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MATRIX METALLOPROTEASES AND CELL MOTILITY IN MALIGNANT MESOTHELIOMA

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To Chunyan and Chi

To my parents

ABSTRACT

Invasion and metastasis of malignant tumor cells requires destruction of and migration through extracellular matrix (ECM) such as basement membrane and interstitial stroma. The ECM-degrading proteases produced by tumor cells and stromal cells and cell motility play a crucial role in this process. Matrix metalloproreases (MMPs) appear to be particularly important because of their capability to degrade components of ECM.

Malignant mesothelioma is an asbestos-associated and highly aggressive tumor arising from mesothelial cell-lined surfaces of body cavities. It is most often present in the pleural cavities. The resulting tumor often forms diffuse thickening of involved surfaces rather than solitary rounded lesions seen in other neoplasms. Malignant mesotheliomas invade and spread through the underlying basement membrane or along serosal surfaces. Metastases occur in up to 75 % of mesothelioma patients. There is no effective and standard treatment for malignant mesothelioma.

The general aim of this thesis is understand why malignant mesotheliomas invade surrounding tissues and metastasize. Using human malignant mesothelioma cell lines as a model, the goal of this thesis was to: (*i*) investigate expression and production of MMPs in malignant mesothelioma cells. All investigated mesothelioma cell lines expressed mRNA for MMP-1, MMP-2, MMP-3, MMP-9 and 6/8 cell lines expressed MMP-7, 3/8 cell lines expressed MMP-10. MMP-11 was not detected in any of mesothelioma cell lines tested. MMP -2 and MMP -9 produced by mesothelioma cells degraded gelatin, whereas MMP-3 degraded laminin, fibronectin and vitronectin; *(ii)* the exposure of malignant mesothelioma cells to different growth factors, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), β-cellulin (BTC), stem cell factor (SCF), insulin-like growth factor (IGF)-I, -II, acidic-fibroblast growth factor (aFGF), basic-FGF (bFGF) and hepatocyte growth factor (HGF), increased production of MMP-9 and/or MMP-3. Production of MMP-2 was not affected by any growth factors used in this study; (*iii*) examine the cell motility (chemotaxis and chemokinesis) induced by multiple growth factors in malignant mesothelioma cells. The growth factors such as EGF, TGF-α, AR, HB-EGF, BTC, IGF-I, IGF-II and SCF stimulated chemotactic and/or chemokinetic motility in mesothelioma cells tested, whereas none of aFGF, bFGF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) induced migration in the same mesothelioma cells; (*iv*) study the role of novel EGF receptor-tyrosine kinase (EGFR-TK) inhibitors (ZD1839, OSI-774, CI-1033) on cell proliferation and invasive behaviour of malignant mesothelioma *in vitro*. All three drugs inhibited TGF-α induced cellular proliferation, cell migration (chemotaxis) and the production of MMP-9 in three cell lines tested.

In conclusion, we have described the expression, production and regulation of MMPs in malignant mesothelioma cells. In addition, our results indicate that MMPs may play a role in malignant mesothelioma cell invasion. Furthermore, cell motility induced by different growth factors may contribute to the highly invasive behaviour of malignant mesothelioma. We have also demonstrated EGFR-TK inhibitors inhibit the proliferation, migration and MMPs production in malignant mesothelioma cells suggesting that these drugs may become an effective treatment strategy for malignant mesothelioma.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text with their roman numbers (I-IV).

I. **Zhiwen Liu,** Anna Ivanoff and Julius Klominek. (2001) Expression and activity of matrix metalloproteases in human malignant mesothelioma cell lines. *Int. J. Cancer.* 91,638-643

II. **Zhiwen Liu** and Julius Klominek. (2003) Regulation of matrix metalloproteases in human malignant mesothelioma cell lines by growth factors. *Thorax*. 58,198-203

III. **Zhiwen Liu** and Julius Klominek. (2004) Chemotaxis and chemokinesis of malignant mesothelioma cells to multiple growth factors. *Anticancer Research.* 24 (3a), 1625-30

IV. **Zhiwen Liu** and Julius Klominek. (2004) Inhibition of proliferation, migration and matrix metalloproteases production in malignant mesothelioma cell lines by tyrosine kinase inhibitors. *Neoplasia.* 6 (6), In press

CONTENTS

LIST OF ABBREVIATIONS

AMF	Autocrine Motility Factor
AR	Amphiregulin
BSA	Bovine Serum Albumin
BTC	β-Cellulin
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
aFGF	acidic-Fibroblast Growth Factor
bFGF	basic-Fibroblast Growth Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HB-EGF	Heparin-Binding EGF-like Growth Factor
HGF	Hepatocyte Growth Factor
IL-6	Interleukin-6
IGF	Insulin-like Growth Factor
MMPs	Matrix Metalloproteases
PDGF	Platelet-Derived Growth Factor
mRNA	Messenger Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCF	Stem Cell Factor
SV40	Simian Virus 40
TK	Tyrosine Kinase
TGF-α	Transforming Growth Factor-α
TIMPs	Tissue Inhibitors of Metalloproteases
TSG	Tumor Suppressor Gene

INTRODUCTION

With rapid advances of cancer research, today, several lines of evidence indicate that the transformation of normal human cells into malignant tumors is a multistep process. As suggested by Hanahan and Weinberg, these processes include six essential alterations in cell physiology, which are: *(1)* self-sufficiency in growth signals; *(2)* insensitivity to antigrowth signals; *(3)* evading apoptosis; *(4)* limitless replicative potential; *(5)* sustained angiogenesis; and *(6)* tissue invasion and metastasis (Hanahan and Weinberg 2000). This thesis focus on various aspects of tumor invasion and metastasis, in particular the mechanisms of the metastatic spread of malignant mesothelioma cells.

TUMOR CELL INVASION AND METASTASIS

Angiogenesis

Angiogenesis, or the generation of new capillary blood vessels, is a crucial mechanism required for tumor growth and metastasis. It is a well-accepted fact that a primary tumor or metastasis can grow to size of approximately 1-2 mm diameter and obtain sufficient nutrition by means of diffusion (Hori et al. 1991). A tumor growth above this size demands oxygen and nutrients by means of angiogenesis. Hypoxia arises early in the process of tumor development because rapidly proliferating tumor cells outgrow the capacity of the host vasculature. Hypoxia may induce the expression of proangiogenic factors through hypoxia-inducible factor-1α (HIF-1α), and if proangiogenic factors are in excess of antiangiogenic factors, it may lead to tumor cell switch to an angiogenic phenotype (Laughner et al. 2001). Angiogenesis consists of sequential processes emanating from microvascular endothelial cells (Folkman 1986). Proangiogenic factors produced by tumor cells which include vascular endothelial growth factor (VEGF), angiopoietins, transforming growth factor-α (TGF-α), basic-fibroblast growth actor (bFGF) and others bind to endothelial cell receptors and initiate the sequence of angiogenesis. When the endothelial cells are stimulated to proliferate, they secrete proteases (mainly matrix metalloproteases (MMPs)) that digest the basement membrane and extracellular

matrix (ECM) surrounding pre-existing capillaries. The dissolution of the ECM also allows the release of proangiogenic factors from the matrix (Bhushan et al. 2002). Then the endothelial cells migrate toward the source of the angiogenic stimulus and undergo morphogenesis to form new capillary.

The ECM components

The ECM can be defined as a complex mixture of collagens, non-collageneous glycoproteins and proteoglycans that are present between clusters of cells in all tissues. ECM is present in two forms: basement membrane and underlying interstitial stroma (Timpl and Dziadek 1986, Timpl 1989, Akiyama et al. 2001). The ECM provides structural and mechanical support to cells and tissues. Basement membrane is a dense, sheet-like matrix of collagen, glycoproteins such as laminin, fibronectin and proteoglycans (Yurchenco and O'Rear 1994). The role of the basement membrane in the tumor microenvironment is not limited to be a barrier against tumor invasion. It is also a reservoir of cell binding proteins and growth factors that affect cell behavior (Klagsbrun and Baird 1991, Roberts et al. 1992).

Collagens

The collagens are a class of at least 25 different proteins that are localized in the ECM (Kalluri 2003). Collagen molecules have three α-chains that form a triple helix (Bork 1992). Collagens type I, II, III, V, and XI are termed ‘fibrillar collagens’ for their capacity to form fibers. They are predominant components of interstitial stroma (Kadler 1994). Collagens type IV, VI, VIII and X do not form fibrils but are cross-linked into a three-dimensional network. They are major components of the basement membrane (van der Rest and Garrone 1991). Collagens provide mechanical stability and strength interacting with one another or with other ECM components. Gelatin is denatured collagen. The previous studies have shown that malignant mesothelioma cells have the ability to synthesize collagen type IV *in vivo* and in culture (Kallianpur et al. 1990, Kataoka et al. 1990, Klominek et al. 1993).

Laminin

Laminin, together with collagen type IV, is one of the main components of the basement membrane. Laminin is known to consist of a family of proteins that are

composed of α, β and γ chains that generate several different isoforms that vary in size, composition and structure (Timpl and Brown 1994). Laminins are synthesized by a wide variety of cells and appear to have variety of biologically functions such as cell adhesion, cell migration, and cell differentiation (Timpl and Dziadek 1986, Beck et al. 1990). In malignancy, laminin has been suggested to mediate tumor cell adhesion, motility, angiogenesis, and contribute to increased the malignant phenotype by inducing proteases (Engbring and Kleinman 2003). In malignant mesothelioma in cell culture, laminin induced cell adhesion, spreading and chemotactic and haptotactic migration indicating its role in tumor invasion (Klominek et al. 1997, Scarpa et al. 1999).

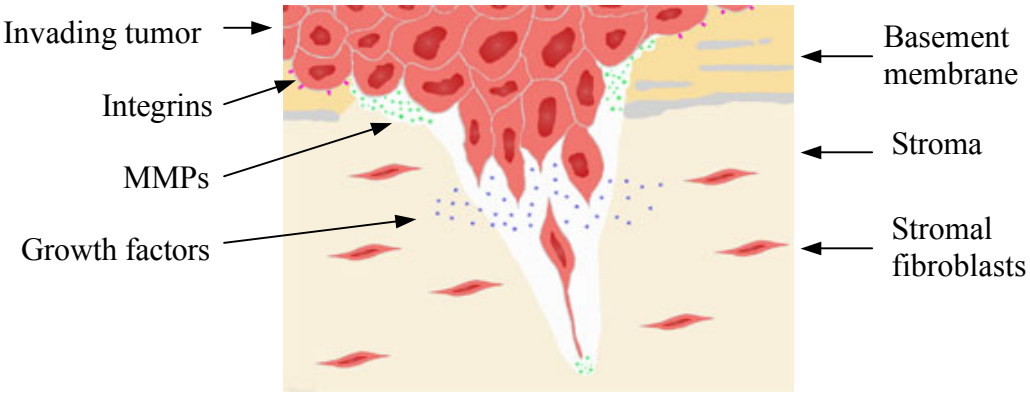
Fibronectin

Fibronectin is a multifunctional glycoprotein that is found in two different forms: soluble and solid. The soluble form, which is produced by hepatocytes, is found in body fluids. The solid form, which is produced by many different cell types including fibroblasts, endothelial cells and others, is found in loose connective tissue and most basement membranes, (Ruoslahti and Vaheri 1974, Hynes and Yamada 1982)**.** Fibronectin acts through several distinct domains to promote cell adhesion, cell migration, and matrix assembly. The primary cell-binding domain is a tripeptide arg-gly-asp (RGD) sequence. In tumors, fibronectin often accumulates in the stroma (Christensen 1992). Cultured malignant mesothelioma cells synthesize and secrete fibronectin *in vitro*. Experiments with malignant mesothelioma cell lines indicate that these cells adhere, spread and migrate in response to both soluble and solid form of fibronectin (Klominek et al. 1993, Klominek et al. 1997, Scarpa et al. 1999).

The invasive and metastatic cascade

Although the genetic basis of tumorigenesis may vary greatly between different cancer types, the cellular and molecular steps required for metastasis are generally similar for all solid tumors (Woodhouse et al. 1997, Liotta and Kohn 2003). Tumor invasion and metastasis is a very complex and dynamic biological process, during which cross-talk between tumor cells, ECM and a variety of host cells take place (Liotta and Kohn 2001). The process of tumor invasion and metastasis can be subdivided into several sequential steps. These include: *(i)* **tumor cell dissociation**:

single tumor cells or cell groups leave the primary tumor; *(ii)* **tumor cell attachment, matrix proteolysis and tumor cell migration**: dissociated tumor cells or cell groups actively invade local microenvironment (Fig.1); *(iii)* **intravasation, survival in circulation, extravasation**: tumor cells move to a distant site via the circulatory system; and *(iv)* **growth of metastases**: metastatic tumor cells proliferate in secondary organ tissue.



(Figure is provided by Julius Klominek)

Fig.1 *Proposed schematic model of tumor cell invasion. Tumor cells attach to the ECM via integrins. MMPs degrade and alter matrix composition. Interaction with matrix components or their degradation products induce cell migration. Growth factors are either released from the matrix upon degradation, secreted by stromal cells or by tumor cells in an autocrine fashion. Released or secreted growth factors act in concert with integrins to additionally propel tumor cell migration. Release of MMPs by tumor cells and stromal cells is modulated by secretion of tissue inhibitors of MMPs. Growth factors also regulate MMP release.*

Tumor cell dissociation

The initial step of tumor metastasis is a dissociation of single tumor cell or cell groups from the primary tumor. This dissociation requires loss of homotypic cell-cell adhesion, which is, mediated by two main classes of cell adhesion molecules (CAMs), the immunoglobulin superfamily and the calcium-dependent cadherin family (Engers and Gabbert 2000).

The important molecules of the immunoglobulin superfamily for cell-cell adhesion are neural cell adhesion molecules (N-CAM), carcino-embryonic antigen (CEA) and the protein of DCC (deleted in colon cancer) gene. Dysregulation of these molecules

by loss of expression or by genetic alterations causes a reduction in intercellular adhesion and facilitates cell detachment from solid tissues (Johnson 1991, Rosales et al. 1995, Yoshioka et al. 1998).

Cadherins are a family of about 30 transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion (Takeichi 1990, 1991). Three of them, E- (epithelial), N- (neural), and P- (placental) cadherin, share a common basic structure but display a unique tissue distribution pattern. Coupling between adjacent cells by E-cadherin bridges results in the transmission of antigrowth and other signals via cytoplastic contacts with β-catenin to the actin cytoskeleton. E-cadherin function is apparently lost in most epithelial cancers, by which mutational inactivation of the E-cadherin or β-catenin gene, transcriptional repression, or proteolysis of the extracellular cadherin domain (Beavon 2000). Reconstitution of a functional E-cadherin/catenin adhesion complex impairs the invasive and metastatic phenotype of many different tumor cell types. Thus, E-cadherin serves as a suppressor of invasion and metastasis by epithelial cancers (Luo et al. 1999, Hsu et al. 2000).

Tumor cell invasion

Cell attachment

In order to cross the ECM (basement membrane and its underlying interstitial stroma), tumor cells use integrins to establish transient attachments with ECM components such as type IV collagen, laminin, fibronectin and vitronectin (Danen et al. 1995). Integrins are transmembrane heterodimeric molecules (composed of an α and β subunit) that bind the ECM through the Arg-Gly-Asp (RGD) peptide sequence present in ECM components (Hynes 1992). Each of integrin subunits has its own binding specificity, but most integrins interact with several components of ECM. Transmembrane signaling by integrins occurs in either inside-out and outside-in direction. Binding to the ECM results in integrins clustering at the cell surface and association of the cytoplasmic tails with adapter proteins, which in turn provide a link to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors (Giancotti and Ruoslahti 1999).

In malignant mesotheliomas, integrins mediate cell attachment to ECM. Experiments revealed that pre-incubation of mesothelioma cells with anti-integrin

antibodies to β1, α2, α5, and α6 subunits inhibited cell attachment to fibronectin, laminin and type IV collagen (Klominek et al. 1997).

Proteolysis of ECM

When tumor cells attach to the ECM, the leading edge of the tumor cells and surrounding stromal cells (e.g. fibroblasts, endothelial cells and inflammatory cells) release ECM-degrading enzymes, leading to proteolytic degradation of ECM (Liotta et al. 1991, McCawley and Matrisian 2000). Many malignant tumors express increased levels of proteolytic enzymes. These enzymes are thought to contribute to tumor cell invasion of the host tissue (Stetler-Stevenson et al. 1993, Crawford and Matrisian 1994). The principal classes of ECM-degrading enzymes are the MMPs; tissue serine proteinases, which include urokinase plasminogen activator (uPA), thrombin, and plasmin; the adamalysin-related membrane proteases (ADAM family); cathepsins and heparanase. The proteolytic activity is tightly regulated by the balance of degrading enzymes, their activators, and their inhibitors (Werb 1997). Among these degrading enzymes, MMPs appear to be particularly involved in the metastatic cascade because of their broad ability to degrade ECM components. In particular, the MMPs include the only enzymes known to be capable of degrading fibrillar collagen (Nagase and Woessner 1999). Malignant mesotheliomas in culture produce several proteases belonging to the MMP family with different substrate specificities (Harvey et al. 2000, Liu et al. 2001).

Cell migration

Migration of tumor cells across the ECM and basement membrane is the crucial step in local invasion. As the invading cell moves forward through ECM barriers, a series of adhesion, de-adhesion and proteolysis takes place. Local attractants include motility stimulating factors, which are secreted either by host cells and tumor cells themselves, and proteolysed ECM fragments, which are recognized by integrins.

Soluble motility factors consist of growth factors, cytokines and chemokines. Some of the ECM proteins that are known to induce motility are fibronectin, laminin, collagen type I and IV, vitronectin, thrombospondin and hyaluronan (Woodhouse et al. 1997, Rein et al. 2003). The ECM proteins fibronectin, laminin, collagen type IV stimulate chemotaxis and haptotaxis of mesothelioma cells in integrin dependent manner (Klominek et al. 1997).

Intravasation, tumor cell arrest and extravasation

After having successful crossing of the ECM, tumor cells interact with the local microvessels (post-capillary veins and lymphatic vessels). This step is called intravasation. This process is similar to the tumor cells invasion of the ECM. The difference is the unique interaction of tumor cells with the endothelial cells, in which the intravasating tumor cells enter the vessel lumen by a non-destructive interaction with the endothelial cells (Glinsky 1993). After having entered the vasculature, tumor cells are passively disseminated in the blood stream. By intravital vidiomicroscopy, circulating tumor cells were found to be arrested in the microcirculation simply by size restriction rather than by rolling along and adhering to the vessel walls. During this arrest, tumor cells are pressed against the endothelium and are mechanically deformed and stretch out along the vascular wall. However, despite strong morphological deformations of tumor cells in the capillary system more than 80% of these cells maintain their membrane integrity, survive, and even successfully extravasate (Chambers et al. 1995, Condeelis and Segall 2003). Besides the mechanical filtering, the circulating tumor cells may also recognize specific terminal organ vessels by expressing cell-cell adhesion molecules during their transition through vasculature (Glinsky 1993). Subsequent to cell-cell adhesion, tumor cells extravasate as described above for the processes of crossing the ECM and intravasation. Indeed, only a very low percentage of circulating tumor cells finally form micrometastases. Metastatic inefficiency of circulating tumor cells seems to result from the control of post-extravasation growth of individual tumor cells (Chambers et al. 1995).

Tumor dormancy and growth of metastases

There are several consequences of tumor cells extravasation. Some tumor cells would not be able to survive in the new matrix environment and would die in the perivascular zone. Some tumor cells such as melanoma and breast cancer often enter a phase of dormancy. During this phase, tumor cells are temporarily unable to respond to local growth factors. However, these dormant tumor cells are capable of re-entering proliferation by appropriate stimuli (Hart 1999). In addition, tumor cells which either have autocrine stimulatory potential or express growth factor receptors

for factors present in that new matrix environment would be able to initiate colony formation and establish metastases (Timar et al. 2001).

Matrix metalloproteases (MMPs)

The MMPs are a family of zinc-dependent metalloendopeptidases that consist of more than 20 human MMPs (Table 1) that are able to degrade ECM components. The MMPs are now divided into subclasses according to their structure. There are eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs) (Egeblad and Werb 2002).

All MMPs are synthesized as inactive zymogens. They are kept inactive by an interaction between a cysteine-sulphydryl group in the propeptide domain and zinc ion bound to the catalytic domain. Activation of MMPs requires proteolytic removal of the propeptide domain (Sternlicht and Werb 2001). Most of the MMPs are activated outside the cell by other activated MMPs and serine proteases (Nagase 1997). MMP activity is tightly controlled by endogenous inhibitors, which are α_2-macroglobulin in tissue fluids (Sottrup-Jensen and Birkedal-Hansen 1989) and tissue inhibitors of metalloproteases (TIMPs). The four TIMPs bind to MMPs in a 1: 1 stoichiometric ratio and reversibly block MMP activity (Gomez et al. 1997).

Table 1. Selected tumor-associated MMPs and their matrix substrates ∗

MMP number	Descriptive name	Principal matrix substrates
MMP-1	Collagenase-1	Fibrillar collagens, gelatin, entactin, aggrecan,
MMP-8	Collagenase-2	tenascin
MMP-13	Collagenase-3	
MMP-2	Gelatinase A	Non-fibrillar collagens, gelatin, elastin, laminin,
MMP-9	Gelatinase B	fibronectin, vitronectin, aggrecan
MMP-3	Stromelysin-1	Laminin, fibronectin, vitronectin,
MMP-10	Stromelysin-2	gelatin, proteoglycans, non-fibrillar collagens,
MMP-7	Matrilysin	fibrin/fibrinogen, entactin, tenascin
MMP-11	Stromelysin-3	Laminin, fibronectin, aggrecan
MMP-14	MT1-MMP	Fibrillar collagens, gelatin, aggregan,
MMP-15	MT2-MMP	fibronectin, laminin, vitronectin
MMP-16	MT3-MMP	

∗ *Not an all-inclusive list.*

The MMP function not only consists of the cleavage of structural ECM components creating a migration path for invading cells; another major consequence of the ECM degradation by MMPs is the release of ECM-sequestered growth factors and cytokines, which in turn affects cellular signalling (Stamenkovic 2000). Recent observation provide evidence that MMP substrates also include non-ECM molecules ranging from growth factor binding proteins and receptors to cell surface adhesion molecules (Levi et al. 1996, Manes et al. 1997, Kajita et al. 2001, Noe et al. 2001).

In general, MMPs are expressed at low levels or not at all in resting-state adult tissue. However, numerous growth factors and cytokines provide stimuli that can rapidly induce MMP expression when there is a challenge to the tissue system, such as embryonic development, tissue remodelling and wound healing in physiological processes, and inflammation, tumor invasion and metastasis in pathological processes (Stamenkovic 2000).

Increased expression of MMPs has been found in almost every type of human cancer, and this is the result of a complex interaction between tumor cells and host stromal cells which all actively participate in the production of these enzymes (McCawley and Matrisian 2000). MMPs are not only associated with tumor invasion and metastasis, but also regulate tumor cell growth, promote tumor angiogenesis and contribute to further tumor progression (McCawley and Matrisian 2000).

Growth factors and cell motility

Cell motility plays a vital role in the process of tumor invasion and metastasis (Wells 2000). Cell motility can be considered as a cyclic series of the biophysical processes of extension, front adhesion, transcellular contraction and rear release of the moving cell (Lauffenburger and Horwitz 1996). This process is preceded by reorganization of the actin cytoskeleton, which in turn is controlled by external signals acting through the soluble motility factors and ECM molecules (Wells et al. 1998).

One group of motility factors that stimulates cells migration and thus facilitates invasion and metastasis consists of growth factors (Levine et al. 1995). Although initially described as growth-promoting agents, these growth factors, including epidermal growth factor (EGF), TGF-α, insulin-like growth factor (IGF)-I, -II, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), bFGF and others, bind to their receptors on the cell surface and induce different types of cell

movement, such as chemotaxis and chemokinesis. Chemotaxis is defined as the directed migration of cells towards a gradient of soluble chemoattractant whereas chemokinesis describes random migration in the absence of a gradient. Moreover, these growth factors also cooperate with ECM adhesion receptors-integrins to promote haptotaxis, which is the directed migration of cells along a gradient of anchored insoluble substrate such as ECM components.

These growth factors normally act in a paracrine fashion, because they cause tumor cells to move toward the cells that produce them. But as tumor progress to malignancy, they may become less dependent on exogenous growth factors. Some tumors are thought to migrate to self-secreted substances called autocrine motility factors (AMF) (Liotta et al. 1986, Klominek et al. 1991, Woodhouse et al. 1997, Bohle and Kalthoff 1999). The mechanism of autocrine stimulation of tumor cell migration was proposed by Liotta who observed that factors produced by tumor cells (such as autotaxin (ATX), TGF-α and IGF-I) could elicit both chemotactic and chemokinetic responses in the producer cells (Liotta et al. 1986, Siegfried 1987, Nakanishi et al. 1988). Multiple growth factors and cytokines, which act in autocrine and paracrine fashion, have been described as motility factors stimulating tumor cell migration (Table 2). Thus, different growth factors produced by tumor cells or host cells may initiate locomotion of tumor cells out of the primary tumor and contribute to the tumor spread.

Table 2. Selected growth factors that effect different sarcoma cell motility

Factors	Cell types	References
AMF	Melanoma	(Liotta et al. 1986)
Autotaxin	Melanoma	(Stracke et al. 1992)
HGF/SF	Melanoma	(Ogasawara and Suzuki 2004)
	Mesothelioma	(Klominek et al. 1998b)
	Glioma	(Badie et al. 1999)
	Osteosarcoma	(Coltella et al. 2003)
IGF-I and/or -II	Melanoma	(Neudauer and McCarthy 2003)
	Mesothelioma	(Liu and Klominek 2004a)
	Neuroblastoma	(Puglianiello et al. 2000)
GM-CSF	Melanoma	(Kohn et al. 1993)
IL-8	Melanoma	(Wang et al. 1990)
PDGF	Mesothelioma	(Klominek et al. 1998a)
	Neuroblastoma	(Pola et al. 2003)
TGF-α	Mesothelioma	(Liu and Klominek 2004a)
HB-EGF	Mesothelioma	(Liu and Klominek 2004a)
β-cellulin	Mesothelioma	(Liu and Klominek 2004a)
Amphiregulin	Mesothelioma	(Liu and Klominek 2004a)
SCF	Mesothelioma	(Liu and Klominek 2004a)
EGF	Mesothelioma	(Liu and Klominek 2004a)
	Glioma	(Berens et al. 1996)
TGF-β	Glioma	(Platten et al. 2000)

MALIGNANT MESOTHELIOMA

The mesothelium, a mesodermally derived single-cell layer, forms the serosal lining of the pleural, peritoneal and pericardial cavities. In the male, it also lines the sac that surrounds the testes. Malignant mesothelioma is a highly aggressive primary tumor arising from mesothelial cells.

The term mesothelium was proposed by Minot in 1890 (Mutsaers 2004). For the primary malignancy derived from mesothelium, the term mesothelioma was first used by Eastwood and Martin in 1921 (Eastwood and Martin 1921). However, malignant mesothelioma did not become a clinical entity until first reported by Wagner in 1960 when he described asbestos as a pathogenic factor for malignant mesothelioma (Wagner et al. 1960).

Incidence and epidemiology

Malignant mesothelioma is a rare disease. In industrialized countries where asbestos was widely used from the end of World War II until the end of 1970s, the incidence of mesothelioma is presently 2 per million in females and 10-30 per million in males per year (McDonald 1987). In endemic regions such as Western Australia, the incidence is as high as 66 per million for men aged 35 or above (Armstrong et al. 1984). Approximately 3000 and 5000 cases occur annually in the United States and Western Europe, respectively. Epidemiological studies indicate that latency period between asbestos exposure and development of mesothelioma is in a range of 30 to 40 years. Therefore, it has been predicted that the number of men dying of mesothelioma each year will almost double in Western Europe over the next 10-20 years (Peto et al. 1995).

The male-female ratio is about 4:1, and 80% of mesotheliomas arise from the pleura (Connelly et al. 1987). The mean age of mesothelioma patients is approximately 60 years, but the disease can occur at any age, including childhood (Grundy and Miller 1972).

Etiology and pathogenesis

Asbestos exposure

A unique feature of malignant mesothelioma is its strong relationship to exposure to asbestos fibers. Many epidemiological surveys have revealed that about 80% of mesotheliomas are attributed to prior exposure to asbestos (Parkes 1973, Chahinian et al. 1982). Animal experiments have confirmed the oncogenicity of asbestos.

The various types of asbestos are divided into two major subgroups: serpentine and amphiboles. The serpentine is curly and flexible, represented by crysotile. The amphiboles are stiff and straight, which include crocidolite, amosite, anthophylite, tremolite, and actinolite. The most commercially important minerals of asbestos are crysotile (white asbestos), crocidolite (blue asbestos), and amosite (brown asbestos) (Fig.2) (Tweedale 2002).

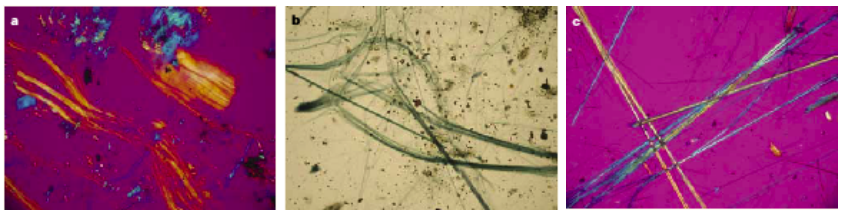


Fig. 2 *Three forms of asbestos. a. Polarized light micrograph of chrysotile (white asbestos). b. Bright-field image of crocidolite (blue asbestos). c. Polarized light micrograph of amosite (brown asbestos).*

The role of serpentine fiber in the pathogenesis of mesothelioma is controversial, whereas the capacity of amphibole asbestos to cause mesothelioma is well documented epidemiologically (Mossman et al. 1990, Nicholson 1991). The serpentines can undergo fragmentation by organic acids and removed by the lymphatic system, whereas the amphiboles may remain unchanged for decades (Churg and DePaoli 1988). The amphiboles have the capacity to reach the pleura either through the lymphatics or by direct penetration, and to cause mesothelioma (Boutin et al. 1996, Mossman and Churg 1998).

The exact mechanisms whereby asbestos can induce malignant mesothelioma have not been fully elucidated. Asbestos may induce deoxyribonucleic acid (DNA) damage either by direct physical interaction (Hesterberg et al. 1986), or by the indirect action

of reactive oxygen species (ROS) produced by inflammatory cells in response to asbestos. These ROS may induce mutations via DNA strand breaks and deletions (Choe et al. 1998, Tanaka et al. 1998). Asbestos may also stimulate the autophosphorylation of the EGF receptor (EGFR) in mesothelial cells, that leads to increases in activator protein-1 (AP-1) activity and cell mitosis or apoptosis (Mossman and Churg 1998, Robledo and Mossman 1999).

Simian Virus 40 (SV40) infection

About 20% of mesotheliomas have no prior exposure to asbestos, and only a fraction of those exposed to asbestos develop mesothelioma. This suggests that other factors may be involved in the etiology of this tumor. SV40 is a DNA tumor virus that can induce mesotheliomas and other tumors in hamsters, and also transform human mesothelial cells *in vitro* (Butel and Lednicky 1999, Carbone et al. 1999). The SV40 proteins associated with transformation and oncogenesis are its early gene products, large T antigen (Tag). Tag is directly mutagenic and causes numerous chromosomal alterations. Tag is also capable of binding and inactivating p53, retinoblastoma protein (pRb) and other tumor suppressor gene (TSG) products. Furthermore, SV40-like DNA sequences and SV40 Tag were detected in 60% of human mesotheliomas by polymerase chain reaction (PCR) analysis and immunohistochemistry, respectively (Carbone et al. 1994). The presence of SV40 sequences in human mesothelioma shows geographic differences, which may be related to the fact that SV40-contaminated polio vaccines were mainly distributed in the United States and parts of Europe (Pepper et al. 1996, De Luca et al. 1997, Hirvonen et al. 1999, Priftakis et al. 2002).

Other etiologic factors

Rare cases of mesothelioma have been associated with radiation exposure. In mesotheliomas, which developed in young adults who had received intensive radiotherapy in their childhood because of Wilm´s tumor, radiation appeared as the only possible causative factor (Austin et al. 1986). Studies in rats support the notion that radiation exposure can cause mesothelioma (Sanders and Jackson 1972).

Familial mesotheliomas in certain villages of Turkey have been reported. The studies revealed the existence of a genetic factor that predisposes affected individuals

to mesothelioma induced by exposure to mineral fiber, erionite in these families (Baris et al. 1978, Baris et al. 1996).

Growth factors

Multiple growth factors, including EGF, TGF-α, IGF-I and II, HGF, PDGF, and keratinocyte growth factor (KGF), may function as autocrine or peracrine growth stimuli of mesothelial cell proliferation, increasing not only the susceptibility of the cells to DNA-damaging agents and genetic instability, but also the expansion of transformed cell populations (Lechner et al. 1989, Mossman and Gruenert 2002).

Genetic alterations

Whatever the cause of mesothelioma, asbestos, SV40, genetics, or radiation, mesothelial cells eventually accumulate a number of genetic alterations and become malignant. It is evident that the most common cytogenetic aberrations in mesothelioma are deletions involving discrete chromosome regions. The accumulated loss and or inactivation of several TSGs in chromosome arms 1p, 3p, 6q, 9p, 13q, 15q, and 22q seem to contribute to the pathogenesis of mesothelioma. Three of these TSGs, which are $p16^{INK4a}$, $p14^{ARF}$ located at 9p21 and NF2 located at 22q12, are frequently deleted in mesothelioma (Murthy and Testa 1999).

Taken together, malignant mesotheliomas are sarcomas in which environmental carcinogens such as asbestos and erionite, SV40 virus, radiation, and genetics interact to cause malignancy. The eventual further isolation of the specific mesothelioma susceptibility gene may represent the key to understanding the precise pathogenesis of mesothelioma.

Pathologic morphology

Malignant mesotheliomas are classified into epithelioid, sarcomatoid and biphasic subtypes based on conventional histological examination (Attanoos and Gibbs 1997). Approximately 50% pleural and 75% peritoneal mesotheliomas are of epithelioid subtype; 30% are biphasic subtype and the remainder is pure sarcomatoid subtype (15% to 20%) (Attanoos and Gibbs 1997).

The epihelioid subtype comprises mesothelioma cells arranged in tubulopapillary or trabecular formations. Epithelioid mesothelioma may also be morphologically

identical to a variety of adenocarcinomas. The sarcomatiod subtype potentially simulates a range of spindle-cell sarcoma morphotypes. Malignant osteoid and cartilage may be present. The biphasic subtype indicates a poor differentiated mesothelioma with features of both epithelial and sarcomatous morphologies (Wick and Mills 2000).

Malignant mesothelioma cells in culture grow as substrate adherent monolayer displaying epithelial, fibroblast or biphasic morphology (Fig.3). The addition of different serum supplements can induce malignant mesothelioma cells to alter their *in vitro* morphology (Klominek et al. 1989).

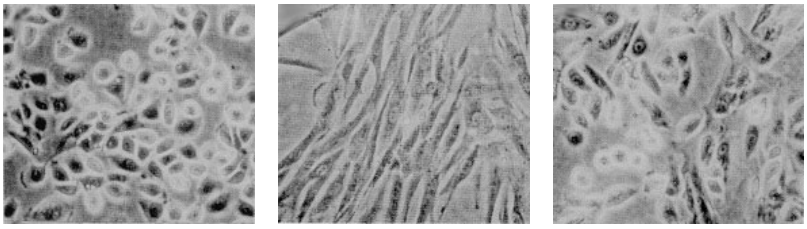


Fig. 3 *epithelioid type* *sarcomatoid type* *biphasic type*

Biological and invasive behavior

Malignant mesothelioma is distinct from other solid tumors. Mesothelioma starts as a localized tumor, but spreads rapidly along mesothelium-lined surfaces to involve pericardium, contralateral hemithorax, and peritoneal cavity by invasion through diaphragm. The resulting tumor often forms diffuse thickening of involved surfaces rather than solitary rounded lesion seen in other neoplasms (Semb 1963).

Invasion through needle biopsy tracts and incision in thoracic wall are typical features in malignant mesothelioma (Chahinian et al. 1982, Hillerdal 1983). Malignant mesotheliomas invade also the underlying basement membrane and produce metastases in up to 75% of patients (Ruffie et al. 1989, Wilson et al. 1992, Moskal et al. 1998). During invasion process, mesothelioma cells must interact with ECM proteins, growth factors embedded in it, and stromal cells, which participate in synthesis and modifications of this microenvironment.

Clinical features and treatment

The onset of mesothelioma is usually insidious; a presenting symptom is often persistent localized pain. In pleural mesothelioma, symptoms such as chest pain, dyspnea, cough and weight loss are frequently found. Pleural effusion with a high level of hyaluronan is present in up to 95% of patients (Chahinian et al. 1982, Roboz et al. 1985, Thylen et al. 2001). In peritoneal mesothelioma, local pain and abdominal distention with ascites are main clinical findings (Chahinian et al. 1982). Median survival ranges from 4 to 12 months depending on the histological subtype, 5-year survival is less than 5% (Ruffie et al. 1989, Boutin et al. 1998).

Malignant mesothelioma is relatively resistant to radiotherapy, but local radiotherapy is recommended to prevent mesothelioma cells from spreading through the needle-biopsy tracks (Rusch et al. 2001). Biological and invasive features of malignant mesothelioma make complete surgical resection impossible. Chemotherapy for malignant mesothelioma continues to be challenging. Despite the fact that various cytotoxic agents have been evaluated in malignant mesotheliomas, few have yielded response rates better than 20% (Baas 2002). In the majority of late stage cases, a palliative treatment approach remains the only choice. However, in a recently published phase III study of pemetrexed (a new antifolate) plus cisplatin, it has been shown that response rates of 41.3% and prolonged survival time in patients with malignant pleural mesothelioma as compared with cisplatin treated alone could be achieved (Vogelzang et al. 2003).

Currently, several novel anticancer agents are being studied in various malignant tumors. Some of these agents target signal transduction from EGFR. EGFR is a transmembrane glycoprotein that is overexpressed in malignant mesothelioma (Dazzi et al. 1990). Ligand binding activates the intracellular tyrosine kinase (TK) domain, triggering EGFR tyrosine autophosphorylation that regulates cell proliferation, survival, angiogenesis, cell movement and metastasis (Ciardiello and Tortora 2001, Yarden and Sliwkowski 2001). ZD1839, OSI-774 and CI-1033 are new low molecular weight EGFR-TK inhibitors that have shown inhibitory effectiveness experimentally in malignant mesothelioma (Janne et al. 2002, Liu and Klominek 2004b). Therefore, the blockade of EGFR signaling pathway may provide a potential therapeutic target for treatment of malignant mesothelioma.

Malignant mesothelioma cell lines

Malignant mesothelioma is a rare tumor. Studies of this tumor have been limited by a paucity of well-characterized *in vitro* human model. In 1984 LaRocca and Rheinwald described difficulties when attempting to establish mesothelioma cell lines and that they were unable to find any published reports of mesothelioma cell lines (LaRocca and Rheinwald 1984). Actually, description of the first mesothelioma cell line appears in the literature in 1982 by Behbehani (Behbehani et al. 1982). After that many investigators reported mesothelioma cell lines being established from pleural effusions and autopsy tissues of mesothelioma patients (Wu et al. 1985, Klominek et al. 1989, Versnel et al. 1989). The establishment of these cell lines represents a rapid and advantageous model for *in vitro* studies of malignant mesotheliomas.

In order to highlight different aspects of cell biology of malignant mesothelioma, nine human malignant mesothelioma cell lines were used in the present thesis: STAV-FCS, ZL34, M14K, ZL5, SPC212, SPC111, M9K, M28K and M38K. These cell lines were established in our laboratory and by others. They represent all three histologic subtypes of mesothelioma (Klominek et al. 1989, Pelin-Enlund et al. 1990, Schmitter et al. 1992).

AIMS OF THE PRESENT STUDY

The ECM-degrading proteases and cell motility plays an important role in tumor invasion and metastasis. In order to understand why malignant mesotheliomas invade surrounding tissues and metastasize, the following aims were formulated:

1. To investigate expression of MMPs and TIMPs, as well as the substrate specificities of these proteases in malignant mesothelioma cells (Paper I).

2. To investigate the effects of different growth factors on production of MMPs in malignant mesothelioma cells (Paper II).

3. To examine the cell motility (chemotaxis and chemokinasis) induced by multiple growth factors in malignant mesothelioma cells (Paper III).

4. To study the role of novel EGFR-TK inhibitors (ZD1839, OSI-774, CI-1033) on cell proliferation, induction of apoptosis, cell migration and MMPs production in malignant mesothelioma cells (Paper IV).

COMMENTS ON METHODOLOGY

Detailed descriptions of methods used in this thesis are presented in the respective publications.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR is a technique for messenger ribonucleic acid (mRNA) transcript detection. Although the obtained RT-PCR results are not quantitative, as compared with Northern blot, RT-PCR is a more sensitive method for mRNA detection. In order to investigate the mRNA expression of different MMP members and growth factor receptors, RT-PCR was chosen and performed on RNA samples from malignant mesothelioma cell lines. The integrity of RNA and complementary DNA (cDNA) synthesis were monitored by amplification of housekeeping gene β-actin mRNA.

Preparation of serum free conditioned medium

In order to investigate the possible presence of ECM-degrading proteases secreted by malignant mesothelioma cells, the serum-free conditioned medium was prepared. The cells were cultured until subconfluent, serum-supplemented medium was removed and cell monolayer was extensively washed to remove remaining serum proteins. Cells were then cultured for 36 to 48 hours in serum-free medium without any other proteins. In some experiments the cells were treated with different growth factors and/or the TK inhibitors. During this process, the ECM-degrading proteases produced by cells were released into the medium. Serum-free conditioned medium was collected (adjusted to the same number cells), dialysed, lyophilised, and then used for substrate zymography.

Substrate zymography

Zymography is a sensitive, quantifiable technique by which a specific enzyme or a class of enzymes can be detected in a protein mixture after electrophoretic separation on a substrate matrix (Heussen and Dowdle 1980). The standard method is based on the sodium dodecyl sulfate (SDS)-polyacrylamide gel embedded a protease substrate

(usually gelatin). Following electrophoresis of the sample containing a protease, SDS is removed from the gel by exchange to Triton X-100. This allows the proteases to renature and autoactivate (Woessner 1995). Coomassie blue staining of the gel reveals sites of proteolysis as white bands on a blue background (Fig. 4). Within a certain range, the proteolytic band intensity can be related to the amount of protease loaded.

To investigate whether malignant mesothelioma cells produce ECM-degrading proteases, and the effects of different growth factors and EGFR-TK inhibitors on production of these proteases, substrate zymography was performed. The substrate degrading enzymes were identified and the intensity of the enzymes was quantified using densitometry (Liu and Klominek 2003).

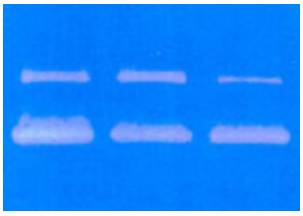


Fig.4 *Gelatin-zymography*

Boyden chamber (cell migration) assay

The migration of malignant mesothelioma cells was studied using a modified Boyden chamber assay (Boyden 1962). This assay is performed in a plastic chamber that is divided into two compartments by a polyvinylpyrrolidone (PVP)-free polycarbonate filter. The size of the pores in the filter has to be chosen so as to let the cells actively squeeze through the pores (Harvath et al. 1980). The use of Boyden chamber assay makes it possible to distinguish between three different motile behaviors: chemotaxis, chemokinesis and haptotaxis (Fig. 5).

For chemotactic assay, the attractant is placed in the lower compartment of the chamber and the cells suspended in medium without attractant are placed in the upper compartment, above the filter. So, in chemotactic experiments there is a chemoattractant gradient across the filter. For chemokinetic assay, different concentrations of the attractant are placed in the lower compartment. Cells suspended in medium containing different concentrations of attractant are placed in the upper

compartment, above the filter. In such an experiment migration of cells can be investigated in the presence of different attractant gradients across the filter as well as in the absence of gradient that is when the same concentration of an attractant is present above and below the filter. Compilation of results or checkerboard analysis allows determination of chemokinetic migration. Chemokinesis occurs if the number of migrating cells in the absence of gradient is greater than when the gradient is present (Zigmond and Hirsch 1973). For haptotactic assay, the attractant (usually an ECM component) is coated onto one surface of the filter. The filter is then placed in the chamber with the coated surface facing the lower compartment. The lower compartment is filled with medium containing a neutral non-attractant protein, usually bovine serum albumin (BSA). The cells suspended in medium containing the same concentration of BSA as in the lower compartment are placed in the upper compartment.

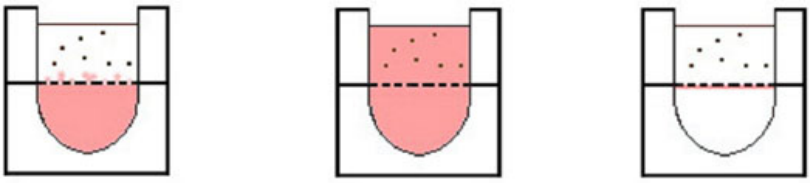


Fig. 5 *chemotaxis* *chemokinesis* *haptotaxis*

(Figure is provided by Julius Klominek)

After the cell suspensions are placed in the upper compartment of the chamber, the chamber is incubated 5 hours at 37°C. Following the incubation the filter is removed, fixed in methanol, and stained with Giemsa. The remaining cells above the filter are wiped off. The cells that migrated through the filter pores are quantited by light microscopy.

RESULTS AND DISCUSSION

Expression and activity of MMPs in malignant mesothelioma cells

Although there is substantial *in vivo* and *in vitro* evidence linking MMP expression to tumor invasion in many different malignancies (Stetler-Stevenson et al. 1993), the production of MMPs by malignant mesothelioma cells has not been thoroughly investigated. In paper I, we investigated the spectrum of MMPs produced in malignant mesothelioma cells by RT-PCR, Western blotting and substrate zymography.

Malignant mesothelioma cells in culture expressed mRNA several MMPs (Liu et al. 2001). These include MMP-1, - 2, -3, -7, -9 and -10, but not MMP-11. In addition to expression of mRNA encoding MMPs, the study also showed that MMP-2 and MMP-9 produced by malignant mesothelioma cells degrade gelatin, whereas MMP-3 degrade laminin, fibronectin and vitronectin (Liu et al. 2001). Our results were in agreement with other studies, in which the production of these MMPs was identified not only in mesothelioma cell lines, but also in mesothelioma patient tissue samples, as determined by gelatin zymography and immunohistochemistry staining (Harvey et al. 2000, Hirano et al. 2002, Edwards et al. 2003). Collagen, fibronectin and laminin are major proteins present in the ECM. Previous studies have shown that malignant mesothelioma cells migrate chemotactically and haptotactically to collagen, fibronectin and laminin (Klominek et al. 1993, Klominek et al. 1997). In the present study, we also show that MMPs produced by malignant mesothelioma cells that have a variable ability to degrade these ECM components. Although our results do not provide specific answer to how these MMPs participate in ECM remodeling *in vivo*, it is apparent that malignant mesothelioma cells produce a broad spectrum of MMPs with multiple substrate degrading specificities and that these MMPs may contribute to highly invasive behavior of this tumor.

Regulation of MMPs in malignant mesothelioma cells

In paper I, we have shown that malignant mesothelioma cells express several members of the MMP family with different substrate specificities. In paper II, we

have further investigated the effects of different growth factors on production of MMP-2, -3 and MMP-9 in malignant mesothelioma cells.

The regulation of MMPs is complex and occurs at both transcriptional and post-transcriptional levels. A number of extracellular factors – including growth factors, cytokines and cell contact with ECM – have been implicated in the regulation of MMP expression in different types of tumor cells (Vincenti et al. 1996, Harvey et al. 2000). The exposure of malignant mesothelioma cells to different growth factors, including EGF, TGF-α, amphiregulin (AR), heparin binding EGF (HB-EGF), β-cellulin (BTC), stem cell factor (SCF), IGF-I, -II, acidic-FGF (aFGF), bFGF, and HGF, increased production of MMP-9 and /or MMP-3. In contrast, the production of MMP-2 was not affected by any of the growth factors investigated. We have also demonstrated mRNA expression of different growth factor receptors in malignant mesothelioma cells that corresponds to the stimulation of their ligands.

A number of studies have shown that several growth factors affect different aspects of cellular behavior such as proliferation, motility, differentiation, and production of ECM proteins (Kondapaka et al. 1997, Bredin et al. 1999). Our recent study has also shown that malignant mesothelioma cells migrate to different growth factors (Liu and Klominek 2004a). Furthermore, it has been shown that a number of growth factors stimulate the synthesis of the ECM molecule hyaluronan and proteoglycans in malignant mesothelioma cells (Tzanakakis et al. 1995). Taken together these results strongly suggest that different growth factors may play an important functional role in the process of malignant mesothelioma cell invasion and metastasis.

Motility of malignant mesothelioma cells

Tumor cell motility plays a vital role in the process of tumor invasion and metastasis (Wells 2000). Substances that stimulate cell locomotion and thus facilitate invasion and metastasis can be presented to tumor cells either in soluble form or as an insoluble matrix (Carter 1967). Several growth factors and cytokines, which are produced either by adjacent cells (paracrine stimulation of cell movement) or by tumor cells themselves (autocrine stimulation of cell movement), as well as ECM components, have been described as inducers of tumor cell migration (Kohn et al. 1990, Engebraaten et al. 1993, Kohn et al. 1993, Sekido et al. 1993, Arihiro et al.

2000). In paper III, we examined a panel of human malignant mesothelioma cell lines for chemotaxis and chemokinesis induced by different growth factors and cytokines.

Using a modified Boyden chamber assay, we found that growth factors such as EGF, TGF-α, HB-EGF, AR, BTC, IGF-I, IGF-II and SCF known to stimulate cell movement in different tumors, stimulated directional (chemotactic) and/or random (chemokinetic) motility in all mesothelioma cell lines tested. Factors such as aFGF, bFGF, granulocyte-macrophage colony factor (GM-CSF) and interleukin-6 (IL-6), also known to stimulate cell movement both in carcinoma and sarcomas, did not induce migration in the same mesothelioma cells.

In previous study we found that some growth factors such as EGF, TGF-α and HB-EGF induced motile response in normal mesothelial cells. But AR, BTC, SCF and IGF-I, -II (which stimulate cell motility in malignant mesothelioma cells) did not induce or induced only a weak motile response in normal mesothelial cells (Palmer et al. 1999). It has been reported that the number of different tumor cells express increasing number of growth factors and their receptors with increasing malignancy grade (Relf et al. 1997, van der Valk et al. 1997, Folkman 2002). In addition, IL-6 can switch from behaving as a paracrine growth inhibitor to an autocrine growth stimulator during malignant progression (Lu and Kerbel 1993). Base on these observations, we suggest that progression from normal mesothelium to malignant mesothelioma is associated with increased motile responsiveness to growth factors.

The effects of EGFR-TK inhibitors on proliferation and invasive behavior of malignant mesothelioma *in vitro*

Malignant mesothelioma is a highly aggressive tumor, with metastasis occurring in up to 75% of patients (Moskal et al. 1998). There is no effective standard therapy for malignant mesothelioma. In paper IV, we investigated the effects of novel EGFR-TK inhibitors (ZD1839, OSI-774 and CI-1033) on proliferation and invasive behavior in malignant mesothelioma cells.

Using [^{3}H] thymidine incorporation, DNA synthesis assay, we found that all three TK-inhibitors inhibited TGF-α induced cellular proliferation in a dose-dependent manner in three cell lines tested. The apoptosis induced by three TK inhibitors was observed in only one cell line by flow cytometry using annexin-V staining. In

addition, all three drugs inhibited TGF-α induced chemotaxis and production of MMP-9 in a dose-dependent manner in three cell lines tested.

The EGFR is a transmembrane glycoprotein. The binding of ligands to EGFR induces receptor dimerization, followed by activation of the intrinsic TK, leading to receptor tyrosine autophosphorylation (Salomon et al. 1995, Olayioye et al. 2000). Activation of EGFR-TK has been identified as an important feature that initiates the cascade of intracellular signaling events that regulate cell proliferation, angiogenesis, cell movement, and metastasis (Ciardiello and Tortora 2001, Yarden and Sliwkowski 2001). In addition, previous studies suggested that EGFR signaling might be involved in asbestos-induced pathogenesis of malignant mesothelioma (Zanella et al. 1996). These studies and our present results indicate that EGFR signaling plays an important role in tumor initiation and progression and the blockade of EGFR signaling pathway may provide a potential therapeutic target for treatment of patients with malignant mesothelioma.

CONCLUSIONS

This thesis has focused on the demonstration of the expression and regulation of MMPs, cell motility and the role of EGFR-TK inhibitors in malignant mesothelioma cells. The obtained results may contribute to a better understanding of the invasive behavior of mesotheliomas, and help to identify therapeutic targets for treatment of malignant mesothelioma. The main conclusions of the studies are:

1. Malignant mesothelioma cells express mRNA of MMPs, and produce MMPs that degrade gelatin, fibronectin, vitronectin and laminin. These results show that the broad spectrum of MMPs produced by malignant mesothelioma cells may contribute to highly invasive behavior of this tumor.

2. The exposure of malignant mesothelioma cells to different growth factors increased production of MMP-9 and /or MMP-3, but not MMP-2.

3. The majority of growth factors that regulate production of MMPs also stimulate chemotactic and/or chemokinetic motility in malignant mesothelioma cells. These results strongly suggest that different growth factors may play an important functional role in the process of malignant mesothelioma cell invasion and metastasis.

4. Three novel EGFR-TK inhibitors (ZD1839, OSI-774, CI-1033) inhibited the proliferation, migration and MMPs production in malignant mesothelioma cells. Our results suggest these drugs may become an effective treatment strategy for malignant mesothelioma.

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