (5 mg/ml),and increasing concentrations of xanthine oxidase in RPMI 1640 and bovine serum albumin (0.5 mg/ml).


Percent Area Retracted
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Xanthine Oxidase Concentration (units/ml)
Percent Release
80
70
60
50
40
30
20
10
0

3.2.1.1 The effect of catalase and buthionine sulfoximine (BSO) on xanthine oxidase-promoted release of ^{3}H-2-deoxyglucose:

The effect of xanthine-xanthine oxidase on the endothelium was mediated primarily by xanthine oxidase generated hydrogen peroxide. Xanthine oxidase-mediated ^{3}H-2-deoxyglucose release and endothelial cell retraction were completely inhibited by the addition of 2000 units/ml of catalase. The addition of heat inactivated catalase to the assays did not block the observed responses (Table 11). Although endothelial cell retraction was found to be a more sensitive measure of the endothelial cell response to xanthine oxidase - generated hydrogen peroxide, the release of ^{3}H-2-deoxyglucose was used as our standard assay in subsequent experiments with the W256 cell. Both the cost of scanning electron microscopy and the time involved in completing a single experiment made the ^{3}H-2-deoxyglucose release assay a more practical assay when measuring the response of endothelial cells to W256 cell - generated hydrogen peroxide.

Tumour cell-dependent ^{3}H-2-deoxyglucose release from human umbilical cord endothelial cells was only observed when the assays were conducted in the presence of 1 mM buthionine sulfoximine (BSO) on cells which had been

Table 11. The effect of catalase on xanthine oxidase-mediate endothelial cell responses

Condition	Percent Above Negative Control		
	^{3}H-2-Deoxyglucose Release	Area Retracted	^{51}Chromate Release
Medium Alone	0.0 ± 1.9	0.0 ± 1.1	0.0 ± 0.2
Xanthine Oxidase	14.4 ± 0.8[a]	48.0 ± 4.1[a]	1.0 ± 0.2
Xanthine Oxidase plus Catalase	0.0 ± 3.5[b]	2.5 ± 5.0[b]	0.6 ± 0.2
Xanthine Oxidase plus Heat Inactivated Catalase	15.4 ± 1.8[a]	50.2 ± 4.5[a]	1.5 ± 0.4

The assay medium contained xanthine (0.2 mM), xanthine oxidase (6.5 10^{-3} units/ml) and soybean trypsin inhibitor (5 mg/ml) at the give concentrations in RPMI 1640 and bovine serum albumin (0.5 mg/ml) Catalase was used in these experiments were indicated at concentration of 2000 units/ml.

[a] $p < 0.05$ when compared to values obtained in the absence of xanthin oxidase

[b] $p < 0.05$ when compared to values obtained in the presence of xanthin oxidase

precultured in 1mM BSO. Both overnight pretreatment of the endothelial cell monolayer and the continued presence of BSO in the reaction media appeared necessary to observe the phenomenum. Under these conditions the specific release of ^{3}H-2-deoxyglucose found in the presence of 6.5 X 10^{-3} units xanthine oxidase was increased from nondetectable levels to 21%. This increase was dependent upon the concentration of BSO (Table 12).

3.2.2 Walker 256 tumour cell-mediated isotope release

Figure 18 illustrates the effect of cancer cell concentration and cell activation state on the specific release of ^{3}H-2-deoxyglucose from human umbilical cord endothelial cells. Maximum values for ^{3}H-2-deoxyglucose release were observed when fMLP - activated W256 cells were incubated with the monolayers. ^{3}H-2-deoxyglucose specific release increased from undetectable levels at a cell concentration of 1.0 X 10^{6} W256 cells/ml to a maximum of 30% at a cell concentrations greater than 2.0 X 10^{6} cells/ml (Figure 18). In the absence of fMLP, there was a small but significant increase in ^{3}H-2-deoxyglucose release at cell concentrations greater than or equal to 4.0 X 10^{6} cells/ml. The incubation of fMLP with endothelial cells in the absence of tumour cells failed to cause an increase in the specific release of ^{3}H-2-deoxyglucose. At concentrations greater than

Table 12. The effect of buthionine sulfoximine on xanthine oxidase-promoted ^{3}H-2-deoxyglucose release.

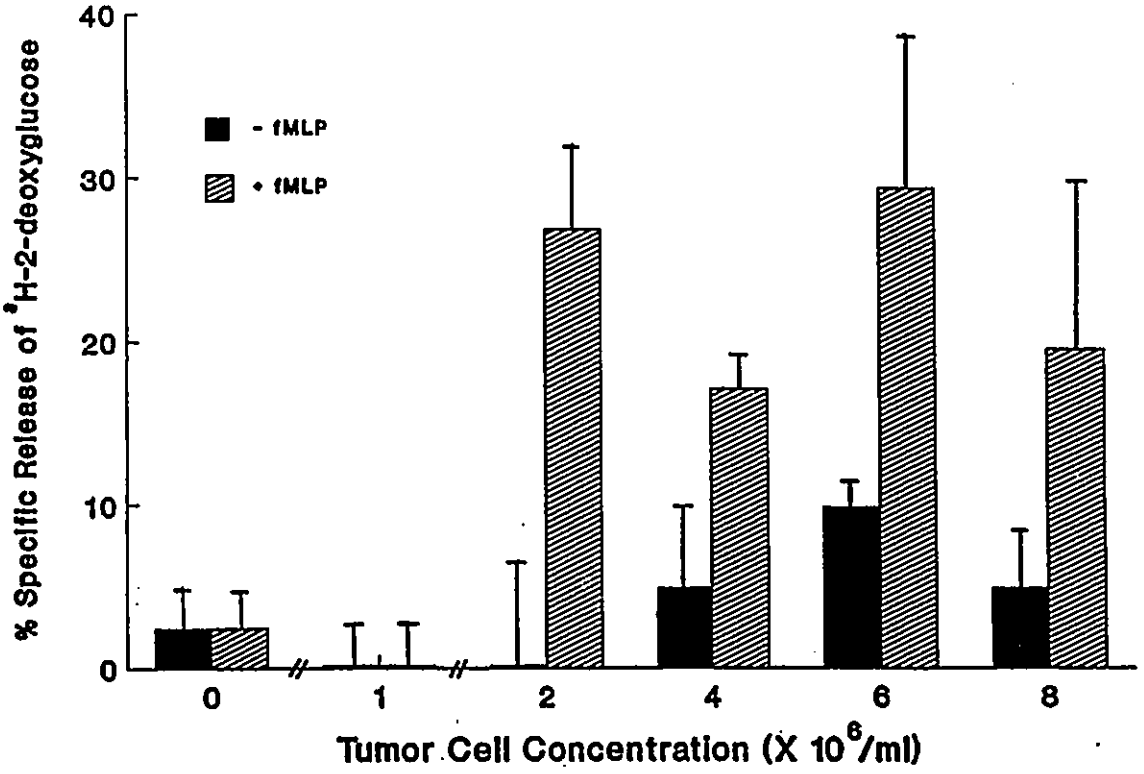
Buthionine Sulfoximine Concentration	Percent Specific ^{3}H-2-Deoxyglucose Release
None	0 ± 2
10 uM	0 ± 3
100 uM	12 ± 6
1.0 mM	21 ± 3[a]

The endothelial cell monolayer was pretreated overnight with varying concentrations of buthionine sulfoximine. The assay medium contained xanthine (0.2 mM), xanthine oxidase (6.5×10^{-3} units/ml), soybean trypsin inhibitor (5 mg/ml), buthionine sulfoximine at the given concentrations in RPMI 1640 and bovine serum albumin (0.5 mg/ml). Isotope release in control cultures was 35 ± 2%.

[a] $p < 0.05$ when compared to release of isotope from endothelium alone.

Figure 18: The effect of W256 cell concentration on the release of ^{3}H-2-deoxyglucose from prelabeled endothelial cells. Endothelial cells were cultured overnight with 1 mM buthionine sulfoximine and 1 μCi/ml ^{3}H-2-deoxyglucose. W256 cells were incubated with the endothelium for 90 minutes in the presence or absence of 1 X 10^{6}M fMLP in RPMI 1640 plus 0.5 mg/ml bovine serum albumin. Isotope release in control cultures was 42 ± 1%.

Figure 18.


40
30
20
10
0
- fMLP
+ fMLP
% Specific Release of ^{3}H-2-deoxyglucose
0
1
2
4
6
8
Tumor Cell Concentration (X 10^6/ml)

2.0 X 10^6 tumour cells/ml, the endothelial cell monolayer appeared completely covered by tumour cells as viewed by light microscopy. It has been calculated that maximum levels of ^{3}H-2-deoxyglucose release were thus obtained with a W256 to endothelial cell ratio between 5-7 : 1. In contrast, significant release of ^{51}chromate was not observed in any experiments regardless of the number of W256 cells tested.

Since, in most experiments, W256 tumour cells were obtained in the form of an ascites, we assessed the contribution of host leukocytes to ^{3}H-2-deoxyglucose release. Cytospins were prepared and differential cell counts performed for each experiment. In a total of 17 experiments, no correlation was found between the extent of ^{3}H-2-deoxyglucose release and the number of contaminating neutrophils ($r = 0.154$). Further, cultured W256 cells stimulated with 10^{-6} M PMA although devoid of contaminating neutrophils, still induced ^{3}H-2-deoxyglucose release from endothelial cells to the same extent as did W256 cells isolated as an ascites. The cultured W256 cells also promoted low levels of ^{3}H-2-deoxyglucose release from endothelial cell monolayers in the absence of PMA (Table 13).

Table 13. The effect of cultured tumour cells on W256 tumour cell promoted ^{3}H-2-deoxyglucose release.

Endothelial Treatment	Expt.1	Expt.2
Phorbol-12-myristate,13-acetate	---	1.0 ± 4.3
Unactivated W256 cells	6.3 ± 4.3	7.8 ± 1.2
Activated W256 cells	19.1 ± 5.2[a]	28.8 ± 3.6[a]

W256 cells obtained from an ascites tumour were cultured in BGJ medium with 10% fetal calf serum and 1% antibiotics for three days prior to the experiment. The culture flask was changed following the first and second day to remove adherent leukocytes. W256 cells were then activated with 1 X 10^{-6}M phorbol myristate acetate since fMLP fails to activate cultured W256 cells (Leroyer et al.,1987). Isotope release in control cultures was 34 ± 2% and 39 ± 1% for experiments one and two respectively.

[a] $p < 0.05$ when compared to release of isotope from endothelium alone.

3.2.2.1 The effect of catalase and superoxide dismutase on W256 tumour cell-promoted release of ^{3}H-2-deoxyglucose

The release of ^{3}H-2-deoxyglucose from prelabeled endothelial cell monolayers, associated with the addition of 5.0 X 10^{6} activated W256 tumour cells/ml was unaffected by the addition of superoxide dismutase at concentrations of 0.1 or 1.0 mg/ml (Table 14). In contrast, the addition of 1000 units/ml of catalase resulted in near complete inhibition of this phenomenon. Catalase concentrations of 100 units/ml did not have an observable effect on tumour cell promoted ^{3}H-2-deoxyglucose release (Table 14).

3.2.2.2 The requirement for cell contact:

Table 15 compares the effect of adding intact tumour cells to endothelial monolayers with the effect of adding the supernatant of these cells. W256 cells were incubated in medium for 90 minutes in the presence or absence of 10^{-6} M fMLP. The cells were then pelleted and the supernatant added to endothelial monolayers to determine its ability to increase ^{3}H-2-deoxyglucose release from them. In parallel cultures, tumour cells from the same population were incubated directly with the endothelium as in the previous experiments. As shown in Table 15, the addition of W256

Table 14. The effect of superoxide dismutase and catalase on tumour cell - promoted ^{3}H-2-deoxyglucose release.

Scavenger	Stimulated W256
None	19.2 ± 4.2
Superoxide dismutase (0.1 mg/ml)	17.2 ± 5.6
Superoxide dismutase (1.0 mg/ml)	18.4 ± 2.3
Catalase (100 U/ml)	25.1 ± 9.5
Catalase (1000 U/ml)	3.3 ± 4.0[a]

Isotope release in control cultures was 24 ± 1%.
In the presence of unstimulated W256 cells the specific release of isotope was 0.8 ± 3.9[a].
[a] $p < 0.05$ when compared to release of isotope in the presence of stimulated W256 cells.

Table 15. Inability of cell-free supernatants to induce release of ^{3}H-2-deoxyglucose from labeled endothelial cells.

Conditions	Percent Specific ^{3}H-2-Deoxyglucose Release
Intact unactivated W256 cells	6.0 ± 2.0
Intact activated W256 cells	16.0 ± 1.0[a]
Supernatant of unactivated W256 cells	3.2 ± 2.0
Supernatant of activated W256 cells	0.0 ± 3.5

W256 cells in suspension culture at a concentration of 5 X 10^{6} cells/ml were activated with 1 X 10^{-6} M fMLP for 90 minutes. The cells were then pelleted and the cell free supernatants added to the labeled endothelial cell monolayers to assess ^{3}H-2-deoxyglucose release. In parallel experiments intact cells from the same original population were added to endothelial cultures. Isotope release in control cultures was 36 ± 2%.

[a] $p < 0.05$ when compared to release of isotope from endothelium alone.

supernatants to endothelial cells failed to significantly increase ^{3}H-2-deoxyglucose release, indicating that a soluble factor released from the tumour cells was not responsible for promoting the release of isotope.

Additional experiments appeared to indicate that contact between the cancer cell and endothelial target was required in order to observe isotope release. Tumour cells were preincubated with 1 μM cytochalasin B before being assayed for their ability to promote ^{3}H-2-deoxyglucose release. Table 16 illustrates the percent inhibition of ^{3}H-2-deoxyglucose release from endothelial monolayers coincubated with cytochalasin B-treated W256 cells. The results from these two experiments clearly indicate that ^{3}H-2-deoxyglucose release is reduced substantially when the tumour cells are pretreated with cytochalasin B. This is in direct contrast to the observed chemiluminescence response of fMLP-activated W256 cells pretreated with cytochalasin B; the pretreatment of W256 cells with 1 μM cytochalasin B, prior to fMLP activation, increased the level of chemiluminescence three fold (Leroyer et al.,1987). Scanning electron micrographs of tumour cells adhered to washed endothelial cell monolayers demonstrated fewer adherent W256 cells when the tumour cells were pretreated with cytochalasin B (Figure 19). Attached cytochalasin B-treated tumour cells had a distorted morphology with a reduction in the number of cytoplasmic processes. These

Table 16. The effect of cytochalasin B treatment of Walker 256 cells on the release of 3H-2-deoxyglucose from endothelial cell monolayers.

	Inhibition of 3H-2-Deoxyglucose Release After Cytochalasin B Treatment	
	(Exp. 1)	(Exp. 2)
Unactivated W256 cell	56 ± 10%[a]	23 ± 17%
Activated W256 cells	55 ± 14%[a]	13 ± 6%[a]

W256 cells were treated with 1 μM cytochalasin B for 10 min, washed, and tested for their ability to promote ^{3}H-2-deoxyglucose release. Inhibition of release was calculated with reference to release caused by W256 cells that had not been treated with cytochalasin B. Activated W256 cells promoted 24% specific isotope release in experiment 1 and 28% in experiment 2.

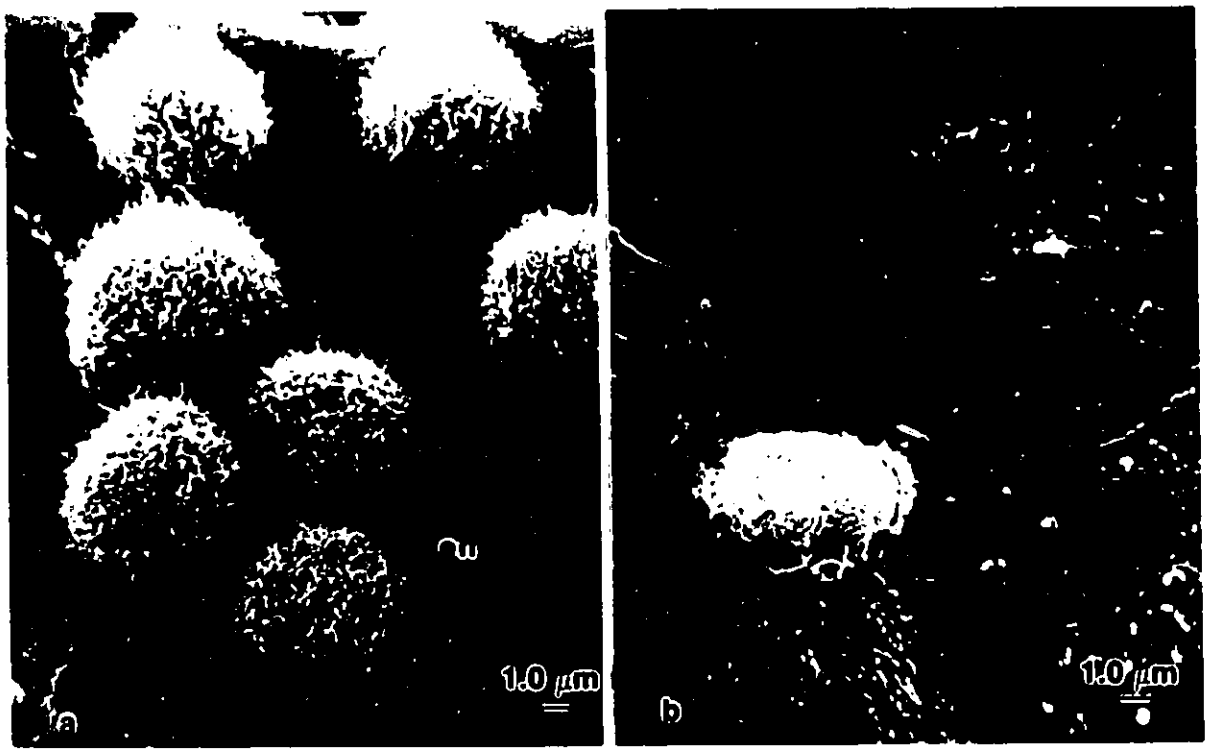
[a] $p < 0.1$ when compared to isotope release promoted by untreated W256 cells.

Figure 19: Scanning electron micrographs of untreated and cytochalasin B treated W256 cells adherent to endothelial cell monolayers. W256 cells were treated with 1μM cytochalasin B for 10 min.,washed, and incubated with endothelial cell monolayers for 90 min. The discs on which the endothelial monolayers were grown were then removed, washed in RPMI medium to remove nonadherent W256 cells, and fixed in 2% glutaraldehyde and 0.1M cacodylate buffer in preparation for electron microscopy.

A/ Untreated W256 cells attached to the endothelial cell monolayer.

B/ Cytochalasin B-treated W256 cell still attached to the endothelial cell monolayer.

Figure 19



combined data suggested that tumour cell attachment to the endothelium was required inorder to mediate the release of isotope.

3.3 The effect of tumour cell adhesion on ^{3}H-2-deoxyglucose release:

In the previous section I demonstrated that activated Walker 256 carcinosarcoma cells could promote the release of ^{3}H-2-deoxyglucose from endothelial cell monolayers. The release of isotope was dependent upon the generation of reactive oxygen species and appeared to be dependent upon the adhesion of W256 cells to endothelial cell monolayers. In the experiments described in this section, I confirm this latter observation directly by demonstrating that both W256 cell-mediated release of ^{3}H-2-deoxyglucose and W256 cell adhesion were partly dependent upon the expression of the vitronectin receptor on the endothelial cell surface.

3.3.1 The effect of W256 cell and endothelial cell activation on tumour cell adhesion and ^{3}H-2-deoxyglucose release

Previous studies have shown that cytokine pretreatment of endothelial cell monolayers increases their

adhesiveness for malignant melanoma and carcinoma cell lines (Rice et al.,1988; Lauri et al.,1989). To determine if the release of ^{3}H-2-deoxyglucose was dependent upon the adhesion of W256 cells to endothelial cell monolayers we treated the endothelial cell monolayers with rIL-1α and assessed both the adhesion of W256 cells and W256 cell-mediated release of isotope.

The adhesion of W256 cells to endothelial cell monolayers was found to be dependent upon the tumour cell activation state and upon cytokine pretreatment of the endothelial cell monolayers (Table 17). Maximum values for adhesion were observed when PMA-activated W256 cells were incubated with monolayers that had been pretreated with 10 ng/ml rIL-1α. When W256 cells were activated by the addition of 10^{-6} M PMA, tumour cell adhesion to the endothelial cell monolayers was increased over control values by 89 ± 35%, $p < 0.05$ (Table 17). Pretreatment of the endothelial cell monolayers with rIL-1α increased the adhesion of PMA-activated tumour cells by an additional 80 ± 13%, $p < 0.005$. The adhesion of unactivated W256 cells to endothelium was not significantly increased by rIL-1α pretreatment of the endothelium alone.

In separate experiments, W256 cells were incubated with ^{3}H-2-deoxyglucose-labelled human endothelial cell monolayers that had been pretreated with rIL-1α. These experiments demonstrated that the release of ^{3}H-2-

Table 17. The effect of PMA and IL-1 on W256 cell adhesion and ^{3}H-2-deoxyglucose release.

Culture Condition	Adherent W256 Cells (X 10^{-4} cells)[1]	^{3}H-2-Deoxyglucose Release (% specific release)[1]
Medium only	------	0 ± 2.1
W256 cells only	1.48 ± 0.16	10.0 ± 2.7
IL-1 treated endothelium	1.76 ± 0.15	12.2 ± 3.3
PMA treated W256 cells	2.79 ± 0.52	20.1 ± 2.7
IL-1 + PMA treatment	5.03 ± 0.39[a]	28.6 ± 2.4[b]

W256 cells obtained from an ascites were cultured in BGj medium with 10% fetal calf serum and 1% antibiotic for 2 days before the experiments. W256 cells used in the adhesion assay were radioactively labeled by the addition of 0.5 μCi/ml of ^{125}I-iododeoxyuridine to the culture medium on day one. Both the W256 cell adhesion assay and the ^{3}H-2-deoxyglucose release assay were performed at 37°C for 90 minutes with 0.5 ml of cultured W256 cells suspended to a concentration of 5 X 10^{6} cells/ml. In some experiments the endothelial cells were pretreated for 4 hours with 10 ng/ml of interleukin-1α and the W256 cells activated with 10^{-6} M phorbol myristate acetate since fMLP fails to activate cultured W256 cells.

[1] Data are expressed as mean ± SEM of four experiments.

[a] $p < 0.005$ when compared with adhesion of PMA-activated W256 cells to untreated monolayers.

[b] $p < 0.05$ when compared with isotope release promoted by PMA-activated W256 cells from untreated monolayers.

deoxyglucose was also dependent upon the tumour cell activation state and cytokine pretreatment of the endothelial cell monolayers. Isotope release was greatest when PMA-activated W256 cells were incubated with rIL-1α pretreated monolayers (Table 17). The specific release of isotope was increased from 10 ± 3% to 20 ± 3% by PMA activation of the tumour cells, $p < 0.01$. rIL-1α-pretreatment of the monolayers further increased the specific release of isotope from 20 ± 3% to 29 ± 2%, $p < 0.05$.When unactivated W256 cells were added to the assay, pretreating the endothelial cell monolayers with rIL-1α had no significant effect on isotope release. In the absence of W256 cells, neither the addition of PMA nor the pretreatment of monolayers with rIL-1α caused a significant increase in the release of isotope. The release of ^{3}H-2-deoxyglucose correlated directly with tumour cell adhesion ($r = 0.98$, $P < 0.005$).

3.3.2 The adherence of Walker 256 tumour cells to endothelial cell monolayers is mediated by vitronectin receptor

Treatment of endothelial cell monolayers with cytokines increases their adhesiveness for cancer cells by inducing the expression of a defined set of adhesive moieties on the endothelial cell surface (Bevilacqua et

al.,1987; Rice and Bevilacqua,1989; Lafrenie et al., in press). Two previously described examples of cytokine inducible moieties involved in tumour cell/endothelial cell adhesion are INCAM-110 (VCAM-1) and ELAM-1 (Bevilacqua et al.,1987; Rice and Bevilacqua,1989). Recently, studies in our laboratory have suggested that the adhesion of A549 human lung carcinoma cells to rIL-1α pretreated monolayers was mediated by the expression of vitronectin receptor (VnR) (Lafrenie et al.,1990; Lafrenie et al, in press). We therefore examined the effect of anti-human VnR antiserum on W256 cell adhesion.

3.3.2.1 Effect of anti-vitronectin receptor antisera on Walker 256 tumour cell adhesion:

Addition of anti-human VnR antiserum to the adhesion assay inhibited W256 cell attachment to endothelial cell monolayers. The inhibitory effects of anti-human VnR antiserum on W256 cell adhesion to the endothelium were dose-dependent (Table 18). Neither the addition of normal rabbit serum (NRS) nor anti-human fibronectin receptor antiserum (FnR) significantly affected W256 cell adhesion to the endothelium (Table 19).

Maximum inhibition was observed in the presence of anti-human VnR antiserum at a titre of 1:3000. At this concentration of antiserum, the adhesion of unactivated W256

Table 18. The effect of anti-vitronectin receptor antiserum on W256 cell adhesion.

Condition	Titre Of VnR	Adherent W256 Cells (X 10^{-3})	
		Duration of Adhesion	
		45 minutes	90 minutes
W256 cells only	Control	0.9 ± 0.1	17.2 ± 1.2
	1:5000	0.8 ± 0.1	14.3 ± 0.7
	1:4000	1.0 ± 0.1	———
	1:3000	0.8 ± 0.1	9.9 ± 2.4a
IL-1-treated endothelium	Control	1.8 ± 0.2	19.6 ± 1.5
	1:5000	1.6 ± 0.4	15.8 ± 1.5
	1:4000	1.7 ± 0.2	———
	1:3000	1.4 ± 0.2	10.3 ± 0.7a
PMA-treated W256 cells	Control	5.4 ± 0.6	21.5 ± 2.8
	1:5000	4.8 ± 0.5	20.6 ± 0.7
	1:4000	3.8 ± 0.4a	———
	1:3000	2.6 ± 0.4a	15.7 ± 1.7a
IL-1 + PMA treatment	Control	20.4 ± 1.0	51.2 ± 2.9
	1:5000	17.0 ± 1.9	44.9 ± 6.9
	1:4000	14.5 ± 0.4a	———
	1:3000	12.0 ± 2.3a	31.4 ± 1.1a

^{125}I-iododeoxyuridine-labeled W256 cells, suspended to a concentration of 5 X 10^6 cells/ml, were incubated with human endothelial cell monolayers for varing lengths of time in the presence of anti-human vitronectin receptor antiserum (VnR). In some experiments the endothelial cells were pretreated for 4 hours with 10 ng/ml of interleukin-1α and the W256 cells activated with 10^{-6}M phorbol myristate acetate.

[a] $p < 0.05$ when compared with tumour cell adhesion from corresponding positive controls.

TABLE 19. The effect of normal rabbit serum and anti-fibronectin receptor antiserum on W256 tumour cell adhesion.

	Adherent W256 Cells ($\times 10^{-4}$)			
	W256 Cells Only	rIL1-α Treated Endothelium	PMA-Treated W256 Cells	rIL-1α + PMA Treatment
Control	0.7 ± 0.1	1.5 ± 0.3	4.2 ± 0.4	5.8 ± 1.3
Normal rabbit serum (1:3000)	1.0 ± 0.2	1.4 ± 0.3	4.2 ± 0.8	6.1 ± 1.5
Control	1.1 ± 0.2	1.2 ± 0.2	3.2 ± 0.6	3.9 ± 0.7
Anti-FnR (1:3000)	0.8 ± 0.1	1.1 ± 0.1	3.1 ± 0.5	4.0 ± 0.3
Control	1.7 ± 0.1	2.0 ± 0.2	2.2 ± 0.3	5.1 ± 0.3
Anti-VnR (1:3000)	1.0 ± 0.2[a]	1.0 ± 0.1[a]	1.5 ± 0.2[a]	3.1 ± 0.1[a]

^{125}I-iododeoxyuridine-labeled cultured W256 cells were incubated with human endothelial cell monolayers for 90 minutes in the presence of normal rabbit serum, anti-human fibronectin receptor antiserum (FnR) and anti-human vitronectin receptor antiserum (VnR). In some experiments the endothelial cells were pretreated for 4 hours with 10 ng/ml of interleukin-1α and the W256 cells activated with 10^{-6}M phorbol myristate acetate.

[a] $p < 0.05$ when compared with tumour cell adhesion from corresponding positive controls.

cells to untreated monolayers was inhibited by 42 ± 14%, p = 0.005. Adhesion to rIL-1α pretreated monolayers was inhibited by 47 ± 3%, p = 0.005. The adhesion of W256 cells activated with 10^{-6} M PMA was also inhibited by the addition of antisera to VnR. The adherance of activated W256 cells to untreated monolayers was inhibited by 27 ± 3%, while adhesion to rIL-1α pretreated-monolayers was inhibited by 39 ± 2%, p < 0.005 (Table 19).

3.3.2.2 The effect GRGDSP peptide on Walker 256 tumour cell adhesion:

We examined the effect of adding GRGDSP peptide to the adhesion assay in order to confirm that an integrin receptor was involved in the attachment of W256 cells to the endothelium. Several integrin receptors eg. $\alpha_V\beta_3$ the VnR, appear to identify their respective ligands by recognizing the tripeptide sequence arginine-glycine-aspartic acid (RGD). Addition of the GRGDSP peptide to the adhesion assay, at a concentration of 100 μM, inhibited W256 cell attachment to endothelial cell monolayers. The adherence of unactivated W256 cells to untreated monolayers was inhibited by 27 ± 6 %, while adhesion to rIL-1α pretreated monolayers was inhibited by 15 ± 3 %, p < 0.05 (Table 20). The adhesion of W256 cells, activated with 10^{-6} M PMA, was also inhibited by the addition of the GRGDSP peptide. The adherence of activated W256 cells to untreated monolayers was inhibited

Table 20. The effect of GRGDSP peptide on W256 tumour cell adhesion

Condition	Adherent W256 Cells (x 10^4)		
	No peptide	GRGDSP peptide	GRGESP peptide
W256 cells only	0.8 ± 0.1	0.6 ± 0.1[a]	0.9 ± 0.1
IL-1-treated endothelium	1.2 ± 0.1	1.0 ± 0.1[a]	1.7 ± 0.3
PMA-treated W256 cells	4.0 ± 0.2	3.0 ± 0.5[a]	4.2 ± 0.4
IL-1 + PMA treatment	9.7 ± 0.8	4.4 ± 1.0[a]	8.5 ± 1.1

^{125}I-iododeoxyuridine-labeled cultured W256 cells were incubated with human endothelial cell monolayers for 90 minutes in the presence of either, 100 μM glycine-arginine-glycine-aspartic acid-serine-proline (GRGDSP) or 100 μM glycine-arginine-glycine-glutamic acid-serine-proline (GRGESP). In some experiments the endothelial cells were pretreated for 4 hours with 10 ng/ml of interleukin-1α and the W256 cells activated with 10^{-6}M phorbol myristate acetate.

[a] $P < 0.05$ when compared with tumour cell adhesion from corresponding positive controls.

by 25 ± 12 %, while adhesion to rIL-1α pretreated monolayers was inhibited by 55 ± 10 %, $p < 0.05$. Under the various assay conditions, addition of the control peptide GRGESP (100 μM) did not affect W256 cell adhesion to the endothelium (Table 20).

3.3.2.3 Modulation of vitronectin receptor (VnR) expression by rIL-1α

Analysis with immunofluorescence flow cytometry demonstrated that only the endothelial cells and not the W256 cells expressed VnR on their cell surface (Figure 20, upper panel, peak 2 vs lower panel peak 2). VnR expression was still not detectable on W256 cells following their incubation with 10^{-6} M PMA for 90 minutes. However, rIL-1α pretreatment of the endothelial cells for 4 hr was found to increase VnR expression (Figure 20, upper panel peak 2 vs 3). Endothelial cell surface expression of the positive control α2-microglobulin, was uneffected by rIL-1α pretreatment.

The results of immunofluorescence flow cytometry were confirmed with antibody binding studies. Figure 21 demonstrates that VnR can be detected on the surface of endothelial cells in the absence of rIL-1α pretreatment. However, VnR expression was increased in a time - dependent manner following the addition of 10 ng/ml of rIL-1α to

Figure 20. Increased vitronectin receptor expression on rIL-1α pretreated endothelial cells as measured by immunofluorescence flow cytometry. In the upper pannel, endothelial cells were grown to confluency in 75 cm^2 flasks. Some monolayers were pretreated with 10 ng/ml rIL-1α for four hours prior to harvesting and processing the cells for immunofluorescence flow cytometry. In the lower panel, W256 cells were maintained in BGJ_b medium with 10% fetal calf serum and 1% antibiotics for 2-3 days prior to the experiment. Some W256 cells were activated with 1 X 10^{-6}M PMA for 90 minutes prior to harvesting and processing the cells for immunofluorescence flow cytometry. Upper pannel, peak 1, untreated endothelial cells with normal rabbit serum; Peak 2, untreated cells with anti-VNR antiserum; Peak 3, rIL-1α - pretreated cells with anti-VNR antiserum. Lower panel, peak 1, untreated W256 cells with normal rabbit serum; Peak 2, untreated cells with anti-VNR antiserum.

FIG. 20

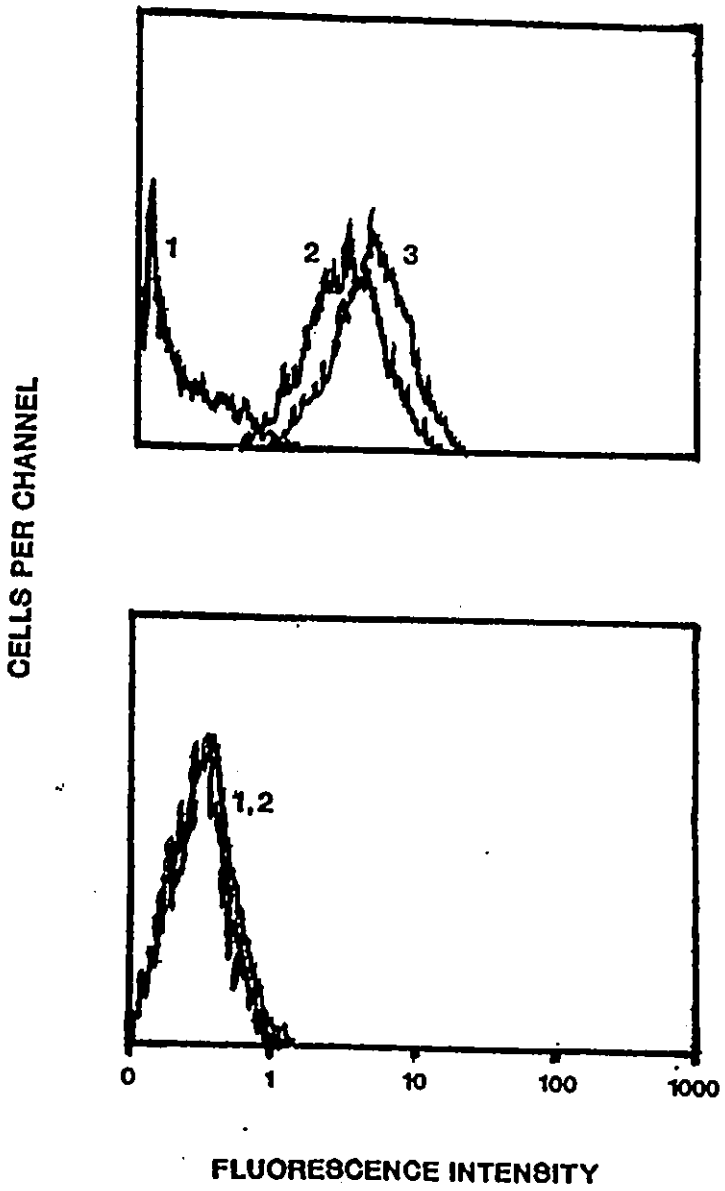
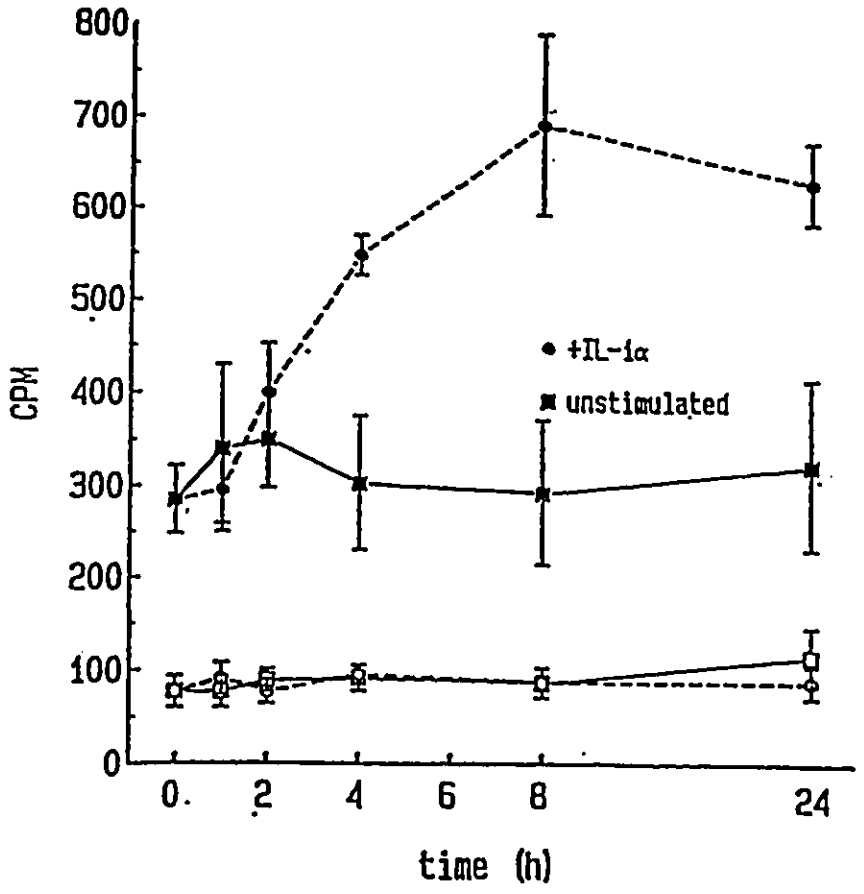

1
2
3
1,2
CELLS PER CHANNEL
0
1
10
100
1000
FLUORESCENCE INTENSITY

Figure 21. Time course for rIL-1α induction of vitronectin receptor expression on human endothelial cells. The monolayers were incubated with 10 ng/ml rIL-1α or an equal volume of medium for various periods of time. They were then incubated for 30 minutes at 4°C with either rabbit anti-VnR polyclonal antiserum at a titre of 1:8000 (closed symbols) or normal rabbit serum (open symbols) at an equivalent titre. Following this incubation, the monolayers were incubated a further 30 minutes with ^{125}I-goat anti-rabbit F(ab)$_2$ fragments (1 mCi/ml) as described in Material and Methods.

Figure 21.


800
700
600
500
400
300
200
100
0
CPM
+IL-1α
unstimulated
0.
2
4
6
8
24
time (h)

endothelial cell monolayers. By this technique rIL-1α pretreatment of the endothelial cells for 4 hrs was found to increase VnR expression 1.9 ± 0.1 fold.

3.3.3 Effect of anti-human vitronectin receptor (VnR) antisera on the W256 cell - mediated release of ^{3}H-2-deoxyglucose from endothelial monolayers

Since W256 cell-dependent ^{3}H-2-deoxyglucose release had correlated directly with tumour cell adhesion ($r = 0.98$) we examined the effect of anti-human VnR antiserum on the release of isotope from prelabeled monolayers. Tumour cell-dependent ^{3}H-2-deoxyglucose release was also inhibited by the addition of anti-human VnR antisera (Table 21). In the presence of unactivated W256 cells, ^{3}H-2-deoxyglucose release was completely inhibited by the addition of anti-VnR antisera, as was the release from rIL-1α-treated endothelial cells after incubation with unactivated W256 cells, $p < 0.005$. Isotope release after incubation with PMA-activated W256 cells was inhibited by 66% in the presence of anti-VnR antisera at a titre of 1 :3000, $P < 0.05$. The (maximal) damage, seen when PMA-activated W256 cells were incubated with rIL-1α - pretreated endothelial cells, was inhibited by 53% in the presence of anti-VnR, $p = 0.005$. W256 tumour cell-promoted damage was not affected by the addition of either normal rabbit serum or anti-human FnR antiserum at

Table 21. The effect of anti-vitronectin receptor antiserum on W256 cell-promoted ^{3}H-2-deoxyglucose release.

Condition	% Specific Release			
	No Antiserum	Normal Rabbit Serum	Fibronectin Receptor Antiserum	Vitronectin Receptor Antiserum
Medium Only	0.0 ± 5.3	0.0 ± 2.1	0.0 ± 2.5	0.0 ± 1.9
W256 cells Only	9.4 ± 2.6	11.2 ± 5.3[a]	10.8 ± 6.6[a]	0.0 ± 1.8[b]
IL-1-treated endothelium	7.9 ± 3.5	2.6 ± 4.0[a]	11.3 ± 5.3[a]	0.0 ± 2.7[b]
PMA-treated W256 cells	19.6 ± 3.5	17.2 ± 3.8[a]	18.9 ± 5.4[a]	6.7 ± 4.4[b]
IL-1 + PMA treatment	27.3 ± 3.2	20.9 ± 5.1[a]	22.3 ± 5.3[a]	12.9 ± 3.5[b]

Cultured W256 cells suspended at a concentration of 5 X 10^{6} cells/ml were incubated with ^{3}H-2-deoxyglucose-labeled human endothelial cell monolayers for 90 minutes in the presence of normal rabbit serum, anti-human fibronectin receptor antiserum or anti-human vitronectin receptor antiserum at a titre of 1:3000. In some experiments the endothelial cells were pretreated for 4 hours with 10 ng/ml of interleukin-1α and the W256 cells activated with 10^{-6}M phorbol myristate acetate.

[a] $p > 0.1$ when compared with release of isotope from corresponding positive controls.
[b] $p < 0.05$ when compared with release of isotope from corresponding positive controls.

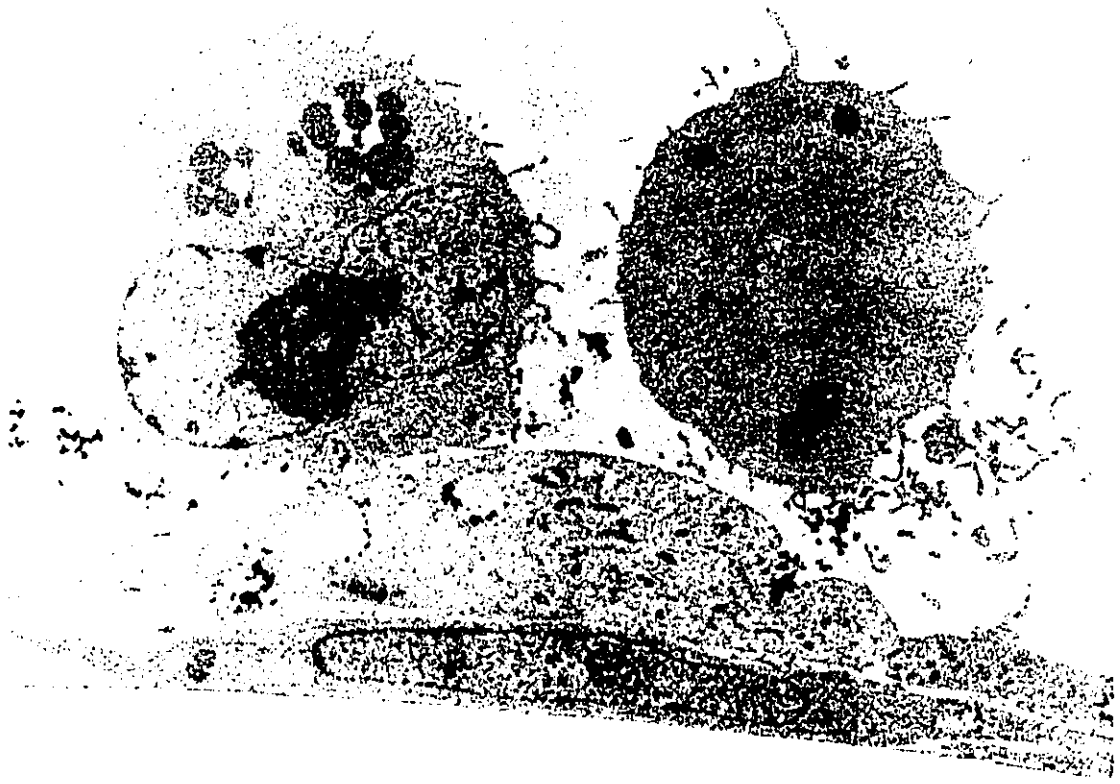
equivalent titres (Table 21).

3.3.4 The detection of CeCl3 precipitates at sites of tumour cell attachment to the endothelium:

The interactions of W256 cells with the endothelium, following a 90 minute incubation, was examined by electron microscopy (Figure 22). $CeCl_3$ was added to the assay in order to observe the generation of hydrogen peroxide. Electron microscopy at sites of tumour cell attachment clearly demonstrated that the tumour cells had adhered to the endothelial cells. In addition, $CeCl_3$-derived precipitates could be observed at sites of tumour cell attachment (Figure 22).

Figure 22. The formation of $CeCl_3$-derived precipitates at sites of W256 cell attachment to endothelial cell monolayers. 0.5 ml of cultured W256 cells, suspended at a concentration of 5 X 10^6 cells/ml in RPMI containing 0.05% bovine serum albumin, were added to human endothelial cell monolayers. Following a 2 minute incubation at 37°C in the presence or absence of 10^{-6}M PMA, an additional 0.5 ml of RPMI containing 0.05% bovine serum albumin and 1 mM $CeCl_3$ was added. The W256 cells and endothelial cells were then incubated together at 37°C for a further 60 minutes. The samples were then fixed at room temperature by the addition of an equal volume of 4% formaldehyde and 5% glutaraldehyde in cacodylate buffer. [x 2500].

Figure 22.



3.4 The effect of reactive oxygen generation on basement membrane degradation

To leave the circulation, blood borne cancer cells must penetrate both the vascular basement membrane and the endothelium. Morphologic studies of metastasis have demonstrated dissolution of the basement membrane at sites of cancer cell/vessel wall attachment (Vlodavsky et al.,1982; Crissman et al,1988) and the ability of cancer cells to degrade basement membrane constituents in vitro has been correlated with their malignancy (Liotta et al.,1980; Nakajima et al.,1987). While direct free radical-mediated damage to endothelial cells has been reported in non-tumour systems (Sacks et al.,1978; Weiss et al.,1981), there is little evidence that oxygen radicals can degrade basement membranes directly. On the other hand, experiments on the acute inflammatory reaction (which shares many similarities with the metastatic cascade) have clearly shown that generation of reactive oxygen metabolites by leukocytes, enhances their proteolytic damage to basement membranes. In this case, free radicals have been postulated to function by inactivating serum protease inhibitors (Weiss and Regiani,1984) or by activating latent proteolytic enzymes

(Weiss et al.,1985; Shah et al.,1987).

3.4.1 Effect of Walker 256 tumour cells on the release of ^{3}H-proline from endothelial cell derived basement membranes:

When W256 cells were added to ^{3}H-proline - labeled basement membranes, the release of ^{3}H-proline was affected by the activation of the tumour cells (Table 22). Maximum values for ^{3}H-proline release were observed when fMLP-activated W256 cells were incubated with the subendothelial matrices. The release of ^{3}H-proline in response to the addition of 5 X 10^{6} W256 cells was increased from 49% to 64% by the addition of 10^{-6}M fMLP. In the absence of tumour cells, the addition of 10^{-6}M fMLP did not effect isotope release. Subendothelial matrices exposed to media alone released 36% of their ^{3}H-proline.

3.4.1.1 The effect of catalase on tumour cell-dependent release of ^{3}H-proline:

Table 23 illustrates the effect of catalase on the release of ^{3}H-proline from subendothelial matrices. The release of ^{3}H-proline associated with the addition of 5 X 10^{6} activated tumour cells was inhibited in a dose-dependent manner by the addition of catalase. The response was

Table 22. The effect of tumour cell activation state on the release of ^{3}H-proline from labeled subendothelial cell matrices.

Condition	Percent of ^{3}H-Proline Released[1]
Medium Only	36.1 ± 2.6
Medium plus fMLP	37.2 ± 1.9
Unactivated W256 cells	48.7 ± 2.5[a]
Activated W256 cells	64.3 ± 2.2[a]

Bovine pulmonary artery endothelial cells were grown in proline-free Dulbeccos MEM with 20% fetal bovine serum, 50 μg/ml ascorbic acid and 5 μCi/ml of ^{3}H-proline. ^{3}H-proline-labeled subendothelial cell matrices were obtained following lysis of the endothelial cell monolayers on day four. W256 cells were obtained from an ascites and suspended at a concentration of 5 X 10^{6} cells/ml in RPMI 1640 containing 10% fetal bovine serum. The assay was initiated by the addition of 0.5 ml of the W256 cell suspension. In some experiments the W256 cells were activated with 10^{-6}M fMLP. Following a 120 minute incubation the medium was removed and its radioactivity along with that retained in the matrix determined using a Beckman LS 1801 scintillation counter. The release of ^{3}H-proline into the assay medium was calculated as a percentage of the total radioactivity.

[1] Data are expressed as a mean ± SEM of six experiments.

[a] $p < 0.05$ when compared to release obtained in the presence of medium alone.

Table 23. The effect of catalase on tumour cell-promoted ^{3}H-proline release in the presence of serum.

Catalase Concentration (Units/ml)	Percent of ^{3}H-Proline Released[1] Activated W256 Cells	Unactivated W256 Cells
0	75.7 ± 8.4	52.0 ± 5.2[a]
250	71.5 ± 4.8	---
500	66.3 ± 6.0	---
1000	37.6 ± 4.9[a]	---
2000	37.1 ± 5.9[a]	34.7 ± 1.1[ab]
2000 (Heat Inactivated)	82.1 ± 4.4	---

W256 cells were obtained from an ascites and suspended to a concentration of 5 X 10^{6} cells/ml in RPMI 1640 containing 10% fetal bovine serum. ^{3}H-proline-labeled subendothelial cell matrices were obtained as described previously. The assay was performed at 37°C for 120 minutes in the presence of increasing concentations of catalase and 0.5 ml of the W256 cell suspension. The W256 cells were activated with 10^{-6}M fMLP. The release of ^{3}H-proline into the assay medium was calculated as a percentage of the total radioactivity in each well.

[a] $p < 0.05$ when compared to the release obtained in the presence of activated W256 cells alone.
[b] $p < 0.05$ when compared to the release obtained in the presence of unactivated W256 cells alone.
[1] Mean ± SEM of two separate experiments.
Note: release with media alone was 29.1 ± 2.3.

completely inhibited by the addition of 2000 units/ml of catalase. ^{3}H-proline release associated with the addition of unactivated W256 cells was also significantly inhibited by the addition of 2000 units/ml of catalase. Under these conditions fMLP-activated W256 cells degraded subendothelial matrices to a similar extent as unactivated W256 cells.

In these experiments the release of ^{3}H-proline associated with the addition of activated tumour cells was inhibited in a dose dependent manner by catalase. The experiments were done in the presence of 10% fetal calf serum. Since free radicals may function by inactivating serum protease inhibitors (Weiss and Regiani, 1984), or by activating latent proteolytic enzymes (Weiss et al.,1985; Shah et al.,1987) we repeated these experiments in the absence of serum. Catalase was still able to inhibit the release of ^{3}H-proline in a dose dependent manner (Table 24). Heat inactivated catalase was unable to inhibit tumour cell-promoted isotope release.

3.4.1.2 Involvement of a metalloprotease in basement membrane degradation:

Members of the metalloproteinase family share a number of characteristics: a) they degrade at least one type of collagen and b) they are inhibited by chelators of

Table 24. The effect of catalase on tumour cell-promoted ^{3}H-proline release in the absence of serum.

Catalase Concentration (Units/ml)	Percent of ^{3}H-proline Released	
	Activated W256 Cells	Unactivated W256 Cells
0	69.4 ± 3.0	50.7 ± 5.1[a]
250	40.5 ± 5.1[a]	---
500	36.1 ± 7.0[a]	---
1000	33.4 ± 7.7[a]	---
2000	42.1 ± 4.1[a]	44.8 ± 3.2[a]
2000 (Heat Inactivated)	65.4 ± 10.8	50.3 ± 2.8[a]

W256 cells were obtained from an ascites and suspended at a concentration of 5 X 10^{6} cells/ml in RPMI 1640 containing 0.05% bovine serum albumin. ^{3}H-proline-labeled subendothelial cell matrices were obtained as described previously. The assay was performed at 37°C for 60 minutes in the presence of increasing concentations of catalase and 0.5 ml of the W256 cell suspension. In some experiments the W256 cells were activated with 10^{-6} M fMLP. The release of ^{3}H-proline into the assay medium was calculated as a percentage of the total radioactivity in each well.

[a] $p < 0.05$ when compared to the release obtained in the presence of activated W256 cells alone.
Release with media alone was 33.1 ± 3.4.

divalent cations such as EDTA. We therefore determined if the addition of 1,10-phenanthroline or EDTA to our assay would inhibit the release of ^{3}H-proline. Tumour cell-dependent release of 3H-proline was inhibited by the addition of the metalloprotease inhibitors 1,10-phenanthroline and EDTA (Table 25).

Since degradation of collagen type IV could involve the secretion of a tumour cell-derived elastase we examined the effect of α1-antitrypsin, on the release of ^{3}H-proline. Tumour cell-promoted release of isotope was unaffected by the addition of α_1-antitrypsin at concentrations as high as 100 μg/ml. In addition, α_2-macroglobulin, an inhibitor of interstitial collagenase activity was ineffective in inhibiting proline release (Table 26).

3.4.2 Activation of the Walker 256 carcinosarcoma cell type IV collagenase/gelatinase proenzyme: Identification of the major conversion products following activation by organomercurials and hydrogen peroxide

Table 27 compares the effect of adding intact tumour cells to subendothelial matrices with the effect of adding the supernatant of these cells. W256 cells were incubated in medium for 60 minutes in the presence or absence of 10^{-6} M fMLP. The cells were then pelleted and the supernatant

Table 25. The effect of 1,10-Phenanthroline and EDTA on tumour cell-dependent release of ^{3}H-proline.

	Percent Release			
	1,10-Phenanthroline		EDTA	
Inhibitor Concentration (μg/ml)	Activated W256 Cells	Unactivated W256 Cells	Activated W256 Cells	Unactivated W256 Cells
0.0	58.0 ± 2.9	40.5 ± 6.4[a]	55.6 ± 4.3	40.9 ± 4.5[a]
1.0	52.9 ± 4.1	----	49.0 ± 5.3	----
10.0	42.9 ± 2.3[a]	----	43.6 ± 3.9[a]	----
100.0	39.9 ± 3.7[a]	32.0 ± 4.7[a]	40.3 ± 3.1[a]	35.5 ± 5.6[a]

W256 cells were obtained from an ascites and suspended at a concentration of 5 X 10^{6} cells/ml in RPMI 1640 containing 0.05% bovine serum albumin. ^{3}H-proline-labeled subendothelial cell matrices were obtained as described previously. The assay was performed at 37°C for 60 minutes in the presence or absence of the metalloproteinase inhibitors 1,10-phenanthroline or EDTA and 0.5 ml of the W256 cell suspension. In some experiments the W256 cells were activated with 10^{-6} M fMLP. The release of ^{3}H-proline into the assay medium was calculated as a percentage of the total radioactivity in each well.

[a] $p < 0.05$ when compared to the release obtained in the presence of activated W256 cells alone.
Release from media alone was 32.8 ± 2.6 and 26.7 ± 4.0 % for the experiments with 1,10-Phenanthroline and EDTA respectively.

Table 26. The effect of α1-antitrypsin and α2-macroglobulin on tumour cell-dependent release of ^{3}H-proline.

Inhibitor Concentration (μg/ml)	Activated W256 Cells	
	α1-Antitrypsin	α2- Macroglobulin
0.0	72.2 ± 3.0	72.2 ± 3.0
1.0	77.8 ± 3.6	66.9 ± 1.7
10.0	73.4 ± 5.4	75.6 ± 6.5
100.0	74.7 ± 2.8	70.8 ± 4.1

W256 cells were obtained from an ascites and suspended at a concentration of 5 X 10^{6} cells/ml in RPMI 1640 containing 0.05% bovine serum albumin. ^{3}H-proline-labeled subendothelial cell matrices were obtained as described previously. The assay was performed at 37°C for 60 minutes in the presence of increasing concentrations of α1-antitrypsin and α2-macroglobulin. In some experiments the W256 cells were activated with 10^{-6}M fMLP. The release of ^{3}H-proline into the assay medium was calculated as a percentage of the total radioactivity in each well. The percent release in the presence of unactivated W256 cells was 61.1 ± 4.4. Release from media alone was 43.9 ± 3.0 %.

Table 27. The ability of cell-free supernatants to induce the release of ^{3}H-proline from labeled subendothelial cell matrices.

Cell Preparation	Percent of ^{3}H-Proline Released	
	Unactivated Cells	Activated Cells
Intact W256 Cell	47 ± 1[a]	70 ± 2
Cell-Free Supernatant	53 ± 1[ab]	78 ± 10[b]

W256 cells were obtained from an ascites and suspended at a concentration of 5 X 10^{6} cells/ml in RPMI 1640 containing 0.05% bovine serum albumin. To obtain cell-free supernants, W256 cells were stimulated with 10^{-6}M PMA for 20 minutes before pelleting the cells by centrifugation. In other experiments the cells were incubated for 60 minutes in the absence of 10^{-6}M PMA before pelleting the cells. Both intact W256 cells and the cell free supernants of activated and unactivated W256 cells were incubated with ^{3}H-proline labeled subendothelial cell matrices for 60 minutes at 37°C. The release of ^{3}H-proline into the assay medium was calculated as a percentage of the total radioactivity in each well.

[a] $p < 0.05$ when compared to the release obtained in the presence of activated W256 cells.

[b] Not statistically different from values obtained in the presence of intact cells.

assayed for its ability to increase ^{3}H-proline release by incubation with subendothelial matrices for 60 minutes. In control cultures, tumour cells from the same population were incubated directly with the matrices as described above. The data was consistent with the hypothesis that W256-mediated matrix damage is caused by metalloproteases released into the culture medium. Cell free supernatants from activated cells had a degradative activity equal to that of cells in direct contact with the basement membrane.

3.4.2.1 Gelatin Zymography:

Since tumour cell-dependent release of ^{3}H-proline was inhibited by the addition of the metalloproteinase inhibitors 1,10-phenanthroline and EDTA, we attempted to identify which metalloproteinases the W256 cells expressed. Members of the metalloproteinase family can be identified by following their migration in gelatin-containing polyacrylamide gels. Two members of this family, the 72 kD type IV collagenase and the 92 kD type IV collagenase are able to degrade type IV collagen as well as gelatin (Collier et al.,1988; Stetler-Stevenson et al.,1989; Wilhelm et al.,1989). Figure 23 demonstrates that, when the cell free supernatants of PMA-activated W256 cells were applied to SDS

Figure 23. The effect of time when incubating 4-aminophenylmercuric acetate with the supernatants of PMA-activated W256 cells. W256 cells obtained from an ascites were suspended in RPMI 1640 at a concentration of 5 X 10^6 cells/ml. Following a 20-minute incubation in the presence of 10^{-6}M PMA, the cells were pelleted by centrifugation and the cell-free supernatants collected. One millilitre aliquots of the cell-free supernatants were then incubated, at 37°C, with 1.0 mM 4-aminophenylmercuric acetate (APMA). At various time points, 40 µl aliquots were removed, mixed with sample buffer and applied directly without prior reduction or heating to 9% polyacrylamide gels containing 0.3% gelatin. Gelatin zymography was then performed as described in the materials and methods, section 2.8.

lanes:

The cell-free supernatants of PMA-activated W256 cells were incubated at 37°C for:

a) for 15 minutes in the absence of APMA.
b) for 15 minutes in the presence of APMA.

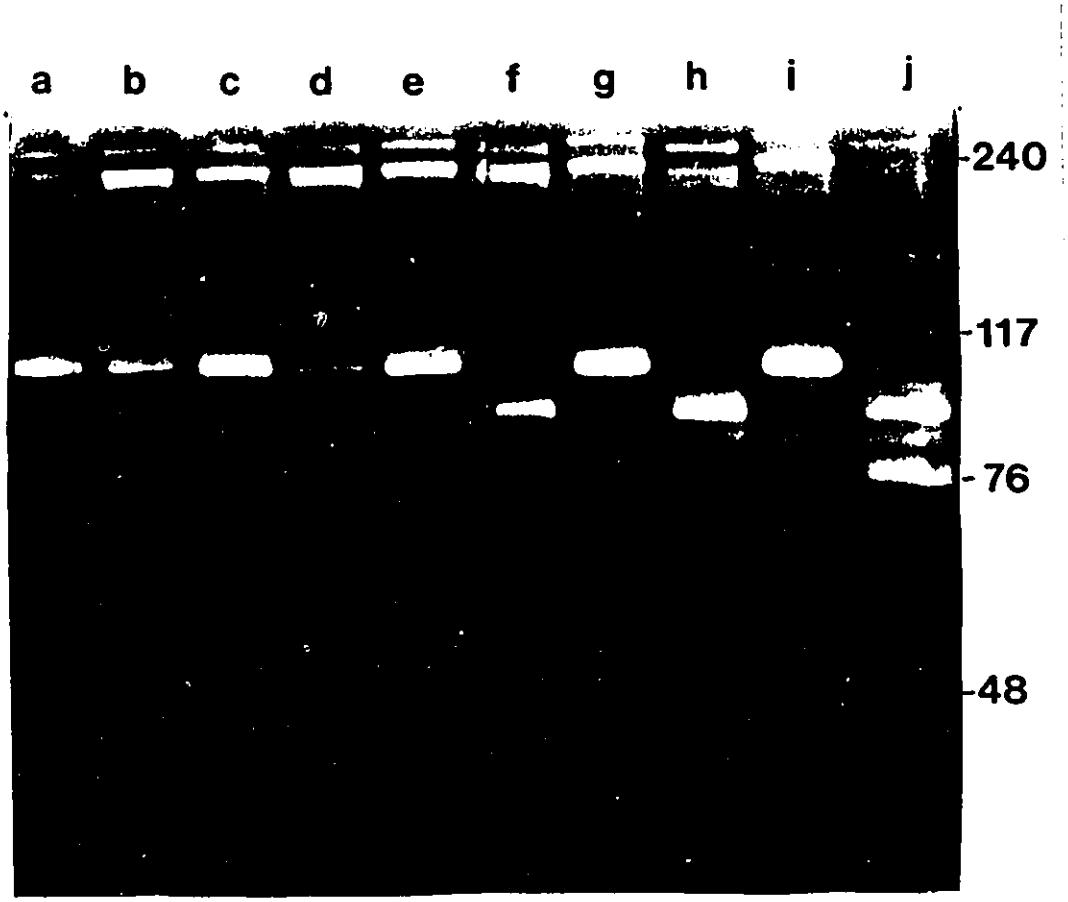
c) for 30 minutes in the absence of APMA.
d) for 30 minutes in the presence of APMA.

e) for 60 minutes in the absence of APMA.
f) for 60 minutes in the presence of APMA.

g) for 120 minutes in the absence of APMA.
h) for 120 minutes in the presence of APMA.

i) for 240 minutes in the absence of APMA.
j) for 240 minutes in the presence of APMA.

Figure 23.



- polyacrylamide gels containing gelatin, five bands of gelatinolytic activity ≥ 94 kD (Mw 94 kD, 130 kD, 225 kD, 230 kD, 274 kD) could be detected (Figure 23, lanes a,c,e,g,i). All gelatinolytic activities were inhibited by developing the zymograms in the presence of 5 mM EDTA (data not shown). The majority of gelatinolytic activity migrated at approx 94 kD (Figure 23, lane a,c,e,g,i).

Members of the metalloproteinase family exist in both a "latent"and "active" form (Stetler-Stevenson et al.,1989). To demonstrate that the 94 kD band (and higher molecular weight bands) represented a latent type IV collagenase/gelatinase which was active in the zymographic assay because of its partial and reversible denaturation by SDS, the supernatants of PMA-activated W256 cells were incubated with 1.0 mM 4-aminophenylmercuric acetate (APMA). Activation of the metalloproteinase family of enzymes with organomercurials such as APMA is associated with a loss in molecular weight as a result of autoproteolysis (Sanchez-Lopez et al.,1988). This step is required in order to attain activity (Sanchez-Lopez et al.,1988). Figure 23 demonstrates that, following a 30 minute incubation of APMA with the supernatants of PMA-activated W256 cells, the 94 kD band (and higher molecular weight bands) exhibited diminished gelatinolytic activity. By 2 hours, at least two new bands of lower molecular weight (Mw 79 and 74 kD) began to appear and the lower molecular weight band at 86kD had intensified

in activity (Figure 23, lane h). By four hours, the 94 kD band was almost completely converted to its lower molecular weight forms (Figure 23, lane j). Under these conditions, three bands of lower molecular weight (Mw 86 kD, 79 kD, 74 kD) could be detected. These bands could also be detected following a 45 minute APMA incubation if the concentration of APMA used ranged from 0.25 to 0.75 mM (Figure 24, lanes b-d). Under these conditions a total of five lower molecular weight bands were observed (Mw 86 kD, 79 kD, 74 kD, 70 kD and 66 kD). We concluded that these lower molecular weight bands represent the active, lower molecular weight, forms of the latent 94 kD gelatinase.

To determine directly if reactive oxygen species could activate the 94 kD type IV collagenase/gelatinase, cell free supernatants of PMA-activated W256 cells were incubated with increasing concentrations of hydrogen peroxide (Figure 25). In these experiments, the cell free supernatants were first concentrated 10 fold by Amicon filtration (Molecular weight cutoff 30 kD). The experiment in Figure 25 shows that in addition to the 94 kD band (and higher molecular weight bands), three bands of gelatinolytic activity (Mw 86 kD, 68 kD and 66 kD) could be observed in the absence of hydrogen peroxide (Figure 25, lane b). The migration of these lower molecular weight bands, at 86 kD and 66 kD, was identical to those formed when the cell-free supernatants of PMA-activated W256 cells were treated with

Figure 24. The effect of 4-aminophenylmercuric acetate concentration on the formation of lower molecular weight bands expressing gelatinolytic activity. W256 cells obtained from an ascites were suspended in RPMI 1640 at a concentration of 5 X 10^6 cells/ml. Following a 20-minute incubation in the presence of 10^{-6}M PMA, the cells were pelleted by centrifugation and the cell-free supernatants collected. One millilitre aliquots of the cell-free supernatants were then incubated, at 37°C, with increasing concentrations of 4-aminophenylmercuric acetate (APMA). Following a 45-minute incubation, 40 µl aliquots were removed, mixed with sample buffer and applied directly without prior reduction or heating to 9% polyacrylamide gels containing 0.3% gelatin. Gelatin zymography was then performed as described in the Materials and Methods, section 2.8.

lanes:

The cell-free supernatants of PMA-activated W256 cells were incubated, at 37°C, for 45 minutes:

a) in the absence of APMA.

b) in the presence of 0.25 mM APMA.

c) in the presence of 0.50 mM APMA.

d) in the presence of 0.75 mM APMA.

e) in the presence of 1.0 mM APMA.

Figure 24.

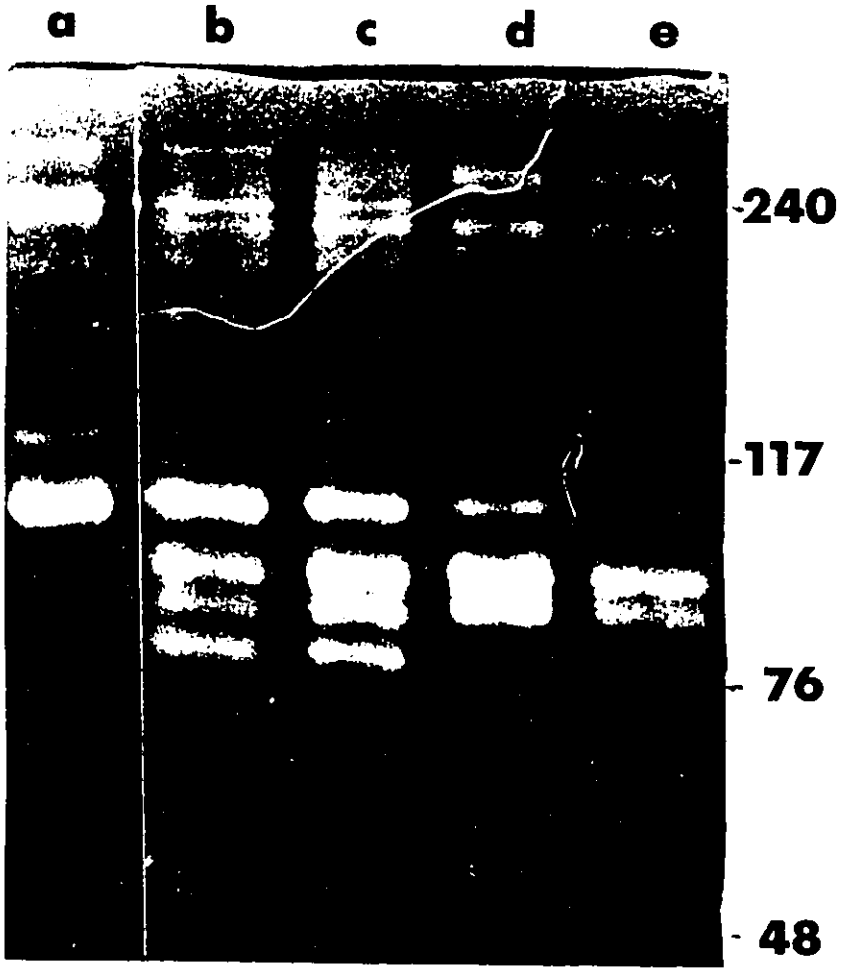


Figure 25. The effect of hydrogen peroxide on the formation of lower molecular weight bands expressing gelatinolytic activity. W256 cells obtained from an ascites were suspended in RPMI 1640 at a concentration of 5 X 10^6 cells/ml. Following a 20-minute incubation in the presence or absence of 10^{-6} M PMA, the cells were pelleted by centrifugation and the cell-free supernatants collected. The cell free supernatants were then concentrated 10 fold by Amicon filtration (Molecular weight cutoff 30kD), pooled, and 0.25ml aliquots were treated with increasing concentrations of hydrogen peroxide. Following a 45-minute incubation at 37°C, 40 µl aliquots were removed, mixed with sample buffer and applied directly without prior reduction or heating to 9% polyacrylamide gels containing 0.3% gelatin. Gelatin zymography was then performed as described in the materials and methods, section 2.8.

lanes:

The cell-free supernatants of unactivated W256 cells (lane a) or PMA-activated W256 cells (lanes b-f) were concentrated 10 fold and incubated, at 37°C, for 45 minutes

a) in the absence of hydrogen peroxide.

b) in the absence of hydrogen peroxide.

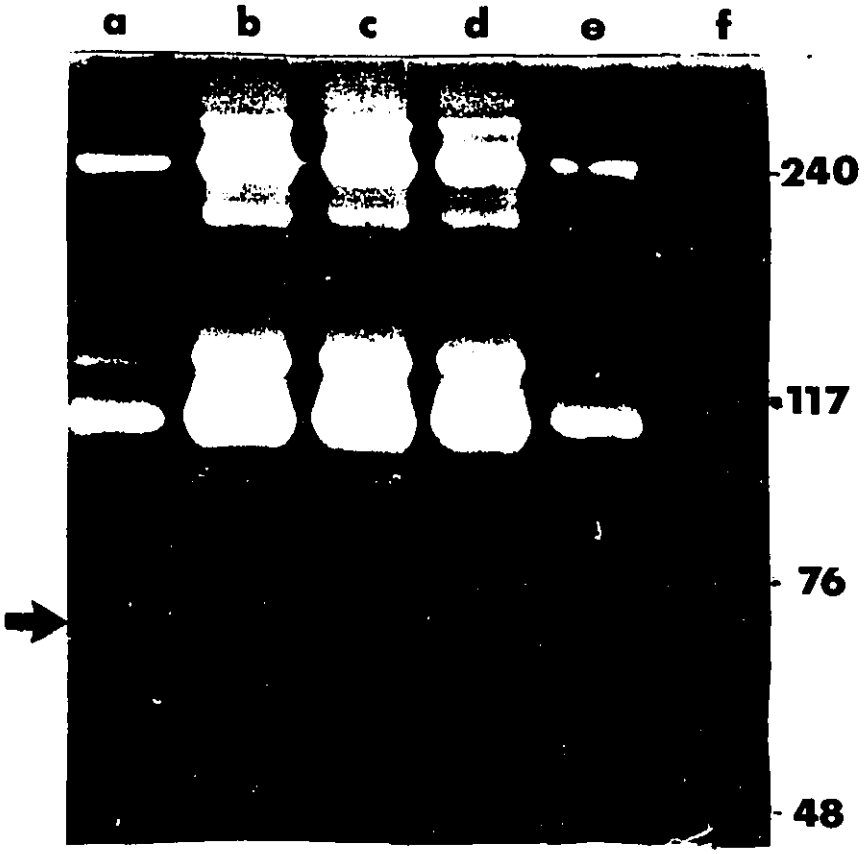
c) with 0.12% hydrogen peroxide.

d) with 0.24% hydrogen peroxide.

e) with 0.48% hydrogen peroxide.

f) with 0.96% hydrogen peroxide.

Figure 25.



APMA. After a 45-minute incubation with hydrogen peroxide (0.12% to 0.24%), the band at 66 kD (arrow) demonstrated a significant increase in its gelatinolytic activity (Figure 25, lane b vs c or d). Densitometric analysis of the band at 66 kD demonstrated that its activity had increased 6.6 ± 0.8 fold (mean of two experiments). Incubation with higher concentrations of hydrogen peroxide resulted in the loss of all gelatinolytic activities (Figure 25, lanes e and f). All gelatinolytic activities were inhibited when the zymograms were developed in the presence of 5 mM EDTA (data not shown). In conclusion, we postulate that the hydrogen peroxide-dependent increase in gelatinolytic activity, found at 66 kD occurs following hydrogen peroxide-mediated activation and autoproteolysis of the 94 kD gelatinase.

4 DISCUSSION

Several models of metastasis/invasion have defined a reproducible sequence of events by which circulating tumour cells may cross the vessel wall prior to forming metastasis (Jones et al.,1971; Kramer and Nicolson, 1979; Crissman et al.,1985; Crissman et al.,1988). First, tumour cells appear to adhere to intact endothelium and then to migrate between the endothelial cells, at sites of retraction (Kramer and Nicolson 1979; Crissman et al.,1988). The tumour cells then adhere to and digest the underlying basement membrane, possibly by secreting metalloproteinases (Crissman et al.,1988; Mignatti et.al.,1986; Reich et.al., 1988). Finally, the tumour cells migrate into underlying tissues were they can proliferate and form metastasis (Crissman et al.,1988).

In this thesis I have provided evidence that W256 carcinosarcoma cells and platelets generate reactive oxygen species and I argue that: a) fMLP-activated W256 cells can perturb endothelial cell monolayers by generating reactive-oxygen species; b) this effect is dependent upon vitronectin receptor-mediated tumour cell adhesion to the endothelium; and, c) W256 cells secrete a latent metalloproteinase which may be activated by reactive oxygen species. A model,

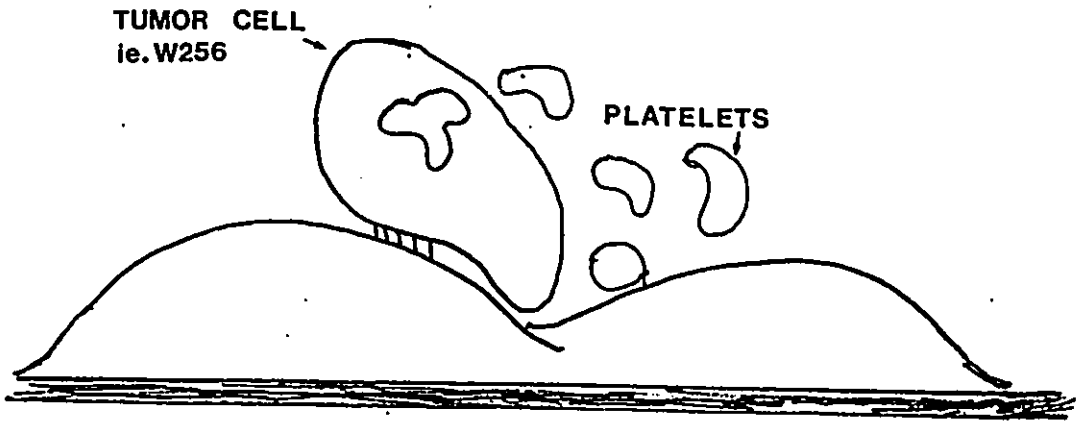
postulating the possible mechanisms by which free radicals may be involved in cancer metastasis is shown in Figure 26. This model has recently been strengthened by the observation that several human melanoma cell lines, carcinomas and a neuroblastoma cell line can also constitutively generate large amounts of hydrogen peroxide (Szatrowski and Nathen, 1991).

A) The effects of W256 cells and xanthine oxidase on endothelial cell monolayers:

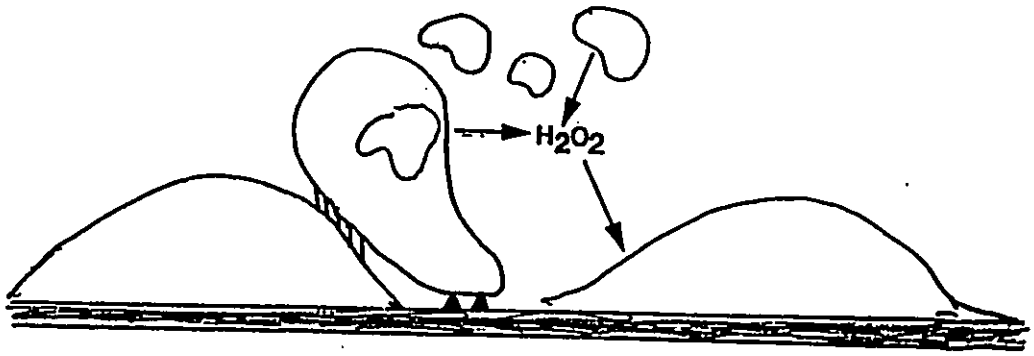
Microvascular walls, consisting of endothelium and basement membrane, act as a barrier to tumour cell extravasation. Although cell migration and the secretion of degradative enzymes have been implicated in cancer cell damage to basement membrane (Mignatti et.al.,1986; Reich et.al., 1988) the mechanisms involved in tumor cell passage through the endothelium have been less well documented. Observations from studies in vitro and in vivo suggest that this might involve active retraction of endothelial cells following cancer cell attachment (Nicolson 1982) and/or in some instances mechanical damage due to intravascular tumor cell proliferation (Baserga and Saffiotti, 1955; Crissman et al., 1988). Here we postulate that some tumor cells, such as

Figure 26. Postulated mechanisms by which free radicals are involved in cancer metastasis. Prior to their extravasation, circulating cancer cells attach to the endothelium via a defined set of adhesion molecules. The results presented in this study, as well as those from other ongoing studies in our laboratory suggest that the vitronectin receptor, $\alpha_V\beta_3$, can function as a endothelial cell-derived adhesive moiety for cancer cells. Following adhesion, cancer cells can perturb the endothelium directly by generating reactive oxygen species. Host cells such as neutrophils and platelets are often found at sites of tumor cell arrest and are also capable of producing reactive oxygen species. The generation these reactive oxygen species may result in endothelial cell retraction and exposure of the underlying basement membrane (section 3.2.1). Cancer cells may then damage the basement membrane by a mechanism which involves both the secretion of proteases and the generation of reactive oxygen species. Reactive oxygen species may activate latent proteases or inactivate serum protease inhibitors. Evidence presented in this study suggests that cancer cells can secrete and then activate latent metalloproteinases by the simultaneous generation of reactive oxygen species.

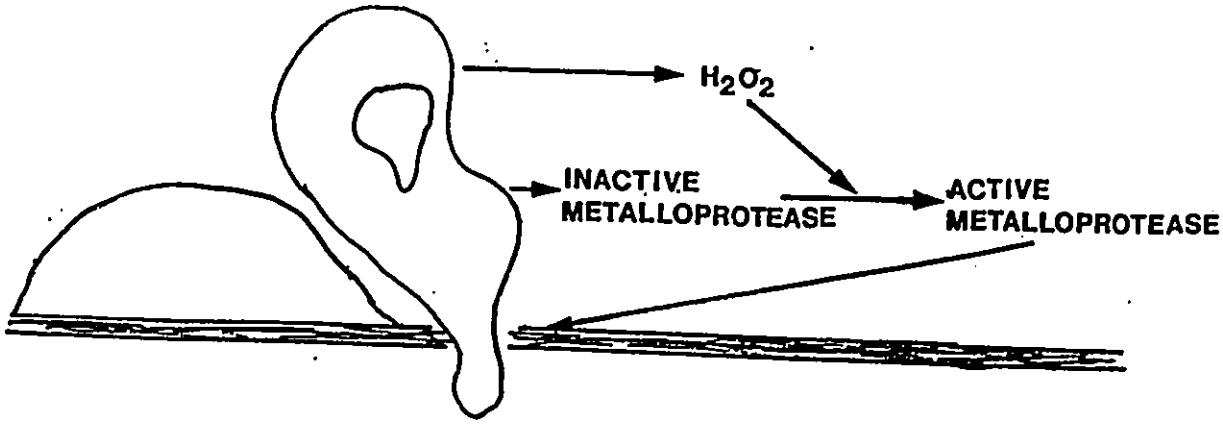
1) TUMOR CELL ADHESION



2) ENDOTHELIAL CELL RETRACTION



3) BASEMENT MEMBRANE DEGRADATION



the W256 cell, may induce endothelial cell retraction and/or injury by the generation of reactive oxygen species and that this may facilitate their metastasis in vivo.

In the present study we demonstrated that both activated platelets and W256 cells generate reactive oxygen species, and that the latter can promote the release of ^{3}H-2-deoxyglucose from pre-labeled endothelial cells (Figure 18). In the presence of activated W256 cells, isotope release was found to be consistent with the generation of reactive oxygen species since: a) pretreatment of the endothelium with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase synthesis, was required to detect significant ^{3}H-2-deoxyglucose release; b) catalase inhibited isotope release; c) the release of isotope was maximal when the tumour cells had been activated with fMLP and correlated with tumour cell-generated chemiluminescence; and, d) the free radical generating system xanthine-xanthine oxidase was also capable of promoting isotope release. The release of ^{3}H-2-deoxyglucose from pre-labeled endothelial cells has been postulated to reflect sublethal injury. Exposure of endothelial cells to reactive oxygen species results in the significant release of 3H-2-deoxyglucose from cells which can still exclude trypan blue (Andreoli et al.,1985; Andreoli et al.,1986; Freeman et al.,1988). In our studies, we demonstrate that the free radical generating system xanthine-xanthine oxidase can promote significant ^{3}H-

2-deoxyglucose release in the absence of either ^{51}chromate or lactate dehydrogenase release (Figure 17). Similiar results were obtained when endothelial cell monolayers were exposed to W256 cells. We conclude that W256 cells can promote the release of 3H-2-deoxyglucose from prelabeled endothelial cells by generating reactive oxygen species and suggest that this may occur as a result of an initial sublethal injury to the endothelium.

It is interesting that the free radical generating system xanthine-xanthine oxidase not only promoted the release of ^{3}H-2-deoxyglucose but also induced endothelial cell retraction (Figure 17). Substantial retraction of the endothelium was observed in the presence of xanthine oxidase, at concentrations, which when compared to the W256 cells, caused equivalent levels of ^{3}H-2-deoxyglucose to be released. This raises the possibility that the W256 cell may also induce endothelial cell retraction by generating reactive oxygen species. In vivo, such a mechanism could facilitate the metatasis of these cells by allowing them access to the basement membrane. Previous studies have suggested that metastasis can be promoted following endothelial damage and subsequent exposure of the underlying basement membrane (Adamson et al.,1986; Orr and Warner, 1987; Adamson et al.,1987). In several of these studies, the involvement of reactive oxygen species in vessel-wall injury and the subsequent promotion of metastasis was suggested

(Adamson et al.,1986; Orr and Warner, 1987; Adamson et al.,1987). Further studies are required to determine if, W256 cells can induce endothelial cell retraction and/or injury by the generation of reactive oxygen species and if this can facilitate the metastasis of these cells in vivo.

B) W256 cell - mediated release of ^{3}H-2-deoxyglucose from labeled endothelial cell monolayers is dependent on vitronectin receptor-mediated W256 cell adhesion to the endothelium:

There is growing evidence that adhesive interactions between the endothelium and cancer cells can regulate the metastatic process. For example, site - specific metastasis may be the consequence of endothelial cell - derived adhesion molecules mediating the attachment of circulating tumour cells within specific microvascular beds (Alby and Auerbach, 1984; Auerbach et al., 1987). To date a number of molecules which mediate tumour cell attachment to the endothelium have been identified. Some of these including, INCAM-110/VCAM and ELAM-1, are only expressed following exposure of the endothelium to cytokines (Rice and Bevilacqua, 1989; Bevilacqua et al., 1987). Here we present evidence which suggests that the VnR, $\alpha_V\beta_3$, previously shown to mediate endothelial cell attachment to von Willebrand factor, fibrinogen, and vitronectin (Cheresh, 1987) also

mediates tumor cell attachment to endothelial cell monolayers.

In the present studies, rIL-1α pretreatment of endothelial cell monolayers was shown to increase the adhesion of PMA-activated W256 cells and to increase the expression of VnR on the endothelial cell surface. The increase in W256 tumour cell adhesion was shown to be at least partially dependent upon endothelial cell VnR expression since adhesion could be blocked by anti-human VnR polyclonal antiserum as well as the GRGDSP peptide. Others in our laboratory have shown that the adhesion of A549 human lung carcinoma cells and M6 human melanoma cells to the endothelium is also enhanced following stimulation of the endothelium with rIL-1α; A549 cell adhesion was also inhibited by the GRGDS peptide (Lauri et al.,1990), anti-human VnR polyclonal antiserum, and by the VnR monoclonal antibody, LM609 (Lafrenie et al.,1990; Lafrenie et al., in press). The latter monoclonal antibody, specifically recognizes the Arg-Gly-Asp dependent adhesion receptor, $\alpha_V\beta_3$, on the surface of several cell types including human umbilical vein endothelial cells (Cheresh and Harper, 1987).

Since tumor cells also express VnR on their surface (Cheresh and Spiro, 1987), we were concerned that the W256 cells may be binding to vitronectin, located within the basement membrane. Evidence that W256 cells had attached initially to the endothelium and not to the underlying

extracellular matrix included: a) scanning electron micrographs (Figure 19); b) the knowledge that the adhesion molecule, VnR is an integral membrane protein, is not located in the basement membrane (Cheresh and Harper, 1987; Cheresh, 1987). In our experiments, flow cytometry demonstrated that only the endothelial cells and not the W256 cells expressed immunologically detectable VnR on their cell surface. Since anti-VnR antiserum blocked W256 cell adhesion, we argue that tumour cell attachment in this model is more likely to be to the endothelium than to the underlying basement membrane.

We have not determined why rIL-1α pretreatment of the endothelium increased the adhesion of PMA-activated W256 cells without increasing the adhesion of unactivated W256 cells. In addition to inducing oxygen radical release, PMA activation of W256 cells has been shown to increase W256 cell adhesion to noncellular foreign surfaces including plastic culture dishes and nylon fibers as well as endothelial cell monolayers (Varani and Fantone, 1982). This response was shown to be an energy dependent process, independent of protein synthesis. The results presented here suggest the possibility that a ligand for vitronectin receptor is expressed on the W256 cell surface following PMA-stimulation. This would be consistent with the observation that PMA-activated W256 cells adhere to IL-1α pretreated endothelial cells in greater numbers than do

unstimulated W256 cells.

Three types of experiments in the present study provided evidence that W256 cell-mediated isotope release was dependent on cancer cell adhesion to the endothelium. In the first experiment, cytochalasin B treatment of the W256 cells inhibited their attachment to endothelial cell monolayers and suppressed ^{3}H-2-deoxyglucose release. Similiar results have been obtained by others in studies using cytochalasin B treated neutrophils (Sacks et al.,1978). A second experiment showed that adhesion of W256 cells to endothelial cell monolayers was increased 1.8 ± 0.1 fold and damage was increased 1.4 ± 0.1 fold after 4-hour pretreatment of the endothelium with rIL-1α. In a third experiment, both the adhesion of stimulated W256 cells to rIL-1α-treated monolayers and ^{3}H-2-deoxyglucose release were inhibited by 40-50% in the presence of polyclonal anti-vitronectin receptor antiserum.

We have not determined why the release of ^{3}H-2-deoxyglucose was dependent on tumour cell adhesion to the endothelium. However, based on the evidence that W256 cell-mediated ^{3}H-2-deoxyglucose release is dependent upon the generation of reactive oxygen species, several explanations can be postulated. First, adhesion of W256 cells to the endothelium might be expected to increase damage by decreasing the diffusion distance between the W256 cell and endothelium. Although highly reactive, these species are

very short lived (Badwey and Karnovsky, 1980). Alternatively, adhesion might focus the generation of reactive oxygen species so that it occurs at sites adjacent to the endothelial cell surface. Indeed, while PMA activated W256 cells in suspension demonstrated cerium chloride-derived precipitates distributed at random on their cell surface (Figure 12), those attached to endothelium demonstrated precipitates mainly at sites that were adjacent to the endothelial cell surface (Figure 22). A similiar observation was made, when neutrophils in suspension were compared with those conjugated to tumour cells or adhered to basement membranes (Vissers 1985; Lichtenstein 1987). Finally, although we have not tested this hypothesis, W256 cell adhesion to endothelial cell monolayers may also increase the amount of reactive oxygen species generated by each attached W256 cell and thereby increase isotope release. Precidence for this suggestion is given by observations showing that the adhesion of neutrophils to cultured endothelium or extracellular matrix components increases the amount of hydrogen peroxide generated by each attached neutrophil (Nathan et al.,1989; Shappell et al.,1990; Herrmann et al.,1990).

C) W256 cells secrete a latent metalloproteinase which is activated by reactive oxygen species:

There is now considerable evidence implicating the metalloproteinase family of degradative enzymes in the processes of invasion and metastasis (Mignatti et.al.,1986; Reich et.al., 1988). Specific inhibitors of metalloproteinases can inhibit the migration of tumour cells across polycarbonate filters coated with matrigel or human amnion (Mignatti et al.,1986; Reich et al.,1988). In addition, in models of experimental metastasis, the formation of metastatic lung lesions is greatly reduced when tumour cells are co-injected with specific metalloproteinase inhibitors (Reich et al.,1988). Two members of the metalloproteinase family, the 72 kD and the 92 kD type IV collagenase preferentially degrade basement membrane type IV collagen and are constitutively secreted by a wide variety of tumour cells (Collier et al.,1988; Stetler-Stevenson et al.,1989; Wilhelm et al.,1989).

Our data indicate that W256 carcinosarcoma cells secrete a 94kD gelatinase which belongs to the metalloproteinase family. Evidence for this includes the following observations: a) the 94kD protease shares substrate specificity with known members of the metalloproteinase family (Stetler-Stevenson et al,1989; Wilhelm et al.,1989); b) the activity of the protease was

inhibited by known inhibitors of metalloproteinases (ie EDTA); and c) incubation of the supernatants of PMA-activated W256 cells with APMA resulted in a loss of the gelatinolytic activity at 94 kD and the appearance of new, lower molecular weight bands with gelatinolytic activity. This latter observation not only demonstrates that the 94 kD gelatinase secreted by W256 cells is a metalloproteinase, but suggests that like other members of the metalloproteinase family it is secreted in a latent form which can be activated by APMA. Based on the observation that the 94 kD gelatinase secreted by W256 cells is a metalloproteinase we postulate that this enzyme is the previously described 92 kD type IV collagenase.

We have presented evidence suggesting that W256 cells secrete a latent metalloproteinase which can be activated by the generation of reactive oxygen species. W256 cells were shown to promote degradation of subendothelial matrices as detected by ^{3}H-proline release from pre-labeled matrices. ^{3}H-Proline release was substantially enhanced by treating the W256 cells with PMA or fMLP. This enhanced isotope release could be inhibited, not only by the addition of metalloproteinase inhibitors such as EDTA or 1,10-phenanthroline but also by catalase. In addition, when the cell-free supernatants of PMA-activated W256 cells were incubated with increasing concentrations of hydrogen peroxide and analyzed by gelatin zymography, a two-fold

increase in the gelatinolytic activity at 66 kD was observed. We have not directly determined if the 66 kD gelatinase obtained from the cell-free supernatant of PMA activated W256 cells can promote the release of 3H-proline from labeled sub-endothelial cell matrices. However, both the activity of the 66 kD gelatinase and the release of 3H-proline are inhibited by EDTA and are increased in the presence of reactive oxygen species.

The mechanism by which hydrogen peroxide increases gelatinolytic activity, at 66 kD, has not been determined. However, we have observed a similiar increase in gelatinolytic activity at 66 kD, following APMA treatment of the W256 cell free-supernatants. In these experiments, the increase in gelatinolytic activity was consistent with APMA-mediated activation and subsequent autoproteolysis of the 94 kD gelatinase. Therefore we postulate that hydrogen peroxide, either directly or indirectly, activates the 94 kD gelatinase, which then autoproteolytically cleaves itself to generate the 66 kD gelatinase. Based on this hypothesis we would expect antiserum raised to the 94 kD gelatinase to recognize, in immunoblot analysis, polypeptides of both 94 kD and 66 kD. There are at least two possible explanations for the ability of hydrogen peroxide to activate the 94 kD gelatinase. The first, is that in a similiar manner to APMA, exposure to hydrogen peroxide can cause a conformational change in the 94 kD gelatinase disrupting the cysteine-Zn^{2+}

interaction and freeing the Zn^{2+} to participate in proteolytic reactions (see section 1.5.3). Exposure to hydrogen peroxide has been shown to alter the conformation of some proteins (Strandberg et al.,1991). The second possibility is that, hydrogen peroxide can indirectly activate the 94 kD gelatinase by inhibiting the activity of plasminogen activator inhibitors (PAIs) (Strandberg et al.,1991). Active PAI forms complexes with exogenous urokinase- or tissue-type plasminogen activators to inhibit plasmin generation (Gaylis et al.,1989). Plasmin in turn can activate latent metalloproteinases (Mignatti et al.,1986; Reich et al.,1988; He et al.,1989). We have not determined if components of the fibrinolytic system are present in our experiments however, it would be of interest to determine if the addition of excess purified PAI-1 or plasmin inhibitors such as α_2-antiplasmin, could inhibit the hydrogen peroxide-mediated increase in gelatinolytic activity at 66 kD.

4.1 Future Studies

A number of key questions raised by this study remain to be resolved, these include:

1) Will human tumour cell lines including melanomas, sarcomas, carcinomas and leukemic cell lines generate luminol-promoted chemiluminescence or cause a loss of scopoletin fluorescence ?

2) Will the addition of catalase, anti-vitronectin receptor antiserum or GRGDSP peptide inhibit the migration of activated W256 cells (or other tumour cells) through endothelial cell monolayers in vitro ?

3) Will the intravenous injection, into rats, of catalase or GRGDSP peptide along with 125IUdr-labeled W256 cells inhibit cancer cell retention in the lungs after 24 hours or decrease metastatic tumour burden after 14 days ? Will pretreatment of the animals with buthionine sulfoximine or antineutrophil antisera affect these results ?

4) Will polyclonal antisera raised to the 94 kD gelatinase of the W256 cell recognize a similiar gelatinase either, in cell extracts or the conditioned media of other human tumour

cell lines ? If so, when analyzed by gelatin zymography, do they show similiar shifts in molecular weight following hydrogen peroxide treatment ? Will their degradation of endothelial cell derived - basement membranes be inhibited by the addition of catalase ?

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