Suppression by Cathepsin L Inhibitors of the Invasion of Amnion Membranes by Murine Cancer Cells

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INTRODUCTION

Considerable progress is being made in uncovering the cellular and molecular processes taking place during invasion by a metastatic cancer cell (1). In order to metastasize, a tumor cell must escape from the primary tumor and establish itself elsewhere in the body, and enzymes that degrade extracellular matrix components have been implicated in these events (2). Liootta (3) proposed that invasion of the extracellular matrix could be dissected into three parts: (a) attachment of the tumor cell to the basement membrane or stroma via cell surface receptors for laminin or fibronectin respectively; (b) degradation of the juxtaposed membrane by proteinases generated by the tumor cell; (c) penetration through the matrix.

A positive correlation between metastatic potential and the extent of enzymatic degradation of collagen type IV has been reported for variants of the B16 melanoma lines (4) and for rat mammary adenocarcinoma lines (5). Laminin stimulates the production of type IV collagenolytic activity by a variety of cell lines (6), and some metastatic cell lines express a high density production of type IV collagenolytic activity by a variety of cell lines (6), and some metastatic cell lines express a high density of laminin receptors (1,3). Inhibitors of metalloproteinases significantly reduce the penetration of the tumor cell; (c) penetration through the matrix.

ABSTRACT

Cysteine proteinases, particularly cathepsins B and L, have been strongly implicated in fostering metastasis in mice. In this work four different inhibitors of cysteine proteinases have been shown to inhibit the invasion of the human amnion by murine melanoma and mammary carcinoma cells in vitro. Two of the inhibitors are synthetic peptides [ZPhePheCHN₂ (benzoylcarbonyl-L-phenylalanylanilinoethylamido)-p-(fluoromethyl)-l-fluoro-2-butanone] and two are thiol proteinase inhibitors (TPIn, TPId) isolated from the skeletal muscle of the hind limbs of normal and dystrophic mice, respectively. The inhibitors (ZPhePheCHN₂, TPId), with apparent selectivity for cathepsin L, blocked invasion as effectively as inhibitors (ZPheAlaCH₂F, TPIn) effective on both metalloproteinases. We suggest that the cysteine proteinases facilitate the action of metalloproteinases (collagenase, gelatinase, and stromelysin), possibly by activating them, in inactivating the tissue inhibitor of metalloproteinases, and/or by making basement membrane matrix more accessible.

MATERIALS AND METHODS

Cell Lines and Media. The SPlneo5 murine mammary carcinoma cell line metastasizes from s.c. tumors to the lung with high efficiency (20). The murine mammary adenocarcinoma lines C3 and C10 have also been described (21). C3 is invasive and metastasizes efficiently whereas C10 displays neither of these traits. In this work we used a subclone of C3, C3-L1, derived from a spontaneous lung metastasis; it also been described (21). C3 is invasive and metastasizes efficiently whereas C10 displays neither of these traits. In this work we used a subclone of C3, C3-L1, derived from a spontaneous lung metastasis; it also been described (21).

Denhardt et al. (17) found that the amount of secreted procathepsin L (major excreted protein, or MEP) correlated with the degree to which murine cells transfected with the ras⁴ oncogene express ras⁴ mRNA and metastasize.

In this research we have sought additional evidence for the participation of cathepsin L in tissue invasion. We have done this by showing that cysteine proteinase inhibitors with varying degrees of specificity for cathepsin L are able to moderate the invasiveness of cells. The peptidyl diazomethyl ketone ZPhePheCHN₂, and likely also the fluoromethylketone ZPheAlaCH₂F, act as affinity labels and alkylate the active center thiol group; they do not affect the activity of the aspartic, metallo, or serine proteinases (18). TPI was purified from mouse skeletal muscle; the inhibitor from normal mice inhibits cathepsins B, H, and L whereas that from dystrophic mice appears to have lost its ability to inhibit cathepsin B (19).

Correlations have also been found between the metastatic potential of various tumor cell lines and their ability to degrade extracellular matrix proteoglycans and sulfated glycosaminoglycans (10-12). Evidence that plasminogen activator can promote tissue invasion and metastasis has been reviewed (13). The cysteine proteinases have also been implicated in tumor metastasis. Human adenocarcinoma explants secrete larger amounts of a cathepsin B-like activity than normal explants (14), and increased levels of lysosomal cathepsin B have been correlated with the enhanced metastatic potential of B16 melanoma lines (15). Compared to nonmetastatic variants, intracellular cathepsin B activity is higher in invasive, metastatic variants of an anaplastic rat sarcoma (16). Denhardt et al. (17) found that the amount of secreted procathepsin L (major excreted protein, or MEP) correlated with the degree to which murine cells transfected with the ras⁴ oncogene express ras⁴ mRNA and metastasize.

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The abbreviations used are: TIMP, tissue inhibitor of metalloproteinases; MEP, major excreted protein; PBS, phosphate-buffered saline; DOC, deoxycholate; ZPhePheCHN₂, benzoylcarbonyl-L-phenylalanylanilinoethylamido-L-alanine ethyl methyl ketone; ZPheAlaCH₂F, 3-(benzoylcarbonylphenylalanylanilinoethylamido)-p-fluoro-2-butanone; TPIn and TPId, thiol proteinase inhibitors from skeletal muscle of normal and dystrophic mice, respectively.
associated with the amnion, the supernatant and pellet fractions of the
was detached, rinsed, and placed in a test tube. The radioactivity
with PBS, and the DOC and PBS washings pooled. Lastly, the amnion
gentle scraping with a policeman. The amnion was then washed 3x
upper chamber.

was associated with the cells. The specific labeling (dpm/cell) was 1.17
complete medium. Under these conditions 35-50% of the radioactivity
were incubated for 24 h in the presence of 0.3 nCi 5-['2iI]iododeoxyu-
mitized nontoxic silicone lubricant (Dow Corning Corp., Midland, MI)
identical to those described by Mignatti et al. (8). Sterile silicone rubber
nines and to the Teflon ring invasion chambers and placed onto the silicone rubber supports in
the culture plates with the stromal side down. Two hundred µl
complete medium, with or without the proteinase inhibitors to be tested,
were placed in the upper chambers and the plates preincubated at room
temperature for 60 min.

Subconfluent cultures of the cells whose invasion was to be assessed
were incubated for 24 h in the presence of 0.3 µCi 5-['2iI]iododeoxyu-
ridine (New England Nuclear, Montreal, 200 Ci/mmol) per 10⁶ cells/
membranes, denuded of the epithelium, were fastened to the Teflon
ring invasion chambers and placed onto the silicone rubber supports in
the culture plates with the stromal side down. Two hundred µl
of complete medium, with or without the proteinase inhibitors to be tested,
were placed in the upper chambers and the plates preincubated at room

RESULTS

It has become evident recently that secretion of procathepsin L (also known as major excrated protein, or MEMP by murine
cells correlates with the cell's metastatic proficiency assessed in
various ways. In transformed mouse fibroblasts MEMP expression
appears to be under the control of ras (28, 29). To augment the
evidence for the involvement of cathepsin L in invasive
phenomena, we have focused our attention on several invasive
cell lines that produce procathepsin L and asked whether inhibi-
tion of the cysteine proteinase diminishes the invasive behavior.

To obtain direct evidence that the cysteine proteinase cathep-
sin L is involved in the invasion process, we investigated
whether inhibitors with some specificity towards cathepsin L
could moderate the invasive process. Cathepsin L preferentially
claves the peptide bond one residue to the carboxyl side of

\(^{9}\) A. H. Warner, unpublished.
\(^{10}\) C. Overall, University of Toronto, personal communication.

\(^{7}\) A. Chambers and D. Denhardt, unpublished.
Inhibitors of the proteinase include ZPhePheCHN₂, ZPheAlaCH₂F, and mouse muscle TPI. The diazomethyl ketone derivative ZPhePheCHN₂ is a much better inhibitor of cathepsin L than cathepsin B (18, 32–34). ZPheAlaCH₂F can be more active against cathepsin L than cathepsin B, but the differential depends on the substrate used in the experiment. Muscle TPI from normal mice (TPIn) inhibits both cathepsin B and L, while the TPI from dystrophic mice, TPId, inhibits only cathepsin L in the range of 10–15 μg/ml (19).

The invasiveness of the cell lines and its sensitivity to the cysteine proteinase inhibitors described above were assessed using the amnion invasion assay. The metastatic C3-L1 and SP1neo5 lines efficiently penetrated into the amnion basement membrane, reaching a maximum by about 72 h; in contrast, the nonmetastatic C10 line was some 5-fold less invasive (Fig. 2). To determine the effect of the inhibitors on invasion, the membrane was incubated with the inhibitor in serum-free PBS for 60 min prior to the addition of the cells; the differential was then continued with the cells added for 72 h. Preincubation is necessary to observe effective inhibition.

Both ZPhePheCHN₂ and TPIn reduced the extent of invasion by 25–50% (Table 1). In comparison, the metalloproteinase inhibitor 1,10-phenanthroline reduced the invasion by about 80%. The radioactivity associated with the membrane in the presence of 1,10-phenanthroline was equivalent to that measured for control noninvasive lines like C10 or Swiss mouse 3T3 fibroblasts. In this experiment, Table 1, ZPhePheCHN₂, and TPIn at concentrations of 5 μM and 2 μg/ml, respectively, were exerting near-maximal effects since 5–10-fold increases in concentration did not produce very much greater inhibition. This argues that the block to invasion is not simply a generalized toxic effect of the inhibitor. ZPhePheCHN₂ has been shown to be an effective inhibitor of lysosomal protein degradation and at high concentrations (>100 μM) to inhibit protein synthesis also (24, 35).

A second more exhaustive study was performed to examine the dose-response curve in more detail and to test the action of ZPheAlaCH₂F and TPId (Fig. 3). Fig. 3 (A and B) shows that ZPheAlaCH₂F inhibited invasion of the amnion by the C3-L1 cells with a dose-response curve similar to that seen with ZPhePheCHN₂; Fig. 3C confirms that TPIn was also an effective inhibitor. The experiment in Fig. 3D was performed to examine the effects of both dipeptide inhibitors and TPId on a second line of cells, B16BL6, which is also a good producer of MEP. TPId inhibited invasion almost as effectively as TPIn or ZPhePheCHN₂, but not as effectively as Trasylol or phenan-

**DISCUSSION**

Metastasis in vivo is a multistep phenomenon; it includes escape of the cell from the primary tumor, penetration through one or more basement membranes, survival in the circulatory system, and establishment at a secondary site. The amnion membrane, possessing a physiologically normal basement membrane, provides an excellent model system for the study of invasion. The basement membrane in various tissues as well as blood vessels is composed of mostly the same components as the denuded (of amnion cells) amnion: collagen type IV, laminin, proteoglycans, elastin, and fibronectin. Collagen type IV appears to be one of the major barriers to invasion of the basement membrane because the extent of invasion often correlates well with the extent of collagen type IV degradation (4, 5). However, Starkey et al. (36) have concluded, from a study of the degra-
Cathepsin L is a lysosomal cysteine proteinase that preferentially cleaves on the C-terminal side of an amino acid with a long aliphatic chain, which may or may not carry a charge, and is joined through its amino group to a hydrophobic amino acid residue. It is distinguishable from cathepsin B and other cysteine proteinases on the basis of its bond specificity as revealed by its activity towards particular synthetic substrates and its sensitivity to specific inhibitors. Thus benzylxoycarbonyl-Phe-Arg-7-(4-methyl)coumarylamide (ZPheArgNH2) is a preferred substrate for cathepsin L in comparison with cathepsin B, and ZPhePheCHN3 is a selective inhibitor (18, 32–34).

The mRNA encoding (prepro) cathepsin L has been cloned in several laboratories. Analysis of the sequence of cDNA clones of MEP and characteristics of the protein have revealed that this secreted murine protein is a catalytically active (at acid pH) precursor of cathepsin L (30, 37, 38). It was also cloned as a mouse cysteine proteinase from a macrophage-like cell line (39) and as a ras-induced transcript by Joseph et al. (29). There appears to be a single gene encoding murine cathepsin L (40), which is initially produced as a 334-amino acid preproenzyme. A putative 17-aa signal sequence is cleaved off during passage into lysosomes or secretory vesicles (39). Under acidic conditions, for example in the lysosome, activation of the enzyme is accompanied by removal of a polypeptide containing 96 amino acids and by other specific proteolytic cleavages. One form of the activated human enzyme consists of two polypeptide chains of approximately M, 25,000 and 5,000 probably held together by disulfide bonds (32). The murine "proenzyme" (MEP) is active in an uncleaved form around pH 5; below pH 5 it is rapidly autocatalytically activated (37, 38).

The cysteine proteinase inhibitors used in this study reduced the invasiveness of the cell lines tested by some 50%. The ability of ZPhePheCHN3, which is a "stronger" inhibitor of cathepsin L than of cathepsin B (18, 33, 34), to inhibit the invasion of the amnion strongly suggests a major role for cathepsin L in facilitating membrane penetration. Supporting this interpretation is the inhibition exhibited by TP1d, which suppresses cathepsin L activity but not cathepsin B activity (19). Tempering this conclusion however are the qualifications that we do not know the effective inhibitor concentrations at their site of action in the basement membrane and that the specificity of the inhibitors was assessed in the test tube using simpler, soluble substrates. Despite these complications in the analysis, the fact that the two agents manifesting comparable inhibition of cathepsins B and L, ZPheAlaCH3F and TP1d, did not abrogate invasion to a greater extent than ZPhePheCHN3 or TP1d argues that cathepsin B is not contributing more to the invasive phenotype than cathepsin L.

The completeness with which the chelating agent 1,10-phenanthroline blocks invasion indicates that the cysteine proteinases are not sufficient in and of themselves; clearly a 1,10-phenanthroline-sensitive metalloproteinase is absolutely essential. Also, the ability of inhibitors (e.g., Trasylol) of serine proteinases (such as plasminogen activator) to inhibit invasion of the amnion by C3-L1 cells (41) implicates serine proteinases in the invasive process of that cell line. We suppose that plasminogen activator (secreted by the tumor cells) cleaves plasminogen, producing plasmin, which in turn serves to activate a prometalloproteinase.

Cathepsin L has been shown to be capable of degrading tendon collagen at acidic pHs in the range of 3–5; like the less effective cathepsin B it acts primarily on the terminal nonhelical portions of type I collagen that are involved in the cross-linking of the molecules (33, 42, 43). It will also attack fibronectin, laminin, and elastin (37, 44) and can inactivate α1-proteinase inhibitor (45). The significant contribution of the cysteine proteinases to invasion may be to make the collagen-cross-linked basement membrane more vulnerable by removing the telomeric cross-links and by inactivating TIMP.

If it is true that increasing the expression of MEP (procathepsin L) enhances the invasiveness of cells, then a mechanism must be found to create the acidic conditions required both for the activity of MEP itself and for its autoactivation. One attractive possibility is that the invasive cell makes close contact with the basement membrane and seals off a portion of the matrix to create a localized microenvironment. The cell could then secrete both protons and proteins into that compartment, perhaps by directing lysosomes or secretory vesicles to empty their contents into it. In these acidic conditions the cysteine proteinases would be able to attack components of the basement membrane. Silver et al. (46) have obtained evidence that adherent macrophages and osteoclasts are capable of creating just such a microenvironment with pH values less than 4, more than adequate to foster cathepsin L activity. A strategy of this kind could facilitate the action of other hydrolytic enzymes and it could be augmented in cases where the tumor cell had receptors on its cell surface that favored a strong interaction between the cell and the membrane.

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