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Role of Cysteine Proteinases in IGF-1R Turnover, Invasion and Metastasis

by

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Summary

Failure of conventional cancer therapy in most neoplastic disease is primarily due to the occurrence of metastases. Metastasis is the process by which single tumor cells break away from the primary tumor, penetrate one or more basement membranes and begin colonization at a new site.

Clinical cancer treatment has focused on the use of cytotoxic agents and/or radiation therapy that target both tumor and normal cells. Consequently, current cancer treatments with chemotherapeutic agents are subject to limitations associated with high toxicity and resistance. There is a need to develop new agents and therapies that will permit long-term administration without compromising the patient. The cysteine proteinases cathepsin B and L have been linked to the invasive steps during the metastatic process and could therefore provide new targets for drug development.

The present work describes our results with a murine Lewis lung carcinoma model which consists of two cell lines, H-59 and M-27, with different patterns of metastasis *in vivo*. Using this model, we found that the cysteine proteinase inhibitor, E-64, significantly inhibited the invasive/metastatic properties of the liver colonising cell line, H-59 both *in vitro* and *in vivo*. PRCB1, a selective inhibitor of cathepsin B, had a partial effect on invasion, suggesting that both cathepsin B and L played a role. Because IGF-1R was identified as a critical mediator of matrix metalloproteinase-2 (MMP-2) synthesis, invasion and metastasis in H-59 cells, the possibility that the cysteine proteinases interfered with receptor for type 1 insulin-like growth factor (IGF-1R) turnover thereby reducing invasion was subsequently investigated. E-64 at non-toxic concentrations

of the tumor cells. Similar effects were also noted with human breast carcinoma MCF-7 cells. A significant reduction was found in the number of IGF-1R in E-64 treated cells. In addition, IGF-1-induced synthesis of MMP-2 was blocked. Concurrent with these changes, there was a noticeable increase in intracellular, tyrosine phosphorylated receptor levels in response to IGF-1 binding, suggesting that inhibition of cysteine proteinase activity disrupted IGF-1 mediated signaling by altering post-ligand binding processing of the receptor.

To elucidate more specifically the role of cysteine proteinases in the process of liver metastasis, cathepsin L expression was inhibited in the H-59 cells by transfection with a plasmid vector expressing a 300 bp cathepsin L cDNA fragment in the antisense orientation. A cloned population stably expressing reduced cathepsin L mRNA levels had reduced cell surface IGF-1 receptor levels, a decreased proliferative response to IGF-1, decreased cloning efficiency and a lower invasive potential as compared to wild type cells. When inoculated via the splenic/portal route *in vivo*, these cells gave rise to less nodules than either the wild-type or mock-transfected cells and most of these nodules were haemorragic. To further investigate the link between IGF-1R expression levels and invasion in these cells, MMP-2 production and activity were investigated. In cathepsin L antisense transfected H-59 cells reduced MMP-2 levels and activity as compare to controls were observed. Together our results suggest that the cysteine proteinases, cathepsin L in particular may regulate the metastatic potential through a role in IGF-1R turnover. The present results provide evidence that metastatic carcinomas which utilize

cysteine proteinases for invasion could potentially be responsive to antimetastatic treatment with cysteine proteinase inhibitors.

Résumé

L ' échec de la thérapie conventionnelle contre le cancer dans le cas de la plupart des maladies néo-plastiques est principalement due à l'occurrence des métastases. La métastase estun processus par lequel les cellules simples de tumeur se détachent de la tumeur primaire, pénètrent une ou plusieurs sous-membranes et commencent la colonisation d'un nouveau site.

Le traitement clinique du cancer s'est concentré sur l'utilisation des agents cytotoxiques et/ou de la thérapie radiologique qui visent la tumeur et les cellules normales. En conséquence, les traitements actuels du cancer avec les agents chimiothérapeutiques sont sujets à des limites associées à la toxicité et à la résistance élevées. Il y a un besoin de développer de nouveaux agents et des thérapies qui permettront la gestion à long terme sans compromettre le patient. Les thiol- protéases cathepsine B et L ont été associées aux étapes envahissantes pendant le processus métastatique et pourraient fournir de nouvelles cibles pour le développement d'un médicament.

Le travail actuel décrit nos résultats avec un modèle murrhin de carcinome de poumon de Lewis qui se compose de deux lignes de cellules, H-59 et M-27, avec des différentes configurations de metastase in vivo. En utilisant ce modèle, nous avons constaté que l'inhibiteur de thiol-protéase, E-64, a sensiblement empêché les propriétés invasives/metastatiques des cellules colonisatrices du foie H-59 in vitro et in vivo. PRCB1, un inhibiteur sélectif de la cathepsine B a eu un effet partiel sur l'invasion, suggérant que les cathepsines B et le L aient joué un rôle. Puisque IGF-1 a été identifié comme un médiateur critique de la synthèse de la métalloprotéase de la matrice 2 (MMP-2), de l'invasion et de la metastase en cellules H-59, la possibilité que les thiol-protéases ont affecté le métabolisme de IGF-1, réduisant de ce fait l'invasion qui a été ultérieurement étudiée. E-64 aux concentrations non-toxiques a supprimé la prolifération des cellules H-59 en réponse à IGF-1 et a réduit l'efficacité de clonage des cellules de la tumeur. Des effets semblables ont été également notés avec les cellules humaines du carcinome MCF-7 de sein. Une réduction significative a été trouvée du nombre d'IGF-1R en cellules traitées par E-64. En outre, la synthèse

de MMP-2 induite par IGF-1 a été bloquée. Simultanément, il y eu une augmentation apparente des niveaux intracellulaires de récepteurs phosphorylés de tyrosine en réponse à la liaison avec l'IGF-1, suggérant que l'inhibition de l'activité de la thiol-protéase a perturbé la signalisation de l'IGF-1, en modifiant le métabolisme du récepteur apres sa liason avec l'IGF-1.

Pour élucider plus spécifiquement le rôle des thiol-protéases sur la metastase du foie, l'expression de la cathepsine L a été empêchée dans les cellules H-59 par transfection avec un vecteur de plasmide exprimant le fragment d' ADN de 300 pb anti-sens de gêne de la cathepsin L. Une population clonée exprimant stablement des niveaux tres bas de ARNm de la cathepsine, avait également des niveaux réduits de récepteurs d'IGF-1, une réponse proliferative à IGF-1 diminuée, l'efficacité de clonage diminuée et un potentiel envahissant inférieur par rapport aux cellules témoin. Une fois inoculée par la voie splénique/portail in vivo, ces cellules ont provoqué moins de nodules que les cellules témoins ou transfecté avec un plasmid contrôl, et la plupart de ces nodules étaient hémoragiques. Pour étudier plus le lien entre les niveaux d'expression d'IGF-1 et l'invasion de ces cellules, la production et l'activité de la MMP-2 ont été étudiées. Dans les cellules transfectés de la cathesine L, de l'anti-sens, une baisse de production et d' activité de la MMP-2, par rapport aux contrôles, a été observée. Nos résultats suggèrent que les thiol-protéases, la cathepsine L en particulier, peut régler le potentiel métastatique, en jouant un rôle dans le métabolisme du IGF-1. Les résultats actuels montrent que les carcinomes métastatiques qui utilisent des thiol-protéases pour l'invasion, pourraient potentiellement être sensibles au traitement antimétastatique avec des inhibiteurs de thiol- protéases.

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Preface

The following excerpt is reprinted from the "Guidelines for thesis preparation" of the Faculty of Graduate Studies and Research of McGill University to inform the reader of Faculty regulations:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly – duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, ***connecting texts that provide logical bridges between the different papers are mandatory****. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.*

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A table of contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, ***the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.*** *Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.*

In accordance with the above guidelines, I have decided to include the following published work as part of the body of the thesis:

Navab, R., Mort, J. S., and Brodt, P. (1997). Inhibition of carcinoma cell invasion and liver metastases formation by the cysteine proteinase inhibitor E-64. *Clin. Exp. Metastasis.* 15: 121-129.

I am responsible for all of the experimental work and analysis carried out in this aforementioned paper with the exception of *in vivo* studies. All of the work was performed in the laboratory of Dr. Pnina Brodt (Department of Surgery, Division of Surgical Research, McGill University) with one of the experiments being done in Dr. John. S. Mort's laboratory (Joint Diseases Laboratory, Shrinner's Hospital for Children).

As well, some of the work described in the papers on which I am a co-author has been included as follows:

Durko, M., **Navab, R.**, Shibata, H.R., and Brodt, P. (1997). Suppression of basement membrane type IV collagen degradation and cell invasion in human melanoma cells expressing an antisense RNA for MMP-1. *Biochemica et Biophysica Acta.* 1356: 271-280.

Long, L., **Navab, R.**, and Brodt, P. (1998). Regulation of the M_r 72,000 type IV collagenase by the type I insulin-like growth factor receptor. *Cancer Research.* 58: 3243-3247.

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Original Contributions to Knowledge

The major novel findings of the present study are as follows:

- Cysteine proteinases such as cathepsin B and cathepsin L are the major proteolytic enzymes for liver metastatic cells, since the inhibition of their activity significantly blocked the invasion/metastatic properties of the liver colonizing cell line, H-59 both *in vitro* and *in vivo*.
- Inhibition of cysteine proteinase activity, disrupted IGF-1 mediated signalling by altering post-ligand binding processing of the receptor. The present finding, provides new insight into the multifaceted role that cysteine proteinases can play in metastasis by implicating them in the regulation of IGF-1R trafficking and turnover.
- Antisense mRNA to cathepsin L completely blocked tumor cell growth and invasion *in vitro* and significantly reduced hepatic metastasis *in vivo*. This is the first report which directly implicates cathepsin L in liver metastases formation.

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List of Abbreviations

DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylene diaminotetraacetate
EGF	Epidermal growth factor
E-64	trans-epoxysuccinyl-L Leucylamido (4-guanidino)-butane
FCS	Fetal calf serum
FGF	Fibroblast growth factor
HBSS	Hank's buffered salt solution
IGF-1	Type 1 insulin-like growth factor
IGF-1R	Receptor for type 1 insulin-like Growth factor
IGFBPs	Insulin-like growth factor binding Proteins
i.s.	Intrasplenic
mAb	Monoclonal antibody
MMP-2	Matrix metalloproteinase-2
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (thiazolyl blue)
PDGF	Platelet derived growth factor
PRCB1	Propeptide of cathepsin B
SFM	Serum free medium
TIMPs	Tissue inhibitor of Metalloproteinases
UPA	Urokinase-type plasminogen activator
UPAR	Urokinase-type plasminogen activator receptor

U24522	[(R,S)-N-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1 -oxopentyl-L- leucyl-L-phenylalaninamide]

CHAPTER 1

Tumor invasion and the extracellular matrix - An overview

1.1. Introduction

Cellular invasion is defined as the ability of cells to cross anatomic barriers, i.e, basement membranes, interstitial stroma, and intercellular junctions, that separate tissue compartments (Bernstein and Liotta, 1994). Under normal physiological conditions cellular invasion occurs during embryogenesis and in the adult organism, during trophoblast implantation, blood vessel formation (angiogenesis), leukocyte extravasation in inflammation and wound repair. Other invasive processes occur under pathological condition, such as rheumatoid arthritis and tumor development (Mignatti and Rifkin, 1993).

During progression from benign to malignant growth tumors acquire an invasive/metastatic phenotype. Metastasis is defined as the process whereby tumor cells detach from the primary site and form metastatic foci at distant locations in the body (Fidler et al., 1978; Sugarbaker, 1981). At the early stage of invasion the tumor cells become detached from the primary tumor and start migrating into the adjacent tissue, presumably guided by chemotactic factors (Tryggvason et al., 1987). In most cases the invading cells reach lymphatic or blood vessels from where they can spread throughout the body, extravasate and then give rise to a metastasis. The formation of metastases is the end result of a continuous invasion process that is associated with dissemination of tumor cells throughout the circulatory system and invasion of a distant host tissue (Poste and Fidler, 1980; Nicolson, 1982; Liotta et al., 1983; Pauli et al., 1983). From a clinical point of view, metastasis is the most serious aspect of cancer, since the lethality of

malignant neoplasms is attributable directly to their ability to develop secondary growths in organs at a distance from the primary tumor mass (Fidler et al., 1978; Sugarbaker, 1981).

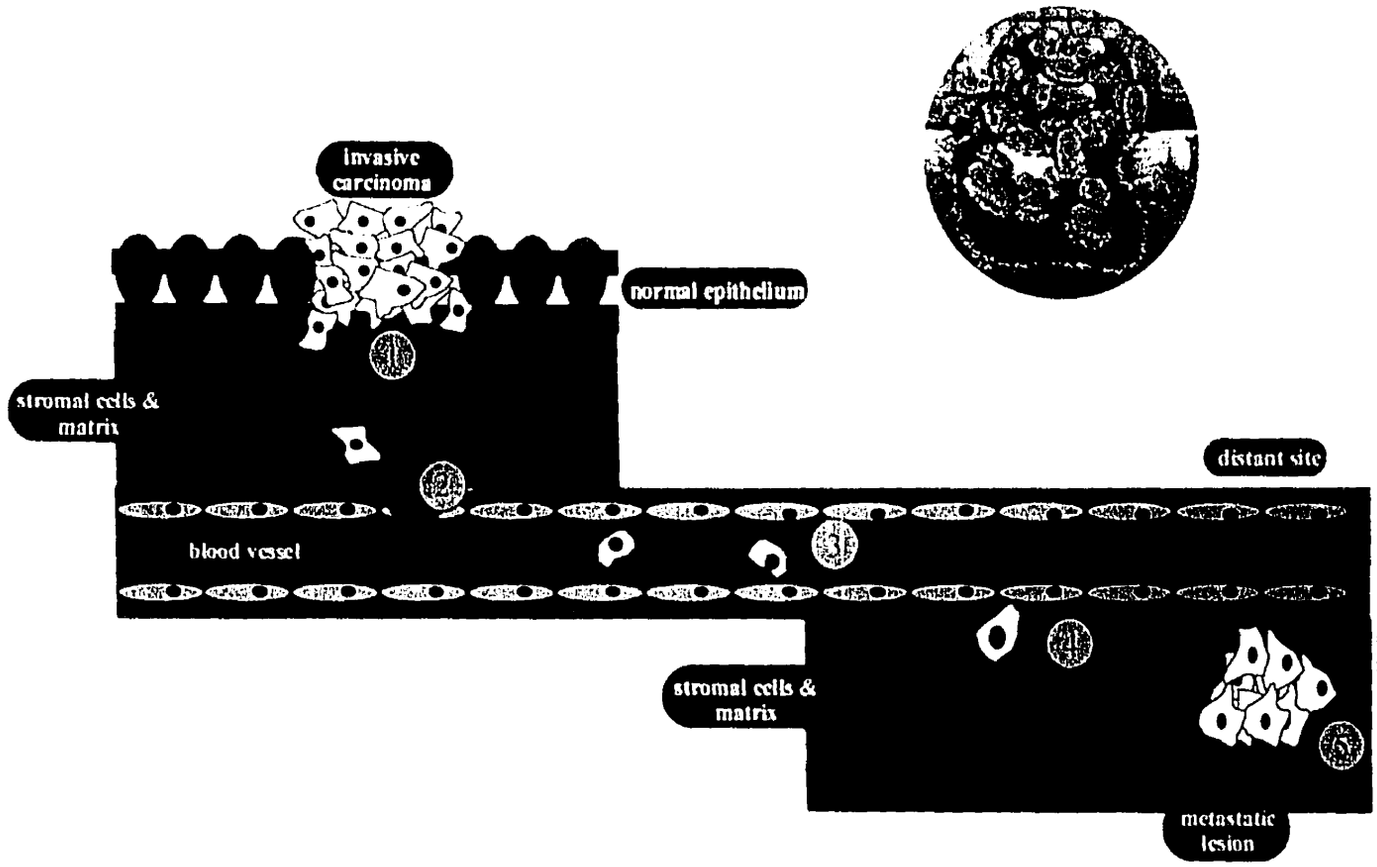
Occult metastatic tumor cells may persist in a dormant state for years after the resection or elimination of the primary tumor (Zajicek, 1987; Meltzer, 1990;). They can then be activated by as yet unidentified stimuli and metastatic foci suddenly develop in an explosive fashion, which often results in the patient's death. Most cancer deaths are due to the metastatic disease that remains resistant to conventional therapies. The primary aim of research into the mechanisms of tumor invasion and metastasis formation is to identify new strategies for more effective therapy against this most deadly aspect of cancer.

One major focus of this research has been an effort to gain an understanding of the molecular mechanisms underlying the development of metastasis. The metastasis is a complex process involving a series of sequential steps, many of which can now be explored at the gene and protein levels. Tumor dissemination requires that cells which have grown to form the primary mass, (a) invade locally with the active or passive penetration of small blood vessels and lymphatics, (b) detach from the site of penetration as either single cells or small emboli, (c) survive potentially lethal traumatic events and both specific and non-specific host immune defenses during passage through the circulation, (d) arrest either at the walls of minor blood vessels or within draining lymph nodes, (e) extravasate and move into surrounding organ parenchyma, presumably by

utilizing the same cellular mechanisms which allow invasion and intravasation , and (f) proliferate at the secondary site to give rise to clinically apparent metastases which can themselves subsequently metastasize (Fig. 1-1) (Fidler et al., 1978).

Fig. 1-1: Tumor cell invasion and metastasis.

(1) Single malignant cells begin to detach from the primary tumor mass, invade local stroma and (2) enter into the vasculature (intravasation). (3) Once in the vasculature, the cancer cell will circulate throughout the body until it finds a site of arrest usually by lodging in a small capillary or lymph node. (4) The cell exits the vasculature (extravasation), invades into the surrounding tissue and begins to proliferate and (5) forms a metastatic lesion.


invasive carcinoma
normal epithelium
stromal cells & matrix
distant site
blood vessel
stromal cells & matrix
metastatic lesion

In order for tumor cell to disseminate, extracellular matrix barriers must be dissociated. The question of how these matrices are degraded to facilitate the migration and proliferation of tumor cells has been the subject of intensive research in recent years.

1.2. The extracellular matrix

The extracellular matrices that are penetrated and degraded during the invasion of tumor cells can be divided into two major categories, basement membranes (BM) and interstitial connective tissue, which differ with respect to composition, location and function (Tryggvason et al., 1987). The BMs consist of uniform thin, continuous acellular sheet-like structures which are present throughout the body, at the interface between epithelial and connective tissues and serve to separate the epithelium and vascular endothelial cells from the interstitial connective tissue (Vracko, 1982). The interstitial connective tissue comprises cells such as fibroblasts, osteoblasts, chondrocytes and macrophages (depending on the tissue type) embedded within a collagenous matrix. The interstitial connective tissue can be divided into several subclasses that form specialized tissues such as tendons, bone, cartilage and loose stroma which differ with respect to function, cell type and chemical composition (Tryggvason et al., 1987).

The BMs serve as a substratum for cell growth and are essential for the maintanance of tissue architecture. They also serve to orient proliferating cells during differentiation in

the embryo and many regeneration processes in vivo such as in epithelialization and renervation in wound repair (Kefalides et al., 1979; Vracko, 1982;). At the electron microscope level the BMs appear as amorphous structures, approx. 100nm thick, in which two layers can usually be identified: an electron- lucent layer, called lamina lucida or rara, adjacent to the plasma membrane of the cells that rest on it, and an electron-dense layer, called lamina densa. In some cases a third layer, the lamina reticularis, can be seen between the basal lamina and the underlying interstitial stroma (Alberts et al., 1989; Mignatti and Rifkin, 1993). The lamina reticularis is probably produced by fibroblasts of the connective tissue. Although the detailed molecular organization of the basal lamina has not yet been fully defined, immunoelectron-microscopic studies indicate that the major constituents of the lamina densa are the collagens (types I, II, III and primarily type IV), with glycoprotein molecules located on either side of the layer. Of the glycoproteins present in the ECM, the best characterized are: laminins, which appear to be present primarily on the plasma membrane aspect of the lamina densa, where it interacts with the cell layer; fibronectin, on the opposite aspect of the lamina densa, bind matrix macromolecules and connective tissue cells; entactin and nidogen. The lamina reticularis consists of collagens type I, II, III, and V, and proteoglycans, and fibronectins (Alberts et al., 1989; Mignatti and Rifkin, 1993).

1.3. Extracellular matrix (ECM)- degrading proteinases

The central role played by tumor associated proteinases in the degradation of the extracellular matrix has been widely documented (Nicolson, 1982; Liotta et al., 1983; Pauli et al., 1983; Saksela, 1985). A large number of reports have demonstrated increased proteinase activity in malignant tissues as compared with normal tissues. Also, an increase in proteinase expression has characteristically been demonstrated in cultured tumor cells or in normal cells transformed by various agents. In some cases a direct correlation between the metastatic potential of tumor cells and the production of certain proteinases has been observed (Liotta et al., 1980; Sloane and Honn, 1984). The origin of tumor proteinases has been traced to the tumor cells themselves, but also to host tissue cells. Therefore, extracellular matrix proteolysis in malignant tissue seems to be a highly complex process involving abnormal expression of proteinase genes both in the transformed cells and in the untransformed host cells.

Proteinases are enzymes that can hydrolyze peptide bonds either as exopeptidases or endopeptidases. These enzymes are grouped into four main classes depending on their catalytic site, optimal pH, cation requirements and susceptibility to inhibitors. Degradative enzymes that most frequently have been associated with the malignant phenotype are the serine, cysteine and aspartic proteinases; these three proteinase families are named according to the residues critical for, and characteristic of, the catalytic mechanism. A fourth family, the metalloproteinases require a metal ion, in most cases Zn^{2+}, in the active site. The metalloproteinases including elastase, the gelatinases, the

stromelysins and interstitial collagenases have all been implicated in the processes of tumor invasion *in vivo*.

1.3.1.The serine proteinases

The serine proteases include the plasminogen activators (PAs), trypsin, chymotrypsin, plasmin, thrombin, leukocyte elastase, and cathepsin G. Of these the former are the best characterized. The urokinase plasminogen activator (uPA) and its receptor (uPAR) constitute an important component of the cell migration apparatus. They play a role in diverse physiological and pathological processes mediating cellular migration and homing (Vassalli et al., 1991). For example, on vascular endothelial cells, the expression of uPA and its receptor are increased on migrating cells in wounded endothelial monolayers suggesting that these proteins may be necessary in physiological processes such as angiogenesis (Pepper et al., 1993). Similarly, these proteins have been implicated in embryogenesis and in inflammation (Vassalli et al., 1991). Urokinase and / or its receptor have also been implicated in invasion and metastasis of several different tumor types including human carcinomas of the breast (Brunner et al., 1994), colon (Pyke et al., 1991; Naitoh et al., 1995;), pancreas (Takeuchi et al., 1993), prostate (Achbarou et al., 1994), and skin (squamous cell carcinoma) (Kook et al., 1994). Evidence for involvement of uPA and its receptor in tumor invasion and metastasis has come mainly from immunohistochemical analysis or *in situ* hybridization. However, more direct methods

have also been used such as genetic manipulation of uPA or uPAR gene expression. For example in the squamous cell carcinoma study, stable transfection with a plasmid vector expressing a 300 bp antisense DNA for uPAR resulted in a reduction in uPAR mRNA and a decrease in invasion as measured using chick chorioallantoic membranes. In SV40-transformed human fibroblasts, the uPAR mRNA and protein expression was inhibited by a uPAR antisense oligomer and this resulted in decreased invasiveness of the cells as measured using Matrigel-coated filters (Quattrone et al., 1995). Overexpression of uPA in a rat prostate cancer cell line transfected with a plasmid expressing a full length rat uPA cDNA resulted in both accelerated and more widespread development of skeletal metastasis in recipient rats (Achbarou et al., 1994). Along different lines of investigation, in a study using ovarian cancer tumor extracts, the levels of uPA strongly correlated with lymphatic metastasis (Kuhn et al., 1994). Moreover, patients with low levels of uPA in the tumor extracts were found to have a better prognosis than those with higher levels. It should also be noted that while the urokinase system can act in an autocrine fashion with all the essential components synthesized by the same cell, cooperatively between different cells, in a paracrine manner, where uPAR-expressing cells bind and utilize uPA secreted by other cells has also been demonstrated. For example, in studies on colon adenocarcinomas, tumor cells which expressed high levels of uPAR were found to bind uPA synthesized by neighbouring stromal cells or tumor-infiltrating macrophages (Grondahl-Hansen et al., 1991; Pyke et al., 1991).

Urokinase converts the enzymatically inactive glycoprotein plasminogen, found in plasma and extracellular fluids to plasmin – a broad spectrum serine protease which can

degrade circulating and tissue proteins and can also contribute to the proteolytic activity of other enzymes such as metalloproteinases, by catalyzing their conversion from latent zymogen to active enzymes (Mignatti and Rifkin, 1993). Urokinase consists of an NH_2-terminal growth factor domain, a kringle domain, and a COOH-terminal region containing the active site (Pöllänen et al., 1991). The enzyme is synthesized and secreted in an inactive single-chain precursor form, the pro-uPA (Skriver et al., 1982). In this form, uPA binds with high affinity through its amino-terminal domain, to a 55-60 kDa membrane receptor (uPAR) and is then converted to a two-chain active enzyme by plasmin and possibly other proteases, such as trypsin and kallikrein (Ellis and Dano, 1991).

The human urokinase receptor is a highly glycosylated 335 amino acid polypeptide. The molecule has no transmembrane domain and is anchored to the cell membrane by glycosylphosphatidylinositol (GPI) (Ploug et al., 1991). The primary structure of the receptor consists of three repeated consensus sequences named domains I, II, and III. Cross-linking studies using uPA and uPAR (Behrendt et al., 1991) demonstrated that domain I is the uPA binding region as antibodies directed to this region prevented uPA binding (Rønne et al., 1991). It is interesting to note that uPA, found in murine mammary and lung carcinomas cells, may be involved in the cleavage of its own receptor thereby specifically releasing the ligand binding domain of the receptor (domain I) while leaving the other two domains (II and III) attached to the cell membrane (Solberg et al., 1994). This finding is relevant as levels of the remaining receptor can be measured and

correlated with uPA activity in the tumor cells. This self-cleavage phenomenon was first observed in U937 – a leukemic monocytic cell line (Hoyer-Hansen et al., 1992).

The PA-producing cells often also express PA inhibitors. The expression of these inhibitors, like PA synthesis, can be modulated by a number of biological agents, including tumor promoters and growth factors. Although PAs can form complexes with several members of the serine proteinase inhibitor (serpin) superfamily (Carrel and Travis, 1985), only three inhibitors have a sufficiently high affinity to be effective in vivo. The first of these, the type I PA inhibitor (PAI-1), is a 45-KDa protein produced by a variety of cell types and is also present in platelets and plasma (Loskutoff and Engingtons, 1977; Hekman and Loskutoff, 1985). The second inhibitor, the type 2 PA inhibitor (PAI-2), is a 46.6-KDa protein expressed most notably by cells of the monocyte-macrophage lineage (Astedt et al., 1985; Kruithof et al., 1986; Kawano et al., 1970). The third inhibitor, protease nexin1 (PN-1), is a 45-KDa protein originally purified from cultured fibroblasts but also produced by several other cell types (Baker et al., 1980; Eaton et al., 1984). A fourth, less characterized inhibitor, called PAI-3, isolated from human urine, is identical to the inactivator of one of the natural anticoagulants, protein C, but is considerably less efficient than the other inhibitors (Heeb et al., 1987).

1.3.2. The matrix metalloproteinases (MMPs)

The ECM-degrading metalloproteinases (Al-Mondhiry, 1984; Matrisian, 1990) share the following characteristics: 1) they contain a zinc ion in their active site and are inhibited by chelating agents; 2) they show consistent sequence homologies; 3) they are all secreted in a latent form and become activated by partial proteolytic cleavage; and 4) they are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs). These enzymes have been grouped into the following three main subclasses: 1) interstitial collagenase (also named type 1 collagenase or MMP-1), the polymorphonuclear (PMN) collagenase (MMP-8) and the collagenase-3 (MMP-13); 2) the gelatinases or type IV collagenases, including a 72-KDa form (72-KDa gelatinase or MMP-2) and a 92-KDa form (92-KDa gelatinase or MMP-9); and 3) stromelysins, which include three members: stromelysin-1 (transin, proteoglycanase, procollagen-activating factor or MMP-3), stromelysin-2 (transin-2 or MMP-10), and matrilysin (PUMP-1 or MMP-7, first identified as a "small metalloproteinase" from uterus) (Chin et al., 1985; Goldberg et al., 1986; Nicholson et al., 1989; Quantin et al., 1989; Sanchez-Lopez et al., 1998; Wilhelm et al., 1987; Wilhelm et al., 1989; and Woessner and Taplin, 1988). More recently, a gene has been identified that shows homology to ECM-degrading metalloproteinases and has therefore been named stromelysin-3 (ST3, MMP-11) (Basset et al., 1990).

The members of the three subclasses of MMPs have different substrate specificities (table.1-1). MMP-1 initiates the breakdown of types I, II and III fibrillar collagens which together constitute the most abundant proteins in the body (Woolley, 1984; Woessner,

1991). In addition to collagens, MMP-1 also has a limited capacity to degrade proteoglycans (Hughes et al., 1991). Fibroblast MMP-1 has also been shown to have some degradative activity towards type IV colagen but the cleavage products are different from those of the type IV collagenase (MMP-2) (Collier et al., 1988). MMP-8 has essentially the same substrate specificity as MMP-1 and cleaves types I, II and III collagens, with a preference for type I collagen (Hasty et al., 1987). MMP-13 was found to degrade type I collagen as the major substrate.

In a second group of the MMPs, are the 92- and 72- KDa forms of type IV collagenase which degrade BM (type IV) collagen, as well as types V, VII, and X collagen. In addition they can degrade gelatins (hence the name gelatinases) and fibronectins but have no effect on interstitial collagens (Wilhelm et al., 1987; Jonat et al., 1990; Hasty et al., 1990; Koklitis et al., 1991; Busiek et al., 1992;). Among the third group of MMPs, stromelysin-1 can degrade a broad range of substrates including fibronectin, laminin, types IV, V, and IX collagens (cleaved in the non-helical region), the protein core of proteoglycans, casein, gelatin and elastin (Wilhelm et al., 1987; Hughes et al., 1991; Koklitis et al., 1991). MMP-10 can degrade fibronectin, casein, and type I, III, IV and V gelatins but has only weak activity against types III, IV and V native collagens (Nicholson et al., 1989; Woessner, 1991;). Matrilysin can degrade casein, gelatins (types I, III, IV, V), fibronectin, laminin, and elastin. The substrate specificity of stromelysin-3 has not to date been conclusively defined.

In addition to these three main groups of matrix metalloproteinase, recently an MMP with a membrane-binding domain (MMP-14 or MT1-MMP) was identified by Sato et al., (Sato et al., 1994) on the surface of human fibrosarcoma HT1080 and mouse fibroblast NIH3T3 cell lines. Subsequently, three other membrane bound MMPs have been described (Hinzmann and Horst, 1995; Takino et al., 1995; Will and Huymann, 1995), MT1-MMP (MMP-14) to MT4-MMP (MMP-17) in this subfamily of proteinases. The MT-MMPs are similar to other MMPs in the basic domain structure but in addition have three characteristic insertions: a 10 – aa insert between the propeptide and the N-terminal domain (Pei and Weiss, 1995); an 8 amino acids peptide at the N-terminus, and a 72 amino acids peptide located after the haemopexin domain in the C-terminus, which contains the hydrophobic transmembrane domain (Shingleton et al., 1996). The first insertion is recognized by a golgi – associated serine proteinase, furin. Furin can activate these MMPs, triggering their secretion from the cell in an active form (Shingleton et al., 1996). Furin specifically cleaves MT1-MMP between Arg^{111}- Tyr^{112} and thus removed the prodomain which resulted in a stimulation of progelatinase A-activation function (Sato et al., 1996). To examine whether the cleavage of MT1-MMP between Arg^{111} and Tyr^{112} facilitates enzymatic activation, progelatinase A-activating capacity was compared between furin-treated and untreated GST-MT (a recombinant MT1-MMP protein which was expressed as a fusion protein with glutathione-S-transferase). Treatment of the recombinant protein with furin enhanced progelatinase A-activation capability in parallel with the cleavage of prodomain (Sato et al., 1996). This may be an important activation step and control point for the MMPs delivering active MMP at the surface of the cell and initiating the activation of other MMPs (Murphy et al., 1992; strongin et al., 1995). The

function of the second insertion is not clear but is thought to be involved in substrate specificity (Shingleton et al., 1996).

Table 1.1. The Metalloproteinase Family

Group	Enzyme	Molecular Mass (kDa)	Substrates	References
I	Type I collagenase Interstitial collagenase MMP-1	57 (latent) 52 (latent) 47 (active) 42 (active)	collagen types I, II, III	Goldberg et al., 1986
	Neutrophil collagenase MMP-8	75 (latent) 57 (active)	collagen types I, II, III	Hasty et al., 1990
	Collagenase 3 MMP-13	66 (latent) 55 (active)	collagen type I	Freije et al., 1994
II	72 kDa type IV collagenase Gelatinase A MMP-2	72 (latent) 66 (active)	collagen types IV, V, VII, X, gelatins, elastin, fibronectin	Collier et al., 1988
	92 kDa type IV collagenase Gelatinase B MMP-9	92 (latent) 84 (active)	collagen types IV, V, VII,X, gelatins,elastin.fibronectin.lamin	Wilhelm et al., 1989
III	Stromelysin-1 MMP-3	60 (latent) 57 (latent)	collagen types IV, V, IX, proteoglycans, Laminin,fibronectin, casein, elastin	Wilhelm et al., 1987
		45 (active) 28 (active)		
	Stromelysin-2 MMP-10	53 (latent) 47 (active) 28 (active)	collagen types III, IV, V, fibronectin gelatins	Nicholson et al., 1989
	Stromelysin-3		Unknown substrate Possibly other?	Pei et al., 1994
	Matrilysin MMP-11 MMP-1 MMP-7	28 (latent) 19 (active)	casein, gelatins, fibronectin, laminin, elastin, proteoglycans	Quantin et al., 1989
IV	Metalloelastase HME MMP-12	54 (latent) 45 (active) 22 (active)	elastin	Shapiro et al., 1993
V	MT-MMP (Membrane-type matrix metalloproteinase) MMP-X1 MMP-14	66 (deduced)	pro-gelatinase A activator	Sato et al., 1994

The production and activity of the MMPs are controlled at three levels namely, through regulation of gene transcription, proenzyme activation and the activity of specific tissue inhibitors (TIMPs). In most cell types MMPs are not constitutively expressed, but transcription can be induced by a number of agents, including tumor promoters, growth factors, and oncogene products (Chin et al., 1985; Salo et al., 1985; Frisch et al., 1987; Kerr et al., 1988; Schonthal et al., 1988; Brenner et al., 1989; Kerr et al., 1990; Masure et al., 1990). In some cells, the expression of more than one MMP can be coordinately regulated, as is the case for the phorbol ester induction of both stromelysin and interstitial collagenase in macrophages and endothelial cells (Mackay et al., 1992). However the expression of these two genes can also be dissociated, indicating the existence of separate regulatory mechanisms (Frisch et al., 1987).

The translational products of the MMP transcripts are rapidly secreted in a proenzyme form. In vitro, latent MMPs can be activated by a number of agents, including organic mercurials, chaotropic agents, and proteinases. In cell cultures the activation of latent MMPs appears to require a cascade of proteolytic activities involving several enzymes, including leukocyte elastase, cathepsin B (Sires et al., 1995), and serine proteinases such as trypsin and plasmin. However plasmin-independent activation of MMPs has also been demonstrated. For example cultured rat mammary carcinoma cells were shown by PA assay and tumor collagenase assay to produce both PA and active collagenase but did not appear to use plasmin for collagenase activation, because inhibition of plasmin or plasminogen activation did not affect the level of active collagenase generated or cause accumulation of procollagenase (O'grady et al., 1981). Also, Mouse Ehrlich ascites cells

were shown to activate procollagenase by a cell surface bound trypsin-like serine proteinase distinct from plasmin (Steven et al., 1980).

The fully activated MMPs can be inhibited by interaction with specific TIMPs. The TIMPs are members of a multigene family. The prototypical member of this family, TIMP-1, is a 26 – 28 KDa protein produced by bone marrow derived cells such as platelets and macrophages (Cooper et al., 1985; Welgus et al., 1985) and by connective tissue cells and found in human body fluids (Murphy et al., 1981; Carmichael et al., 1986). It has also been detected in conditioned media derived from different cell cultures (Murphy et al., 1985; Herron et al., 1986; Edwards et al., 1987; Lokeshwar et al., 1993) but its production is markedly reduced in cultures of neoplastic cells (Lokeshwar et al., 1993). The second member of the TIMP family, TIMP-2, is a 21-KDa protein isolated from human melanoma cells (Stetler-Stevenson et al., 1989). TIMP-2 shows a significant (66%) sequence homology to TIMP-1. Two functional domains have been identified in both TIMP-1 and TIMP-2 (Hahnel et al., 1993), the amino-terminal, inhibitory domain which is generic and interacts with all open MMP active sites and the carboxyl-terminal domain which is gelatinase specific. The secreted form of TIMP-1 consists of 184 a.a. residues, contains two sites of N-linked glycosylation and 12 cysteine residues which have been assigned to six disulfide bonds (Carmichael et al., 1986; Docherty and Murphy, 1990). The secreted TIMP-2 unlike TIMP-1 is not glycosylated (Stetler-Stevenson et al., 1990). Although similar in composition and structure, the two proteins are immunogenically distinct as antibodies raised against TIMP-1 did not react with TIMP-2 (Goldberg et al., 1989; Stetler-Stevenson et al., 1990). Both TIMP-1 and

TIMP-2 bind noncovalently to active MMPs in a 1:1 molar ratio and specifically inhibit their activity. However, TIMP-1 forms a complex with the latent proform of the 92-KDa type-IV collagenase (progelatinase B), whereas TIMP-2 appears to form a complex specifically with the latent form of the 72-KDa type-IV collagenase (progelatinase A) (Goldberg et al., 1989; Stetler-Stevenson et al., 1989;). By deletion mutagenesis, it has been shown that interactions between the C-terminal domains of TIMPs and MMPs influence the rate of complex formation. Ionic interactions involving the negatively charged C-terminus of TIMP-2 are thought to be important in the rapid binding to the C-terminus of progelatinase A (Willenbrock et al., 1993). Evidence from studies involving truncated enzymes and cross-linking methods suggests the following model: the N-terminal domain of TIMP is inhibitory and the C-terminal domain confers binding specificity. Therefore, although the N-terminal domain of TIMP-1 and TIMP-2 bind to the N-terminal domain of active gelatinase A, only the C-terminal domain of TIMP-2 will bind specifically to the C-terminal domain of progelatinase A (Yu et al., 1996a). This indicates a possible specificity in the TIMP-MMP interactions. The TIMPs also appear to be involved in the control of pro-MMP activation. In human fibroblasts, the plasma membrane-mediated activation of the 72-KDa progelatinase is inhibited by TIMP-2, but only poorly by TIMP-1 (Ward et al., 1991). Therefore it was proposed that TIMP-2 has a dual regulatory role: the inhibition of metalloproteinase activity as well as the inhibition of activation of 72 kDa progelatinase by a membrane metalloproteinase leading to progelatinase self-cleavage (Ward et al., 1991).

TIMP-1 and –2 are independently regulated *in vitro* and *in vivo* (Stetler-Stevenson et al., 1990). For example in colorectal carcinoma specimens, the levels of TIMP-1 but not TIMP-2 transcripts were found to be elevated relative to the adjacent normal mucosa (Stetler-Stevenson et al., 1990). In contrast to TIMP-1 which can be up-regulated by TGF β in normal and malignant cells, TIMP-2 transcripts are down-regulated by this mediator in tumor cells but not in normal fibroblasts (Stetler-Stevenson et al., 1990). Phorbol esters which were consistently found to induce transcription of TIMP-1 (Murphy et al., 1985; Stetler-Stevenson et al., 1990), only slightly increased TIMP-2 transcript levels in a normal fibroblast cell line and either failed to modulate or reduced transcript levels in melanoma cells and fibrosarcoma cells, respectively (Stetler-Stevenson et al., 1990).

The third member of this family, TIMP-3 was first isolated as a transiently expressed 21 kDa protein in the extracellular matrix of transformed chick fibroblast cultures (Blenis and Hawkes, 1983; Blenis and Hawkes, 1984). Although referred to initially as the 21 kDa protein (Blenis and hawkes 1983), the present consensus is that the unmodified mature protein migrates at 24-25 kDa (Kishnani et al., 1995; Sun et al., 1995). TIMP-3 shares a 40.1% amino acid sequence identity with TIMP-1 and 44.9% with TIMP-2. In contrast to TIMP-1 and TIMP-2, TIMP-3 has poor aqueous solubility and a specific localization in the ECM (Pavloff et al., 1992; Blenis and Hawkes, 1983). In cultured cells, TIMP-3 is present in the substratum (i.e., the ECM) but is absent from conditioned medium of cell cultures (Leco et al., 1994). It is possible that the more variable C-terminal part of the TIMPs may confer distinctive properties on each TIMP and that the

C-terminal half of TIMP-3 contains the ECM-binding domain. The ECM ligand (s) of TIMP-3 have not yet been identified, but it has been suggested that one ligand might be hyaluronic acid (Yang and Hawkes, 1992). TIMP-3 and TIMP-1 are equipotent in their ability to inhibit MMP-1 , MMP-2, MMP-3, and MMP-9 (Anand-Apte et al., 1996).

Recently a new member of the TIMP family has been identified using the expressed sequence tag sequencing approach (Greene et al., 1996). It was designated TIMP-4 on the basis of its high sequence homology to the TIMP family, the 12 conserved cysteine residues, and the MMP inhibitory activity. The open reading frame encodes a 224-amino acid precursor including a 29-residue secretion signal. The predicted polypeptide sequence of the new protein has 37% identity with TIMP-1 and 51% identity with TIMP-2 and –3. In Northern analyses, only the adult heart showed abundant TIMP-4 transcripts with a 1.4 - kb predominant transcript band. Very low levels of the transcripts were detected in the kidney, placenta, colon, and testes, lung, thymus, and spleen. This unique expression pattern suggests that TIMP-4 may function in a tissue-specific fashion in extracellular matrix homeostasis. Greene et al., (1996) cloned and sequenced this novel human TIMP gene (TIMP-4). They analyzed the MMP inhibitory activity of the expressed rTIMP-4 from the conditioned media of transfected MDA-MB-435 human breast cancer cells and as they expected, rTIMP-4 proteins expressed from human breast cancer cells possess an inhibitory activity against MMP and are secreted extracellularly.

1.3.3. Matrix metalloproteinases in cancer invasion and metastasis.

Extracellular matrix (ECM) turnover is a tightly regulated process. Much of the normal (physiological) or abnormal (pathological) degradation of the ECM is catalyzed by a class of proteases known as the matrix metalloproteinases (MMPs) or matrixins. Matrixins are a family of homologous Zn^{++} atom dependent endopeptidases that are usually secreted from cells as inactive zymogens. Net degradative activity in the extracellular environment is regulated by specific activators and inhibitors. One member of the matrixin family, gelatinase A, is regulated differently from other MMPs, suggesting that it may play a unique role in cell-matrix interactions, including cell invasion. In fact, it was the requirement for tumor cells to degrade type IV collagen-rich basement membranes that led to the discovery of 72 KDa type IV collagenase, which was later renamed gelatinase A (Liotta et al., 1979). It has since been found that gelatinase A is not only tumor specific, as the conversion from the 72 KDa progelatinase A to the active 62 KDa species may be a key event in the acquisition of invasive potential, but also is involved in ECM remodelling in a wide range of non-neoplastic processes, including embryonic development, trophoblast invasion, angiogenesis, and wound healing.

Evidence that gelatinase A is required for tumor invasion and metastasis has come from a number of experimental studies correlating enzyme activity with the metastatic potential of tumor cell lines (Liotta et al., 1980; Turpeenniemi-Hujanen et al., 1985; Nakajima et

al., 1987; Nakajima et al., 1989;). In a recent study by Imren et al. (Imren et al., 1996), the use of retroviral constructs to over-express TIMP-2 in experimental tumors in nude mice was found to reduce the growth rate of tumors, reduce local invasion, and lead to the formation of a thick connective tissue capsule around the tumor. These effects are likely to be at least partly due to inhibition of gelatinase A by TIMP-2. Studies on human tumor tissues provide further evidence that gelatinase A has an important role in tumor invasion. For example, immunocytochemical and in situ hybridization studies have shown increased gelatinase A expression in many different human tumors, including carcinomas of the colon (Poulsom et al., 1992; Pyke et al., 1993), pancreas (Gress et al., 1995), prostate (Boag and Young, 1994), bladder (Davies et al., 1993), skin (squamous and basal cell carcinoma) (Pyke et al., 1992), breast (Davies et al., 1993; Polette et al., 1994), and ovary (Autio-Harmainen et al., 1993).

In almost all carcinomas studied by in situ hybridization, gelatinase A mRNA appeared to be localized to the tumor stroma. Exceptions to this rule include prostate cancer, where both mRNA and protein were detected mainly in the neoplastic epithelium (Boag and Young, 1994), and also carcinomas of the hypopharynx and pancreas, in which gelatinase A mRNA was localized to both neoplastic cells and tumor stroma (Gress et al., 1995; Miyajima et al., 1995). For carcinomas of the colon, breast, and ovary, in situ hybridization studies detected gelatinase A mRNA in the connective tissue stroma (Poulsom et al., 1992; Autio-Harmainen et al., 1993; Poulsom et al., 1993; Pyke et al., 1993; Polette et al., 1994). Surprisingly, gelatinase A protein was detected by

immunocytochemistry mainly in the neoplastic epithelium of these tumors (Levy et al., 1991; Autio-Harmainen et al., 1993; Polette et al., 1994). One explanation for this discrepancy could be that the half-life of the gelatinase A mRNA, and (or) the efficiency of translation, differ between the stromal cells and neoplastic epithelial cells. Another explanation is that the enzyme is synthesized predominantly in the tumor stroma but that on secretion it is bound mostly by a putative receptor on the surface of neoplastic epithelial cells (Yu et al., 1996a).

Grigioni and co-workers (Grigioni et al., 1991) found that increased immunostaining for gelatinase A in hepatocellular carcinomas was associated with poorer survival. Increased immunostaining of breast cancer tissue has also been correlated with a higher frequency of local recurrence (Daidone et al., 1991). In a study of gastric cancer patients, TIMP-2 expression was found to be lower in advanced cases while gelatinase A expression was either increased or unchanged (Grigioni et al., 1994). In addition, patients who died from their primary gastric tumor had higher percentages of gelatinase positive cells and lower percentages of TIMP-2 positive cells compared with survivors. In patients with stage III ovarian cystadenocarcinoma, tissue levels of gelatinase A appeared to be a prognostic indicator (Garzetti et al., 1995). Tissue gelatinase A levels in these ovarian tumors were shown to correlate with risk of recurrence and appeared to be independent of histological grade, lymph nodal status, and residual disease after debulking surgery. Although this evidence is suggestive of a role for MMP-2 in tumor progression, the level of active enzyme will probably be more informative in that it is the activated enzyme that could

contribute to the invasive phenotype. Quantitative gelatin zymography has so far been used in a small number of studies to assess the level of active gelatinase A in tumors. In breast cancer, the proportion of gelatinase A in the active form has been found to increase with higher tumor grade (Davies et al., 1993), although another group found no correlation with staging or other existing prognostic markers (Brown et al., 1993). In colon cancer, increased levels of both active and proenzyme forms of gelatinase A were detected in neoplastic cells microdissected from invasive areas as compared with an equal number of normal epithelial cells (Emmert-Buck et al., 1994).

Recently, it is found that MT1-MMP or MMP-14 expressed in human colon, breast, head, and neck carcinomas (Okada et al., 1995) and gastric carcinomas (Nomura et al., 1995) and it is important in the activation of MMP-2 in tumor invasion (Tokuraku et al., 1995).

1.3.4. The lysosomal proteinases

The ubiquitously expressed lysosomal proteinases include members of the cysteine (cathepsin B, L, H, S, K and O) and aspartic (cathepsin D) proteinases. Specialized cells such as the polymorphonuclear leukocyte and the mast cell contain in addition lysosome-like granules with unique serine proteinases (cathepsin G, elastase, chymases and tryptases).

In general, all cysteine proteinases are true intracellular enzymes, usually found in the cytosol or in lysosomes. Although cysteine proteinases are lysosomal in origin, their release from living cells into the extracellular space has been documented in a few pathological conditions. Some malignant tissues secrete a "cathepsin B-like" cysteine proteinase, which is thought to be involved in ECM degradation and invasion and/ or in facilitating cell detachment from primary tumors (Sloane et al., 1981). Cathepsins have also been found in the conditioned media of some cultured tumor cells and breast carcinoma organ cultures (Poole et al., 1978; Recklies et al., 1982).

Similarly to the metalloproteinases, the cysteine proteinases are also synthesized as inactive precursors. The amino acid residues critical for peptide bond hydrolysis are absolutely conserved in all members of this family and there is a high level of homology in regions around these residues (Kamphuis et al., 1985; Bond and Butler, 1987). Other parts of the protein show a greater sequence diversity. Detailed sequence analysis of the family of lysosomal proteinases has led to further division of its members into two sub-families (Karrer et al., 1993). Cathepsin L, H and S are most similar to the founder member, papain. They have long propeptide domains (~100 residues) and the length of the mature protein is approximately the same (~200 residues). Cathepsin B is distinct, as it contains a shorter propeptide (62 residues) but has a larger mature protein. The proregion is contiguous to the N-terminal signal peptide and can act as a specific inhibitor of enzymatic activity (CB) (Fox et al., 1992). This region must be proteolytically cleaved to generate the active enzyme. However, binding is only effective at neutral pH and the

complex dissociates on acidification, leading to degradation of the propeptide. Lysosomal proteinase precursors are glycoproteins which are tagged for transport to the lysosome by substitution of their N-linked oligosaccharides with mannose-6-phosphate (Man-6-P). Receptors (MPRs) in the endosomes of the trans Golgi network then modify this sugar and transport the precursor to the lysosome. As the vesicle contents acidify, the enzyme is released from the receptor which is then recycled. In this way the endosomal enzymes are segregated from the proteins that are destined for secretion. Two mannose-6-phosphate receptors have been described, one being cation dependent, the other cation independent. The former mediates intracellular transport to the lysosome, while the latter is involved in re-uptake of secreted precursor forms. The cation-independent mannose-6-phosphate receptor is identical to the insulin-like growth factor II (IGF-II) receptor (Kornfeld and Mellman, 1989). In addition, this sorting process appears to be complemented by other receptors which bind to the lysosomal proteinase precursors in a mannose-6-phosphate independent manner (McIntyre and Erickson, 1991). A small number of lysosomal enzymes fail to bind to the receptors and are secreted along with secretory proteins. These enzymes may bind to surface MPRs in coated pits and be internalized into the prelysosomal compartment. Concomitant with transport, acidification also triggers activation of the enzyme by proteolytic cleavage of the propeptide, in most cases apparently by autoprocessing.

Unscheduled proteolytic activity of lysosomal cysteine proteinases is restricted by two mechanisms. A series of inhibitors (the cystatins) are present both in the cytoplasm and

in the extracellular milieu. In addition, the mature forms of the cysteine proteinases, cathepsin B, cathepsin H and cathepsin L have a built in "self-destruct" mechanism whereby they undergo spontaneous denaturation when they are exposed to neutral - alkaline pH on release from the acidic environment of the lysosome (Barrett et al., 1988).

1.3.5. The cysteine proteinases

The family of cysteine proteinases includes endo- and exopeptidases which depend on the reactive thiol group of a Cys residue for catalytic activity. In all the members of this family, the amino acid residues around the Cys residue in the active site and an essential His are highly conserved (Bond and Butler, 1987), whereas other regions of the polypeptide show more sequence diversity.

The substrate specificity of these proteinases is of great interest because it provides an important guide for inhibitor design. Generally these proteinases can be assayed using dipeptide-based substrates having a blocked N-terminus and a chromogenic or fluorogenic break up group. Some examples are carbobenzyloxy-phenylalanyl-arginine-p-nitroalanilide(z-P-Arg-pNA) and carbobenzyloxy-argininyl-arginine-4-methyl-7-coumarylamide (z Arg-Arg-MCA) which have been used for analysis of cathepsin L and B activity (Barrett and Kirschke, 1981).

Some cysteine proteinaes are able to activate procollagenase or directly degrade collagen and can digest the protein core of proteoglycans (Etherington, 1976; Eeckhout and Vaes, 1977; Delaisse et al., 1980; Gohda and Pitot, 1981). In addition, cathepsin B, but apparently not other cathepsins, can activate both the soluble and the receptor-bound forms of pro-uPA (Kobayashi et al., 1991), thereby contributing to proteolytic cascades required for ECM degradation.

Cathepsin L. Cathepsin L is considered to be one of the more potent lysosomal proteinases. It is a 25 kDa endopeptidase which can degrade proteoglycans, collagen and elastin (Mason et al., 1986; Maciewicz et al., 1990; Nguyen et al., 1990;). The activity of this enzyme has been analyzed using the synthetic substrates, z-phe-Arg-pNA and z-Arg-Arg-MCA. The mature form of the enzyme is highly susceptible to denaturation at neutral to alkaline pH. In 1985 Gal et al observed that transformed fibroblasts secrete large amounts of an activatable acid protease which they termed MEP (Major Excreted Protein) and this was identified as procathepsin L (Gal and Gottesman, 1986). MEP was subsequently identified in other transformed cell lines (Gal and Gottesman, 1986) suggesting that in these cells, some of the enzyme is routed to lysosomes, while a large proportion is secreted (Gal et al., 1985). The proportion of MEP which is secreted by cultured mouse 3T3 cells could be augmented by phorbol esters, and cellular transformation (Gottesman and Sobel, 1980; Nilsen-Hamilton et al., 1981; Scher et al., 1983; Doherty et al., 1985; Frick et al., 1985). Recent cloning of cDNAs encoding mouse, rat, and human cysteine proteases confirmed that MEP is in fact homologous to

the lysosomal form of cathepsin L (Portnoy et al., 1986; Ishidoh et al., 1987; Troen et al., 1987; Joseph et al., 1988).

In quiescent NIH 3T3 fibroblasts, MEP/cathepsin L is synthesized as a 39 kDa precursor which is processed to lower molecular weight forms upon delivery to lysosomes (Gal et al., 1985; Dong et al., 1989). The secreted procathepsin L was shown to degrade extracellular matrix proteins such as type I and IV collagen, fibronectin and laminin in the presence of glycosaminoglycans (GAGs) (Ishidoh and Kominami, 1995). The extracellularly processed Cathepsin L also participates in the degradation of type I and IV collagen, fibronectin and laminin but for fibronectin the proteolytic degradation products produced by the two enzyme forms were found to be distinct indicating that procathepsin and cathepsin L may have different substrate specificities (Ishidoh and Kominami, 1998). The recycling of extracellular procathepsin L is less efficient than that of other proforms of lysosomal enzymes due to a lower degree of N-linked oligosaccharides (Dong and Sahagian, 1990). Targeting of cathepsin L to the lysosomes is mediated by mannose 6-phosphate (Man-6-P) receptors which recognize phosphorylated asparagine-linked oligosaccharides of lysosomal proteins during their biosynthesis (Poole et al., 1978; Recklies et al., 1980; Hasnain et al., 1992; Guinec et al., 1993; Hasnain et al., 1993).

Cathepsin B. Of the many lysosomal cysteine proteinases so far characterized, cathepsin B has received the most attention. It is unique in that it can function as both an endopeptidase and a peptidyldipeptidase (removing dipeptides from the c-terminus of substrate proteins). The three dimensional structure of the mature 30 kDa protein provided the structural basis for this dual activity (Musil et al., 1991). The molecular structure of human liver cathepsin B was resolved by X-ray crystallography (Musil et al., 1991). The overall folding pattern of cathepsin B and the arrangement of the active site residues are similar to the related cysteine proteinases papain, actinidin and calotropin DI. However, several large insertion loops are accomodated on the molecular surface and modify its properties. Some of the primed subsites which accept substrate residues are occluded by a novel insertion loop. Two histidine residues (His 110 and His 111) in this 'occluding loop' provide positively charged groups that can act as an acceptor for the free negative charge on the carboxy terminus present on the exopeptidase substrate. These structural features explain the well-known dipeptidyl carboxypeptidase activity of cathepsin B. The above mentioned histidine residues, but also the buried Glu171 might represent the group with a pk_a of ~ 5.5 near the active site, which governs endo - and exopeptidase activity (Musil, 1991).

Using synthetic substrates, cathepsin B was found to be active over a wide pH range (Hasnain et al., 1992). Activity in the neutral – alkaline pH range is however severely limited by the instability of the enzyme. Among some of the known substrates of this enzyme are several ECM proteins including proteoglycans such as hyaluronic acid and

chondroitin sulphate, fibronectin and collagen (Maciewicz et al., 1990; Nguyen et al., 1990; Guinec et al., 1993).

A role for extracellular cathepsin B in tumor invasion has been recognized for almost two decades (Poole et al., 1978; Recklies et al., 1980; Recklies et al., 1982;). Both procathepsin B and an active form, stable at neutral pH, have been identified in tumor cell and organ cultures (Mort and Recklies, 1986). In addition, elevated levels of CB have been associated with the metastatic phenotype of melanoma, hepatoma, colon adenocarcinoma (Sloane et al., 1981; Sloane et al., 1986; Rozhin et al., 1987; Sloane et al., 1987; Moin et al., 1989; Rozhin et al., 1990), prostate carcinoma (Sinha et al., 1995) and mammary adenocarcinoma cell lines (Rozhin et al., 1990). More recently a cell membrane - associated form of CB has been described (Sloane et al., 1986) and an alternatively spliced CB mRNA lacking exon 2 has been reported which codes for a form of the enzyme lacking the signal peptide and seven residues of the propeptide (Gong et al., 1993). It was postulated that the translational product of this truncated mRNA modified intracellular targeting properties and is therefore secreted (Gong et al., 1993). Alterations in trafficking of cathepsin B were observed in MCF-10 human breast epithelial cells transfected with oncogenic ras. In these cells, there was an increased association of cathepsin B activity and cathepsin B protein with plasma membrane/endosomal fractions (Sloane et al., 1994), which suggests that alterations in cathepsin B trafficking occur at the point of transition between the pre-neoplastic and neoplastic state.

1.3.6. Cysteine proteinases in cancer invasion and metastasis

Malignantly transformed cells are associated with increased release of a number of proteolytic enzymes which are thought to facilitate tumor cell growth and invasiveness (Liotta et al., 1986; Tryggvason et al., 1987; Laiho and Keski-Oja, 1989).

Lysosomal vesicles contain a variety of cysteine cathepsins and constitute a proteolytic 'package' with a hydrolytic potential that depends on the set of enclosed cathepsins. Lysosomes may differ not only in histologically different types of cells, but also in the same cell type under different physiologic and pathologic conditions (Heuser, 1989; Honn et al., 1994; Sameni et al., 1995). The redistribution of lysosomal enzymes may play an important role also in tumor progression (Honn et al., 1994; Keppler and Sloane, 1996).

Tumor cells produce and release type-IV-collagen specific collagenases which function at neutral pH (Salo et al., 1983; Irimura et al., 1987; Starkey et al., 1987), but the micro-environment of the tumor-host junction may favour the activity of collagenolytic enzymes that act at acidic pH rather than at neutral pH. Collagenolytic enzymes acting at acidic pH include the lysosomal cysteine proteinases, cathepsin B, L, N and S (Etherington, 1977; Ducasting and Etherington, 1978; Kirschke et al., 1982; Maciewicz et al., 1987; Maciewicz and Etherington, 1988). The ability to release hydroxyproline from basement membranes has been shown qualitatively for cathepsin B at acidic pH (Etherington, 1977; Davies et al., 1978) and more quantitatively for cathepsin L in the pH

range of 3.0 to 5.5 (Baricos et al., 1988). Both cathepsin B and L have been shown by cell culture studies to be synthesized as precursors which are thought to be enzymatically inactive (Mort and Recklies, 1986; Mc Donald and Kadkhodayan, 1988; Maciewicz and Etherington, 1988; Nishimura et al., 1988). A pre-malignant human colorectal epithelial cell line secreted as much, or more, of both cathepsins B and L precursors as did the malignant, carcinoma-derived cell line, thus indicating that the invasive potential of a tumor may be related to its ability to process extracellularly the secreted precursor enzyme to a mature and consequently active enzyme, rather than to the amount of proteinase synthesized and/or secreted (Maciewicz et al., 1989).

In some tumor cells, the cathepsin precursors are secreted in large amounts, avoiding the lysosomal targeting and normal processing (Rochefort et al., 1990). In others, lysosomes, normally localized to the perinuclear area, were found in proximity to and possibly merging with the plasma membrane, with release of active forms of cathepsins (Sloane et al., 1994a,b; Sameni et al., 1995).

In addition to altered trafficking, increased mRNA, protein and activity levels have been reported for Cathepsin D, Cathepsin B and Cathepsin L in numerous animal and human tumors (Sloane et al., 1994a). The induction of various cathepsins is clearly selective (Budihna et al., 1995; Lah et al., 1995; Lah et al., 1996a) and is most likely associated with specific tumor cells and related to specific steps in tumor progression (Berquin and

Sloane, 1995). In addition to directly hydrolyzing protein components of the extracellular matrix (Schmitt et al., 1992a) and/or modifying other proteins relevant to tumor progression, cathepsins have also been suggested to activate precursors of other proteinases, initiating proteolytic cascade reactions (Schmitt et al., 1992b).

One of the first association of cathepsin B with malignancy was made by Poole and colleagues (Poole et al., 1978) in studies on explants of human breast carcinoma. Immunocytochemical analyses revealed high levels of expression of cathepsin B in human colon carcinoma which correlated with tumor progression and shortened patient survival (Campo et al., 1994). In human breast carcinoma, high levels of total cathepsin B in tumor cytosols or strong immunostaining in stromal components (myofibroblastic and endothelial cells) were shown to correlate with reduced overall patient survival, suggesting that cathepsin B levels may have prognostic significance (Lah et al., 1996b). Expression of cathepsin B in tumors is not homogeneous. In fact, the highest levels of expression are at the invasive edges of tumors (Emmert-Buck et al., 1994). Cathepsin B activity is eight fold greater at the invasive edges of human breast carcinoma than in matched normal epithelial cells (Buck et al., 1994). In the case of the B16 murine melanoma, activity and mRNA levels for carthepsin B were shown to increase with metastatic potential (Sloane et al., 1982; Moin et al., 1989). Only the levels of cathepsin B correlate with experimental metastasis while activities of cathepsins D and H and mRNA levels for cathepsins D, H, L, and S remained unchanged (Sloane et al., 1982; Qian et al., 1989). Subsequently, the increased level of cathepsin B transcripts in the

highly metastatic B16 subline was found to be due to an increased transcription rate (Qian et al., 1994). In addition, increased expression (mRNA, protein and activity), membrane/endosomal association and/or release of cathepsin B have been observed in transformed murine 3T3 fibroblasts (Achkar et al., 1990) and transformed human breast epithelial cells (Sloane et al., 1994b) and in several malignant murine and human tumors, including human breast (Poole et al., 1978), colon (Murnane et al., 1991) and prostate tumors (Sinha et al., 1993). In prostate specimens, increases in immunostaining and in in situ hybridization for cathepsin B in epithelial cells parallel malignant progression and are observed at the leading, invasive edges of prostate carcinomas (Sinha et al., 1993). Such increases in cathepsin B activity in malignant tumors may reflect alterations in synthesis, in activation and processing, and/or in intracellular trafficking and delivery as well as in the endogenous inhibitors of cathepsin B. Increases in mRNA transcripts for cathepsin B have been identified, but an association of multiple transcripts with malignancy has not been confirmed. Cathepsin B precursors found in human malignant ascites fluid do not possess mannose-rich carbohydrates suggesting that a defect in the post translational processing of carbohydrate moieties on tumor cathepsin B may be responsible for the release of cathepsin B observed in many tumor systems. However, the intracellular trafficking of cathepsin B responsible for its association with plasma membrane/endosomal systems and for its release will require further study as both latent, precusor forms of cathepsin B and native forms of cathepsin B are involved (Sloane et al., 1990a,b).

Similarly to cathepsin B, both the expression (mRNA) and release of a precursor cathepsin L (procathepsin L or MEP) parallel malignant transformation of murine 3T3 fibroblasts (Kane and Gottesman, 1990). Several growth factors such as PDGF, EGF, as well as PMA were shown to upregulate procathepsin L synthesis in non-transformed or v-ras-transformed NIH3T3 cells (Ishidoh and Kominami, 1998). Denhardt and coworkers (Denhardt et al., 1987) reported that the levels of mRNA for cathepsin L in murine cells transfected with the ras oncogene correlated with their metastatic potential *in vivo*, suggesting that procathepsin L might participate in tumor metastasis.

In transformed and malignant cells, an alteration in the trafficking of cathepsin B and L has consistently been observed (Rozhin et al., 1989; Kane and Gottesman, 1990; Sloane, 1990a; Sloane et al., 1990b; Sloane et al., 1992;). For example, in murine and human melanomas, membrane/endosomal association of cathepsin L parallels malignancy (Rozhin et al., 1989). The increased expression and the altered trafficking of cathepsin B and L in malignant cells probably reflect modifications in more than one step in the pathway that normally leads to their delivery to lysosomes (i.e., alteration in transcription/translation, co- or post-translational processing, and/or in intracellular sorting and targeting).

1.3.7. Cysteine proteinase inhibitors as potential anti-metastatic agents

The cysteine proteases present a relatively new therapeutic challenge in the treatment of neoplasms and, as such, the development of therapeutically useful inhibitors to specific members of the mammalian cysteine proteases is still in its infancy (Michaud and Gour, 1998). However, active research in this field over the past few years has resulted in the production of several novel inhibitors with both increased potency and selectivity for particular members of the cysteine protease family (Poste and Fidler, 1980; Yu et al., 1996b).

The activities of all cysteine proteinases are controlled intracellularly and extracellularly by a family of specific cysteine proteinase inhibitors, the cystatins (Lumkowski et al., 1997) which are widely distributed intracellularly and in the extracellular fluid. One of these endogenous cysteine proteinase inhibitors, cystatin A (stefin A), is affected by malignant transformation. Reduced expression (mRNA and protein) of stefin A is found as well as a reduction in its inhibitory activity against cathepsin B (Heidtmann et al., 1997). One limitation of the data which support a functional role(s) for cathepsin B and its endogenous inhibitors in cancer progression is that they are mainly correlative. Experimental approaches utilizing well-defined model systems in conjunction with genetic manipulation of cathepsin B and its endogenous inhibitors are needed to provide convincing evidence that cathepsin B has an important role in cancer.

Substantial progress has been made in the development of specific low molecular weight inhibitors that could be used therapeutically. Screening of microorganisms yielded pepstatin, a potent general inhibitor of the aspartic proteinases, and E-64, a naturally occurring epoxysuccinyl, which is a potent specific irreversible inhibitor of the cysteine proteinases of the papain family, and was first isolated from a culture of Aspergillus japonicus (Hanada et al., 1978). Modification of the peptidyl portion of E-64 has allowed the introduction of selectivity for cathepsin B. Also by esterifying the charged groups on the inhibitor, prodrugs have been produced which pass through cell membranes allowing entry into the intracellular compartment. De-esterification then leads to intracellular generation of active inhibitor that allows differentiation between intracellular and extracellular proteinase activity.

E-64 was able to abrogate the invasion process in several tumor cell lines: inhibition of cathepsin B alone was postulated to be directly responsible for the abrogation of invasion in the EJ transitional cell carcinoma of the bladder (Redwood et al., 1992). In the HOC-1 ovarian cancer model, cathepsin B inhibition was shown to prevent the activation of prourokinase (pro-uPA) to active uPA, thereby blocking plasmin production and abrogating invasion (Kobayashi et al., 1992). In a study with human prostate cancer cell lines, inhibition of cathepsin B is believed to have prevented the activation of the aspartyl protease cathepsin D, resulting in an overall decrease in the invasiveness of the cell line (Weiss et al.,1993).

The development of specific inhibitors for the individual members of the cysteine protease family has been slow. These specific inhibitors are necessary not only for a true understanding of the individual roles of each of the members of the cysteine protease family but also in order to minimize the toxic effects arising from the lack of inhibition specificity.

CHAPTER 2

The IGF-I receptor: structure, function, and role in malignancy

2.1. The cell cycle: overview

Over the past few years cell biologists have made remarkable progress in identifying the molecular mediators which drive the cell cycle: the carefully choreographed series of molecular events which culminate in cell division. In doing so they have not only provided a better understanding of one of the most fundamental of the cell functions, they have also opened a new direction for research aimed at pinpointing the genetic changes that lead to cancer. The reason for this intriguing convergence is that accumulating evidence indicates that derangements in the cell cycle machinery are likely at the core of the uncontrolled growth characteristic of cancer. Many cancer causing oncogenes, for example, turn out to encode components of the pathways through which growth factor-derived signals feed into the cell cycle to stimulate cell division. The tumor suppressor genes, which normally help keep cell growth in check, may also operate through the cell cycle. The recent demonstration that the protein encoded by the p53 tumor suppressor gene apparently inhibits cell growth by turning on the production of a protein that blocks the cell cycle is a case in point (Marx, 1993).

The cell cycle consists of four major phases: (1) the presynthetic phase, G_1; (2) the phase of DNA synthesis, S; (3) the premitotic phase, G_2; and (4) mitosis, M (Pardee, 1989; Lowe, 1991) (Fig. 2-1). Competence factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are necessary in order for quiescent cells, i.e., cells which are out of the cell cycle (G_0), to enter G_1 . In the case of Balb/c-3T3 cells, ~ 12 hr is required, after induction of competence, for the cell to progress from G_0 through

G_1 to S phase. Progression into S phase cannot occur in the absence of progression factors such as IGF-1 (Baserga et al., 1997) and epidermal growth factor (EGF). On the basis of these studies it was proposed that subphysiologic concentrations of IGF-1, in addition to EGF, are required for competent cells to progress from the G_0 to the V point—a restriction point which occurs~6 hr into G_1 and at which point cells will be arrested in their progression in the absence of essential nutrients. The only growth factor required for progression from the V point to the S phase is IGF-1 at physiological concentration. An additional checkpoint, the W point, occurs later in G_1 at the G_1/S border (Fig. 2-1). Studies from the group of Renato Baserga demonstrated that fibroblast Balb/c-3T3 cells stably transfected with expression vectors for IGF-1 and the IGF-1 receptor are able to grow in serum-free medium without the addition of any exogenous growth factor. When transfected only with the IGF-1 receptor plasmid, the only supplement required by the cells was IGF-1. These experiments clearly indicate that an interaction between IGF-1 and the IGF-1 receptor is sufficient for these cells to grow. PDGF and EGF, which are required by the parental cells, are not necessary to support growth of the overexpressing clones (Pietrzkowski et al., 1992a). A hypothesis which emerges from these experiments postulates that the only function of PDGF and/or EGF is to induce enough IGF-1 and IGF-1 receptor to elicit the growth response (Baserga, 1992; Baserga and Rubin, 1993). In line with this hypothesis, the same group showed that EGF stimulates the expression of IGF-1 mRNA in cells overexpressing the IGF-1 receptor, and that this effect can be abolished using antisense oligodeoxynucleotides to IGF-1 receptor mRNA. Furthermore, the modest growth of cells overexpressing EGF and the EGF receptor in serum-free media can be inhibited with antisense oligomers directed to

the IGF-1 receptor. These results can thus be interpreted to suggest that the role of EGF is to activate an autocrine loop based on the IGF-1—IGF-1 receptor interaction (Pietrzkowski et al., 1992b). In addition, PDGF was shown to increase the expression of the IGF-1 receptor gene by augmenting its promoter activity (Rubini et al., 1994). Alternatively, it is possible that activation of the IGF-1 receptor in the overexpressing clones may induce the transphosphorylation of the EGF receptor (Baserga and Rubin, 1993). However, for many cell types, PDGF or EGF alone can induce transition to S-phase (Fig.2-1). EGF has been demonstrated to be a primary mitogen for hepatocytes and hepatoma cells and stimulates hepatocyte DNA synthesis (Fausto et al., 1995). EGF has been implicated as a hepatotrophic factor during liver regeneration. Indeed, circulating levels of EGF, TGFα, and HGF increase dramatically after partial hepatectomy, a procedure which results in large scale hepatocyte proliferation (Diehl and Rai, 1996). These are strong inducers of DNA synthesis in cultured hepatocytes. The EGFR may be involved in liver regeneration and development through the interaction with TGFα. Overexpression of TGFα induces liver neoplasia, pancreatic metaplasia and abnormal mammary gland development in transgenic mice (reviewed by Fausto et al., 1995).

In addition to growth factors, progression through the cell cycle is dependent on the sequential activation of a family of serine-threonine-specific protein kinases, whose activities are regulated by accessory proteins called cyclins. Nine cyclins and ten cyclin-dependent kinases (cdk) have been identified to date in mammalian cells (Hartwell and Weinert, 1989; Enoch and Nurse, 1991). Some of these cyclins are expressed during G_1

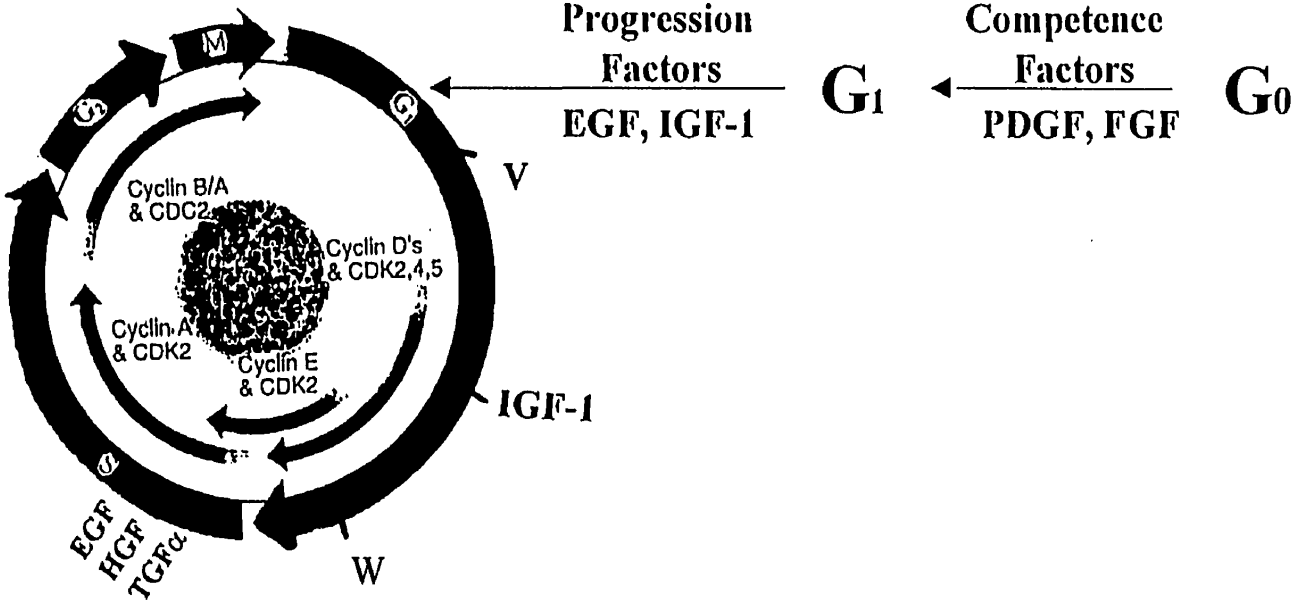
(Fig. 2-1), which led to the suggestion that the critical event regulated by growth factors is the modulation of G_1 cyclin expression. Among G_1 cyclins, the D-type cyclins in particular emerge as important players in the control of cellular replication. D-type cyclins are rapidly induced by colony-stimulating factor-1 (CSF-1) and PDGF in responsive macrophages and fibroblasts, respectively (Matsushime et al., 1991; Won et al., 1992). Cyclin-D-cdk complexes are capable of phosphorylating the retinoblastoma gene product (Hinds et al., 1992) and, in addition, alterations in cyclin D expression occur in certain tumors (Lammie et al., 1991; Motokura et al., 1991). While cyclin D has received the most attention, it is not the only cyclin whose expression is deranged in cancer cells. Keyomarsi et al., (1994) found that the gene for cyclin E, which also becomes active during the G1 phase of the cycle, is overexpressed in cultured breast cancer cell lines and in primary breast tumors. Cyclin E production was also detected in a wide variety of other cancers, including lung, colon, and ovarian cancers. Cyclin A may also be involved in oncogenesis and like cyclins D and E, is important for completion of G1 and passage into the DNA-synthesizing stage of the cell cycle (Pagano and Draetta, 1991).

IGF-1 has been implicated in the upregulation of several cyclins namely, cyclins D1, E, A, and B in viable postinfarcted myocytes (Reiss et al., 1997). In these cells, cyclin E- and A- associated histone H1 kinase activity and cyclin D1-associated retinoblastoma protein-associated kinase but not the cyclin B-associated kinase activity also increased. These changes in the activation of cyclins and kinases were reduced by more than 50% by IGF-1R antisense oligodeoxynucleotide. Interestingly in these cells, IGF-1 did not

induce mitotic cell division (Reiss et al., 1997) suggesting that the growth-promoting effect of IGF-1 is regulated by the density of IGF-1 receptors. IGF-1 also induced an increase in cyclin-D1 expression as shown in MG63 human osteosarcoma cells (Furlanetto et al., 1994). This effect occurs early in G_1, the portion of the cell cycle in which IGF-1 is active. Cyclin B1, cdc2 and cdk2 are increased later in the cell cycle following IGF-1 administration. This effect of IGF-1 on cyclin D1 early in G_1 phase may represent a major effect or mechanism of this growth factor on cell cycle progression.

Fig. 2-1: Role of growth factors in the cell cycle.

The role of growth factors in the cell cycle is primarily to stimulate quiescent cells which are out of the cycle (G_0) to enter into G_1 and to promote progression through G_1 into S phase. Competence factors such as PDGF and FGF are required by the cell to enter into the cell cycle, whereas progression from G_0 to the V point will not occur in the absence of progression factors such as EGF and IGF-1. The only growth factor required for progression from the V point (a restriction point at which cells will be arrested in their progression in the absence of essential nutrients) to the S phase is IGF-1. The cyclins with their CDKs move cells through the cycle until they divide in M (mitosis) phase.


Progression
Factors
EGF, IGF-1
G1
Competence
Factors
PDGF, FGF
G0
M
G2
G1
S
V
W
IGF-1
EGF
HGF
TGFα
Cyclin B/A
& CDC2
Cyclin D's
& CDK2,4,5
Cyclin A
& CDK2
Cyclin E
& CDK2

2.2. Growth factors and receptors

Extracellular growth factors play an essential role in cell cycle regulation. They may act locally by autocrine and paracrine stimulation or by endocrine pathways. The binding of a growth factor to its specific receptor generates a cascade of intracellular signals that eventually leads to transcriptional activation, the synthesis of regulatory proteins, DNA replication, and ultimately mitosis.

Hydrophobic molecules cross the cell membrane and carry out their effects by directly interacting with intracellular molecules. Hydrophilic factors, including growth factors and polypeptide hormones, can not penetrate the cell membrane and must therefore interact with specific receptors on the cell surface that transmit extracellular signals to the interior of the cell. Among these receptors are cytokine receptors, serine/threonine kinase receptors, and receptor tyrosine kinases (RTKs). All RTKs have single transmembrane domains, which separate the molecules into extracellular and intracellular parts (Fantl et al., 1993; Van der Geer et al., 1994). RTKs can be classified into different subfamilies on the basis of the structure of their extracellular domains. RTKs are type I transmembrane proteins with the N-termini outside the cell, a single membrane-spanning domain and a catalytic intracellular domain. The extracellular domain of RTK is composed of several hundred amino acids that contain characteristic patterns of structural motifs. Most RTK extracellular domains are modified by N-linked glycosylation. The transmembrane domain consists of a stretch of hydrophobic residues and the cytoplasmic domain consists of a juxtamembrane region, the kinase domain and a C-terminal region.

The kinase domain is approximately 250 residues in length and carries out the phosphotransfer reaction. It is closely related to that of cytoplasmic protein tyrosine kinases and protein-serine/threonine kinases (Hanks and Hunter, 1995). The C-terminal domain varies in length from a few up to 200 residues and its function varies among members of the RTKs. Moreover, the kinase domains of members of a certain subfamily are more closely related to each other than to members of other RTK receptor subfamilies (Van der geer et al., 1994). There is also often a structural similarity between ligands for receptors within a subfamily and there are several examples of a ligand binding to more than one related receptor. In addition, a single receptor can bind more than one related ligand. The characteristic features of some of the different RTK receptor subfamilies are described below.

The epidermal growth factor receptor family. The epidermal growth factor receptor (EGFR) was the first RTK to be purified (Cohen et al., 1982) and cloned (Ullrich et al., 1984). It is the prototype of a subfamily of four members, EGFR itself, ErbB2, ErbB3 and ErbB4 (Carraway and Cantley, 1994), which are characterized by the presence of two cysteine rich domains extracellularly, and an intracellular part with a long C-terminal tail, on which most of the autophosphorylation sites are located. The ligands for the members in this receptor class include epidermal growth factor (EGF), transforming growth factor alpha (TGFα), Neu differentiation factor (also called heregulin), amphiregulin, heparin binding EGF, cripto, Schwannoma derived growth factor and betacellulin, which all contain a conserved EGF like domain and are synthesized as transmembrane precursor

molecules. The specificities for receptor binding have not been fully elucidated, but several of the ligands can induce heterodimeric receptor complexes (Groenen et al., 1994).

The insulin receptor family. The insulin receptor family has threee members—the insulin receptor, the insulin like growth factor 1 receptor and the insulin related receptor (White and Khan, 1994). These receptors are disulphide bonded heterotetramers of two α and two β chains. The α and β chains are formed by proteolysis of a common precursor. The α chains constitute most of the extracellular parts of the receptors and contain a cysteine rich domain and two fibronectin type III (FN III) domains, whereas the β chains contain the transmembrane domains and the cytoplasmic RTK domains. The IR can bind to insulin-like peptides including the insulin-like growth factors, IGF-1 and IGF-2, but the affinity for these ligands is ~ 100-fold less than that for insulin. The receptor for type 1 insulin like growth factor (IGF-1) has been identified as a critical regulator of cell growth and development (Werner et al., 1991a; Lowe, 1991). There are three known ligands of this receptor and they bind with decreasing affinities IGF-1>IGF-2>insulin. Ligand binding induces autophosphorylation of three closely located tyrosine residues inside the kinase domain, which leads to an increase in the catalytic efficiency of the kinase (White et al., 1988). There are few other autophosphorylation sites in the receptor molecules, but receptor induced phosphorylation of insulin receptor substrate 1 (IRS-1) provides docking sites for several SH2 domain containing signal transducing molecules (White, 1994) (see below).

The platelet derived growth factor receptor family. This receptor family includes platelet derived growth factor (PDGF) α and β receptors, colony stimulating factor 1 (CSF 1) receptor (also known as the proto-oncogene product Fms), stem cell factor (SCF) receptor and Flt3/Flk2 (Fantl et al, 1993; van der Geer et al, 1994). Each of these receptors has five immunoglobulin (Ig) like domains extracellularly, which splits into two parts by an inserted sequence of 60-100 amino acid residues without homology to kinases. The ligands for members of this receptor family are all dimeric molecules (Westermark and Heldin ., 1993). For example, PDGF is made up as dimers of A and B polypeptide chains which are combined to generate the three isoforms of PDGF (AA, AB, BB). These bind with different specificities and affinities to two types of cell surface receptors (the αPDGF receptor and the βPDGF receptor). PDGF receptor subunits can associate as three dimer forms (αα, αβ, ββ) with variable binding specificities for the PDGF ligand dimers (Hart and Bowen-Pope, 1990). The αPDGF receptor can bind to both A and B subunits of the PDGF, while the βPDGF receptor, can bind only to B subunit of PDGF. PDGF (AA) combines αα, PDGF (AB) makes dimers of αα and αβ, and PDGF (BB) can make three types of dimers, αα, αβ, and ββ. These dimeric PDGFRs are active forms and phosphorylate its own domain and other neighbor specific proteins (Hart and Bowen-Pope, 1990).

The fibroblast growth factor receptor family. The fibroblast growth factor receptor (FGFR) family contains four gene products, each characterized by the presence of two or three Ig domains and a sequence of acidic residues extracellularly and an intracellular RTK domain with a short inserted sequence (Fantl et al, 1993; van der Geer et al, 1994).

The fibroblast growth factor (FGF) family of ligands currently contains nine structurally related members, all of which bind with high affinity to heparin (Mason, 1994).

The hepatocyte growth factor receptor family. There are three members in this family: the hepatocyte growth factor (HGF) receptor (Met), Ron and Sea. They are characterized by having large extracellular domains that undergo proteolytic cleavage near the N-terminus; the short N-terminal fragment remains disulphide bonded to the major part of the receptor (Rubin et al., 1993). In the case of the HGF receptor, this cleavage is necessary for ligand binding to occur. HGF, the ligand for the HGF receptor, is also known as "scatter factor" in view of its scattering effect on epithelial cells (Gherardi et al., 1989).

There are also other RTK families such as the vascular endothelial growth factor receptor, the neurotrophin receptor, the Eph like receptor, the Axl/Ark/Ufo receptor, and the Tie receptor .

2.3. Signalling by RTK: overview

Ligand induced dimerization or oligomerization appears to be a general mechanism whereby RTKs are activated (Heldin, 1995). The ligand induced receptor autophosphorylation serves two important functions. First, it leads to an increase in the

catalytic activity of the receptor kinases. For example, the phosphorylation of conserved tyrosine residues inside the kinase domains of the insulin receptor (White et al., 1988) or the HGF receptor (Naldini et al., 1991) increases the V_{max} of the kinases. Second, the phosphorylated tyrosine residues provide docking sites for downstream signal transduction molecules that contain conserved domains recognizing the phosphotyrosine in a specific environment. The SH2 domain is a 100 amino acid motif, which folds to form a surface that recognizes phosphotyrosine and three to six C-terminal amino acid residues (Pawson, 1995). Phosphotyrosine- binding domains (PTB) are more recently discovered motifs that recognize phosphotyrosine and N-terminal amino acid residues (Kavanaugh and Williams, 1994). The differences in preferences between different SH2 and PTB domains regarding amino acid residues surrounding the phosphorylated tyrosine residues account for specificity in the interactions. The interaction with specific SH2 or PTB domain containing signal transduction molecules initiates the activation of signal transduction pathways, which ultimately lead to cell division, chemotaxis and cell shape changes.

The events that activate the SH2 domain - containing signal transduction molecules is in some cases the actual binding to autophosphorylated regions in the RTKs. One example is PI3-kinase, the enzymatic activity of which increases after the regulatory subunit has bound to phosphorylated peptides (Backer et al., 1992). Another example is protein tyrosine phosphatase (PTP-1D), which increases its phosphatase activity after binding to phosphorylated peptides (Lechleider et al., 1993); for full activation, both SH2 domains need to bind to phosphotyrosine residues (Pluskey et al., 1995). A second possibility for

activation of SH2 domain - containing molecules involves their phosphorylation on tyrosine residues by the RTKs. One example is PLCγ, the enzymatic activity of which is increased after phosphorylation (Nishibe et al.,1990). Also the adaptor Shc is phosphorylated on tyrosine residues after binding to RTKs. This phosphorylation allows the binding to Shc of other SH2 proteins, for example, Grb2 (Downward, 1994). A third possibility for activation is a translocation of an already active signal transduction molecule. One example appears to be Grb2, which occurs in a complex with the constitutively active nucleotide exchange factor SOS1; autophosphorylation of activated RTKs or tyrosine phosphorylation of substrates recruits the Grb2/SOS1 complex to the cell membrane (Aronheim et al., 1994).

2.4. The IGF-1/IGF-1R system

2.4.1. Overview

The normal process of growth and differentiation results from the genetically programmed action of a number of different cellular and extracellular factors. Derangement in the function of one or more of those agents can result in a pathologic phenotype, including neoplastic growth. A family of growth factors shown to be intimately involved in the regulation of cell growth as well as in cellular transformation is the IGF family. IGF-1 and IGF-2 are mitogenic polypeptides produced in the largest

amounts in the liver and secreted into the circulation where they mediate the effects of growth hormone (GH) on longitudinal growth. In addition to this endocrine axis, IGFs are produced by most extrahepatic organs where they are involved in autocrine/paracrine types of activities (Daughaday and Rotwein, 1989; Sara and Hall, 1990; Werner et al., 1994).

The biological actions of the IGFs are mediated by a family of transmembrane receptors which include the insulin, the IGF-1, and the IGF-II/mannose-6-phosphate (M-6-P) receptors (Werner et al., 1991b; Nissley and Lopaczynski, 1991). The first two receptors contain a tyrosine kinase domain in their cytoplasmic portion, whereas the IGF-II/M-6-P receptor lacks this type of enzymatic activity. Although a certain degree of crosstalk exists between the various ligands and their receptors, it is widely accepted today that most of the effects of the IGFs on growth and differentiation result from the ligand-dependent activation of the IGF-1 receptor.

The important role of the IGF system in embryonic and post-natal growth has been elegantly demonstrated by studies from the Efstratiadis group, they showed that targeted disruption of the IGF-II gene results in viable mice that weight 60% of their normal littermates at the time of birth and that develop into fertile proportionate dwarfs (DeChiara et al., 1990). On the other hand, some null mutants for the IGF-1 gene die shortly after birth, while others survive and reach adulthood. These animals are infertile, showing in addition delays in the ossification process, underdeveloped muscle tissue, and

poorly organized lungs (Liu et al., 1993a). The most severe growth retardation occurs in mice lacking the IGF-1 receptor gene. These mice, weighing 45% of controls, die invariably at birth. They exhibit generalized hypoplasia, delayed bone development, impaired skin formation, and abnormal central nervous system morphology (Werner and LeRoith, 1996). Taken together, these findings demonstrate that the IGFs and their receptors are essential components in major proliferative and differentiation pathways in the processes of embryonic and postnatal growth in mammals.

In addition to ligands and receptors, the IGF system comprises a third category of molecules which bind IGFs in the circulation and in extracellular compartments. Six IGF-binding proteins (IGFBPs) have been characterized to date. By affecting the ratio of free to bound IGFs, IGFBPs can modulate IGF action in both positive and negative ways.

2.4.2. The IGF-1R ligands

The IGF-1R ligands include IGF-1, IGF-2 and insulin. The receptor binds IGF-1 with high affinity (Kd 1nM) and IGF-2 and insulin with considerably lower affinities (10-fold and 100-fold lower affinities, respectively) (Ullrich et al., 1986). The gene and protein structures of IGF-1 and IGF-2 have been extensively studied. IGF-1 and IGF-2 share about 70% sequence homology and have close structural homology to insulin. The mature 70 amino acid IGF-1 peptide, like insulin, has an A and B domain. In addition, a

C domain connects the A and B domains, and a unique 8-amino acid D domain is present at the carboxy-terminal end (Sara and Hall, 1990; Humbel, 1990). The human IGF-1 gene is located on chromosome 12 and is composed of at least six exons (Rotwein et al., 1986; Shimatsu and Rotwein, 1987). Transcription of the mammalian IGF-1 gene and processing of its primary transcript are elaborate, with alternative leader exons 1 and 2 encoding mutually exclusive 5' untranslated regions (UTRs) and distinct N-termini of the signal peptide. Northern blot analysis of IGF-1 mRNA reveals a complex pattern, with multiple transcripts ranging in size from ~1 kb to more than 7 kb. The expression of the IGF-1 gene is developmentally regulated, with levels of IGF-1 mRNA in most tissues increasing 10- to 100- fold between birth and adulthood (Adamo et al., 1989). In addition, both hepatic and extrahepatic production of IGF-1 mRNA are controlled by GH at the level of transcription (Roberts et al., 1986).

The mature IGF-2, similarly to IGF-1, contains 4 domains (BCAD) and is 67 amino acids in length. The human IGF-2 gene is located on the distal end of the short arm of chromosome 11, contiguous to the insulin gene, and it encompasses ~30 kb of chromosomal DNA (Tricoli et al., 1984). The gene includes nine exons, and the coding sequence of the mature peptide is encoded by exons 7-9. IGF-2 mRNA range in size from ~2.2 to ~6.0 kb. Unlike IGF-1 mRNA, IGF-2 mRNA levels in all tissues are high during late fetal and perinatal periods and decline thereafter (in humans, however, IGF-2 can be detected in the circulation at adult stages) (Adamo et al., 1991).

2.4.3. The IGF-1 receptor

The IGF-1 receptor was originally isolated from human placenta (Bhaumick et al., 1981; Bhaumick et al., 1982) and was found to strongly resemble the insulin receptor but had a relatively low affinity for insulin (Chernausek et al., 1981; Massague and Czech, 1982). The human IGF-1 receptor cDNA was cloned in 1986 by Axel Ullrich and colleagues (Ullrich et al., 1986). The rat IGF-1 receptor cDNA has recently been characterized (Pedrini et al., 1994). The human IGF-I receptor cDNA consists of an open reading frame of 4101 nucleotides that encodes a protein of 1367 amino acids. The IGF-1 receptor belongs to the small family of homologous receptors that includes the insulin receptor and an orphan receptor termed the insulin receptor-related receptor (Shier and Watt, 1989). The insulin receptor-related receptor has mitogenic potential (Zhang and Roth, 1992), but because the ligand is still unknown, its possible relationship to the IGF-I receptor is presently unclear.

The IGF-1 receptor is synthesized as a single polypeptide chain, which is then glycosylated and proteolytically cleaved at a tetrabasic arg-lys-arg-arg sequence at position 707-710 (Ullrich et al., 1986) into α- and β- subunits. The receptor exists as an α_2-β_2 heterodimer, with several α-αand α-β disulfide bridges. The ligand binding domain is located on the extracellular α-subunit. Approximately one-third of the β-subunit is extracellular and is connected to the intracellular portion by a single transmembrane domain. The tyrosine kinase catalytic site and the ATP-binding site are located on the cytoplasmic portion of the β-subunit. The tyrosine kinase domains of the

IGF-1 and insulin receptors share sequence homology greater than 80%, whereas there is greater divergence in the ligand binding domains.

IGF-1 receptors are present in nearly all tissues and cells in culture. Northern blot analysis of IGF-I receptor RNA reveals a major 11 Kb transcript with minor 7 Kb transcripts in some tissues (Ullrich et al., 1986). In the adult rat, IGF-1 receptor RNA is heavily expressed in the brain but is also found in many other tissues, including heart, lung, kidney, and testes (Werner et al., 1989; Werner et al., 1992). The liver has exceedingly low levels of IGF-I receptor mRNA. However, IGF-1 receptor RNA expression and IGF-1 binding sites increase upon liver regeneration and are also found in fetal rat liver (Caro et al., 1988; Santos and Yusta, 1994).

Although highly homologous, the physiologic functions of the IGF-1 and the insulin receptor differ substantially. Activation of the IGF-1 receptor is primarily mitogenic, whereas the insulin receptor mediates principally metabolic functions.

Several key findings support the conclusion that the IGF-1 receptor can account for most of the mitogenic properties of IGF-1, IGF-2 and insulin: (a) Overexpression of the IGF-1 receptor in several cell types (Steele-Perkins et al., 1986; Kaleko et al., 1990; McCubrey et al., 1991; Pietrzkowski et al., 1992a) can bypass the requirement for all growth factors

other than IGF-1, IGF-2, and insulin at supraphysiologic concentrations; (b) Prevention of the binding of ligands to the insulin or IGF-2 receptors does not interfere with the mitogenic actions of their respective ligands, whereas blockage of the IGF-1 receptor inhibits mitogenesis (Furlanetto et al., 1987; Osborne et al., 1989; Adashi et al., 1990).

2.4.4. IGF-1R signalling

Current analysis of the molecular events that follow growth factor receptor activation comprises both the identification of the cytosolic and nuclear mediators that lead to transcriptional activation and the subsequent expression of regulatory gene products. The evidence currently available indicates that upon ligand binding the β subunit of the receptor is autophosphorylated. This leads to phosphorylation of IRS-1 (insulin receptor substrate-1) (Sun et al., 1992; Giorgetti et al., 1993; Myers et al., 1993). This protein although it does not contain SH2 or SH3 domains, has potential binding sites for SH2-containing proteins and appears to act as a multi-site "docking" protein serving as a link between downstream substrates and the receptor. Recently the nuclear proto-oncogenes c-Jun and c-Fos which had been identified as regulators of collagenase (MMP-1, MMP-3, MMP-9, and MMP-10) were reported as nuclear substrates downstream of the IGF-1R signaling pathway suggesting that some of the receptor functions are mediated via transcriptional activation of AP-1 binding sequences (Rosenzweig et al., 1993; Heidenreich, 1993). IRS-1 also associates with other SH2 domain-containing proteins involved in growth factor signaling pathways, including Grb-2, Nck (also an adaptor

protein), and Syp (also known as PTP1D, PTP2C, or SHPTP2, a phosphotyrosine phosphatase) (Sun et al., 1993). Syp becomes tyrosyl-and threonine-phosphorylated in response to PDGF and EGF stimulation (Lechleider et al., 1993). Syp is also known to associate with IRS-1 via its SH2 domain which interacts with specific phosphotyrosine-containing sequences of IRS-1 (Sugimoto et al., 1994).

Another downstream substrate of IGF-1 receptor signaling is the Shc (src-homology/collagen) protein (Giorgetti et al., 1994; Sasaoka et al., 1994). Like IRS-1, upon tyrosine phosphorylation, Shc associates with Grb2, and subsequently activates Ras via a Grb2-mSOS complex. Since the Shc gene has transforming properties (Pelicci et al., 1992), it may play an important role in the transforming ability of the IGF-1 receptor. The IGF-1R also directly phosphorylates Crk (Beitner-Johnson and LeRoith, 1995), a cellular homologue of v-crk. Members of the Crk family bear SH2 and SH3 domains, share homlogy with Grb2 and Nck, and interact with the ras-binding protein mSOS (Matsuda et al., 1994). The relative importance of this pathway in signal transduction by IGF-1R remains to be elucidated.

2.4.5. Mutational analysis of the IGF-1R

Mutational analyses of the β-chain of the IGF-1R have indicated that the three functions mentioned before, mitogenesis, transformation, and protection from apoptosis, map on

separate domains of the receptor (Hongo et al., 1996; O'Connor et al., 1997; Surmacz et al., 1995). As expected, the data demonstrate that receptor tyrosine kinase activity is required for most signaling functions. A mutation of lysine-1003, the ATP binding site, completely disrupts receptor function in transfected NIH 3T3 fibroblasts (Kato et al., 1993). Minimal tyrosine phosphorylation occurred in response to IGF-1, and ligand binding did not trigger activation of PI3-kinase, glucose uptake, or DNA synthesis (Kato et al., 1993). Receptors containing β-chain truncated at amino acid 952, and thus lacking the kinase domain, did not transmit growth-promoting signals and acted as dominant-negative inhibitors when transfected into cells expressing wild-type receptors (Prager et al., 1994). A similarly non-functional receptor was created by substitution of tyrosines –1131, -1135, and –1136 with phenylalanine (Gronborg et al., 1993; Kato et al., 1994). Alterations of all three tyrosines, which are the major sites of receptor autophosphorylation in the IGF-1R, resulted in no autophosphorylation, no phosphorylation of cellular substrates, and no short-term, or long-term biological effects (Gronborg et al., 1993; Kato et al., 1994). Mutation of individual residues in this triple tyrosine cluster also caused a decrease in the extent of autophosphorylation and diminished IRS-1 and Shc phosphorylation (Li et al., 1994; Fernandez-Sanchez et al., 1995; Stannard et al., 1995). While substitution of phenylalanine for tyrosine at residues 1131 or 1135 did not inhibit induction of DNA synthesis or cellular proliferation in response to IGF-1 in receptor-deficient fibroblasts transfected with each mutant, modification of tyrosine –1136 did reduce replication (Li et al., 1994). Taken together, these results demonstrate that each tyrosine in this cluster is not equivalent and indicate

that a fully tyrosine phosphorylated and presumably fully functional receptor is required for anchorage-independent growth but not for mitogenesis.

Tyrosine-950 is essential for binding and phosphorylation of IRS-1 (Yamasaki et al., 1992; Hsu et al., 1994; Miura et al., 1995a). Substitution of phenylalanine for tyrosine at this site did not alter autophosphorylation or ligand-activated receptor internalization, but blunted phosphorylation of IRS-1 and blocked other biological effects (Yamasaki et al., 1992; Miura et al., 1995a). This tyrosine also has been found to bind to another signaling intermediate, the Ras GTPase-activating protein (GAP) (Seely et al.,1995). More extensive mutations, deleting residues 947-950 or 944-965 of the juxtamembrane domain, additionally abrogated IGF-1-stimulated receptor internalization (Yamasaki et al., 1992; Hsu et al., 1994).

Mutation of tyrosine-1316 near the carboxyl-terminal of the IGF-1R also interrupts protein-protein interactions between the activated IGF-1R, the regulatory subunit of PI3-kinase p85, and the tyrosine phosphatase SH-PTP2 (Seely et al., 1995; Blakesley et al., 1996). The functional consequences of these mutations have not been elucidated.

Two tyrosines at positions 1250 and 1251 of the IGF-1R are not found in the insulin receptor (Ullrich et al., 1986). Substitution of either or both amino acids with

phenylalanine had a minimal effect on autophosphorylation and substrate phosphorylation in receptor-deficient fibroblasts transfected with these mutants, and did not alter cellular proliferation in response to IGF-1 (Miura et al., 1995b). In contrast, the double mutation or substitution for tyrosine-1251 alone caused a profound decrease in the efficiency of cellular transformation, as measured by diminished colony growth in soft agar (Miura et al., 1995b; Blakesley et al., 1996). These results demonstrate that the mitogenic and transforming properties of the IGF-1R can be dissociated and present an experimental model to test the hypothesis that distinct signal-transduction pathways mediate each biological effect.

Additional determinants within the carboxy-terminal tail of the IGF-1R may be involved in mediating cellular transformation. After transfection into receptor-deficient fibroblasts, receptors lacking the last 108 amino acids showed ligand-dependent autophosphorylation, mediated phosphorylation of IRS-1, activated PI3-kinase, and stimulated cellular proliferation, but did not enhance colony formation in soft agar (Surmacz et al., 1995). These studies also indicate that signal transduction pathways responsible for the mitogenic and transforming properties of the IGF-1R may be separable.

2.4.6. IGF-1R function

The insulin-like growth factor 1 receptor (IGF-1R) plays an important role in the regulation of cellular proliferation in at least three ways: (I) it is required, in cooperation with other growth factors, for the optimal growth of cells in vitro (Stiles et al., 1979) and in vivo (Baker et al., 1993; Liu et al., 1993b); (ii) it is necessary for the establishment and maintenance of the transformed phenotype, in a variety of cell types (Baserga, 1995); and (iii) it protects cells from apoptosis (Prisco et al., 1997). Site directed mutagenesis of the receptor provided evidence that these functions namely mitogenesis, transformation, and protection from apoptosis (programmed cell death), map to separate domains of the receptor as discussed in the previous section (Surmacz et al., 1995; Hongo et al., 1996; O'Connor et al., 1997). In addition to, and distinctly from its role as a positive regulator of cell growth (details in section 2.1.), IGF-1 is also a survival factor. The molecular basis of the anti-apoptotic effect of IGF-1 is still poorly understood (Sell et al., 1995). IGF-1 could prevent apoptosis induced by overexpression of the c-myc oncogene in rat-1 fibroblasts (Harrington et al., 1994). It could also inhibit apoptosis of IL-3-dependent hematopoietic cells folowing removal of IL-3 (Rodriguez-Tarduchy et al., 1992). The evidence for an antiapoptotic function of the IGF-1R is substantial. Thus, an overexpressed IGF-1R allowed the growth of the hematopoietic, IL-3 dependent FDC-P1 cells in the absence of IL-3 (McCubrey et al., 1991). These cells could also be protected from IL-3 depletion induced apoptosis by the addition of IGF-1 (Rodriguez-Tarduchy et al., 1992). When the function of the IGF-1R was impaired by antisense strategies or by expression of dominant negative mutants, tumor cells were found to undergo massive

apoptosis, an effect more prominently seen both *in vitro* and *in vivo* (Resnicoff et al., 1995a,b; D'Ambrosio et al., 1996). In fact, several tumor cell lines expressing antisense IGF-1R mRNA were found to be non-tumorigenic in vivo and this may be due to rapid death due to apoptotic mechanisms (Resnicoff et al., 1995a,b; Sell et al., 1995).

Recently, Werner and collaborators have identified a link between the IGF-1R and p53, by demonstrating that wild-type p53, a multifunctional protein that plays a major role in apoptosis, can supress IGF-1R promoter functions, thereby decreasing receptor levels (Werner et al., 1996). The biological significance of this effect was described by Prisco and coworkers (1997). They investigated the relationship between p53 and the IGF-1R in IL-3 withdrawl induced apoptosis in the murine hematopoietic precursor 32D cells which express low endogenous levels of IGF-1R and no IRS-1 or IRS-2. 32D cells which stably express the mutant temperature-sensitive (ts) mouse p53 protein (this protein assumes the wild-type conformation at 32°C and a mutant, inactive conformation at 39°C) were transfected with one of two plasmids, expressing the wild-type human IGF-1R cDNA (Ullrich et al., 1986), under the control of the cytomegalovirus (cmv) or the rat IGF-1R promoter. In these cells wild-type p53 reduced the number of IGF-1receptors expressed under the control of the rat IGF-1R (but not the cmv) promoter. IL-3 withdrawal induced apoptosis in these cells but not in cells expressing the receptor under the control of a viral promoter. Suggesting that in these cells IGF-1R is a physiological target of p53.

In view of the central role of the IGF-1 receptor in cell proliferation, it was of interest to determine whether aberrant expression of IGF or the IGF-1R can lead to cellular transformation. Macaulay (1992) reviewed the spectrum of malignancies in which overexpression of IGF-1, IGF-2 or the receptor could be observed and found that malignancies of the lung, breast, thyroid, gastrointestinal tract, liver, pancreas, kidney, neuroendocrine cells, and others. For breast cancer, estrogen receptor expression directly correlated with expression of the IGF-1R (Macaulay, 1992). However, IGF-1 or IGF-2 receptor expression has not been definitely correlated with prognosis in breast cancer or other malignancies. IGFs are also potent mitogens for a wide range of tumor cell types in vitro, and the activation of the IGF-1 receptor promotes the autocrine growth of many tumor cell lines.

Overexpression of the IGF-1R in fibroblasts resulted in the acquisition of tumorigenicity when the transfected cells were injected into immunodeficient nude mice (Kaleko et al., 1990). This transforming effect could be blocked by expression of a truncated receptor lacking a tyrosine kinase domain (Fernandez-Sanchez et al., 1995; Li et al., 1994; Prager et al., 1994), possibly due to formation of heteromeric complexes consisting of both wild-type and mutant $\alpha\beta$-heterodimers. IGF-1R$^-$ fibroblast could not form tumors in nude mice (Sell et al., 1994), nor did cells expressing receptors with mutations in the tyrosine cluster at amino acids 1131, 1135, and 1136 (Gronborg et al., 1993; Kato et al., 1994), or the carboxy-terminal tyrosine-1251 (Miura et al., 1995b; Blakesley et al., 1996), thus indicating that intact receptors are required to mediate tumorigenesis.

Reduction in IGF-1R expression has been shown to inhibit cellular transformation and tumorigenicity (Resnicoff et al., 1994a,b; Shapiro et al., 1994). Anti-IGF-1R reagents such as antibodies and antisense oligonucleotides were shown to block tumor growth and may have potential therapeutic applications (Arteaga, 1992; Sell et al., 1993; Pietrzkowski et al., 1993; Resnicoff et al., 1994b; Resnicoff et al., 1994c; Resnicoff et al., 1995b; Resnicoff et al., 1996).

2.5. The IGF - Binding Proteins

The physiologic action of the IGFs are modulated by their association with IGF binding proteins (IGFBP). To date, six IGFBP have been identified which have varying affinities for IGF-1 and IGF-2 (Clemmons, 1991; Drop et al., 1992; Clemmons et al., 1993; Cohick and Clemmons, 1993). They are encoded on different chromosomes, and the corresponding cDNA have been cloned. The IGFBPs vary in length from 216 to 289 amino acids and are composed of shared cysteine rich amino- and carboxyl-terminal domains and unique central regions (Clemmons, 1991). BP1-6 bind both IGF-1 and IGF-2 with high affinities. The presentation of IGF-1 to cells by IGFBP may account for various reports wherein IGFBP alternatively enhance or inhibit the mitogenic potency of IGF-1 (Elgin et al., 1987; De Mellow and Baxter, 1988; Ritvos et al., 1988; La Tour et al., 1990; Clemmons et al., 1993; Jones et al., 1993). The IGFBPs regulate the biological accessibility and activity of IGFs in several ways which can be summarized as follows:

they transport IGFs from the circulation to peripheral tissues (a function of BP-1, -2, and –4); maintain a reservoir of IGFs in the circulation (a function of BP-3), potentiate or inhibit IGF action and mediate IGF-independent biological effects (Jones and Clemmons, 1995). Reeve et al., (1995) have presented evidence that the transformation of human fibroblasts by SV 40 T Ag is associated with the loss of IGFBP-5 expression, thereby increasing the sensitivity of the cells to autocrine IGF-1 stimulation. IGFBP-3 could inhibit the growth of Hs 578T human breast cancer cells (Oh et al., 1993a) and mouse 3T3 cells (Cohen et al., 1993) in medium lacking IGF-1. Oh et al. (1993b) have identified a specific 55-KDa IGFBP-3 binding site that may function as a receptor to modulate cell growth. The local functions of IGFBPs may be modulated by interactions with the extracellular matrix (ECM) and with the cell surface. For example the association of BP-5 with the ECM, is thought to provide a mechanism for IGF-1 binding and sequestration in the ECM where it can be exposed during processes such as wound healing, tissue repair and tumor cell invasion (Jones et al., 1993). IGFBP-1 in turn has an Arg-Gly-Asp (RGD) sequence known to play a role in integrin-mediated adhesion (Lee et al., 1997). Several IGFBPs are known to undergo proteolytic degradation by specific proteinases including the metalloproteinases MMP-2 and MMP-9, a process thought to increase IGF-1 bioavailability (Fowlkes et al., 1994a,b). There is presently conflicting evidence regarding the role of IGFBP in growth regulation as they were shown to either enhance or inhibit the mitogenic potency of IGF-1 (Clemmons et al., 1993) for example, transgenic mice overexpressing BP-1 showed mild growth retardation and modest hyperglycemia (Rajkumar et al., 1995), mice lacking BP-2 by targeted gene disruption were reported to

were reported to be normal (Wood et al., 1993) and overexpression of BP-3 caused selective increases in heart, liver, and spleen weights (Murphy et al., 1995).

2.6. IGF-2 receptor

The IGF-2R is a single transmembrane polypeptide. It is a large, 250 kDa protein, containing 15 contiguous repeats in its extracellular portion, each with a similar pattern of 8 cysteine repeats, and a small region homologus to the collagen-binding domain of fibronectin. The cytoplasmic tail is relatively short and does not contain a tyrosine kinase domain (Morgan et al., 1987), although some studies suggest that it may be linked to a G protein signaling pathway (Morgan et al., 1987; Lobel et al., 1988; MacDonald et al., 1988). The IGF-2R is identical to the cation-independent mannose 6-phosphate receptor. The two ligands (IGF-2 and M6P) do not appear to recognize the same site on IGF-2R, as M6P does not inhibit IGF-2 binding (Morgan et al., 1987). In addition to its role in the clearance of IGF-2 (Sessions et al., 1987), the IGF-2/M6PR plays a central role in targeting newly synthesized mannosylated enzymes from the Golgi apparatus to lysosomes during tissue remodeling (von Figura and Hasilik, 1986). Cathepsin-D, a glycoprotein produced in abundance by breast carcinoma cells, appears to bind to this receptor through its mannose side chains (Rochefort et al., 1986; Lobel et al., 1987;). The precursor form of another peptide growth factor, TGF-β1, has been shown to bind this receptor, again through mannose 6-phosphate containing moieties which are added to the parent peptide posttranslationally (Kovacina et al., 1989). IGF-2 is also internalized by

the small number of cell surface IGF-2/M6PR for targeting to the lysosomal compartment and degradation by lysosomal enzymes. A recent study on mutant mice lacking the IGF-2/M6PR gene has shown that this receptor may play different roles during embryogenesis and post-natally (Wang et al.,1994). In the embryo, it may serve essentially to promote growth. During post-natal life, however, it may act primarily to maintain the enzyme activity of lysosomes.

IGF-2 interacts with both the IGF-1 and the IGF-2/M6P receptors. The mitogenic effects of IGF-2 are mediated by the IGF-1R in IGF-2 overexpressing malignancies, such as Wilm's tumor and rhabdomyosarcoma (Gansler et al., 1986; El-Badry et al., 1990; Neilsen et al., 1991). The significance of the interaction between IGF-2 and the IGF-2R is more controversial. A number of studies have concluded that ligand bound IGF-2R can stimulate cellular responses, including proliferation (Nishimoto et al., 1987) and motility (Minniti et al., 1992). In a limited number of cell lines, IGF-2 binding to the IGF-2/M6PR has been shown to induce Ca^{2+} influx into the cells, to generate inositol phosphate (IP3) and diacylglycerol, and to stimulate cellular proliferation. These effects are mediated by a G protein signaling pathway (Nishimoto et al., 1989; Okamoto et al., 1990). However, a study by Korner et al. (Korner et al., 1995) has challenged this conclusion, because they were unable to demonstrate an interaction between IGF-2R and G proteins (Korner et al., 1995). Ellis and coworkers (Ellis et al., 1996) developed retrovirus vectors expressing IGF-1 and –2 mutants with altered IGF-1R and IGF-2R affinities and used them to investigate the role of the IGF-1R and IGF-2R in modulating

IGF-2 signaling in MCF-7 breast carcinoma cells expressing both receptors (Osborne et al., 1989; Cullen et al., 1990; Stewart et al., 1990;). They found that extracellular IGF accumulation and the level of IGF-1R-dependent signaling was reduced by high IGF-2R affinity (Ellis et al., 1996). Because When IGF-2 binds to the IGF-2R, it is internalized and degraded, making it unavailable to activate the IGF-1R (Neilsen et al., 1991; Neilsen et al., 1993). Through this mechanism, the IGF-2R acts as an IGF-2 antagonist. IGF-2R-mediated negative regulation of IGF-2 has a critical role in IGF-2-dependent embryonic growth, since IGF-2 accumulates in IGF-2R-deficient mice, causing excessive growth and fatal organ hyperplasia (Lau et al., 1994; Wang et al., 1994).

CHAPTER 3

The internalization of macromolecules and receotor tyrosine kinases (RTK) traffic

3.1. Introduction

Cells exchange not only small molecules, such as inorganic ions or sugars, with their environment, but also macromolecules—particularly proteins and even particles several micrometers in size. This process of binding and internalizing macromolecules and particles from the microenvironment is known as endocytosis. The model of ligand mediated receptor endocytosis is often considered to be based on studies of the low-density lipoprotein (LDL) internalization pathway (Goldstein and Brown, 1977; Brown and Goldstein, 1979; Goldstein et al., 1979; Brown et al., 1982; Anderson and Kaplan, 1983; Brown et al., 1983). It was based on the findings that LDL receptors (LDLRs) are located almost exclusively in coated pits and that entry of LDL into the secondary lysosomes involves membrane-bound structures. The general application of the LDL pathway to the endocytosis of insulin, and other peptide hormones led to the identification of differences in the processing of different ligands by the cells. These differences can be found at the level of cell surface localization as well as in the intracellular compartments where ligand/receptor complexes accumulate and are concentrated prior to intracellular processing and sorting (Bergeron et al., 1985).

3.2. Receptor-mediated endocytosis

Receptor-mediated endocytosis allows the selective uptake of these extracellular proteins and small particles. Receptor proteins on the cell surface bind specific ligands with high

affinity and specificity. Most receptors diffuse within the plasma membrane; receptor-ligand complexes cluster in small regions of the membrane, which are then internalized. Endocytosis of these specific complexes usually occurs at specialized depressions on the cell surface known as coated pits (Fig. 3-1). Coated pits are large structures, often vesicular and often with associated tubules. The evidence that this compartment is receptor-enriched and contains intact ligand (especially insulin and prolactin) has been documented by subcellular fractionation (Josefsberg et al., 1979; Khan et al., 1982; Quintart et al., 1984). These structures called Clathrin-coated pits, are cell surface microdomains where randomly moving membrane proteins are segregated from resident plasma membrane proteins, and are internalized into the cell. Ligands and membrane proteins are selectively taken up while other proteins of the plasma membrane are prevented from being internalized. Coated pits are relatively uniform in size (~100-150 nm in diameter) and were first observed by Roth and Porter (Anderson et al., 1977) as bristle coated invaginations of the plasma membrane. Subsequently the major protein component of the coat was identified as clathrin (Pearse, 1976). The purification of coated vesicles permitted identification of the major structural units of the coat, namely, clathrin, triskelion and adaptors. The characteristic ultrastructural hexagon and pentagon lattice of coats is made up of clathrin while the inner shell of the coat consists of adaptor proteins that interact with the cytoplasmic domains of the receptors in the membrane of the pit/vesicle (Schmid, 1997).

In electron micrographs, a visible proteinaceous layer on the cytosolic side gives a coated appearance to these parts of the plasma membrane and, initially, to the vesicles that form

from them to produce coated vesicles (Schmid, 1997). The number of coated pits and vesicles at the PM as well as the dynamics of their formation have been studied by several investigators. Biochemical analyses of receptor internalization kinetics revealed that following ligand stimulation, receptors such as EGFR and IR— known to use the coated pit pathway, are rapidly lost from the PM ($t_{1/2} \sim 1$ min) (Burgess et al., 1992; Di Guglielmo et al., 1994). These vesicles lose their coats after endocytosis, forming smooth-surfaced vesicles called endosomes. Endocytosis also occurs through a pathway that is independent of clathrin. Clathrin-independent endocytosis has been demonstrated in unperturbed cells and may contribute up to half of the total membrane and fluid-phase uptake of the cell. The coated pit and clathrin-independent pathways of EGFR endocytosis can be distinguished by their characteristic kinetics with rapid internalization occuring via the coated pit pathway (especialy at low cell surface receptor occupancy) while the clathrin-independent pathway is less rapid. Constitutive internalization occurring via a clathrin-independent pathway, may be responsible for the uptake of molecules that do not use coated pits, such as GPI-anchored proteins, or this process may play a role in turnover of membrane proteins.

Clathrin-independent endocytosis is mediated by caveolae. Caveolae are plasma membrane invaginations involved in transcytosis which are present in most cells, except fresh monocytes and neutrophils (Bohuslav et al. 1995), while caveolae are virtually undetectable in liver (Scherer et al., 1994), in cultured hepatocytes caveolin is expressed and has been co-localized to caveolae with dynamin, a major component of the

membrane buddding mechanism (Henley et al., 1998). These differences may be a reflection of the process of adaptation to cell culture conditions where cells have to adopt a non-polarized morphology and express gene products not present in quiescent cells in the organism.

Early studies suggested that caveolae play a role in molecular transport across the membrane; caveolae are capable of moving tracers, introduced into the blood, across the endothelial cell by transcytosis (Simionescu, 1983). Other, more recent studies have proposed that they are specialized microdomains that have many attributed functions, including concentration of glycosylphosphatidylinositol (GPI)-linked receptors and signalling proteins. These receptors and proteins are recruited to the caveolae upon clustering or activation (Okamoto et al., 1998).

The main structural component of caveolae is caveolin, a 22-kDa protein. Caveolin has a putative 30-40 amino acid membrane-spanning sequence, which may pass the membrane once or twice (Okamoto et al., 1998). Recent studies have suggested regulatory as well as strructural functions for caveolin; caveolin may directly regulate numerous signaling proteins in caveolae (Couet et al., 1997; Feron et al., 1997; Liu et al., 1997). A 20 amino acid residue N-terminal cytosolic domain, the 'caveolin scaffolding domain', associates with an assortment of signalling molecules, such as the Gα subunits of heterotrimeric G proteins, Ha-Ras and Src-family tyrosine kinases, this has led to the idea that caveolin

plays a central role in the orchestration of signalling events in caveolae (Couet et al., 1997; Feron et al., 1997; Liu et al., 1997).

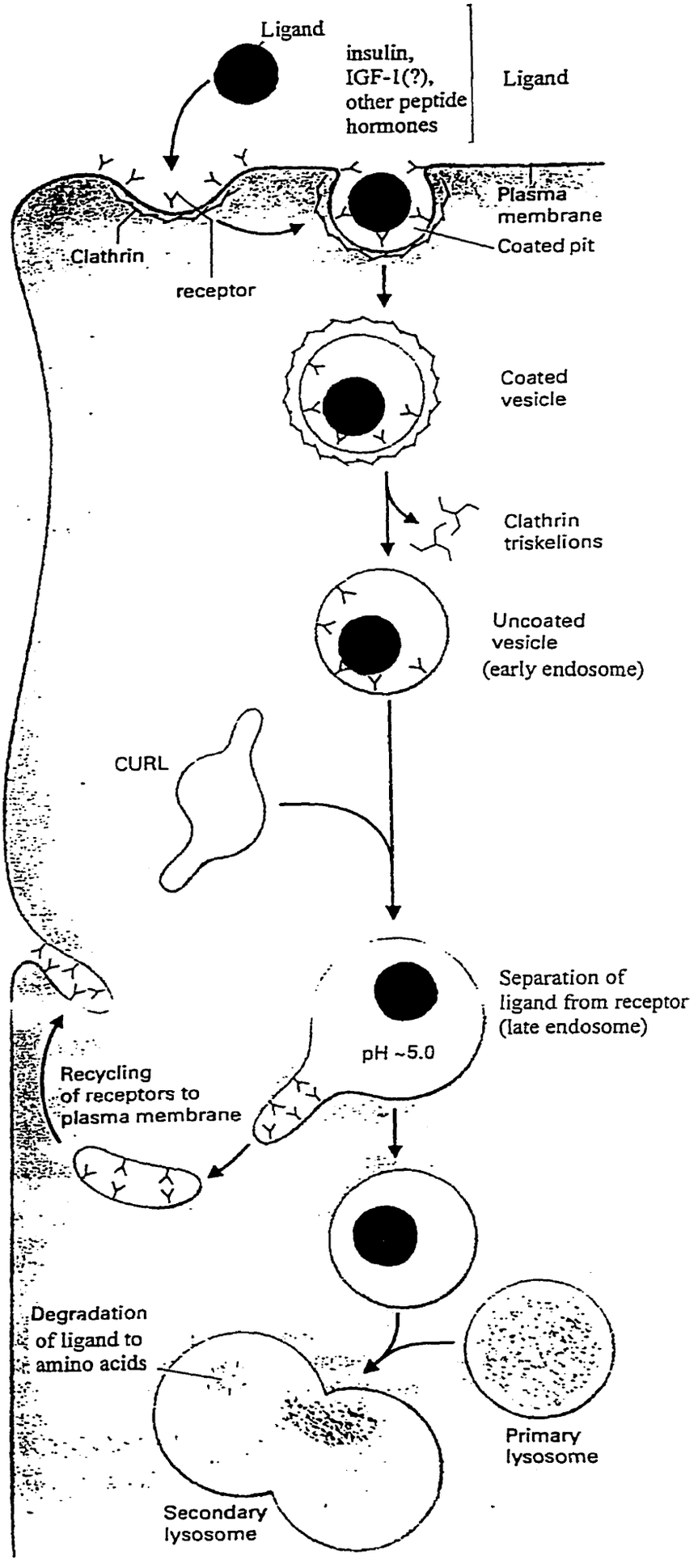
Caveolin and caveolae might also play a role in ECM-mediated promotion of cell proliferation or terminal differentiation, depending on the cell type and matrix composition. The signalling pathways involved in these processes are not fully understood but are likely to be mediated by integrins. A major advance in this area came with the finding that mAb-mediated ligation of certain β1 integrins, as well as αvβ3, caused recruitment of the adaptor protein Shc, leading to activation of the mitogen-activated protein kinase (MAPK) pathway (Wary et al., 1996). This led to the compelling idea that integrin-dependent cell survival results in one of two outcomes depending on the ability of the engaged integrins to activate Shc. Adhesion mediated by integrins that activate Shc promotes cell cycle entry and cell proliferation, whereas adhesion mediated by integrins that do not activate Shc results in cell cycle exit and differentiation. The ability to recruit Shc is specified by the same membrane-proximal extracellular and transmembrane domains of the integrin α subunit that interact with caveolin (Wary et al., 1996), implicating caveolin, and potentially caveolae, in this integrin-Shc interaction. Recently, Yamamoto and coworkers using cell fractionation analysis showed that the IR is enriched in caveolae and demonstrated that the scaffolding domain of caveolin interacts with the IR, leading to an increase in IR kinase activity (Yamamoto et al., 1998).

Whether endocytosis is mediated via coated or uncoated pits and vesicles, the receptor is usually recycled intact to the cell surface. This process involves several types of intracellular vesicles. After internalization of ligand/receptor complexes by coated vesicle, the clathrin coat depolymerizes to triskelions, resulting in an uncoated vesicle called endosome. The endosomal apparatus is positioned both temporally and physically between the plasma membrane (PM) and the lysosome (Fig. 3-1). Based on the time required for an internalized receptor to accumulate in the endocytic apparatus, two main compartments have been defined and termed early and late endosomes. Receptor-ligand complexes are delivered into early endosomes (within 2-5 min) which are located at the cell periphery and consist of weakly acidic tubular elements (pH 6-6.5) (Authier et al., 1994a,b). For some ligand-receptor complexes such as insulin and its receptor, endosomal acidification results in the dissociation of the ligand from its receptor (Fig. 3-1). Ligand degradation may then occur in this compartment (Authier et al., 1994a,b; Authier et al., 1998). It is early in the endosomal pathway that a mechanism exists which sorts the receptors for recycling to the PM from those targeted to the lysosomes for degradation (Lai et al., 1989). This mechanism is dependent, at least to some extent, on receptor occupancy levels. Early endosomes fuse with an uncoupling vesicle known as the compartment of uncoupling of receptor and ligand (CURL), characterized by an internal pH of ~5.0 to form late endosomes. The late endosomes (10-20 min) consists of tubulovesicular structures of varying sizes located in the Golgi-lysosome area of the cell. Some receptor recycling may occur at this level also. The free receptors congregate in one membrane region of these uncoupling vesicles, which ultimately bud off to form a separate elongated vesicle to recycle the receptor back to the plasma membrane. The

ligand, in contrast, is segregated into a different type of vesicle that ultimately fuses with a lysosome; there, lysosome proteases and other hydrolytic enzymes degrade the ligand (Fig. 3-1) (Tikkanen et al, 1996). However, The precise fate of ligand-receptor complexes within the endosomal apparatus appears to vary depending on the particular ligand and receptor (Bergeron et al., 1995).

Fig. 3-1: Receptor-mediated endocytosis.

Based in part on direct evidence and on homologies with the insulin signalling pathway, it is believed that IGF-1 binding initiates the migration of IGF-1 receptors to clathrin-coated pits and the subsequent formation of early endosomes containing internalized but still active receptors. The ligand-receptor complex ultimately becomes dissociated and inactivated in the acidic environment of late endosomes, where ligands and receptors are sorted for degradation in lysosomes or recycling to the cell surface. The same pathway is followed by other ligands. Modified from: Molecular Cell Biology (James Darnell, Harvey Lodish, and David Baltimore, eds.,— 2d ed.). 1990.


Ligand
insulin,
IGF-1(?),
other peptide
hormones
Ligand
Plasma
membrane
Coated pit
Clathrin
receptor
Coated
vesicle
Clathrin
triskelions
Uncoated
vesicle
(early endosome)
CURL
Separation of
ligand from receptor
(late endosome)
pH ~5.0
Recycling
of receptors to
plasma membrane
Degradation
of ligand to
amino acids
Primary
lysosome
Secondary
lysosome

3.3. Insulin receptor endocytosis

In the rat liver hepatocytes, IRs in their unbound state (ligand-free) are preferentially associated with surface microvilli, and excluded from coated pits (Bergeron et al., 1979). Studies by Carpentier and McClain (Carpentier and McClain, 1995) revealed that the C-terminus of the IR functions to anchor the unoccupied receptors to the microvillar membrane. Insulin binding to the receptor results in the activation of the RTK which initiates internalization by releasing this constraint. How this occurs is unclear but it may be controlled by the dileucine motif (Haft et al., 1998). In H35 hepatocytes, occupied receptors appeared to be excluded from coated pits (Knutson, 1991). Likewise, in HepG2 hepatoma cells, where coated pit formation was inhibited by the depletion of potassium under hypotonic conditions, insulin-IR complexes endocytosed normally even in the absence of clathrin coats. While these observations were made under in conditions of high receptor occupancy, internalization was inhibited by potassium depletion under more physiological conditions of low insulin concentrations and low receptor occupancy suggestng a coated pit dependent mechanism. These results, and those obtained with derived from CHO cells, suggest that the insulin-IR complex may be internalized by both smooth and coated pits in a manner that may be related to receptor occupancy (McClain and Olefsky, 1988; Backer et al., 1991).

Insulin mediated endocytosis requires specific amino acid residues found in the juxtamembrane domain (encoded by exon 16) of the receptor. This region, capable of

inducing endocytosis of an IR lacking the portion encoded by exons 17-22 (Carpentier and McClain, 1995), contains two endocytic sequences; a strong motif, GPLY^{965} and a weak code, NPEY^{972} (Rajagopalan et al., 1991). Mutation of these codes to APLA and APEA, respectively reduced internalization to 32% and 87% respectively, as compared to wild type IR internalization. Both motifs form a tight β turn structure exposing a tyrosine residue, an essential recognition motif which is present in the internalization motifs of many receptors. In addition to these endocytic sequence motifs, efficient internalization of the IR requires the activation of its catalytic kinase activity and tyrosine phosphorylation (Backer et al., 1991). An active IR kinase may be required to induce a conformational change which would result in the exposure of internalization codes, as suggested for the EGFR (Cadena et al., 1994) (see above). Alternatively, an active receptor tyrosine kinase may be required to phosphorylate substrates involved in mediating receptor internalization.

3.4. IGF-1 receptor endocytosis

Rat-1 fibroblasts in culture express high levels of IGF-1R (Zapf et al., 1994). In a recent study, the kinetics of IGF-1 internalization in rat-1 fibroblasts were compared with those of insulin in the same cells overexpressing the IR. A marked difference in the endosomal dissociation of the cognate ligands was observed. Whereas insulin was dissociated and degraded rapidly after internalization, IGF-1 was more resistant to acid-induced dissociation from its receptor, resulting in a prolonged (up to 120 min) accumulation of

intracellular (endosomal) IGF-1 (Zapf et al., 1994). This lead to speculations that the differences in endosomal ligand dissociation between these receptor-ligand complexes may be a factor in their distinct physiological roles.

In CHO fibroblasts, IGF-1-induced receptor internalization was inhibited by mutation of the C-terminal region of the IGF-1 receptor (Tyr to Phe substitution at residue 1310), by incubation of cells at low temperature (15 °C), or by treatment with the endocytosis inhibitor dansylcadaverine (Chow et al., 1998). With each of these different experimental approaches, there was a significant decrease in ligand induced receptor internalization and a corresponding decrease in IGF-1-induced stimulation of Shc phosphorylation. Thus it appears that tyrosine phosphorylation of Shc occurs predominantly by activated IGF-1 receptors in endosomes, or at least by receptors present in vesicles which have not moved past the point in the endocytotic pathway which is inhibitable by dansylcadaverine.

Objectives of the present study

1) To assess the role of ECM degrading proteinases in H-59 tumor cell invasion and metastasis using natural and synthetic inhibitors.

2) To analyze more specifically the role of cysteine proteinases cathepsin B and L in metastasis and determine whether these cysteine proteinases cathepsin B and L regulate the metastatic phenotype by mechanisms other than direct ECM proteolysis.

3) To elucidate the role of cysteine proteinases in IGF-1 receptor turnover.

CHAPTER 4

The role of cysteine proteinases in metastasis of the Lewis lung carcinoma cells

4.1. Overview

Results of my studies on the role of cysteine proteinases in cancer metastasis are presented in part in this chapter in the form of a published paper. I was responsible for all of the experimental work except the *in vivo* studies that were assisted by Mrs. Fallavollita, L.

All of the studies were carried out with the murine Lewis lung carcinoma model which consists of two sublines with divergent metastatic properties namely, carcinoma H-59 cells which are highly and preferentially metastatic to the lymph nodes and liver but also to lung, adrenal gland, kidneys and even the heart, while M-27 cells are moderately and specifically metastatic to the lung. Both tumor sublines retained the characteristic morphology of the parent line originally described as a " poorly differentiated epidermoid carcinoma" (Brodt, 1986; Brodt, 1989). In addition differences were noted in the repertoires of basement membrane degrading enzymes produced by the two sublines. H-59 cells were found to secrete higher levels of the 72 kDa collagenase (MMP-2, gelatinase A) and cathepsin L whereas M-27 cells were found to express higher levels of the urokinase type plasminogen activator (uPA) and cathepsin B, suggesting that invasion by these cells is differentially regulated (Brodt et al., 1992). These findings are summarized in table. 4-1.

In the following paper, H-59 and M-27 cells were used to study the role of matrix degrading enzymes particularly cysteine and metallo-proteinases in regulating the ability of these tumor cells to form invasion and metastasis.

Table 4.1: Common and unique properties of sublines H-59 and M-27 cells.

	Metastasis			Adhesion		Expression of Matrix Degrading proteinases			
3LL subline	Liver	Lung	LN[a]	LN	Hep[b]	MMP	Cathep[c].B	Cathep.L	uPA[d]
H-59	++	+	++	+	+	++++	+	++++	+
M-27	-	++	-	-	-	+	++++	+	++++

a. LN, lymph node
b. Hep., hepatocyte
c. Cathep., cathepsin
d. UPA, urokinase plasminogen activator

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Inhibition of carcinoma cell invasion and liver metastases formation by the cysteine proteinase inhibitor E-64

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Cysteine proteinases, in particular cathepsins B and L, have been implicated in tumor invasion and are thought to be important mediators of metastasis. Using two clonal sublines of the Lewis lung carcinoma with distinct patterns of metastasis, we previously reported that H-59 carcinoma cells, which are highly invasive and preferentially metastatic to the liver, express high levels of cathepsin L and lower levels of cathepsin B whereas M-27 cells which are less invasive and only moderately metastatic to the lung express cathepsin B only. In the present study, the role of these enzymes in invasion and metastasis, in particular the involvement of cysteine proteinases in liver metastasis of H-59 cells was further investigated. Using a reconstituted basement membrane (Matrigel) invasion assay we found that the cysteine proteinase inhibitor, E-64, blocked the invasion of H-59 cells under conditions which did not affect cell viability. A more minor but significant inhibitory effect (up to 32%) was also seen with the propeptide of cathepsin B, implicating this enzyme in the invasion process. Furthermore, treatment of H-59 cells with E-64 inhibited experimental liver metastases formation by up to 90%. On the other hand, invasion of M-27 cells could not be blocked by cysteine proteinase inhibitors even under conditions which resulted in complete abrogation of intracellular enzymatic activity, as assessed using synthetic substrates. Together, these results confirm our previous conclusion that the two carcinoma sublines utilize distinct proteolytic mechanisms for invasion and identify the cysteine proteinases as key mediators of H-59 carcinoma invasion and metastasis.

Keywords: cathepsin, cysteine proteinase, cysteine proteinase inhibitors, invasion, metastasis

Introduction

Metastasis, the process whereby cells detach from the primary lesion, migrate through lymph or blood vessels and form new foci at distant sites, is a critical aspect of cancer pathology and its suppression would represent a major breakthrough in the development of a curative cancer treatment. The multistep metastatic process depends, at different stages, on the ability of tumor cells to actively invade through different extracellular matrix (ECM) barriers [1].

Several families of proteolytic enzymes and their natural inhibitors have been implicated in tumor invasion and metastasis. They include the matrix metalloproteinases (MMPs) such as interstitial collagenase (MMP1), gelatinase A and gelatinase B (MMP2 and MMP9, also known as type IV collagenases and the 72 kDa and 92 kDa gelatinases, respectively) and matrilysin (MMP7), the cysteine

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proteinases (CP), in particular, cathepsins B and L, and the serine proteinases, urokinase and tissue plasminogen activator [2].

In vivo, the pericellular activity of proteinases is regulated by endogenous inhibitors. The increased proteolysis mediated by malignantly transformed cells is therefore a consequence of an imbalance in the production of active proteinases relative to the level of their inhibitors [3, 4]. The production of functional proteinases requires several independently regulated steps including transcription, translation and secretion of the proenzymes and their subsequent processing to yield the active enzymes. Conversion of proenzymes to their mature forms occurs by limited proteolysis which may be autocatalytic or may involve the action of other proteinases as part of an activation cascade [5, 6].

Previously we reported that H-59 cells, a subline of the Lewis lung carcinoma selected for metastatic preference for the liver, express elevated levels of cathepsin L (as compared to the lung-metastasizing subline M-27) and lower but detectable levels of cathepsin B [7]. The objective of the present study was to investigate further the role of these proteinases in invasion and metastasis in this model. To this end, two exogenous cysteine proteinase inhibitors were used, namely, the natural product E-64 [*trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane] which is a specific irreversible inhibitor of cysteine proteinases [8], and for further selectivity, the propeptide of cathepsin B, which was recently shown to be a potent specific inhibitor of this cysteine proteinase [9].

We show here that these inhibitors significantly reduced H-59 invasion *in vitro* in a reconstituted basement membrane (Matrigel) model. Furthermore when the cells were pretreated with E-64, the most potent inhibitor *in vitro*, their ability to form experimental liver metastases following an intrasplenic/portal injection was drastically reduced. On the other hand, M-27 invasion was not significantly affected by these inhibitors. The results confirm that these cells utilize distinct proteolytic mechanisms for invasion and implicate the cysteine proteinases in liver metastases formation.

Materials and methods

Animals

Female C57BL/6 mice, 8–12 weeks old, were supplied by Charles River (St. Constant, Quebec, Canada). Animals were regularly screened for the presence of common laboratory pathogens including murine hepatitis virus.

Tumor cells

The origin and metastatic properties of sublines H-59 and M-27 of the Lewis lung carcinoma were described in detail previously [7]. The tumors were maintained *in vivo* by s.c. implantation of hepatic or pulmonary metastases, respectively. *In vitro* monolayer cultures were prepared from the dispersed solid tumors and maintained in culture for up to 4 weeks only. In order to maintain uniformity of experimental conditions, the experiments performed throughout this study were carried out with cells in the second *in vitro* passage.

Proteinase inhibitors

E-64 [*trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane] was obtained from Sigma Biochemicals. Leupeptin was obtained through the US–Japan Medical Sciences Program. PRCB1, the 56 residue propeptide of rat cathepsin B, was prepared by solid phase peptide synthesis and purified as described previously [10]. The synthetic peptidylhydroxamate metalloproteinase inhibitor U24522 [(R,S)-N-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl-L-leucyl-L-phenylalaninamide] [11] was generously provided by Dr G. Dipasquale (Stuart Pharmaceuticals, Wilmington, Delaware).

Toxicity assays

To determine the effect of the inhibitors on cell viability, 5×10^4 tumor cells in 100 μl of RPMI medium containing 0.2% BSA were incubated with or without different concentrations of inhibitors for 48 h at 37°C in a 5% CO_2 atmosphere saturated with H_2O. Cell viability was then assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (thiazolyl blue) assay [12]. To each well, 10 μl of MTT (Sigma) were added and the plates were incubated at 37°C for 4 h. The plates were centrifuged for 5 min at low speed and 150–200 μl of supernatant were removed from each well. To the wells, 160 μl of DMSO[5] were added and the plates placed on a platform shaker for mixing. Absorbance was measured with an ELISA reader (model 3550 microplate reader, BIO-RAD, Richmond, CA, USA) at 540 nm.

RNA isolation and Northern blot analysis

Total RNA was extracted from H-59 and M-27 cells using the acid guanidinium thiocyanate–phenol–chloroform method of Chomczynski and Sacchi [13]. The RNA (30 μg of total RNA) was separated by electrophoresis through a 1.1% agarose gel containing 2 M formaldehyde and transferred to a

nylon membrane (Hybond N, Amersham, Oakville, Canada) by capillary action. Hybridization was for 48 h at 42°C, with a 600 bp cDNA probe [14] for murine MMP-2 (a kind gift from Dr Ann F. Chambers, London Regional Cancer Center, London, Ontario, Canada) which was radiolabeled with [^{32}P]dCTP by random primer extension [15]. The blots were washed twice at room temperature with 1 × SSC containing 0.1% SDS and twice at 55°C with 0.1 × SSC–SDS and exposed for autoradiography for 2–7 days at –70°C. As a control for RNA loading, the blots were subsequently probed with a ^{32}P-labeled oligonucleotide probe for 18S rRNA [16]. The relative amounts of the mRNA transcripts were analysed by laser densitometry using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer and normalized relative to the internal 18S control.

Cell invasion assay

Tumor cell invasion was determined *in vitro* by the reconstituted basement membrane (Matrigel) invasion assay, essentially as described previously [7]. Briefly, 60 μl of Matrigel (Collaborative Research, Bedford, MA, USA) diluted to a concentration of 0.23 mg/ml were applied to 8 μm filters. These filters were dried overnight, reconstituted with serum-free RPMI and placed in 24-well plates. To each filter 5 × 10^4 cells in 100 μl of RPMI medium containing 0.2% BSA were added with or without the indicated concentrations of the inhibitors. Rat fibronectin (5 μg/ml; Gibco BRL) was used as a chemoattractant in the lower chamber. Following a 48-h incubation at 37°C, the cells on the upper surface of the filter were removed with a cotton swab and the filters fixed in 0.1% glutaraldehyde and stained with 0.2% crystal violet. For each filter 20 random fields were counted using a Nikon inverted microscope (× 100) and duplicate samples were analysed for each assay condition. The average numbers of (untreated) H-59 and M-27 cells which were counted per filter were approximately 3000 and 800, respectively. In each experiment, control filters were coated with 7.5 μg/filter of human placental type IV collagen (Sigma) to control for changes in cell migration. In previous experiments we found that, under these conditions, there was no significant difference between the migration levels seen with highly and poorly invasive H-59 and M-27 cells, respectively [7].

Analysis of E-64 uptake by tumor cells

Confluent cultures of H-59 or M-27 cells in serum-free RPMI were exposed to E-64 (10–100 μg/ml) or PRCB1 (40–50 μg/ml) for 4 h at pH 7.4. The medium was removed, the monolayers washed repeatedly to remove excess inhibitor, and the cells cultured in the absence of the inhibitor for the duration indicated in the text. The cells were then dispersed, centrifuged and lysed in a 0.1% Triton X-100 solution containing 50 mM sodium acetate and 1 mM EDTA, pH 5.0. Cysteine proteinase activity in the cell lysates was measured fluorimetrically using the synthetic substrates Z-Phe-Arg-MCA [benzyloxycarbonyl-phenylalanyl-arginine-7-(4-methylcoumarylamide)] [17] or the more selective cathepsin B substrate Z-Arg-Arg-MCA [benzyloxycarbonyl-argininyl-arginine-7-(4-methyl-coumarylamide] [18]. The enzymatic reactions were carried out at 25°C in a solution containing 50 mM sodium phosphate, pH 6.0, 1 mM EDTA, 1 mM dithiothreitol, 0.025% Brij-35, 3% DMSO and 20 μM substrate. Addition of E-64 to the assay confirmed that all the activity measured was due to the action of cysteine proteinases.

Liver colonization assay

Animals were injected with 2 × 10^5 H-59 cells by the intrasplenic/portal route (i.s.) and then immediately splenectomized as we described previously [19]. For treatment with E-64, 10–100 μg/ml of the inhibitor were added to H-59 monolayers for a 4-h incubation at 37°C. The medium was removed, the cells dispersed with PBS-EDTA, adjusted to the correct cell density in HBSS and mixed for a second time with the identical concentration of E-64, immediately prior to injection. Some of the animals received in addition, two direct i.v. injections of 100 μg E-64 in 0.1 ml HBSS 4 and 18 h after tumor cell injection. The animals were sacrificed 14–21 days later, the livers removed and the metastases enumerated immediately [20].

Statistics

The Mann–Whitney test was used to analyse differences in the numbers of the metastases.

Results

The role of cysteine proteinases in tumor cell invasion

To assess the role of cysteine proteinases in Matrigel invasion in our tumor system, two inhibitors were used. E-64, a broad spectrum inhibitor of CP activity and PRCB1, a synthetic peptide corresponding to the proregion of rat cathepsin B which was recently shown to be a potent, specific inhibitor of the active form of this enzyme [9]. The results shown in Figure

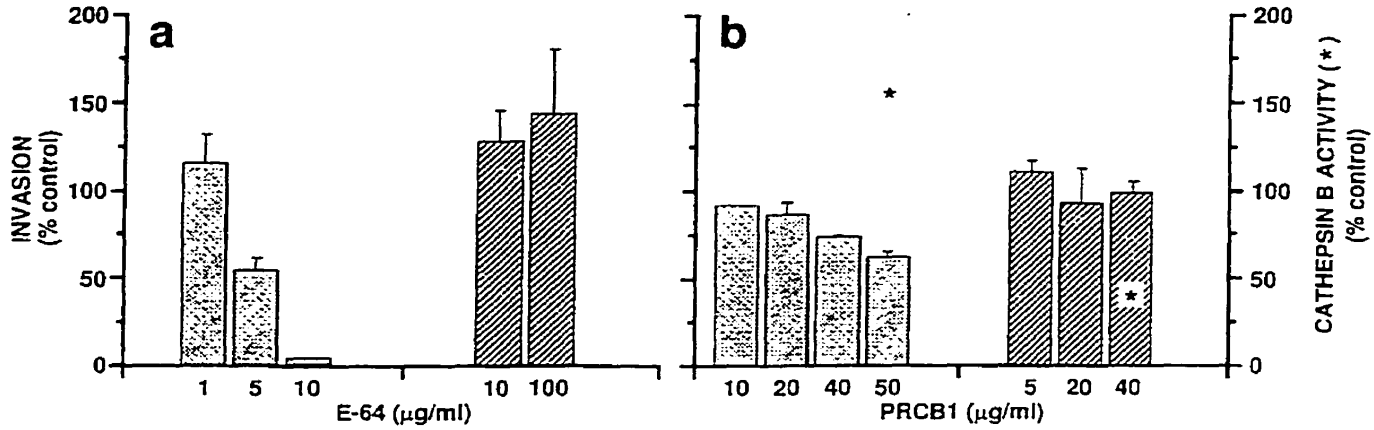

Figure 1. The effect of the cysteine proteinase inhibitors E-64 and PRCB1 on tumor cell invasion. H-59 (solid bars) and M-27 (hatched bars) cells (5×10^4 in 100 μl medium) were plated on Matrigel-coated filters together with the indicated concentrations of E-64 (a) or PRCB1 (b) and incubated for 48 h at 37°C. In each of the experiments, untreated cells were used as controls. Results are based on two experiments carried out in duplicate and are presented as percentage of invasion relative to control untreated cells. Bars denote SD. (*) indicates the level of intracellular cathepsin B activity in cells treated for 4 h with 40 (M-27) or 50 (H-59) μg/ml PRCB1 as measured using the synthetic substrate Z-Arg-Arg-MCA.

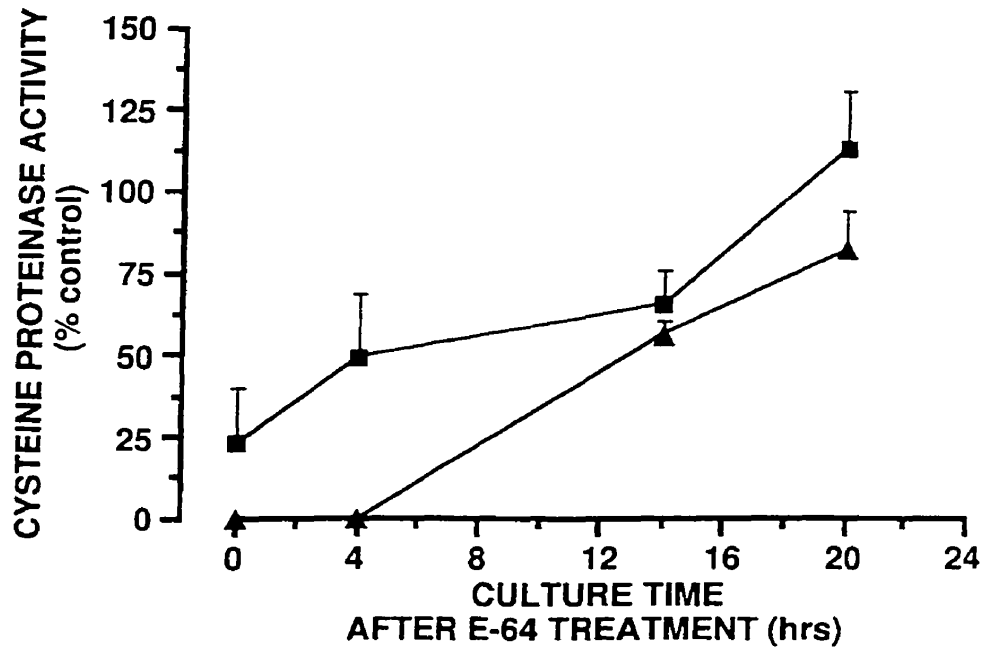

Figure 2. Modulation of intracellular cysteine proteinase activity by E-64. H-59 (■) and M-27 (▲) cells were incubated with E-64 (10 and 100 μg/ml, respectively) for 4 h. The medium was removed and the cells cultured in the absence of E-64 for the duration indicated. Cysteine proteinase activity in the cell lysates was measured using the synthetic substrate Z-Phe-Arg-MCA. Results are expressed as percentage of enzymatic activity in control cells cultured in the absence of E-64. They are based on three to four experiments. Bars denote SD.

I demonstrate that E-64 inhibited H-59 invasion in a dose-dependent manner with a maximal inhibition of 97% at a concentration of 10 μg/ml which was non-toxic. Cell migration as measured with filters coated with 7.5 μg/filter type IV collagen was reduced by only 25% suggesting that the cysteine proteinases played a more minor role in cell migration in the absence of a basement membrane barrier. On the other hand M-27 invasion was not significantly affected by treatment with E-64 even at concentrations as high as 100 μg/ml (Figure 1a).

PRCB1 had a more moderate effect on the invasion of H-59 cells, reducing it by up to 32% at a concentration of 50 μg/ml (Figure 1b). Interestingly, PRCB1 had no effect on M-27 invasion despite the high cathepsin B levels found in these cells (Figure 1b) [7].

These results raised the possibility that H-59 and M-27 cells differed in their ability to internalize the inhibitors. To measure inhibitor uptake and internalization by the two cell types, intracellular enzymatic activity was analysed at different time intervals after E-64 or PRCB1 were added using either a non-specific synthetic CP substrate Z-Phe-Arg-MCA [17] or the substrate Z-Arg-Arg-MCA which more specifically measures cathepsin B activity [18]. Using a fluorimetric assay to quantitate proteolytic cleavage of these substrates, we found a marked reduction in enzymatic activity in the cell lysates of both tumors 4 h after treatment with E-64 and this activity began to recover 4–12 h later (Figure 2). PRCB1 significantly reduced the intracellular cathepsin B activity in M-27 cells for up to 8 h but surprisingly, had no measurable effect on the enzymatic activity measured in the H-59 lysates (Figure 1b). This confirmed that the observed lack of effect of these inhibitors on M-27 invasion was not due to deficient uptake and/or internalization of the inhibitors, and provided further evidence that the ECM-degrading mechanisms in the two tumor types were distinct [7].

In addition to differences in the expression of the cysteine proteinases cathepsins B and L, we also previously reported that H-59 cells produced higher levels of MMP-2 than M-27 cells. In the present study the increased expression of MMP-2 in H-59 cells was also confirmed at the mRNA level as indicated by results of a Northern blot analysis shown in Figure 3a. Since metalloproteinases have been shown to act in concert with other ECM-degrading proteinases, including cysteine proteinases [21, 22], it was of interest to assess the role of MMP2 in Matrigel invasion in our model. To this end, the synthetic metalloproteinase inhibitor U24522 was

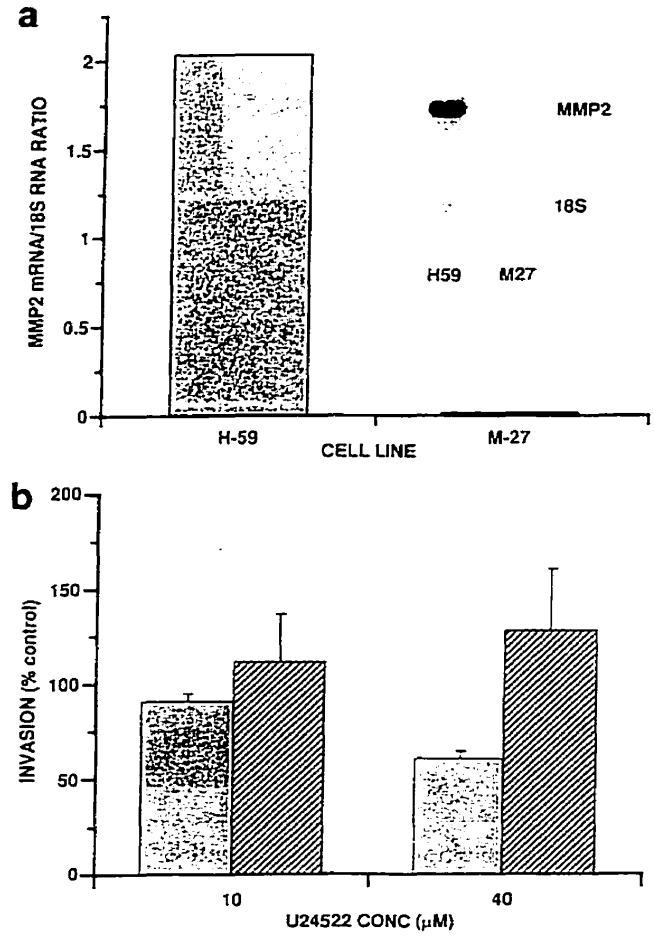

Figure 3. MMP-2 expression and role in invasion of H-59 and M-27 cells. Results of a Northern blot analysis of MMP-2 mRNA are shown in (a): 30 μg of total RNA from H-59 and M-27 cells were size fractionated by electrophoresis on 1.1% formaldehyde-agarose gels. The blots were probed first with a ^{32}P-labeled MMP-2 600 bp cDNA and then with a ^{32}P-labeled oligonucleotide probe for 18S rRNA. The intensity of the bands was measured by laser densitometry and is expressed relative to control bands of 18S rRNA. The effect of the metalloproteinase inhibitor U24522 on H-59 (solid bars) and M-27 (hatched bars) invasion is shown in (b): assay conditions were as described in the legend to Figure 1. Results are expressed as percentage of invasion relative to control cells and are based on two experiments in which duplicate samples were analysed. Bars denote SD.

used in the Matrigel invasion assay. We found that U24522 could inhibit invasion of H-59 cells by up to 40% at a concentration of 40 μM (Figure 3b) which did not affect cell viability or proliferation. Under identical conditions this inhibitor had no significant effect on the invasion of M-27 cells, suggesting that M-27 invasion could proceed despite the reduction in metalloproteinase activity.

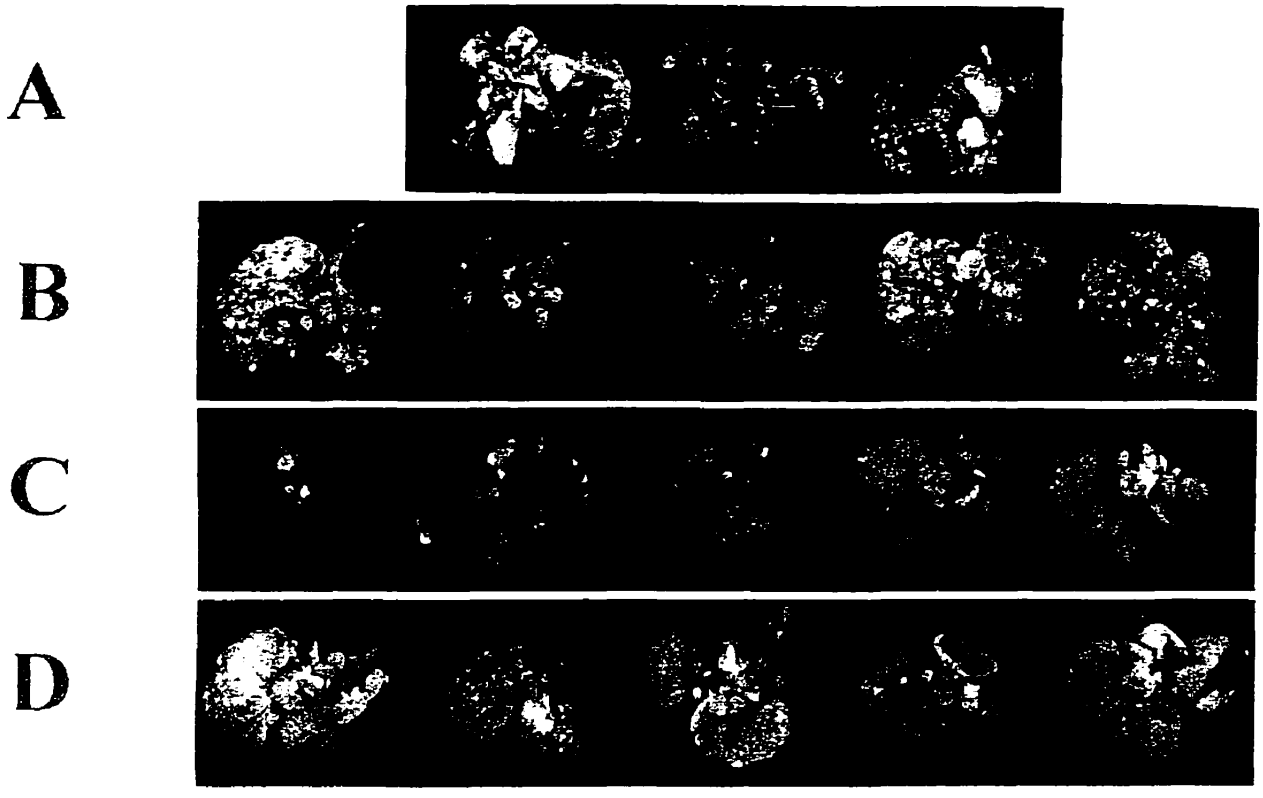

Figure 4. Inhibition of liver colonization by E-64. Representative livers from experiment 1 (Table 1) are shown. (A) Animals inoculated with untreated cells. (B) Cells were untreated but animals received two i.v. injections of E-64. (C) Cells were pretreated with 100 μg/ml E-64. (D) Cells were pretreated as in (C) and animals inoculated i.v. twice with 100 μg E-64.

The profound effect that E-64 had on H-59 invasion in the Matrigel assay prompted us to investigate its effect on liver metastases formation in this model. The *in vitro* analyses described above (Figure 2) suggested that incubation of H-59 cells with the inhibitor for 4 h resulted in a significant inhibition of intracellular cysteine proteinase activity. The tumor cells were therefore pretreated for 4 h with different concentrations (10–100 μg/ml) of E-64 prior to injection via the intrasplenic/portal route. For some animals this was followed with two i.v. injections of 100 μg E-64 each, at 4 and 18 h following tumor inoculation. When hepatic colonies were enumerated 2–3 weeks later, we found that pretreatment with E-64 inhibited significantly and in a dose-dependent manner the ability of these cells to colonize the liver. The highest concentration used (100 μg/ml) caused a reduction of 82–89% in the median number of experimental liver metastases (Table 1 and Figure 4). Injections of 100 μg E-64 i.v. without pretreatment of the tumor cells had no significant effect on liver colonization. However when combined with tumor cell pretreatment, i.v. injections appeared to further increase the reduction in the number of liver colonies (Table 1).

Discussion

In an earlier report we described the differences in the repertoires of proteolytic enzymes produced by two sublines of the Lewis lung carcinoma which are metastatic preferentially to the liver (carcinoma H-59) or lung (carcinoma M-27) from local primary sites [7]. We proposed that there may be a relationship between the patterns of metastasis of these cells and their repertoires of ECM-degrading proteinases. Because one of the major differences we detected between the two cell lines was in the production of the cysteine proteinases cathepsins B and L, it was of interest to evaluate the role of these

Table 1. The cysteine proteinase inhibitor E-64 blocks liver colonization by carcinoma H-59 cells[a]

Tumor cell treatment concentration of E-64 (μg/ml)	No. of metastases/liver median (range)		
	Exp. 1	Exp. 2	Exp. 3
—	64 (32–91)	78 (63–86)	211 (26–320)
—[b]	64 (12–84)[c]		
10	35 (12–72)[c,d]	73 (51–113)[c]	323 (220–333)[c]
50	33 (14–48)[c,e]		123 (80–242)[c]
100	17 (8–25)[f]		
100	8 (0–25)[c,f]	14 (2–17)[c,f]	23 (9–43)[c,g]

[a] Experimental hepatic metastases were enumerated 14 (Exp. 1 and 2) and 21 days (Exp. 3) following i.s. inoculation of 2×10^5 H-59 cells. Results are based on 5–10 animals per treatment group.

[b] Animals received two i.v. injections of 100 μg E-64, 4 and 18 h following tumor inoculation.

[c] Not statistically significant in comparison to control.

[d] $0.05 < P < 0.1$.

[e] $P = 0.05$.

[f] $P = 0.001$.

[g] $0.01 < P < 0.025$.

enzymes in tumor cell invasion and metastasis in this model, through the use of specific proteinase inhibitors.

Our results show that the invasion of H-59 but not M-27 cells through reconstituted basement membrane (Matrigel) could be blocked completely by the general cysteine proteinase inhibitor, E-64 and partially by the propeptide of cathepsin B which inhibits specifically the activity of the mature form of this enzyme. The effect on H-59 cells suggests that cysteine proteinases play a role in the invasion of these tumor cells. On the other hand, the failure of either of these inhibitors to block M-27 invasion despite evidence that they are internalized by the cells and block intracellular enzymatic activity suggests that M-27 invasion (which is significantly lower than that of H-59) can probably proceed in the absence of cathepsin B activity.

Several studies have implicated cysteine proteinases in invasion and tumor progression [23–25] and their altered expression, processing and/or cellular localization have been demonstrated in various malignantly-transformed cell types [23, 24, 26]. The evidence suggests that cathepsins B and L may participate in invasion by direct degradation of extracellular matrix proteins [27, 28]. It has also been shown that cysteine proteinases can be engaged in proteolytic cascades with other proteinases such as the serine proteinase urokinase plasminogen activator (uPA) and metalloproteinases such as MMP-1 [29]. It is conceivable that in the present model, cathepsins B and/or L mediate ECM degradation directly or they may be involved in direct or indirect activation of another proteinase required for invasion such as MMP-2, an enzyme highly expressed in H-59 cells. Indeed we found that the metalloproteinase inhibitor U24522 reduced H-59 invasion in Matrigel (40%) whereas it had no effect on M-27 invasion. Since M-27 (but not H-59) cells express high levels of uPA [7], it is possible that the low level of invasion of these cells [7] could proceed in the absence of cathepsin B activity because an alternative, uPA-mediated mechanism of proteolysis is in place. This is supported by our recent finding (not shown) that treatment of M-27 cells with the plasmin inhibitor E-ACA (ε-amino-*n*-caproic acid) [30] inhibited Matrigel invasion by up to 66%. The possible role of proteolytic cascades involving metallo-, serine- and cysteine proteinases in ECM degradation by these tumor cells is currently under investigation.

We found that in H-59 cells PRCB1 had no measurable effect on the intracellular activity of cathepsin B as measured with a specific cathepsin B substrate (Figure 1b). The reason for this lack of inhibition is not immediately clear and could be related to an inefficient uptake of the inhibitor by these cells. The observation that this inhibitor could still block H-59 invasion in the Matrigel assay suggests that it probably interfered with the extracellular activity of the enzyme. This is in line with other reports that in malignant cells cathepsin B can translocate to the cell surface or is secreted into the extracellular milieu [25, 26].

In addition to cathepsins B and L, several other cysteine proteinases have been identified including

cathepsins H, S, K and O [see review in 31]. Although some of these proteinases (e.g. cathepsin O) have been identified in carcinoma cells [32], their role in ECM degradation and in invasion remains to be confirmed [31]. The involvement of one or more of these enzymes in H-59 invasion cannot at present be ruled out.

Our finding that the cysteine proteinase inhibitors also reduced cell migration by approximately 20% in the absence of a basement membrane type barrier, suggests that these enzymes may play a role in cell migration. This is in accord with other reports which implicate cysteine proteinases in regulation of chemotaxis and motility [22, 33, 34].

Of particular importance is our finding that pretreatment of H-59 cells with E-64 inhibited metastases formation following intrasplenic/portal injection, suggesting that cysteine proteinases are involved in liver colonization in this model. This, to our knowledge, is the first report which directly implicates the cysteine proteinases in liver metastases formation and one of only few to demonstrate an inhibitory effect of E-64 *in vivo*. Recently, Redwood *et al.* [34] reported that E-64 blocked motility and invasion of human bladder tumor cells and appeared to reduce lung metastases in nude mice. Other studies suggest however that the inhibitory effect of E-64 on metastasis is variable and it appears to be tumor and organ-site specific [35].

Our data based on *in vitro* studies (Figure 2) suggest that in H-59 cells, E-64 could inhibit cysteine proteinase activity for up to 16 h after treatment. Taken together with the metastasis studies, these results imply that cysteine proteinase activity was probably required within less than 24 h following cell arrest in the hepatic circulation. Intravital microscopy results have shown that tumor cell extravasation from the hepatic sinusoidal lumen occurs within 24–36 h of cell injection [36]. Thus in this model cysteine proteinase activity appears to have been rate-limiting during the pre-extravasation phase when the tumor cells were still within the microvascular space. These enzymes may therefore be involved in degradation of collagen-containing extracellular matrix deposits which are found in association with the sinosoidal endothelium and in the space of Disse [37]. We can also not rule out the possibility that E-64 blocks other cellular functions which are essential for metastases formation such as mitosis [38] and motility [33].

Multiple hepatic metastases which are inaccessible to surgical resection are generally incurable and fatal. Our results suggest that E-64 could have an inhibitory effect on liver metastases when inoculated directly into tumor-bearing mice because the number of experimental metastases we observed following tumor cell pretreatment with 10 μg/ml of E-64 was significantly reduced only when two additional i.v. injections of the inhibitor were administered 4 and 18 h after tumor inoculation (Table 1). When taken together with other reports [22,30,34] the present results provide evidence that metastatic carcinomas which utilize cysteine proteinases for invasion could potentially be responsive to antimetastatic treatment with cysteine proteinase inhibitors.

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CHAPTER 5

The role of cysteine proteinases in IGF-1R turnover

5.1. Overview

The results presented in this chapter are in the form of a submitted manuscript. I was responsible for all the experiments. Dr. DiGuglielmo, G. M. (Dept. of Anatomy) assisted in some of the immunoprecipitation analyses and Mrs. Giannias, B. (Dept of Surgery) assisted in flow cytometric analyses.

Cysteine proteinases in particular cathepsins B and L, have been implicated in tumor invasion and are thought to be important mediators of metastasis. However, it is not yet clear how these intracellular enzymes contribute to cellular invasion. Previously we reported that a cysteine proteinase inhibitor, E-64, blocked invasion and metastasis of the highly invasive murine Lewis lung carcinoma subline H-59 cells by up to 90%. In the present study, we have shown that treatment of H-59 and MCF-7, a human breast carcinoma cell line, with E-64, abolished their proliferative response to IGF-1. This prompted us to investigate the role of cysteine proteinases in the regulation of cell growth.

IGF-1R was identified as a critical mediator of cellular transformation and the acquisition of the malignant phenotype. Previously our laboratory reported that IGF-1 is involved in the regulation of MMP-2 synthesis (Long et al., 1998b). This added new insight into the role that IGF-1R plays in tumor progression because it implicated in the regulation of late

events in the process, namely, the acquisition by transformed cells of an invasive/metastatic phenotype. In line with our finding on the inhibition of IGF-1-induced mitogenesis by E-64, we also found that IGF-1 induced synthesis of MMP-2 was significantly reduced in H-59 cells treated with E64. This identified the cysteine proteinases as modulators of cellular proliferation, transformation and invasion regulated by IGF-1/IGF-1R system. Interestingly, we observed an increase in the accumulation of intracellular tyrosine phosphorylated IGF-1R in the absence of cysteine proteinase activity. The present finding that cysteine proteinases such as cathepsin B and L could alter IGF-1R expression and function, to our knowledge is the first report implicating cysteine proteinases in IGF-1R turnover.

Altered IGF-1R expression and function in tumor cells treated with the cysteine proteinase inhibitor E-64

R. Navab, G.M. Di Guglielmo, J.J.M. Bergeron and P. Brodt

Abstract

The receptor for IGF-1 (IGF-1R) was identified as a critical mediator of cellular transformation and the acquisition of the malignant phenotype in different cell types. Using carcinoma H-59, a highly metastatic subline of the Lewis lung carcinoma, we recently established that this receptor regulates several cellular functions which can impact on the metastatic potential of the cells, including cellular proliferation and invasion. Because invasion and metastasis of these cells could be blocked by the cysteine proteinase inhibitor E-64, we sought to determine here whether cysteine proteinases played a role in regulating the cellular responses to IGF-1. Using H-59 and human breast carcinoma MCF-7 cells, we found that E-64, at non-toxic concentrations, abolished the proliferative responses of these cells to IGF-1 and caused a reduction of 7-10 fold in their cloning efficiency in semi-solid agar. A ligand-binding assay and FACS analysis revealed a 2-fold reduction in cell surface IGF-1R expression in the E-64 treated cells. In addition, IGF-I induced synthesis of MMP-2 was also reduced. Concurrent with these changes, we found in these cells a 4 fold increase in the intracellular levels of tyrosine phosphorylated receptor β subunit following stimulation with the IGF-1 ligand. The results suggest that inhibition of cysteine proteinase activity disrupted IGF-1 mediated functions by altering post ligand- binding processing of the receptor.

Key Words: cysteine proteinases, IGF-1R internalization, MMP-2, endocytosis.

Introduction

The IGF-1 receptor is synthesized as a single polypeptide chain, which is then glycosylated and proteolytically cleaved into α- and β-subunits (Ullrich et al., 1986). The receptor consists of two 130-135 KDa α and two 90-95 KDa β chains, with several α-α and α-β disulfide bridges (Massague and Czech, 1982). The ligand binding domain is located on the extracellular α subunit. Approximately a 1/3 of the β subunit is extracellular and it is connected to the intracellular portion by a transmembrane domain. The intracellular region of the β subunit has a binding site for phosphorylation substrates, an ATP-binding site, a tyrosine kinase domain and several tyrosines in the carboxy domain, known to be essential for some of the receptor's biological functions (Rubin and Baserga, 1995).

The ligands for IGF-1R include IGF-1, IGF-2 and insulin which bind to the receptor with decreasing affinities IGF-1> IGF-2 > insulin. Cellular responses to the type 1 and type 2 insulin-like growth factors (IGF-1 and IGF-2) depend on their binding to the tyrosine kinase receptor IGF-1R. Early events triggered in response to binding of these ligands include rapid activation of the receptor tyrosine kinase (White and Kahn, 1986), autophosphorylation of several tyrosine residues in the receptor β subunit (Tornqvist et al., 1988; White et al., 1988) and rapid intertnalization of ligand-receptor complexes by receptor-mediated endocytosis into the membrane-bound endosomes (Brown et al., 1983; Helenius et al., 1983). Based in part on direct evidence and in part infered from studies

on the insulin signalling pathway (Furlanetto, 1988; Carpentier, 1994), it is believed that ligand binding initiates the migration of IGF-1 receptors to clathrin-coated pits and the subsequent formation of early endosomes containing internalized but still active receptors. The ligand-receptor complex is ultimately dissociated and inactivated in the acidic environment of late endosomes, where ligands and receptors are sorted for degradation in lysosomes or for recycling to the cell surface (Beisiegel et al., 1981; Watts, 1985; Fine and Ockleford, 1984). Endosomal proteolysis of internalized complexes determines the intracellular fate of ligand and receptor and may be important in terminating signal transduction (Bergeron et al., 1995; Blum et al., 1993; Authier et al., 1994c).

Endosomal proteinases have been implicated in the processing of polypeptide hormones such as insulin (Doherty et al., 1990; Backer et al., 1990; Authier et al., 1994a) and parathyroid hormone (Diment et al., 1989), growth factors such as the epidermal growth factor (Renfrew and Hubbard, 1991) as well as plant toxins (Blum et al., 1991; Fiani et al., 1993) and protein antigens which undergo endocytosis and processing for major histocompatibility class II presentation (Guagliardi et al., 1990). In addition to these, Authier et al., (1995) reported that hepatic endosomes contain endopeptidases that are membrane-associated and that process glucagon to multiple peptides at low pH. In their report, the major proteinases identified in endosomes were the active (28 KDa) and inactive (45 KDa) forms of the cysteine proteinase cathepsin B, the 37 KDa inactive form of cathepsin L and the active form of the aspartic proteinase, cathepsin D(34 KDa). The

selective enrichment of these hydrolases in endosomes is likely to be related to their association with endosomal membranes via hitherto undefined receptors (Hasilik and Neufeld, 1980; Kornfeld, 1986; Kornfeld and Mellman, 1989; Von Figura and Hasilik, 1986). These putative receptors could provide the strong association observed between the precursor and mature forms of cathepsin B and the endosomal membranes (Authier et al., 1995).

While it has been shown that after binding, the IGF-1R/IGF-1complex undergoes endocytosis, and that intracellular ligand-receptor dissociation occurs in the acidified endosome (Furlanetto, 1988; Carpentier, 1994), the proteinases involved have not yet been identified.

The IGF-1R and its ligand play a critical role in the regulation of cellular proliferation, apoptosis, and transformation (Brodt et al., 1992). Reduction of IGF-1R expression has been shown to inhibit cellular transformation and tumorigenicity (Briozzo et al, 1988; Saiga et al., 1987; Philips et al., 1987). Anti-IGF-1R reagents such as antibodies and antisense strategy have shown the potential therapeutic application in the future (McPherson, 1983a,b; Long et al., 1994; Nip et al., 1995). Also, it was shown that in our murine carcinoma model, the invasive and metastatic potentials of the cells correlated with IGF-1R expression and MMP-2 levels and it was suggested that IGF-1R can regulate the expression of MMP-2 which implies that in addition to its growth-

modulating effects, IGF-1 can also impact the metastatic potential by increasing the collagenolytic activity of the cells (Long et al., 1998b).

Previously we reported that a cysteine proteinase inhibitor, E-64, blocked invasion and metastasis of the highly invasive, murine Lewis lung carcinoma subline H-59 cells (Navab et al., 1997). The objective of the present study was to determine whether the inhibition of the cysteine proteinases altered IGF-1R expression and function.

Materials and Methods

Cell Lines. tumor H-59 is a subline of the Lewis lung carcinoma which is highly and preferentially metastatic to the liver. Its origin, metastatic properties and method of propagation were described in detail previously (Brodt et al., 1992). Human breast carcinoma line MCF-7 (Briozzo et al., 1988) was a kind gift from Dr. Mader (Dept of Biochemistry, university of Montreal, Mtl, Quebec, Canada).

Reagents and antibodies. E-64 [trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane], Protein A-sepharose beads and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (thiazolyl blue) were purchased from Sigma (St Louis, MO). [^{3}H] thymidine (2.0 Ci/mmol) was from Du Pont Canada (Mississauga, Ontario, Canada). ^{125}I-labeled IGF-I (2000 Ci/mmol) was obtained from Amersham Canada (Oakville, Ontario, Canada). A 1.1- Kb type IV collagenase cDNA fragment was kindly provided by Dr. W. Stetler-Stevenson (NIH, Bethesda, MD). A 700-bp IGF-IR cDNA fragment was a kind gift from Dr. M. Pollak (Lady Davis Research Institute, Jewish General Hospital, Montreal, Quebec, Canada). The following antibodies were used: rabbit antiserum to MMP-2 (Ab-45), a kind gift from Dr. William Stetler-Stevenson (NIH), a mAb to phosphotyrosine clone PT-66 from Sigma (St. Louis, MO), a mAb to the murine IGF-IR β subunit (C-20) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mab αIR3 to human IGF-1R (Ab-1) from Calibiochem (cambridge, MA), horseradish peroxidase (HRP)- conjugated goat anti-mouse and goat anti-rabbit IgG

antibodies from Bio-Rad (Mississauga, Ontario), alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG from Bio/Can Scientific, (Mississauga, Ontario).

Tumor Cell Proliferation Assay. Semiconfluent cultures of H-59 or MCF-7 cells growing in RPMI 1640 with 5% FCS were washed twice with serum free (SF)-RPMI and cultured in SF-medium for 24 h with or without different concentrations of E-64. The cells were dispersed with phosphate-buffered saline-EDTA (PBS-EDTA), and $2x10^3$ cells/well were seeded onto 96-well polystyrene plates (Falcon) with different concentrations of IGF-I and with or without increasing concentrations of E-64. Following a 54-h incubation, the cells were pulsed with 0.1 mCi/ml of [^{3}H] thymidine for 18 h. The cells were lysed by repeated freezing and thawing, the cell lysates harvested onto paper filters using the Micromate 196 harvester (Packard Instrument Company, Inc., Meriden, CT) and [^{3}H] Thymidine incorporation monitored using the Beckman LS 8000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Soft agar cloning Assay. To measure anchorage-independent growth, a modification of the standard soft agar cloning assay was used (Saiga et al., 1987). Briefly, tumor cells were mixed with a solution of 0.8% agar (Difco Laboratories Inc., Detroit, MI) added to an equal volume of a 2x concentrated RPMI-FCS medium in the presence or absence of 10 μg/ml of E-64, and plated in six- well plates (Fisher Scientific, Montreal, Quebec) on solidified 2% agar at a concentration of 10^4 cells/well. The overlay was allowed to

solidify and then supplemented with 1 ml RPMI-FCS. The medium with or without 10 μg/ml of E-64 was replenished on alternate days for 12 days. Colonies which exceeded 250 μm in diameter were enumerated using an inverted microscope (Diaphot-TMD Inverted, Nikon Canada).

Ligand-Binding Assay. IGF-1 binding sites were quantitated using the method described by Phillips et al (1987). Briefly H-59 cells were cultured with RPMI-FCS in 24-well plates for 2-3 days. The culture medium was removed and replaced with fresh medium with or without 10 μg/ml E-64. The binding assay was carried out 24 h later. To each well, 8-1500 pM of ^{125}I- labeled IGF-1 in binding medium (SF-RPMI containing 1 mg/ml BSA and 1μg/ml leupeptin) were added, with or without graded concentrations of unlabeled IGF-1 for a 1 h incubation at 37°C. The cells were rinsed twice with ice-cold binding medium and solubilized in 0.01 N NaOH containing 0.1 % Triton X-100 and 0.1% SDS. The number of cells/well at the time of the assay was determined from triplicate control wells which were manipulated in the same manner. An aliquot was removed from each well and the radioactivity was measured in an LKB gamma counter. The number of IGF-1 binding sites were calculated using the Ligand program (McPherson, 1983a,b).

Immunocytofluorometry. MCF-7 cells were cultured in RPMI-SF with or without E-64 (10 μg/ml) for 24 h, dispersed and seeded into 96-well plates (Falcon, Lincoln Park, NJ)

at a density of 10^5 cells/well. The cells were stimulated with 10 ng/ml of IGF-1 for 10 min. IGF-1 was washed off three times with RPMI-SF medium and incubated at 37°C for 30 min then incubated for 1 h on ice with 5 μg/ml of mAb αIR3. After extensive washing with cold medium, the cells were incubated with FITC-conjugated goat anti-mouse IgG (diluted 1:50) for 1hr on ice, washed and fixed in PBS containing 1% formalin. The labeled cells were analyzed by flow cytofluorometry using a FACS Calibur System (Becton-Dickinson, San Jose, California).

Northern Blot Assay. The Northern blot assay was performed essentially as described previously (Long et al., 1994). The ^{32}P-labeled 1.1-Kb human MMP-2 cDNA fragment, 700-bp human IGF-IR and 800-bp fragment of rat cyclophilin cDNA (Nip et al., 1995) were used as hybridization probes. The relative amounts of mRNA transcripts were analysed by laser densitometry using an Ultroscan XL enhanced laser densitometer and normalised relative to the internal cyclophilin controls.

Immunoprecipitation. Immunoprecipitation was performed essentially as we described previously (Long et al., 1998a). Tumor cells were cultured in the presence or absence of 10 μg/ml E-64 for 4-48 hr and then with 10 (H-59) or 50 (MCF-7) ng/ml IGF-1 for 10 and 5 min respectively. The cells were lysed by incubation in HBS (0.15 M Nacl, pH 7.5 containing 50 mM HEPES, 1% Triton X-100, 0.5% deoxycholate and 10% glycerol) for 30 min at 4°C. The lysates were centrifuged at 12,000 g for 5 min in a Brinkmann

microfuge . To the supernatants 10 μg of mAb αIR3 (for MCF-7 cells) or mAb C-20 (for H-59 cells) were added for a 2 h incubation at 4°C. Immune complexes were precipitated by incubation with protein A sepharose beads for 60 min at 4°C. The beads were washed repeatedly with the lysis buffer, resuspended in SDS sample buffer and boiled for 10 min. The eluted proteins were electrophoresed on 8% SDS-polyacrylamide gels under reducing conditions.

Western blotting. Proteins were separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose filters (Xymotech, Mt Royal, Quebec, Canada). The blots were incubated in TNT buffer (0.15 M Nacl, pH 7.5 containing 0.05% Tween-20, 10mM Tris) containing 5% skimmed milk or 2% BSA (for detection of phosphotyrosine) and probed with anti-IGF-1R or anti-phosphotyrosine mAbs at dilutions of 1:1000. To visualize the bands, blots were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG antibodies and developed with enhanced chemiluminescence detection reagents (Amersham) or they were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG at a dilution of 1:2000 and developed using NBT-BCIP (Sigma) as substrate. Densitometry was performed on the film with SciScan (tn)5000 (United States Biochemical Corporation).

Gelatin Zymography. The gelatinolytic activity of MMP-2 was analyzed by zymography as described previously (Durko et al., 1997). The concentrated conditioned

media (×60) from H-59 cells which were cultured for 48 h with or without 10μg/ml E-64 in the presence or absence of 10 ng/ml IGF-1 were electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. The gels were stained with Coomassie Blue and destained with 10% acetic acid-50% methanol until the desired color intensity was obtained. The gelatinolytic activity seen as a clear zone on the blue background was quantitated by densitometry using photographic negatives of the gel.

Results

To investigate the effect of the inhibition of cysteine proteinase activity on IGF-1R-mediated function, tumor cells were treated with E-64 and their proliferation in response to IGF-1 as well as their ability to form colonies in semi-solid agar were measured. E-64 at the non-toxic concentration of 10 μg/ml (Carmichael et al., 1987; Navab et al., 1997) reduced by factors of 7 and 10 respectively, the cloning efficiency of MCF-7 and H-59 cells in semi-solid agar (Fig.1). It also completely abolished the IGF-1 induced proliferation of both cell types (Fig. 2).

We previously reported that MMP-2 synthesis in H-59 cells was regulated by IGF-1 (Long et al., 1998b). When the effect of E-64 on MMP-2 mRNA expression was investigated using Northern blotting, we found a reduction of 2 fold in MMP-2 transcripts in the treated as compared to non-treated cells (Fig.3). This was reflected in decreased MMP-2 synthesis and function as determined by Western blotting and gelatin zymography respectively (Fig. 3).

To determine whether E-64 treatment altered IGF-1R synthesis, we first analyzed cell surface receptor expression by a ligand binding assay (for H-59 cells) and flow cytometry (for MCF-7 cells). The ligand-binding assay revealed a 2 fold reduction in the number of IGF-1 binding sites on E-64 treated as compared to non-treated H-59 cells (table. 1). A

similar reduction was seen in the number of positively labeled MCF-7 cells as analyzed using mAb αIR3 to the human receptor (Fig. 4). In neither of these cells did E-64 treatment cause a reduction in IGF-1R mRNA levels (Fig. 5) or in the total level of immunoprecipitable receptor (Fig. 5). This suggested that the reduction seen in cell surface IGF-1R expression and function was not due to a decrease in receptor production and raised the possibility that E-64 treatment altered receptor turnover or signalling.

One of the earliest molecular events in IGF-1R ligand-induced signalling is the autophosphorylation of tyrosine residues on the receptor β subunit. To determine whether E-64 treatment affected early events in IGF-1R signalling, cell lysates prepared from IGF-1 stimulated and E-64 treated (or non-treated) MCF-7 cells, were immunoprecipitated with αIR3 and the immunoprecipitates analysed by Western blotting with an anti-phosphotyrosine mAb. Results of these analyses, shown in Fig. 6A, revealed that the total amount of tyrosine phosphorylated β subunit increased by 2.7 fold in E-64 treated as compared to non-treated cells. A similar trend was seen with H-59 cells but the magnitude of the increase in tyrosine phosphorylated β subunit in these cells was lower (Fig. 6B). To distinguish between plasma membrane and intracellular receptor pools, E-64 treated and IGF-1 stimulated MCF-7 cells were subjected to a brief treatment with 0.05% trypsin. In these cells we found an increase of 4-fold in immunoprecipitable tyrosine phosphorylated β subunit levels as compared to non-treated cells (Fig. 7) suggesting that E-64 treatment while decreasing the number of extracellular receptors

caused an increased accumulation of tyrosine phosphorylated intracellular (trypsin-insensitive) receptors.

Discussion

The lysosomal cysteine proteinases cathepsins B and L have been implicated in tumor progression as mediators of cancer invasion and metastasis (Sloane et al., 1994c). In these processes they are generally thought to be involved extracellularly as facilitators of matrix dissolution, either directly by cleavage of ECM proteins (Buck et al., 1992) or indirectly by participating in proteolytic cascades leading to the conversion of proenzymes involved in ECM proteolysis (e.g. metalloproteinases) into their catalytically active forms (Weiss et al., 1993). The present findings implicate these cysteine proteinases in intracellular, post-ligand binding mechanisms involved in IGF-1R turnover and signalling and thereby add a new dimension to their role in the regulation of the malignant phenotype.

Our results show that the inhibition of cysteine proteinases by the specific inhibitor E-64 abolished the proliferative response to IGF-1, reduced serum-induced cloning efficiency in semisolid agar and decreased MMP-2 production in mouse and human carcinoma cell lines. Taken together with our previous studies which identified IGF-1R as a regulator of anchorage-independent growth, cellular proliferation, MMP-2 synthesis and invasion (Long et al., 1998a,b), these results suggest that intact cysteine proteinase activity is essential for IGF-1R-dependent cellular functions.

Following ligand binding, some receptor tyrosine kinases (RTK) such as the receptors for EGF and PDGF are targeted to lysosomes for degradation while others, such as the insulin and IGF-1 receptors are processed in the endosomes and recycled to the plasma membrane (Bergeron et al., 1985). Cysteine proteinases (cathepsins B and L) have been localized to early and late endosomes (Authier et al., 1995). The endosomal lumen was shown to be progressively more acidic, providing an environment necessary for optimal activity of these enzymes and the dissociation of internalized ligand-receptor complexes (Bergeron et al., 1985).

We found that cells treated with E-64 had a 2 fold reduction in the number of cell surface IGF-I binding sites and a concomitant increase in the intracellular (trypsin insensitive) pool of IGF-1R, consistent with altered receptor processing and sorting in these cells. When taken together with other studies, these results implicate the E-64-sensitive cysteine proteinases cathepsins B and /or L in intracellular (endosomal) processing of the IGF-1R / IGF-1 complex. This is in agreement with earlier studies where the treatment of osteosarcoma cells with the cysteine proteinase inhibitor, leupeptin was shown to increase intracellular levels of internalized IGF-1 (Furlanetto, 1988).

Interestingly, we found that in E-64 treated MCF-7 cells which were stimulated with IGF-1, there was an increase in the intracellular level of tyrosine-phosphorylated IGF-1R β subunit, despite the reduction in the total number of cell surface receptors and the

complete abrogation of IGF-1 mediated functions. These findings suggest that in these cells, ligand-mediated signal transduction was disrupted as a result of altered receptor processing and raise the possibility that in the absence of cysteine proteinase function, receptor-ligand complexes remain "trapped" in a subcellular compartment where the receptor tyrosine kinase or its substrates may be inaccessible to downstream transducers of the IGF-1R signal.

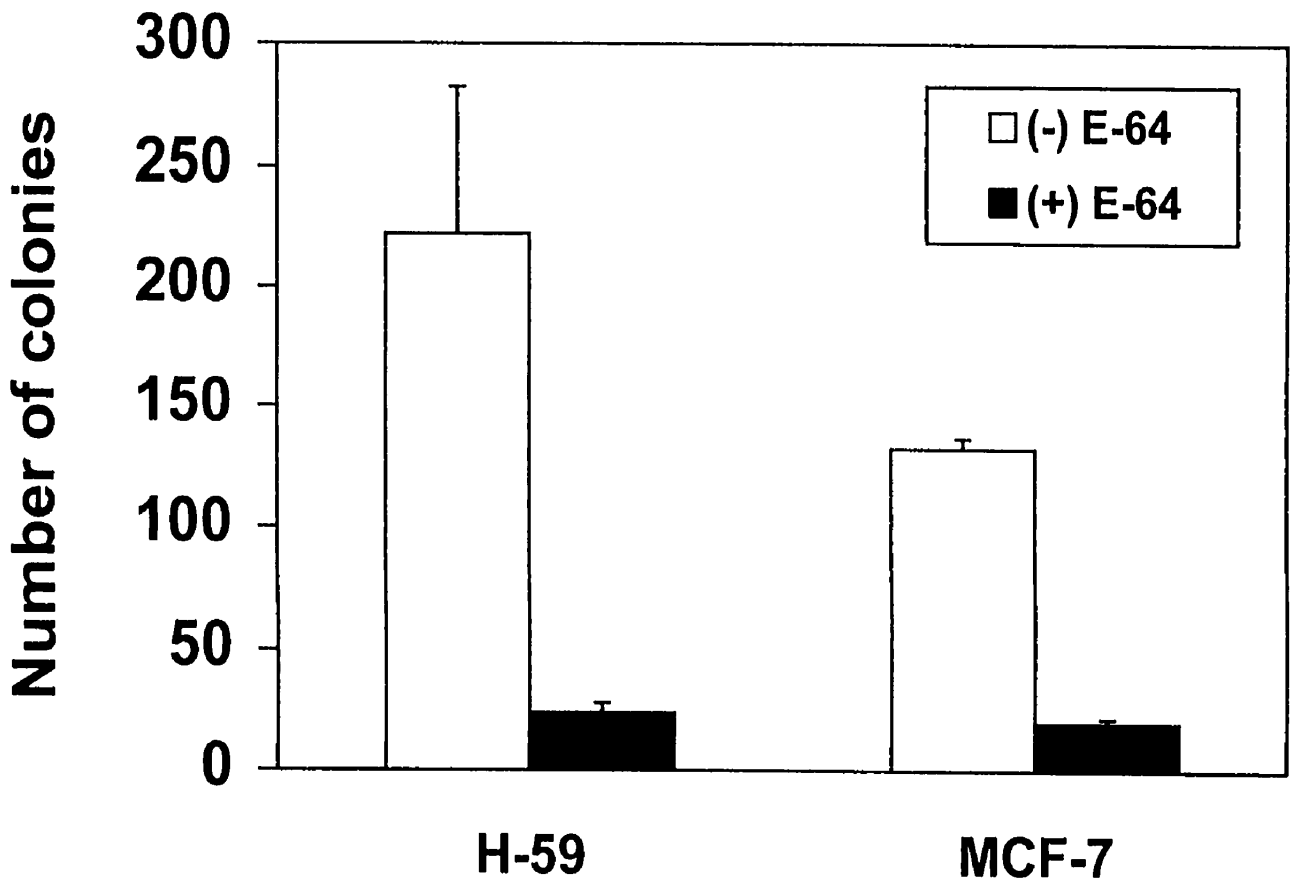
The activated intrinsic tyrosine kinase of the IGF-I receptor catalyzes receptor autophosphorylation and the tyrosine phosphorylation of early signaling intermediates, including insulin receptor substrate-1 (IRS-1) and the Shc (Src-homology/collagen) proteins (Chuang et al., 1993; Myers et al., 1993; Giorgetti et al., 1994). IRS-1 serves as a docking site for a number of signaling molecules, including phosphatidylinositide (PI)3-kinase (Myers et al., 1992), while the Shc proteins, which include several isoforms (46,52 and 60 KDa) can associate with Grb 2 and link IGF-1R to the Ras /MAP kinase pathway (Pelicci et al., 1992; Sasaoka et al., 1994). In a recent study, Chow et al using an IGF-1R mutant, low temperature (15^0C) or the inhibitor Dansylcadaverine to reduce IGF-1R internalization have shown that phosphorylation of IRS-1 and the association of IRS-1 with PI-3K did not require receptor internalization whereas activation of the Shc/MAPK pathway was dependent on receptor internalization (Chow et al., 1998). Our results suggest that signal transduction downstream of the internalised receptor is essential for the mitogenic and tumorigenic functions of IGF-1R and that the signalling process requires intact cysteine proteinase activity. The molecular event in the receptor signalling

pathway which is disrupted through the inhibition of cysteine proteinase activity remains to be elucidated and is the object of our present investigation.

Fig. 1: E-64 treated tumor cells have a reduced cloning efficiency in semi-solid agar.

A. H-59 and MCF-7 cells were cultured in semi-solid agar for 9 days in the presence or absence of 10 μg/ml E-64. MTT assay performed on control monolayer cultures treated in the same manner showed no reduction in the number of viable cells. Colonies which exceeded 250 μm in diameter were enumerated using a microscope equipped with an ocular grid (Diaphot-TMD Inverted, Nikon Canada). Results represent total number of colonies/plate and are expressed as means and SD of three plates per cell type. Light microscopic view of the agar colonies is shown in B. Representative fields of non-treated and E-64 treated H-59 (a and b) and MCF-7 (c and d) cells (×250) are depicted.

A



B

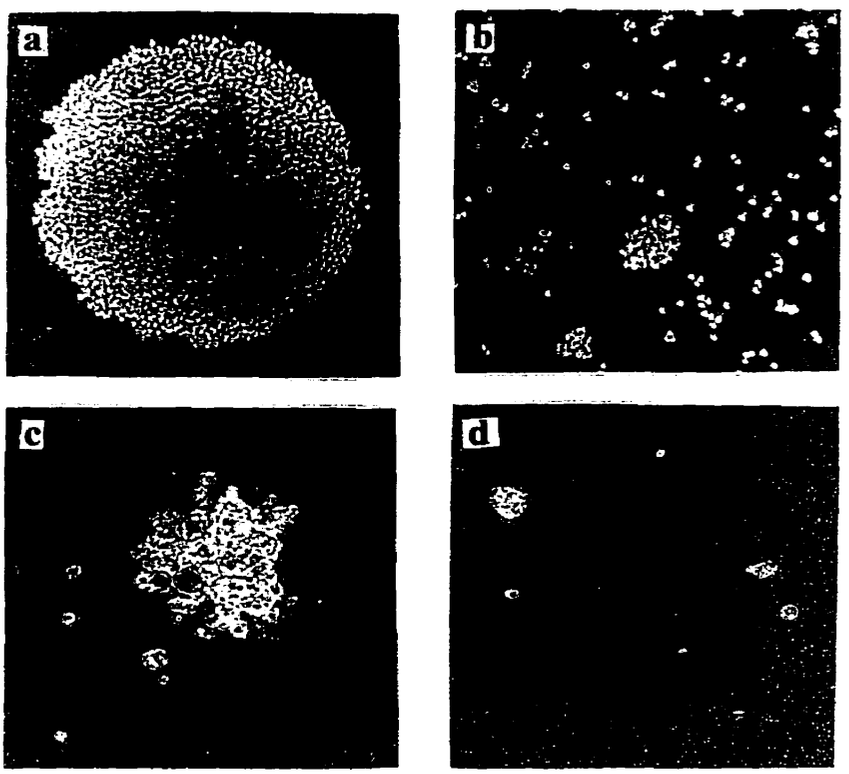


Fig. 2: Inhibition of the proliferative response to IGF-1 in E-64 treated tumor cells.

H-59 (A) and MCF-7 (B) cells were treated with different concentrations of E-64 for 24 h, seeded in 96-well microtiter plates in serum free medium containing 10 μg/ml E-64 and then incubated for 72 h with or without the indicated concentrations of IGF-1. The results represent means and SD of three experiments and are expressed as the increase in [^{3}H] thymidine incorporation relative to cells incubated without IGF-1.

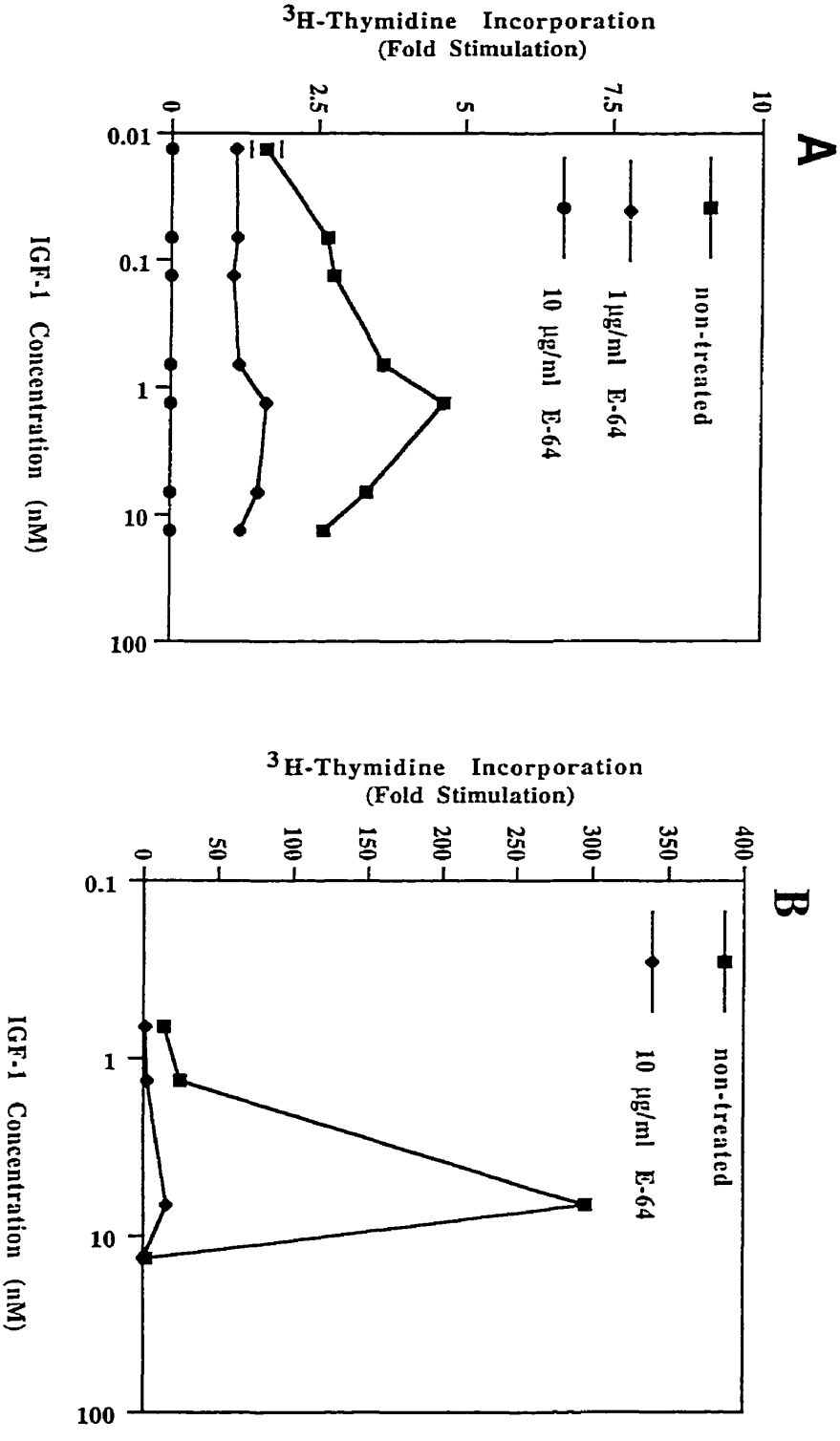
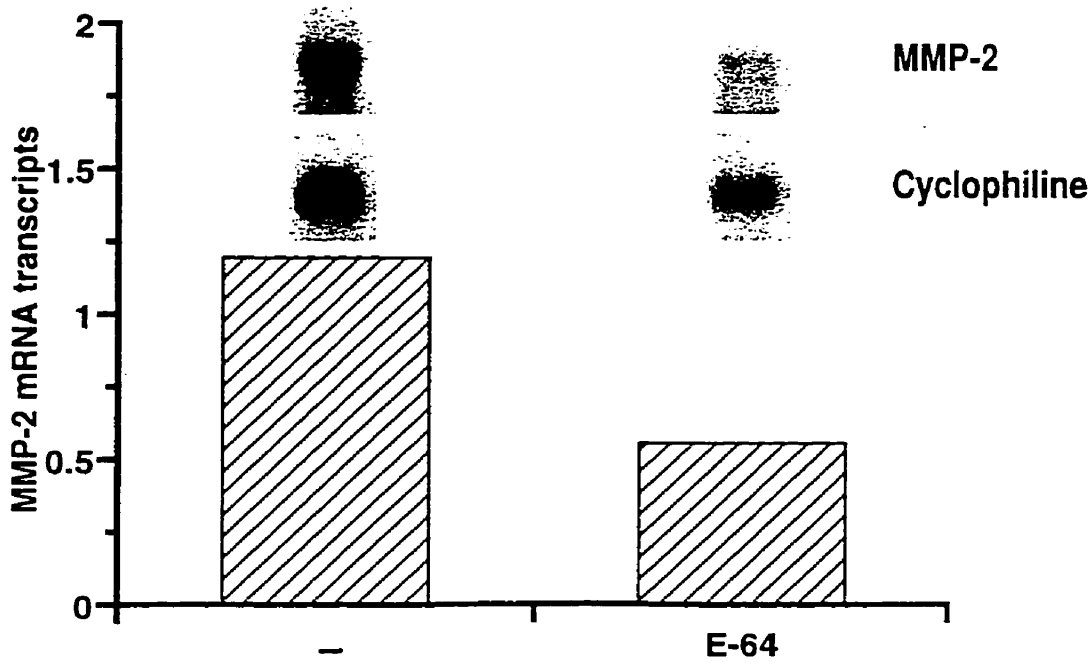

A
^{3}H-Thymidine Incorporation
(Fold Stimulation)
0
2.5
5
7.5
10
0.01
0.1
1
10
100
IGF-1 Concentration (nM)
non-treated
1 µg/ml E-64
10 µg/ml E-64
B
^{3}H-Thymidine Incorporation
(Fold Stimulation)
0
50
100
150
200
250
300
350
400
0.1
1
10
100
IGF-1 Concentration (nM)
non-treated
10 µg/ml E-64

Fig. 3: Reduced MMP-2 expression in E-64 treated tumor cells.

A. Northern blot analysis: H-59 cells were cultured for 48 h in the presence or absence of 10 μg/ml E-64. Thirty μg of total RNA were loaded per lane. Blots were probed consecutively with ^{32}P- labeled 1.1-Kb human MMP-2 cDNA and 800-bp rat cyclophiline cDNA fragments. The intensity of the bands was measured by laser densitometry and is expressed as a ratio relative to the intensity of the cyclophiline bands.

B. Western blot analysis: H-59 cells were cultured for 48 h with or without 10 μg/ml E-64 in the presence or absence of 10 ng/ml IGF-1. Concentrated (×60) conditioned media were separated by electrophoresis on 8% polyacrylamide gels and the bands probed with a mAb to MMP-2 (Ab-45) diluted 1:500 which was detected with a alkaline phosphatase conjugated affinity purified goat anti-rabbit IgG diluted 1:2000. C. Gelatin zymography: Concentrated condition media were separated by electrophoresis on 10% polyacrylamide gels containing 1 mg/ml gelatin. Shown are results obtained with control H-59 cells (a), H-59 cells treated with E-64 (b), untreated H-59 cells stimulated with IGF-1 (c) and E-64 treated H-59 cells stimulated with IGF-1 (d).

A



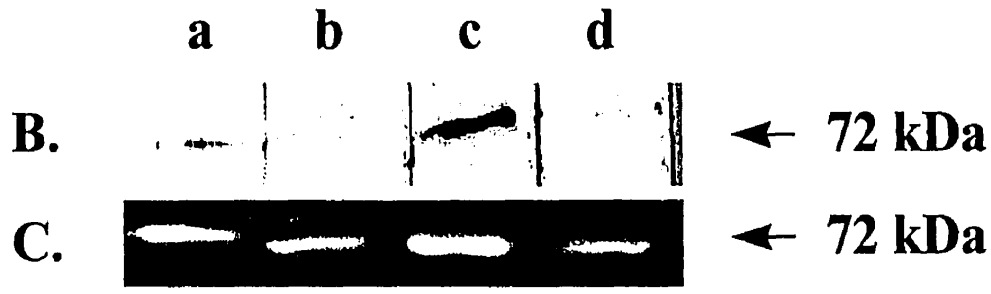

a
b
c
d
B.
72 kDa
C.
72 kDa

Fig. 4: Reduction of IGF-1 binding site in E-64 treated MCF-7 cells after IGF-1 stimulation.

MCF-7 cells were serum starved in RPMI-SF with or without E-64 (10 μg/ml) for 24 h. Cells were dispersed and seeded into 96-well plates at a density of 10^5 cells/well. The cells were stimulated with 10 ng/ml IGF-1 for 10 min. IGF-1 was washed off and the cells were incubated for 30 min at 37°C. The cells were incubated with mAb αIR3 at a concentration of 5 μg/ml. After several washing steps, cells were incubated with FITC-conjugated goat anti-mouse IgG and fixed in formalin. The labeled cells were analyzed by flow cytofluorometry. Numbers on the right indicate the proportion of positively labeled cells (%) and MIF indicates the mean intensity of fluorescence of the positive cells.

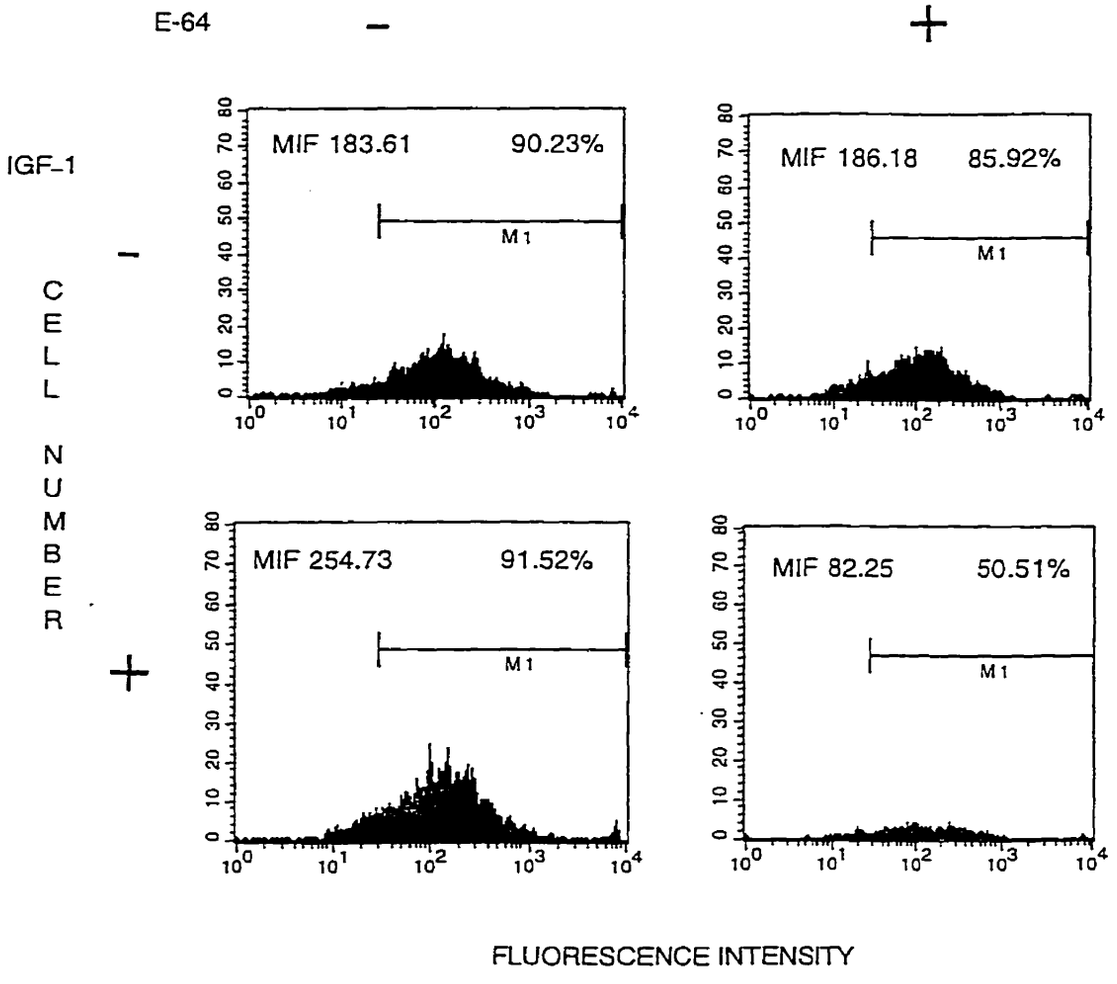

E-64
-
+
IGF-1
-
+
CELL NUMBER
MIF 183.61
90.23%
M1
MIF 186.18
85.92%
M1
MIF 254.73
91.52%
M1
MIF 82.25
50.51%
M1
FLUORESCENCE INTENSITY

Fig. 5: E-64 treatment does not affect IGF-1R synthesis.

A. Northern blot analysis was performed on H-59 and MCF-7 cells cultured for 24 h in the presence or absence of 10 μg/ml E-64 using the conditions described in the legend to Fig 3. The ^{32}P-labeled 700-bp human IGF-1R and 800-bp rat cyclophiline cDNA fragments were used as hybridization probes. Laser Densitometry showed no difference in the relative intensities of the mRNA bands obtained from non-treated or E-64 treated cells. B. Immunoprecipitation analysis and Western blotting of the IGF-1R β subunit with mAb C-20 was performed with H-59 and MCF-7 cells treated as indicated above.

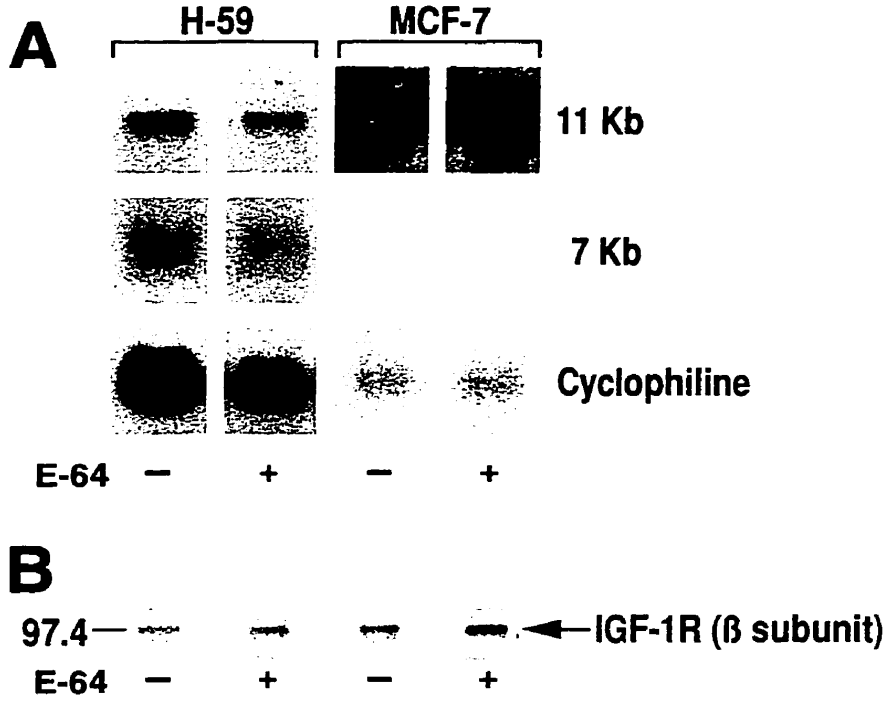

A
H-59
MCF-7
11 Kb
7 Kb
Cyclophiline
E-64 − + − +
B
97.4
IGF-1R (ß subunit)
E-64 − + − +

Fig. 6A: Increased levels of tyrosine phosphorylated IGF-1 receptor in E-64 treated MCF-7 cells.

Serum starved MCF-7 cells were treated with 10 μg/ml E-64 and stimulated for 5 min with 50 ng/ml IGF-1. The cells were lysed, the proteins immunoprecipitated using 10 μg of mAb αIR3 and Western blotting was performed using mAb PT-66 to phosphotyrosine at a dilution of 1:1000 and an HRP-conjugated goat anti-mouse IgG antibody diluted 1:10000 as a secondary antibody. Shown are the results obtained with: non-treated (-), E-64 (E), IGF-1 (I) and E-64 + IGF-1 (E+I) treated cells. The duration of the E-64 treatment is indicated on the bottom of panel A.

A

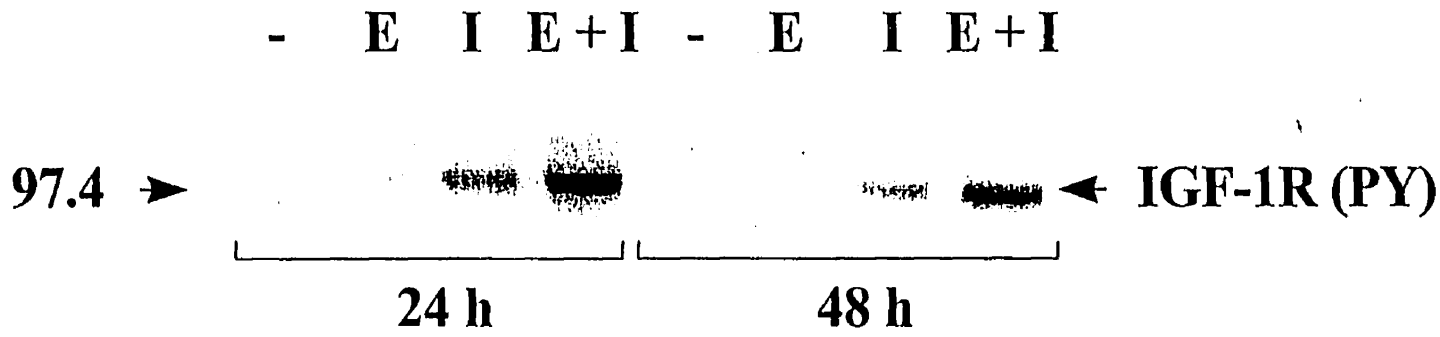


Fig 6 B: Increased levels of tyrosine phosphorylated IGF-1 receptor in E-64 treated H-59 cells.

Serum starved H-59 cells were treated with 10 μg/ml E-64 for 24 h and stimulated for 10 min with 10 ng/ml IGF-1. The cells were lysed, the proteins immunoprecipitated using 10 μg mAb C-20 and Western blotting was performed using mAb PT-66 to phosphotyrosine at a dilution of 1:1000 and an HRP-conjugated goat anti-mouse IgG antibody diluted 1:10000 as a secondary antibody. Shown are the results obtained with: non-treated (-), E-64 (E), IGF-1 (I) and E-64 + IGF-1 (E+I) treated cells.

B

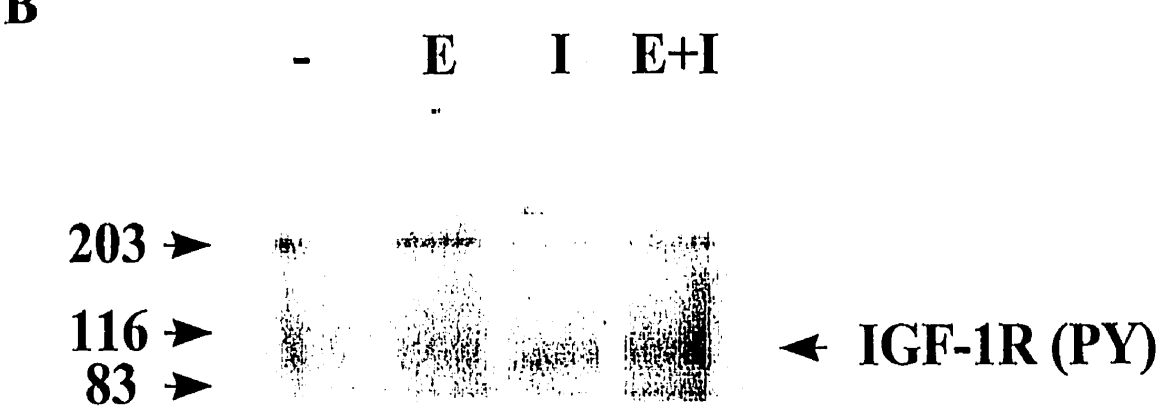
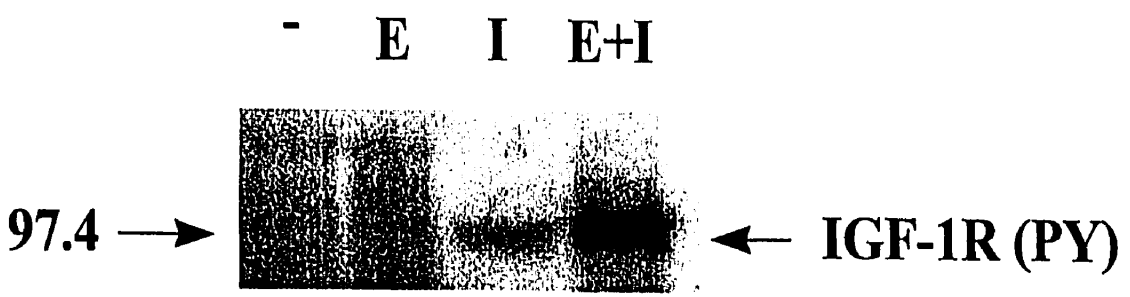


FIG. 7: Selective increase in the levels of intracellular tyrosine phosphorylated IGF-1 receptor in E-64 treated cells.

Serum starved, untreated or E-64 treated MCF-7 cells were stimulated with IGF-1 and treated for 1 min at R.T. with 0.05% trypsin. The cells were lysed and the lysate proteins mmunoprecipitated with mAb αIR3 to the receptor α subunit and the immunoprecipitated proteins probed with a murine anti-phosphotyrosine mAb (clone PT-66) diluted 1:1000 and a HRP-conjugated goat anti-mose IgG antibody diluted 1:10000. The Bands were visualized with enhanced chemiluminescence detection reagents.


-
E
I
E+I
97.4
IGF-1R (PY)

Treatment	H-59 Binding Sites/cell
–	3.9×10^5
10 μg/ml E-64	1.8×10^5

Table. 1: Reduction of IGF-1 binding site in E-64 treated cells.

H-59 cells in 24 well plates were treated for 24 h with 10 μg/ml E-64 prior to the ligand binding assay. To each well ^{125}I-IGF-1 was added at concentrations ranging from 8-1500 PM for a 1 h incubation at 37°C. Triplicate wells were used for each concentration. The number of IGF-1binding sites/cellcalculated from the means using the ligand program (34,35).

Chapter 6

The role of cathepsin L in H-59 tumor invasion-manuscript in preparation

6.1. Overview

The results in this chapter are presented as manauscript in preparation. Mrs. Fallavollita, L., assisted in the in vivo studies and Dr. Khatib, A., assisted in ligand binding assay.

The ability of malignant cells to form metastases in secondary sites remain a major obstacle to the curative treatment of cancer. Previously, we found that the cysteine proteinase inhibitor, E-64, blocked by 97% the invasion of H-59 cells, using a Matrigel invasion assay. A more minor but significant inhibitory effect (up to 32%) was seen with the propeptide of cathepsin B, implicating the major role of cathepsin L in H-59 tumor invasion. Here, especifically the role of cathepsin L was further investigated using H-59 cells transfected with a plasmid vector expressing CL cDNA in the antisense orientation. The transfected clone (CLAS-1) had a reduced expression and synthesis of cathepsin L and lost the ability to invade through Matrigel in an *in vitro* assay. When injected *in vivo*, these cells showed a significant reduction in liver metastases. These cells showed the same pattern of response to IGF-1 as we have seen before for H-59 cells treated with E-64, which is an inhibitor of cysteine proteinases such as cathepsin B and L. In addition we found the reduction of MMP-2 in these cells.

Based in part on our previous results, we have shown that the activities of IGF-1R and MMP-2 are coordinated at both the transcriptional and functional levels and in this coordination, cysteine proteinases such as cathepsin B and L are important mediators of

IGF-1R turnover, and that together they play a central role regulating the metastatic phenotype. Taken together with these findings, our present data in this chapter demonstrate the critical role of cathepsin L in the regulation of carcinoma metastasis through the regulation of IGF-1-dependent cellular growth.

Abstract

Several lysosomal proteinases including the cysteine proteinase cathepsin L, have been implicated in malignant progression of tumors. Many investigators have demonstrated correlations between increases activity of cathepsin L and increased metastatic capability of animal tumors or malignancy of human tumors. Here, the role of cathepsin L in metastasis was further investigated using H-59 cells transfected with a plasmid vector expressing CL cDNA in the antisense orientation. Among the transfectant clones, a few and mostly one clone (CLAS-1) showed reduction in both expression and synthesis of cathepsin L. These cells markedly reduced invasion in a reconstituted basement membrane (98%) as compared with that of controls. These cells had a significant decrease in MMP-2 synthesis as assessed by gelatin zymography. The CLAS-1 cells had a reduction in IGF-1 binding sites and lost the ability to respond to IGF-1. When injected *in vivo*, directly into the microvasculature of the liver (experimental metastasis), these cells reduced the number of metastases under conditions which allowed wild-type or control transfectants to form multiple hepatic metastases. The results demonstrate that cathepsin L can play a critical role in the regulation of carcinoma metastasis.

Introduction

The ability of the cancerous cells to invade adjacent tissue and disseminate to distant sites i.e. to metastasize, is the primary cause of death for most patients with cancer. The past thirty years have seen dramatic increases in our understanding of the metastatic process. Research has demonstrated that metastasis is not a random process but rather a series of sequential steps, the individual outcome of which depends on the interactions of the cancer cells with their environment (Fidler, 1990). The steps in the metastatic process are interrelated and failure at any one of these stages aborts the process (Poste and Fidler, 1979). Recent advances have led to identification of molecular mediators and mechanisms underlying the process of metastasis, these include isolation and characterization of families of molecules involved in regulation of angiogenesis, cell-cell and cell-matrix adhesion, proteolysis, migration and growth. This improved understanding of the complex process of cancer progression has been the impetus for a recent worldwide effort to develop new diagnostic tools and therapeutic reagents targeting molecular mediators of metastases.

One step crucial for invasion and metastasis is the proteolytic degradation of the extracellular matrix (ECM) (Liotta et al., 1986). Among several families of proteolytic enzymes implicated in this degradative process, are the lysosomal cysteine proteinases cathepsin B and L (reviewed in Rozhin et al., 1989; Sloane, 1990a,b).

In the past decade, inhibitors of the cathepsins, in particular, cathepsins B, L and D have been developed as potential anti-metastatic agents. Human tumors generally express higher levels of these enzymes than normal tissues (Kane and Gottesman, 1990). As evidence continues to accumulate on factors distinguishing highly metastatic cells from those with lower or non-invasive properties, it has become clear that the more invasive cell types have both increased cysteine proteinase activity and decreased levels of endogenous cysteine protease inhibitors (Lumkowski et al., 1997). The proteolytic events necessary for tumor invasion not only depend on upregulated production of proteinases and downregulated production of inhibitors but also on mechanisms of proteinase secretion and activation at the cell surface. Proteinases are normally activated through proteolytic cascades requiring the coordinated participation of members of several proteinase families (Sameni et al., 1995; Murnane et al., 1991).

Tumor H-59 is a highly metastatic variant of the Lewis lung carcinoma which produces high levels of cathepsin L and MMP-2 but low levels of cathepsin B (Brodt et al., 1992). Previously, we have shown that E-64, a natural specific inhibitor of cysteine proteinases inhibited liver colonization by these tumor cells, whereas PRCB1 a specific inhibitor of cathepsin B (Navab et al., 1997) had no effect this suggested that cathepsin L is the major cysteine proteinase affected by the treatment with E-64. A second study suggested that E-64 treatment altered IGF-1R internalization and signaling in these cells. To further investigate the role of cathepsin L in H-59 invasion, the tumor cells were transfected with a plasmid vector in which cathepsin L cDNA is constitutively expressed in the antisense

orientation relative to the SV40 promoter. We show here that the antisense transfectants lost the ability to respond to IGF-I and had reduced tumorigenicity *in vitro* and altered metastatic phenotype *in vivo*.

Materials and Methods

Cell Lines. Tumor H-59 was established from a hepatic metastases of the parent line 3LL (Ulrich et al., 1986). The tumor was maintained *in vivo* by s.c. implantation of liver metastases derived from tumor-bearing mice into new recipient animals. *In vitro* monolayer cultures of the tumor were maintained in RPMI containing 10% FCS as detailed elsewhere (Yeatman and Nicolson, 1993).

Construction of cathepsin L plasmids. An XbaI-EcoRI fragment corresponding to the first 300 base pairs of the cathepsin L cDNA was ligated into the EcoRI-XbaI site of the PSVK3 plasmid vector (Pharmacia) in the antisense orientation relative to the SV40 early promoter gene. This Plasmid also expresses a neomycin resistance (Neo^R) gene under the control of an SV40 promoter that confers resistance to G418 sulfate. Cloning of the cathepsin L cDNA in the antisense orientation was confirmed by restriction analysis.

Transfections. The plasmid designed to produce antisense cathepsin L, was introduced into H-59 cells by coprecipitation with calcium phosphate (Graham et al., 1980) and then cultured in RPMI 1640 containing 10% FCS, which was supplemented from day 2 onward with 100 μg/ml G-418 (GIBCO-BRL, Burlington, Ontario, Canada). Stable G418- resistant transformants were isolated 12-14 days later.

Northern Blot Analysis. Cellular RNA was extracted from H-59 and transfected cells by Trizol (Verhofstede et al., 1996). A ^{32}P-labeled 1.19-Kb mouse cathepsin L cDNA fragment (a kind gift from Dr. Ann F. Chambers, London Regional Cancer Center, London, Ontario, Canada) and an 800-bp fragment of rat cyclophilin cDNA (Nip et al., 1995) were used as hybridization probes. The relative amounts of mRNA transcripts were analyzed by laser densitometry using an Ultroscan XL enhanced laser densitometer and normalized relative to the internal cyclophilin controls.

Western blot analysis. Western blot analysis was essentially as we described previously (Wang et al., 1991). Briefly, serum-free conditioned media (60 x concentrated) from transfected and non-transfected H-59 tumor cells, were separated on a 12.5 % SDS-polyacrylamide gel and the proteins electrophoretically transferred onto nitocellulose filters (0.2mm). The blots were probed with a rabbit antiserum to human recombinant procathepsin L (721,a kind gift from Dr. John. S. Mort) at a dilution of 1:100. As an standard, human cathepsin L was run on a separate well (1 μg/μl). Alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG (Bio/Can Scientific, Mississauga, Ontario) was used as a second antibody at a dilution of 1:1000.

Gelatin Zymography. The gelatinolytic activity of MMP-2 was analyzed by zymography as described previously (Brodt et al., 1992). The concentrated conditioned media (×60) from transfected and non-transfected clones which were cultured for 48 h

were electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. The gels were stained with Coomassie Blue and destained with 10% acetic acid-50% methanol until the desired color intensity was obtained. The gelatinolytic activity seen as a clear zone on the blue background was quantitated by densitometry using photographic negatives of the gel.

Soft agar cloning Assay. To measure anchorage-independent growth, a modification of the standard soft agar cloning assay was used (Saiga et al., 1987). Briefly, tumor cells, transfected and non-transfected, were mixed with a solution of 0.8% agar (Difco Laboratories Inc., Detroit, MI) added to an equal volume of a 2x concentrated RPMI-FCS medium and plated in six- well plates (Fisher Scientific, Montreal, Quebec) on solidified 2% agar at a concentration of 10^4 cells/well. The overlay was allowed to solidify and then supplemented with 1 ml RPMI-FCS containing G418. The medium was replenished on alternate days for 12 days. Colonies were enumerated using an inverted microscope (Diaphot-TMD Inverted, Nikon Canada).

Tumor Cell Proliferation Assay. H-59 cells and transfectants were cultured in SF-RPMI for 24-h and then dispersed and seeded into 96-well plates (Falcon, Lincoln Park, NJ) at a density of $2x10^3$ cells/well and incubated for 54 h with medium containing IGF-I as we described previously (Long et al., 1994). The cells were pulsed with 0.1 mCi/ml of

[^{3}H]thymidine (Du Pont Canada, Mississauga, Ontario, Canada) for 18 h, and thymidine incorporation was monitored as detailed elsewhere (Long et al., 1994).

Ligand-Binding Assay. IGF-1 binding sites were quantitated using the method described by Phillips et al (Phillips et al., 1987). Briefly transfected and non-transfected H-59 cells were cultured with RPMI-FCS containing G418 in 24-well plates for 2-3 days. The culture medium was removed and replaced with fresh medium. The binding assay was carried out 24 h later. To each well, 8-1500 pM of ^{125}I- labeled IGF-1 in binding medium (SF-RPMI containing 1 mg/ml BSA and 1μg/ml leupeptin) were added, with or without graded concentrations of unlabeled IGF-1 for a 1h incubation at 37°C. The cells were rinsed twice with ice-cold binding medium and solubilized in 0.01 N NaOH containing 0.1 % Triton X-100 and 0.1% SDS. The number of cells/well at the time of the assay was determined from triplicate control wells which were manipulated in the same manner. An aliquot was removed from each well and the radioactivity was measured in an LKB gamma counter. The number of IGF-1 binding sites were calculated using the Ligand program (McPherson, 1983a,b).

Cell invasion assay. Tumor cell invasion was determined *in vitro* by the reconstituted basement membrane (Matrigel) invasion assay, essentially as described previously (Navab et al., 1997). Briefly, 60 μl of Matrigel (Collaborative Research, Bedford, MA, USA) diluted to a concentration of 0.23mg/ml were applied to 8 μm filters. These filters

were dried overnight, reconstituted with serum-free RPMI and placed in 24-well plates. To each filter $5x10^4$ cells in 100 µl of RPMI medium containing 0.2% BSA were added. Rat fibronectin (5 µg/ml; Gibco BRL) was used as a chemoattractant in the lower chamber. Following a 48-h incubation at 37°C, the cells on the upper surface of the filter were removed with a cotton swab and the filters fixed in 0.1% glutaraldehyde and stained with 0.2% crystal violet. For each filter 20 random fields were counted using a Nikon inverted microscope (x100) and duplicate samples were analysed for each assay condition. In each experiment, control filters were coated with 7.5 µg/filter of human placental type IV collagen (Sigma) to control for changes in cell migration.

Liver colonization assay. Animals were injected with $2x10^5$ transfected and non-transfected H-59 cells by the intrasplenic/portal route (i.s.) and then immediately splenectomized as described previously (Asao et al., 1992). The animals were sacrificed 14-21 days later, the livers removed and the metastases enumerated immediately (Wexler, 1966).

Results and Discussion

In the present study we analysed the role of cathepsin L in the invasion and metastasis of a highly invasive murine lung carcinoma subline H-59 cells, in which the constitutive expression of cathepsin L was suppressed by stable transfection with a plasmid vector expressing a 300 bp antisense fragment of cathepsin L cDNA in the antisense orientation relative to the promoter. One clone (CLAS-1) was isolated in which cathepsin L mRNA expression was 50% reduced relative to non or mock-transfected cells (Fig. 1) with a corresponding loss in protein synthesis (Fig. 2). These cells had a significantly reduced invasion (99%) as measured in the reconstituted basement membrane (Matrigel) model (Fig. 3), as well as a significantly reduced (87%) migration on uncoated or 7.5 μg type IV collagen coated filters. When the colonigenicity of these cells was measured in semi solid agarose plates we found an 82% reduction in their cloning efficiency relative to control cells (Table. 1, Fig. 4). In monolayer cultures these cells lost their proliferative response to IGF-I (Fig. 5) associated with a 56-66% reduction in the number of IGF-I binding sites compared to controls as assessed by the ligand binding assay (Table. 2). When the function of MMP-2 was investigated in antisense transfected CLAS-1 cells, we found a significant decrease in the level of MMP-2 mediated gelatinolytic activity, as assessed by gelatin zymography (Fig. 6). Taken together with our previous studies which identified IGF-1R as a regulator of anchorage-independent growth, cellular proliferation, MMP-2 synthesis and invasion (Long et al., 1998a,b), in these cells, the results implicate cathepsin L activity in the regulation of the IGF-1R / IGF-1 system cellular functions.

In vivo studies revealed that CLAS-1 cells had a significantly reduced ability (up to 70% reduction) to form hepatic metastasis following the intrasplenic/portal injection of $2x10^5$ cells, suggesting that cathepsin L is involved in regulation of liver colonization in this model (Table. 3, Fig. 7). Interestingly, we observed in livers of CLAS-1 — injected mice, small haemorragic lesions which were absent in liver of animals injected with mock-transfected cells and never observed in control H-59 — injected animals (Fig. 7). This is the first report which directly implicates cathepsin L in liver metastases formation.

The inhibitory effect of antisense cathepsin L in metastasis was also observed by other studies. Injection of *in vitro* antisense cathepsin L inhibited transformed NIH 3T3 cells, showed reduction in lung colonization in nude mice (Zhang and Schultz, 1992). In these cells which were transformed by modified yeast RAS1 gene sequence, the cathepsin L gene expression was constitutively elevated (Zhang and Schultz, 1992).

An essential role for proteases in metastasis has long been suggested, but evidence from the literature for a role of a particular protease has often appeared confusing for several reasons. Most of the observations are correlative, often the conclusions are extrapolations from *in vitro* models, or conclusions are made from a variety of different tumors and cell lines among which comparisons are difficult. Direct *in vivo* evidence for a role of a particular protease in metastasis comes from only a few experiments in which specific inhibitors of the proteolytic activity are utilized or from *in vivo* molecular biology

experiments in which a particular protease gene expression can be selectively increased or decreased. These types of *in vivo* experiments are difficult and have been successfully carried out in only a few examples. Our data are the first direct evidence for a role of cathepsin L in experimental metastasis. Taken together these results are encouraging and it seems that new candidates for clinical trials will soon emerge.

Fig. 1: Reduced expression of cathepsin L in antisense transfected clone (CLAS-1).

Using Northern blot analysis, Thirty μg of total RNA were loaded per lane. Blots were probed consecutively with ^{32}P-labeled 1.19- kb mouse cathepsin L cDNA and 800-bp rat cyclophiline cDNA fragments. The intensity of the bands was measured by laser densitometry and is expressed as a ratio relative to the intensity of the cyclophiline bands.

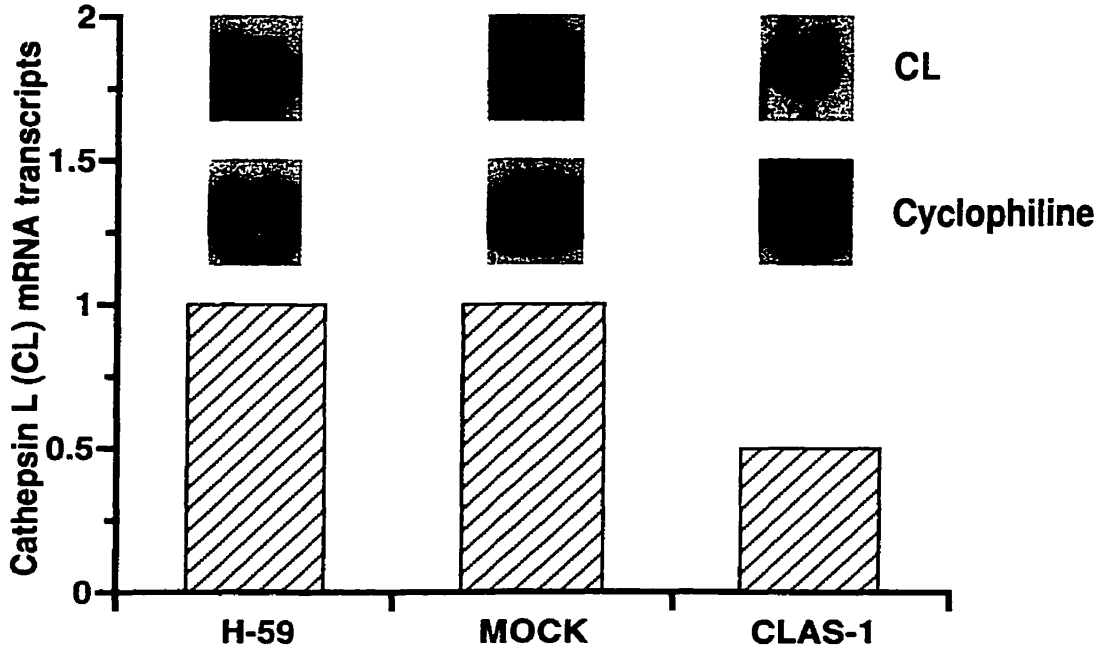

Cathepsin L (CL) mRNA transcripts
2
1.5
1
0.5
0
CL
Cyclophiline
H-59
MOCK
CLAS-1

Fig. 2: Western blot analysis of cathepsin L synthesis in the antisense transfected cells.

Conditioned media derived from wild-type H-59, Mock transfected clone and antisense transfected clone (CLAS-1), were concentrated (60x) and the proteins (60 μg per lane) resolved on 12.5% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were probed with a rabbit antiserum to human recombinant procathepsin L and normal human cathepsin L (CL) was used (1 μg/ml) as a control. The position of the procathepsin L is indicated with an arrow on the left.

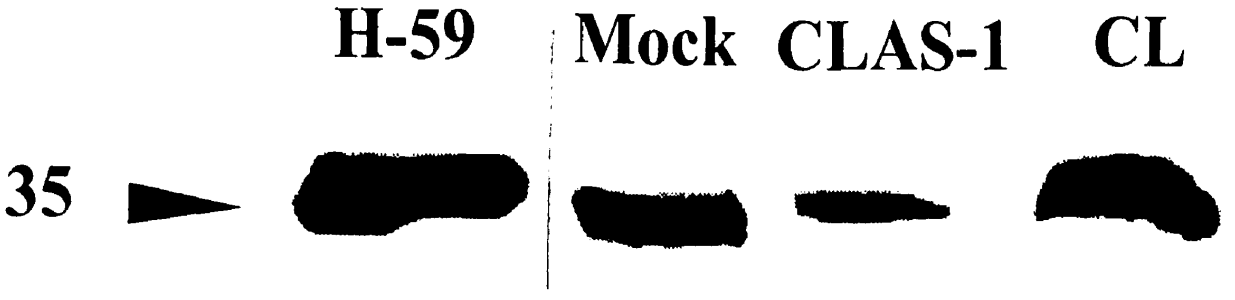

H-59
Mock
CLAS-1
CL
35

Fig. 3: Inhibition of H-59 invasion by antisense cathepsin L transfectant cells.

Transfected and non-transfected H-59 cells ($5x10^4$) were plated on Matrigel-coated filters and incubated for 48 h at 37 °C. In each of the experiments, control filters were coated with human placental type IV collagen to control for changes in cell migration. Results are based on four experiments carried out in duplicate and are presented as percentage of invasion relative to control non-transfected cells.

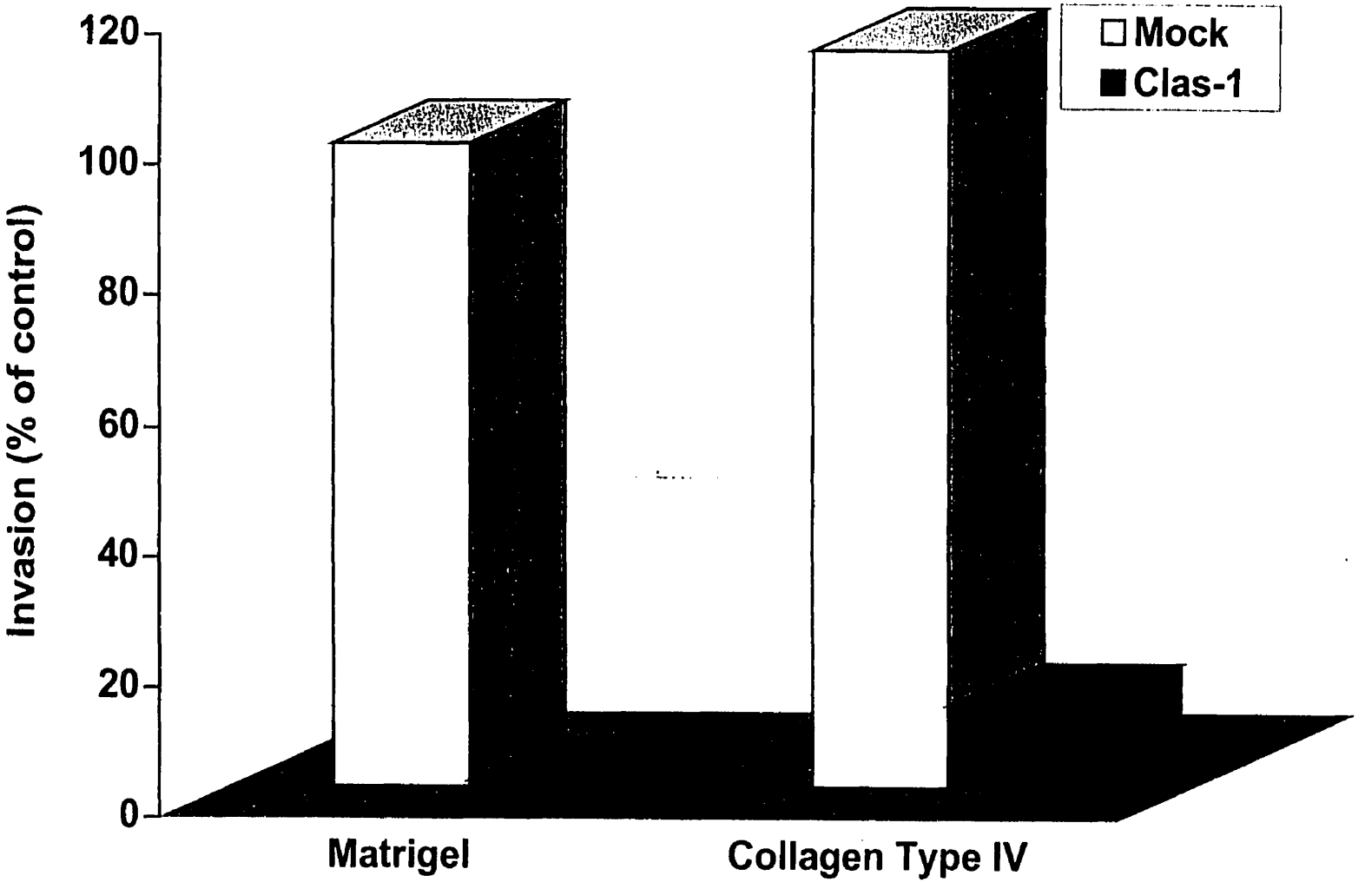

120
100
80
60
40
20
0
Invasion (% of control)
Mock
Clas-1
Matrigel
Collagen Type IV

Fig. 4: Reduction in the cloning efficiency of antisense transfected clone.

Light microscopic view of the agar colonies from table. 1. Representative fields of control (a,b) and antisense transfected (c) cells (x 250) are depicted.

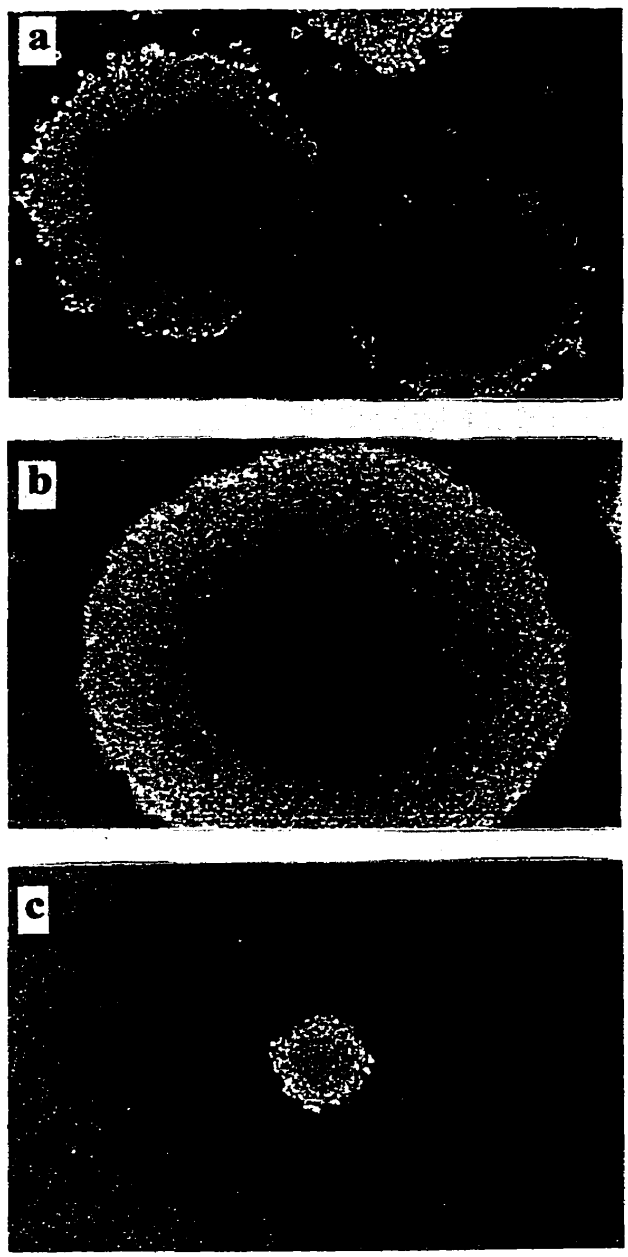

a
b
c

Fig. 5: Inhibition of the proliferative response to IGF-1 in cathepsin L antisense transfected cells.

H-59 and transfected cells were seeded in 96-well microtiter plates in serum free medium and then incubated for 72 h with or without the indicated concentrations of IGF-1. The results represent means and SD of three experiments and are expressed as the increase in [^{3}H] thymidine incorporation relative to cells incubated without IGF-1.

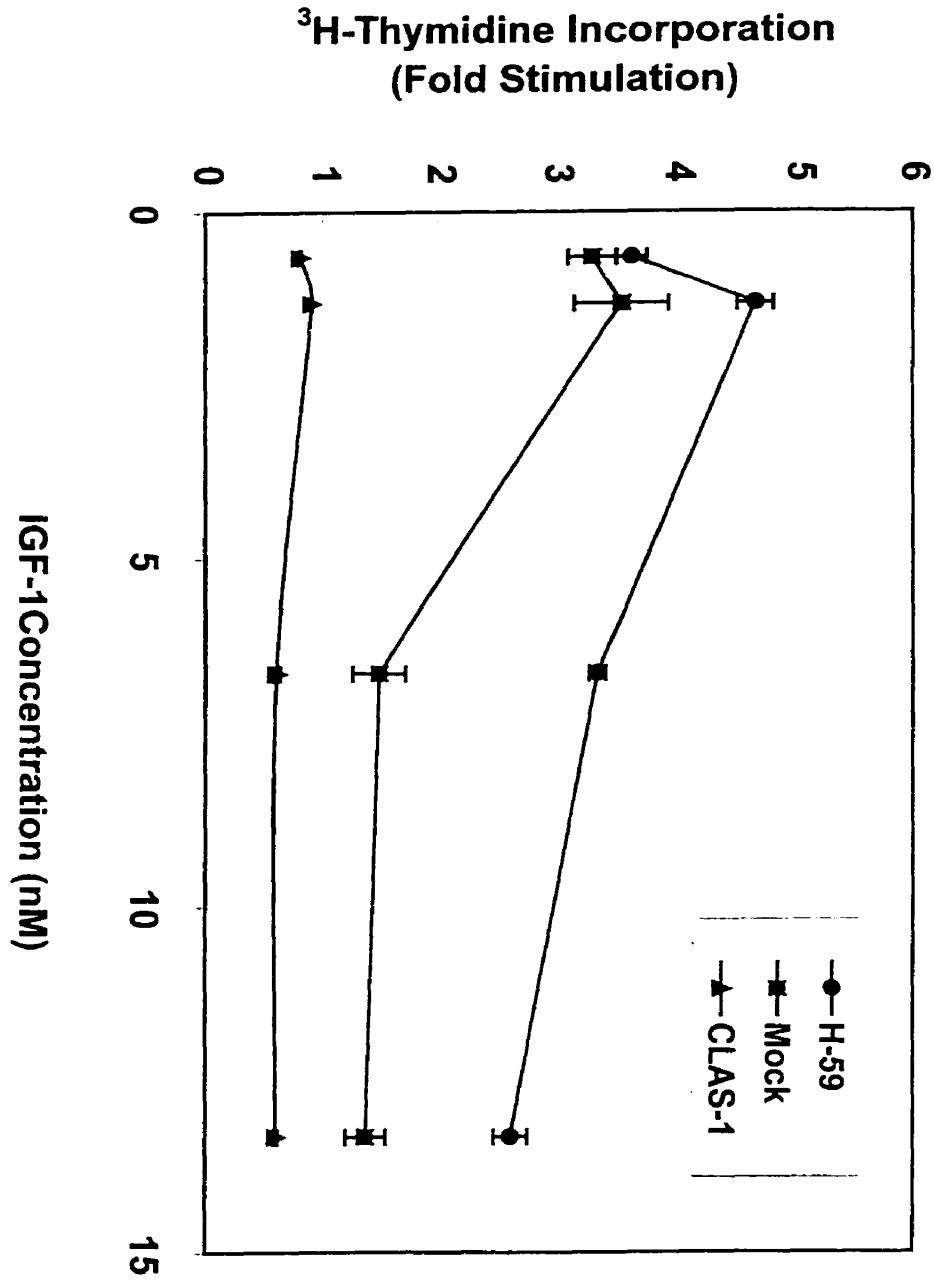

^{3}H-Thymidine Incorporation
(Fold Stimulation)
0
1
2
3
4
5
6
0
5
10
15
IGF-1Concentration (nM)
H-59
Mock
CLAS-1

Fig. 6: Zymographic analysis of MMP-2 activity in cathepsin L antisense transfected clone.

Concentrated condition media (x60) were separated by electrophoresis on 10% polyacrylamide gels containing 1 mg/ml gelatin. Shown are results obtained with antisense transfected and control H-59 cells.

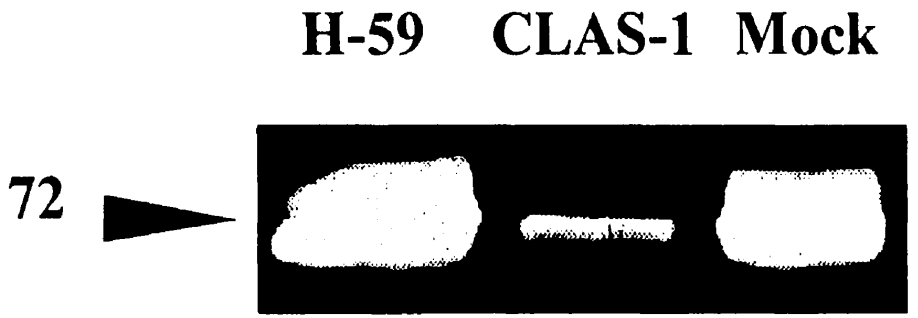
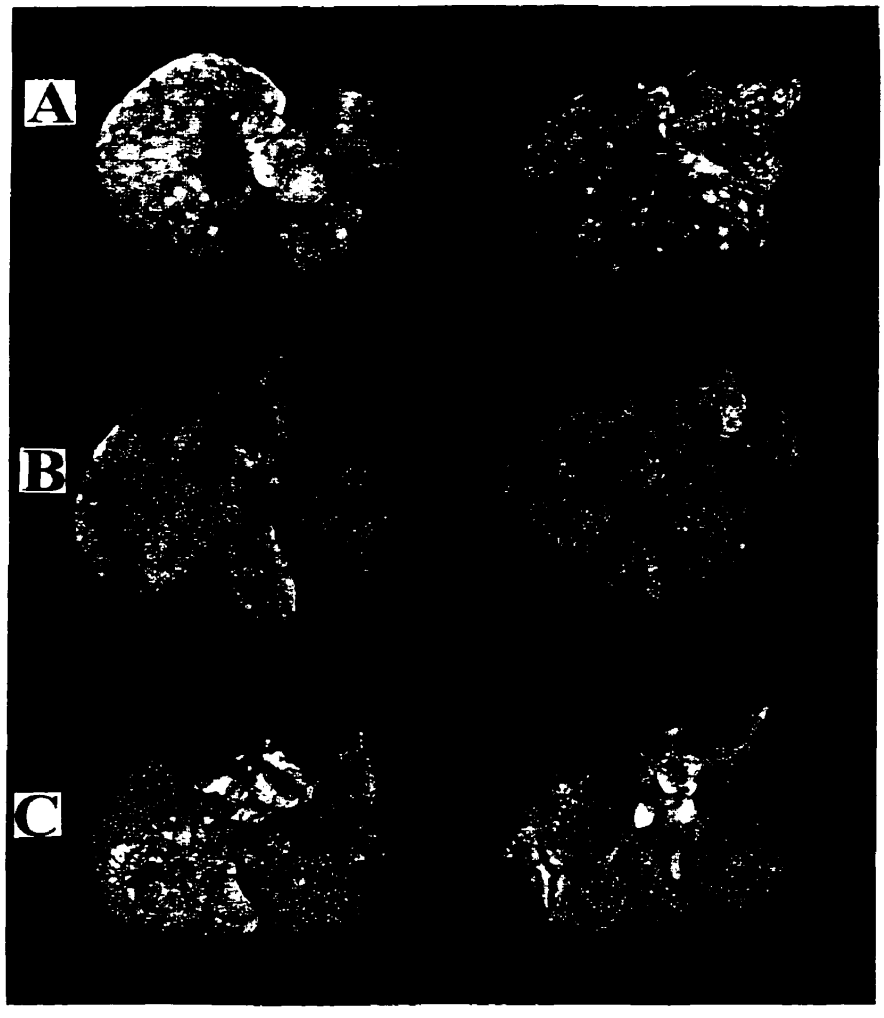

H-59
CLAS-1
Mock
72

Fig. 7: Inhibition of liver colonization by cathepsin L antisense transfected cells.

Representative livers from table 3 are shown. (A) non-transfected cells. (B) Control transfected cells (Mock). (C) Antisense cathepsin L transfected cells (CLAS-1).


A
B
C

	Number of colonies
H-59	**287±16.97**
Mock	**266.7±42.67**
CLAS-1	**52.5±13.44**

Table 1: Cathepsin L antisense transfected H-59 cells have a reduced cloning efficiency in semi-solid agar.

H-59 and antisense transfected cells were cultured in semi-solid agar for 12 days. Colonies which exceeded 250 μm in diameter were enumerated using a microscope equipped with an ocular grid. Results represent total number of colonies/plate and are expressed as means and SD of three plates per cell type.

	Binding sites/cell
H-59	**$5.1x10^5$**
Mock	**$3.96x10^5$**
CLAS-1	**$1.75x10^5$**

Table 2: Reduction of IGF-1 binding sites in cathepsin L antisense transfectants clone (CLAS-1).

Non-transfected and transfected H-59 cells were cultured in 24-well plates. To each well ^{125}I-IGF-1 was added at concentrations ranging from 8-1500 PM for a 1h incubation at 37°C. Triplicate wells were used for each concentration. The number of IGF-1 binding sites/cell calculated from the means using the ligand program.

	Median # of nodules
H-59	112.5 (36-147)
Mock	149.5 (56-200)
CLAS-1	43.5 (29-84)*

Table. 3: Cathepsin L antisense transfected H-59 carcinoma cells block liver colonization.

Experimental hepatic metastases were enumerated 14 days following i.s. inoculation of $2x10^5$ tansfected H-59 cels. Results are based on 5-10 animals per each group.

*. P= 0.004 relative to H-59 and P= 0.008 relative to Mock.

CHAPTER 7

Discussion

During tumor progression proteolysis is a key, multistep process in the invasion of host tissue by cancer cells (Reich et al., 1988; He et al., 1989; Liotta et al., 1991; Liotta and Stetler-Stevenson, 1991; Kobayashi et al., 1993; Mignatti and Rifkin, 1993; Monsky et al., 1994; Ruppert et al., 1994; Sivaparvathi et al., 1995). Histopathological studies and *in vitro* studies of cultured cancer cells with metastatic potential have revealed that matrix metalloproteinases (Liotta et al., 1980; Reich et al., 1988; Monsky et al., 1994; Emmert-Buck et al., 1994), plasminogen activators (Ossowki and Reich, 1983; Mignatti et al., 1986; Reich et al., 1988; Sier et al., 1994) and cathepsins (Maciewicz et al., 1989; Inoue et al., 1994; Rempel et al., 1994; Lah et al., 1995; Elliott and Sloane, 1996) are involved.

The lysosomal cysteine proteases are the most active and abundant proteases within mamalian cells. They are normally located in the lysosome, where they hydrolyze extracellular proteins taken up into cells by endocytosis and intracellular proteins taken up by autophagy (Lloyd and Mason, 1996). In pathological processes, cathepsin B and L play important roles in diseases that are associated with aberrant protein turnover. Levels of expression of cathepsins B and L are reported to be higher in invasive than noninvasive tumor cells, implicating these enzymes in the invasive process (Sloane et al., 1981; Sloane et al., 1986; Denhardt et al., 1987; Lah et al., 1991).

The present studies demonstrate that the invasion of H-59, but not M-27 cells through Matrigel could be blocked completely by E-64- a potent, specific, irreversible inhibitor of cysteine proteases and only partially by the propeptide of cathepsin B which specifically inhibits the activity of the mature form of this enzyme. Of particular importance is our

finding that pretreatment of H-59 cells with E-64 inhibited metastases formation following intrasplenic/portal injection, suggesting that cysteine proteinases are involved in liver colonization in this model. The results described provide the first indication that cysteine proteinases are involved in liver metastases formation and are among the few reports to demonstrate an inhibitory effect of E-64 *in vivo*. Our results are consistent with findings that *in vivo* administration of E-64 into nude mice reduced the metastatic capability of EJ transitional cell carcinoma of the bladder (Redwood et al., 1992) and decreased the number of spontaneous metastases in ovarian sarcoma M5076 - bearing mice (Leto et al., 1994). Studies using rat models also showed that E-64 and a related analogues could prevent the osteolysis associated with the early stages of malignant hypercalcaemia (Insogna et al., 1984).

Several mechanisms may account for the involvement of lysosomal cysteine proteinases in ECM degradation. Several studies have implicated cathepsins B and L in invasion by direct degradation of extracellular matrix proteins (Buck et al., 1992; Cardozo, 1992). The proteolytic events necessary for tumor cell migration not only depend on the production of active proteases but also on their continuous secretion and activation at the cell surface in order to degrade the components of the extracellular matrix. In normal cells, cathepsin B is confined to the lysosome but cancerous cells were found to have a 4-30 fold increases in cathepsin B levels in the plasma membrane (Sylven et al., 1974). Changes in localisation of cathepsin B can be brought about by the movement of lysosomal vesicles to the periphery of the cell and/or by altered trafficking of the enzyme

to the cytoplasm followed by association with the membrane (Keppler et al., 1996). A tumor-generated acidic extracellular environment may also be responsible for the overall observed increase in the secretion of cathepsin B from malignant cells. A slightly acidic extracellular pH has been shown to trigger the release of active cathepsin B from cells with an invasive phenotype (Rozhin et al., 1994). Very recently, Van Noorden et al., (1998) reported that cathepsin B is functional not only in the lysosomes of normal and cancer cells but also at the plasma membrane of cancer cells.

Cysteine proteinases can also be involved in proteolytic cascades with other proteinases such as the serine proteinase urokinase plasminogen activator (uPA) and the metalloproteinase MMP-1 (Sires et al., 1995). Receptor bound pro-uPA, is normally activated by serine proteinases such as plasmin, trypsin or plasma kallikrein but can also be activated by the cysteine proteinases cathepsins B or L. The enzymatically active uPA can then convert cell surface bound plasminogen to plasmin, a wide-spectrum proteinase which can cleave components of the tumor stroma (e.g. fibrin, fibronectin, proteoglycans, laminin) and may also activate type IV- procollagenase to degrade type IV-collagen, a major component of the basement membrane (Schmitt et al., 1992b). Hence, cysteine proteinases can play multiple roles both direct and indirect in ECM degradation and invasion.

The present study provides new insight into the multifaceted role that cysteine proteinases can play in metastasis, by implicating them in the regulation of IGF-1R trafficking and turnover. Thus, our results show that the inhibition of cysteine proteinases by E-64 abolished the proliferative response to IGF-1, reduced anchorage-independent growth in semisolid agar and decreased MMP-2 production by H-59 and MCF-7 cells. This reduction in IGF-1-induced responses coincided with a 2-fold reduction in the number of IGF-1Rs as assessed by the ligand binding assay (for H-59) and FACS analysis (for MCF-7). Interestingly, we found an increase in the intracellular level of tyrosine-phosphorylated IGF-1R β subunit in E-64 treated MCF-7 cells stimulated with IGF-1, suggesting that the cysteine proteinase involved played a role in post-ligand binding signaling by regulating post-tyrosine phosphorylation events.

Many ligands and their receptors are internalized by receptor-mediated endocytosis into membrane-bound vesicles called endosomes (Brown et al., 1983; Helenius et al., 1983). One of the functions of the internalization process is dissociation and degradation of ligand (Brown and Greene, 1991). During the internalization process, receptors cluster in clathrin-coated vesicles, and pass through an acidified endosome. In this sorting endosome (Geuze et al., 1983), ligands and receptors are sorted for degradation in lysosomal compartments, for recycling to the cell surface (Brown et al., 1982; Stein and Sussman, 1986) or for intracellular storage. Based in part on direct evidence and on homologies with the insulin signaling pathway (Furlanetto, 1988; Carpentier, 1994), it is believed that IGF-1 binding initiates the migration of IGF-1Rs to clathrin-coated pits and

the subsequent formation of early endosomes containing internalized but still active receptors. The ligand-receptor complex ultimately becomes dissociated and inactivated in the acidic environment of late endosomes, where ligands and receptors are sorted for degradation in lysosomes or recycling to the cell surface (Furlanetto, 1988; Carpentier, 1994). The acidic pH of the endocytic compartment causes the dissociation of insulin from its receptor. Several studies have demonstrated that the endosome is a major site of degradation of insulin (Backer et al., 1990; Doherty et al., 1990; Authier et al., 1994a). Insulin degradation is initiated in rat liver endosomes as rapidly as 1 min after insulin administration to hepatic circulation. The degradation of insulin appears to be carried out by an endosomal acidic insulinase (EAI), found in the lumen of hepatic endosomes (Authier et al., 1994a,b). In contrast to insulin, IGF-1 does not dissociate from its intracellular receptor as easily as insulin in the acidifying endosome and, therefore, it is likely that IGF-1 can return to the cell surface via a recycling receptor (retroendocytosis; the phenomenon of intact ligand returning to the cell surface) (Zapf et al., 1994). Cysteine proteinases (cathepsins B and L) have been localized to early and late endosomes (Authier et al., 1995). The endosomal lumen was shown to be more acidic, providing an environment necessary for optimal activity of these enzymes and the dissociation of internalized ligand-receptor complexes (Bergeron et al., 1985).

Our results are consistent with the postulate that the increased accumulation of intracellular tyrosine-phosphorylated IGF-1R β subunit levels in the absence of cysteine proteinase activity disrupted ligand-mediated signal transduction as a result of altered

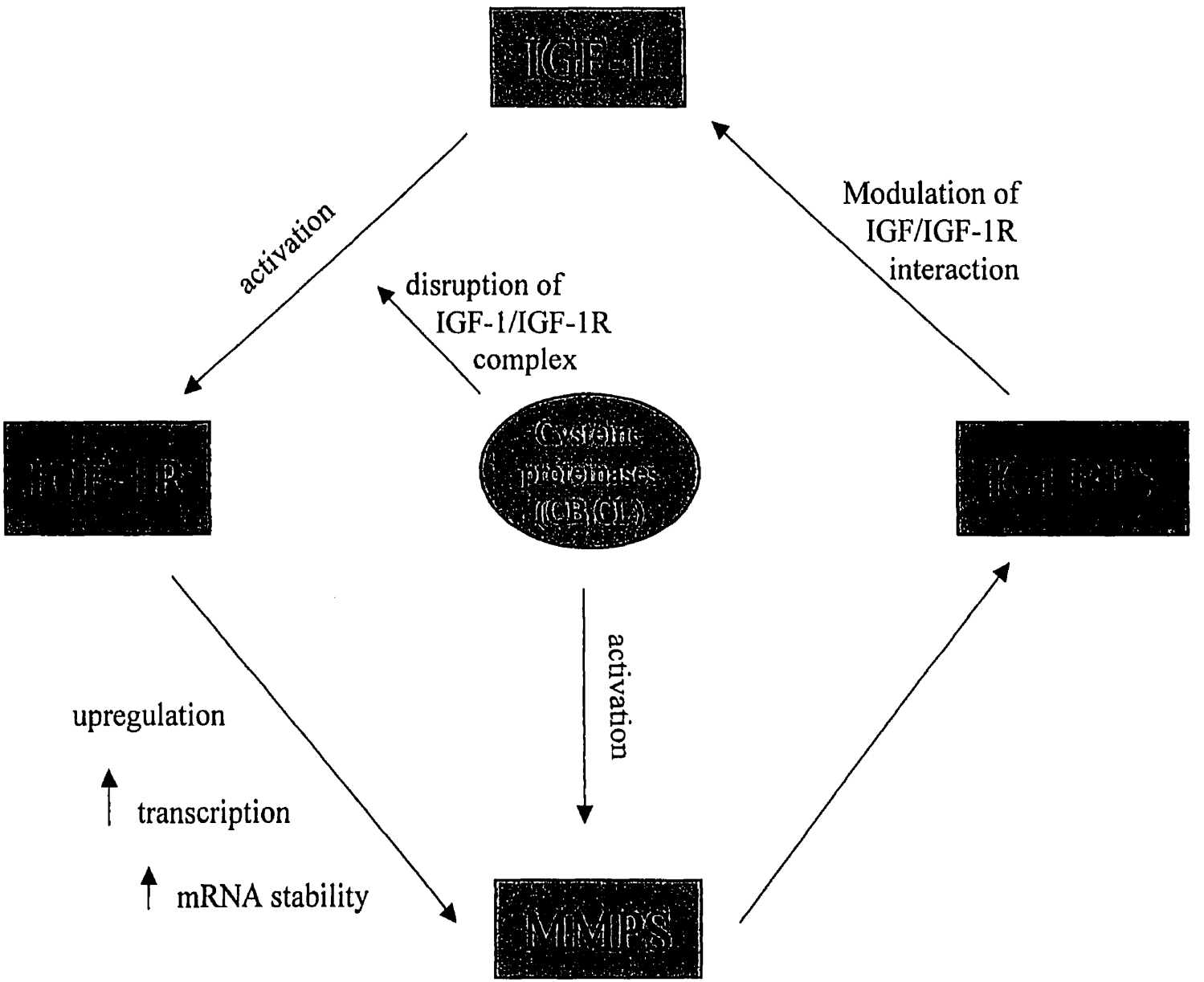
receptor processing. This in turn led to decreased IGF-1 dependent MMP-2 expression (Long et al., 1998b) and the reduced cellular invasion noted *in vitro* (Matrigel assays) and *in vivo* (liver metastases). This is suggestive of a possible cysteine proteinases:IGF-1/IGF-1R:MMP-2 cascade critical for the invasive potential (Figure. 7-1). Recent evidence that MMP-2 is involved in the processing of IGF binding proteins (Fowlkes et al., 1994a,b; Jones and Clemmons, 1995; Thrailkill et al., 1995) thereby modulating IGF bioavailability and receptor-binding add to the complexity of this cascade and suggest the existance of a regulatory loop in the IGF-1R/MMP-2 link (Fig. 7-1). Our finding that cathepsin L antisense mRNA expressing cells had a 98% reduction in invasive potential relative to controls, reduced anchorage-independent growth and IGF-1 dependent proliferation suggest that this enzyme may be the major cysteine proteinase involved in IGF-1R/IGF-1 processing.

Our findings are in agreement with other studies where the treatment of MG-63, an IGF-1-responsive human osteosarcoma cells with the cysteine proteinase inhibitor, leupeptin, was shown to increase intracellular levels of internalized IGF-1 (Furlanetto, 1988). It was shown that this internalization is mediated through the type 1 IGF receptor and not through the type 2 IGF receptor or the IGF binding protein(s), which are also present on these cells. These conclusions are based on the observation that treatment of MG-63 cells with agents that inhibit endosomal and lysosomal function resulted in the intracellular accumulation of [^{125}I] IGF-1 and that this accumulation could be inhibited by other agents, such as high dose insulin and αIR-3, which specifically inhibit ligand binding to the IGF-1R. In a recent study, Chow et al by using an IGF-1R mutant, low temperature

(15°C) or dansylcadaverine, an endocytic inhibitor have shown that phosphorylation of Shc but not IRS-1 requires IGF-1R internalization. The Shc/MAPK signaling cascade is involved in important cellular processes such as transcription and protein synthesis. Therefore, our results suggest that signal transduction mechanisms downstream of the internalized receptor which are essential for the mitogenic and tumorigenic functions of IGF-1R depend on intact cysteine proteinase activity. In other words, disruption of endosomal processing of IGF-1/IGF-1R complex by E-64, blocked signalling, suggesting that a mechanism such as decoupling of receptor-ligand may be necessary for receptor translocation to a compartment where it is accessible to down stream substrates.

Fig. 7-1: A schematic illustration of the proposed loop.

IGF-1 can upregulate the expression of MMP-2 through the activity of cysteine proteinases. Active MMP-2 in turn is involved in the processing of IGF binding proteins which can modulate the interaction of the IGFs with their receptors.


IGF-1
activation
disruption of
IGF-1/IGF-1R
complex
Modulation of
IGF/IGF-1R
interaction
IGF-1R
Cysteine
proteinases
(CB/CL)
MMPs
activation
upregulation
transcription
mRNA stability
MMPs

Interestingly, the *In vivo* results obtained following injection of CLAS-1 cells through intrasplenic/portal route were different from those seen following injection of E-64 treated cells. While pretreatment of H-59 cells with E-64 reduced experimental liver metastases by up to 90%, CLAS-1 cells showed a reduction of 70% in the number of hepatic metastasis. Moreover in CLAS-1 injected mice, small haemorragic hepatic lesions were observed which were absent in livers of animals injected with E-64 treated cells. Several mechanisms may be postulated to explain these differences. It is possible that the more profound effect of E-64 on liver metastases formation was due to the fact that this inhibitor blocks the activities of both cathepsin L and B while in CLAS-1 cells only cathepsin L expression was reduced and the reduction was partial. This suggest that cathepsin B and L play complementary roles in the invasive/metastatic proccess. On the other hand in CLAS-1 cells the reduction in the number of IGF-1 binding sites was 1.4 fold greater than in E-64 treated H-59 cells. This additional decrease in IGF-1 binding sites may have resulted in accelerated apoptosis in CLAS-1 metastatic lesions giving rise to small, necrotic lesions. Indeed other studies have shown that when the function of the IGF-1R is decreased or otherwise impaired by antisense strategies or by dominant negative mutants, tumor cells undergo massive apoptosis, which is more prominent *in vivo* than *in vitro* (Resnicoff et al., 1995a,b; D'Ambrosio et al., 1996) and in preliminary analyses we have observed in these deformed lesions apoptosis using Tunnel assay (not shown).

A review of the literature on the effects of proteinase inhibitors on tumor growth fails to clearly answer the question: are cysteine proteinase inhibitors potentially useful in tumor therapy? Although in some studies anti-tumor effects were clearly demonstrated, in most other studies, limited or no effects were observed. For example, in a study with leupeptin, a non-selective inhibitor of both serine (Umezawa, 1996) and cysteine proteinases (Saito et al., 1980) it was shown that a regime of 50 mg leupeptin/kg, injected twice daily by the intraperitoneal route into rats inhibited the formation of experimental metastases following intravenous injection of Yoshida ascites hepatoma cells (Redwood et al., 1992). It is interesting that in this system the drug prevented the formation of fibrin clots around the metastasizing cells, suggesting that prevention of thrombus formation may be one of the effects of leupeptin. Elastatinal and chymostatin which inhibit elastase and chymotrypsin, respectively were ineffective (Redwood et al., 1992). On the other hand, oral administration of leupeptin had no effect on N-butyl-N-(4-hydroxybutyl)nitrosamine induction of bladder tumors (Arai et al., 1978). Other lines of evidence however implicate the cysteine proteinases in tumor progression. For example, direct involvement of cathepsin B in metastatic liver colonization was recently confirmed by Van Noorden et al., (1998). Metastases were reduced by the use of a cathepsin B specific fluoromethyl ketone based inhibitor. Inhibition was limited to extracellular cathepsin B as the inhibitor was incapable of crossing cell membranes. This suggested that effective inhibitors could be designed to act on cell surface cathepsin B without interfering with the cytoplasmic or lysosomal forms of the enzyme (Van Noorden et al., 1998). In the HOC-1 ovarian cancer cell line, cathepsin B inhibition prevented the activation of prourokinase (pro-uPA) to active plasminogen, thereby abrogating invasion (Kobayashi et al., 1992). While in

human prostate cancer cell lines, cathepsin B inhibition resulted in an overall decrease in the invasiveness of the cell lines possibly because it blocked activation of the aspartyl protease cathepsin D (Weiss et al., 1993).

As presently more specific inhibitors for the individual members of the cysteine protease family are not widely available, the roles of individual enzymes cannot be fully elucidated. Our results provide compelling evidence that cathepsin L also plays a critical role in invasion and metastasis probably through its involvement in IGF-1R signaling. Therefore our results provide a rationale for the development of specific, anti-metastatic drugs which target cathepsin L synthesis or activity.

7.1. Suggestions for future research

The following aspects require further investigation:

1. Elucidating the effect of E-64 on IGF-1R signaling pathways.
2. Tracing post-ligand binding receptor trafficking in normal and E-64 treated cells using molecular marker-tagged receptors.
3. Evaluating the beneficial antimetastatic effects of cathepsin L-targeted antisense-based gene therapy.

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