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Cathepsin B and its endogenous inhibitors: the role in tumor malignancy

Bonnie F. Sloane, Kamiar Moin, Evzen Krepela and Jurij Rozhin
Department of Pharmacology, Wayne State University, Detroit, MI 48201, USA

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Summary

Several lysosomal proteinases including the cysteine proteinase cathepsin B have been implicated in malignant progression of tumors. Many investigators have demonstrated correlations between increased activity of cathepsin B and increased metastatic capability of animal tumors or malignancy of human tumors. 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 observed in both murine and human tumors and multiple 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, precursor forms of cathepsin B and native forms of cathepsin B are involved. We speculate that malignant tumor cells adherent to basement membrane are capable of forming a digestive microenvironment in which lysosomal proteinases such as cathepsin B function optimally, a microenvironment similar to that formed between adherent osteoclasts and bone. One of the endogenous cysteine proteinase inhibitors, stefin A, also is affected by malignancy. Reduced expression (mRNA and protein) of stefin A is found as well as a reduction in its inhibitory capacity against cysteine proteinases. The data to date at both the molecular and protein levels supporting a functional role(s) for cathepsin B and its endogenous inhibitors in cancer progression are only 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.

Introduction

Within the past year the lysosomal cathepsins have received international attention in both the scientific and popular press due to the apparent correlation between release of precursor forms of cathepsin D, a lysosomal aspartic proteinase, and an unfavorable prognosis for women with node-negative human breast carcinoma [1, 2]. As noted in a letter to the editor of the *New England Journal of Medicine* [3], human breast carcinoma cells as well as other malignant cells release lysosomal proteinases of the cysteine proteinase class as well as of the aspartic proteinase class suggesting possible common mechanisms for both their release and their association with malignancy. In this review we will concentrate on recent findings about the relationship between malignancy and the lysosomal cysteine proteinase cathepsin B and the endogenous low M_r inhibitors of cysteine proteinases, the cyst-

atins. Since other reviews are available [4–10], this review will not attempt to be comprehensive in its coverage of the literature, but rather will attempt to provide a hypothetical framework for further study of the mechanisms by which cysteine proteinases and cystatins may influence tumor invasion and metastasis.

Cathepsins

The use of the name cathepsin has often been a point of confusion for researchers in other fields since the name can designate proteinases or endopeptidases of more than one class. Cathepsin itself is derived from a Greek word meaning to digest and was used originally to designate acidic proteinases [11]. The letters D, G, B, L, etc. designate individual enzymes. Cathepsin D as indicated above is an aspartic proteinase, cathepsin G a serine proteinase and cathepsins B and L are cysteine proteinases. Although several of the cathepsins have collagenolytic activity, cathepsins of the metalloproteinase class have not been identified.

In general, cathepsins are found inside cells in digestive vesicles called lysosomes. Lysosomes, the cellular equivalents of the digestive tract, participate in normal turnover of cellular components as well as the degradation of molecules taken up from the extracellular environment. In addition to cathepsins, lysosomes contain hydrolytic or digestive enzymes with the ability to degrade polysaccharides, nucleic acids, etc. The cathepsins like other lysosomal enzymes are glycoproteins with phosphomannosyl residues. Newly synthesized cathepsins are sorted from other newly synthesized proteins by virtue of their ability to bind via these phosphomannosyl residues to mannose-6-phosphate receptors (MPR). This MPR binding results in delivery of lysosomal enzymes to lysosomes (for review see Ref. [12]). However, in some disease states or cell types the normal intracellular trafficking of lysosomal enzymes is altered resulting in secretion of lysosomal enzymes. For example, lysosomal enzymes are released from activated macrophages, from osteoclasts and from fibroblasts obtained from patients with I-cell disease. The release of lysosomal proteinases from tumor cells suggests that intracellular trafficking of these enzymes may be altered during malignant conversion of cells.

Cathepsin B

Cathepsin B was the first lysosomal cysteine proteinase purified to homogeneity and consequently has been more thoroughly studied than other cysteine proteinases. The characteristics of cathepsin B and two related lysosomal cysteine proteinases, cathepsins L and H, have been summarized by Barrett and Kirschke [13]. Depending on species and tissue of origin, mature cathepsin B exists in a single chain form, a double chain form or as both the single and double chain forms. In the double chain form, the active site cysteinyl residue of cathepsin B is found in the light chain. The nucleotide sequence indicates that cathepsin B is synthesized as a preproenzyme of 339 amino acids, consisting of a 17 residue prepeptide sequence, a 62 residue propeptide sequence, a 6 residue extension at the carboxy terminus and the 254 residues of mature single chain cathepsin B [14]. Mature double chain cathepsin B contains only 252 residues as a two amino acid linkage between the two chains is removed during processing. Based on an average mass of 110 daltons for each amino acid, the predicted molecular masses for the preproenzyme are 37.3 kDa, for the proenzyme 35.4 kDa, for the mature single chain enzyme 27.9 kDa and for the mature double chain enzyme a 22.4 kDa heavy chain and a 5.2 kDa light chain. The molecular masses determined by biosynthetic labeling of cathepsin B in rat macrophages, rat hepatocytes and human fibroblasts [15–18] correspond well with the predicted molecular masses. In rat macrophages and hepatocytes procathepsin B is 39 kDa (unglycosylated = 35 kDa), whereas in human fibroblasts procathepsin B is 44.5–46 kDa (unglycosylated = 39 kDa). In rat macrophages and hepatocytes mature cathepsin B appears first as a 29 kDa single chain that is converted to the double chain form over an extended time period. Hanewinkel *et al.* [15] reported that there are two active forms of cathepsin B of 33 kDa and 27 kDa in human fibroblasts, the 33 kDa form

being significantly more active. However, their activity measurements were not performed on purified samples so the relative activities of the two forms of human fibroblast cathepsin B have not been demonstrated conclusively. Analyses of cathepsin B processing in the same cell types from several species should clarify whether the molecular masses and activities of cathepsin B forms remain consistent within a single species or a single cell type.

Cathepsin B purified from many tissues and many species has been shown to be a broad spectrum endopeptidase with limited activity as a peptidyl dipeptidase against selected substrates (for review see Ref. [13]). A possible exception is cathepsin B purified from porcine spleen which has been shown to be an exopeptidase [19]. Cathepsin B from porcine liver does appear to be an endopeptidase as defined by its ability to cleave the bait region of α_2-macroglobulin, i.e., a cleavage site only available to an endopeptidase [20]. The broad spectrum of bonds cleaved by cathepsin B suggests that there may be many natural substrates for this enzyme. Routine, sensitive assay of cathepsin B activity is accomplished most readily with synthetic substrates consisting of a blocked peptide with a leaving group that can be detected colorimetrically or fluorometrically (the latter being preferable due to its greater sensitivity). In our laboratory we use Z-Arg-Arg-NHMec for assay of cathepsin B (e.g., see Ref. [21]). The cysteine proteinase cathepsin H has essentially no activity against this blocked di-Arg substrate [13], whereas cathepsin L and S have activity more than 2 orders of magnitude less than that of cathepsin B against this subtrate [22]. However, proteinases other than cysteine proteinases may cleave the Z-Arg-Arg-NHMec substrate and therefore we assay cathepsin B activity in the presence and absence of E-64, an irreversible inhibitor and active site titrant for cysteine proteinases that is ineffective against aspartic, metallo and serine proteinases [23]. Although cathepsin B is inhibited by thiol blocking reagents such as iodoacetic acid and $HgCl_2$ and the microbial inhibitors leupeptin and antipain, these are less specific than are E-64 or the peptidyl diazomethyl ketones, peptidyl fluoroketones, peptidyl semicarbazones and peptidyl sulfonium salts. Furthermore in these latter groups of inhibitors, the peptide residues can be modified to increase their selectivity for individual cysteine proteinases [24–28]. Such inhibitors are often used to discriminate the activity of cathepsin L from that of cathepsin B in biological samples since both cathepsin B and cathepsin L readily cleave the Z-Phe-Arg-NHMec substrate used for routine assay of cathepsin L [22]. Inhibitors that discriminate the activity of cathepsin B from that of cathepsin L are in the developmental stage and are not yet routinely available [29, 30]. Therefore, for accurate quantitation of cathepsin B activity in biological samples, it is important to use a substrate selective for cathepsin B rather than the Z-Phe-Arg-NHMec substrate that is readily cleaved by both cathepsins B and L. To further ensure accurate quantitation of cathepsin B activity in biological samples such as subcellular fractions, we determine activities as V_{max} in order to minimize interactions with the endogenous reversible inhibitors of the enzyme that may also be present in these biological samples [21].

Since the normal intracellular environment for cathepsin B is the lysosome, one might assume that cathepsin B is most active and most stable at acid pH. In general, the pH profiles for cathepsin B activity have acid optima, particularly for protein substrates. Against synthetic substrates the optimum pH is somewhat higher. Cathepsin B appears to be stable below pH 7.0, but not above, as determined by measuring its activity against synthetic substrates in stopped assays. However, there are exceptions to this rule. For example, if activity of cathepsin B against synthetic substrates is monitored continuously, one can observe significant activity of cathepsin B even at pH 8.0 [31], and if activity against large protein substrates such as laminin is assessed, one can observe significant activity for times up to 12 h at pH 7.4 [32]. In the latter case, the large protein substrates seem to stabilize the enzyme [8]. Procathepsin B is more stable at neutral or alkaline pH than is mature cathepsin B [33]. Procathepsin B is released from tumor cells [33] and once activated extracellularly may be stable in its active form due to the presence of large protein substrates such as extracellular matrix pro-

teins [8]. Therefore, procathepsin B released from tumor cells may serve as a 'proteolytic reservoir' ready to participate in focal degradation of extracellular matrix proteins adjacent to invading or adherent tumor cells.

Cathepsin B and malignancy

Over the last decade, a number of laboratories including our own have reported an association between tumor malignancy and the cysteine proteinase cathepsin B (for further review see Ref. [8]). Table 1 is a partial listing of the evidence linking cathepsin B to malignancy. As is obvious from the list, cathepsin B has been associated with malignancy of tumors from many tissues including ones of mesenchymal and epithelial origin. Much of the work in our laboratory has focused on metastatic variants of the B16 melanoma. Using these variants, we have demonstrated that their metastatic capability correlates with increases in cathepsin B activity in subcutaneous tumors [34, 35], tumor cells in primary or secondary culture [35], tumor cells isolated by centrifugal elutriation [36] and tumor plasma membrane fractions [21, 36–39] as well as increases in cathepsin B mRNA levels [39]. Similar correlations between metastatic capability of B16 melanomas and cathepsin B activity [40] and mRNA levels [41] have been observed by others. Elevated cathepsin B activity is found in all metastatic B16 melanomas, nevertheless the correlation between cathepsin B activity and metastatic capability often appears to be a qualitative one rather than a quantitative one.

The qualitative nature of this correlation may reflect the fact that cathepsin B activity in malignant tumors can be regulated at many levels. Alterations in activity of cathepsin B may reflect changes in its synthesis [39, 41], in activation and processing [33, 40], in intracellular trafficking and delivery [33, 38, 42, 43] and in the endogenous inhibitors or cystatins [44, 45]. Alterations in intracellular trafficking may result in delivery of cathepsin B to endosomal/plasma membrane fractions and/or to release of the enzyme from tumor cells. If the release of cathepsin B is enhanced in malignant cells, measurements of cellular cathepsin B in these same cells may lead one to the erroneous conclusion that cathepsin B activity is the same or less than in comparable non-malignant cells. In all the human and rodent malignant tumors we have examined to

Table 1. Cathepsin B: Correlations with malignancy

Experimental system	Finding
Murine B16 melanoma	mRNA, activity and membrane association correlate with metastatic capability.
Murine liver normal vs. hepatoma	mRNA, activity and membrane association correlate with malignancy.
Cloned low-metastatic murine Lewis lung carcinoma cells	Activity correlates with induction of increased metastatic capability.
Murine fibrosarcoma: MCA-F and clone 9.4	Membrane association correlates with metastatic capability.
Viral transformation: murine BALB/3T3 fibroblasts	Release of latent form correlates with transformation.
Rat sarcoma: LW13K2 and RPS	Release correlates with metastatic capability.
ras transfection: rat embryo fibroblasts and human MCF-10 mammary epithelial cells	Membrane association correlates with malignant conversion.
Human breast tumors	Release of high M_r forms of enzyme correlates with malignancy.
Human prostate: benign prostatic hyperplasia vs. carcinoma	Membrane association correlates with malignancy.
Human lung squamous cell carcinoma and adenocarcinoma	Activity elevated in tumor tissues as compared to matched normal parenchyma.
Human colon tumors	mRNA and activity elevated in carcinoma tissue as compared to matched normal mucosa.
Human colorectal tumor cell lines	Release of mature enzyme correlates with malignancy.

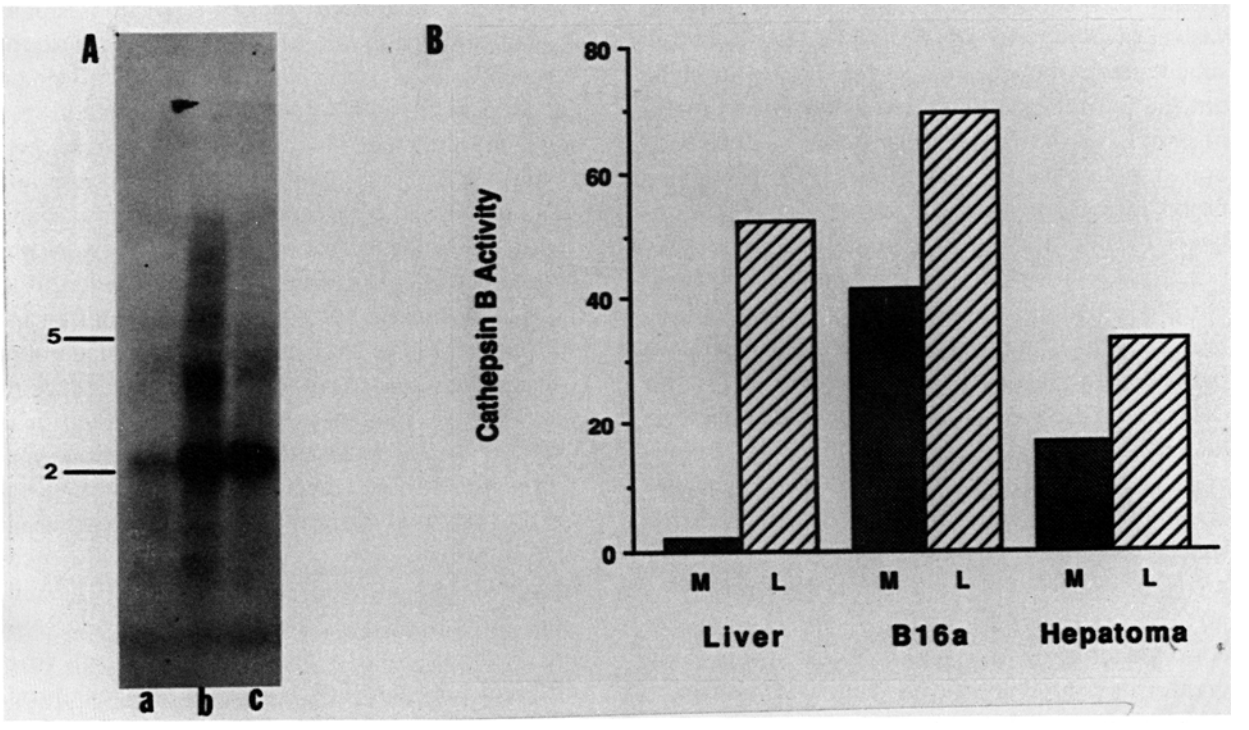

Fig. 1. Cathepsin B specific RNA transcripts and cathepsin B activities in subcellular fractions of murine liver and tumors (Adapted from Ref. [39]). A. Northern blot hybridization of RNAs to human cathepsin B cDNA. Nylon membrane containing total RNAs (30 μg/lane) was hybridized to the cathepsin B cDNA labeled with ^{32}P and subjected to autoradiography. Lanes a, b and c represent normal liver, B16 amelanotic melanoma and Hepa cl 9 hepatoma RNA, respectively. Ribosomal RNA sizes (kb) and their locations are indicated at the left. B. Distribution of cathepsin B activities in plasma membrane/endosomal (M) and lysosomal (L) fractions isolated by Percoll density gradient centrifugation as described previously [37]. Activity is expressed as nmoles/min·mg protein and was determined as V_{max} using our published protocols [21].

date (e.g., in melanoma, hepatoma, colon adenocarcinoma [21, 37–39, 45], prostate carcinoma (Rozhin, Sinha and Sloane, unpublished data) and mammary adenocarcinoma [45]), we have shown that there is cathepsin B activity associated with plasma membrane/endosomal fractions separated by sequential differential and Percoll density gradient centrifugation. Furthermore, we have shown that regulation of cysteine proteinase activity by the stefin A subfamily of cystatins may be impaired in malignant tumors [44]. Thus cathepsin B activity in malignant tumors is affected at more than one level.

Cathepsin B: Synthesis

Indirect evidence had indicated that the enhanced activity of cathepsin B seen in many different tumors might be due to increased synthesis. Koppel *et al.* [46] had reported that cycloheximide reduces the cathepsin B content of a metastatic rat anaplastic sarcoma and Recklies *et al.* [47] had reported that cycloheximide inhibits cathepsin B release from malignant human breast tumors. We and others have now provided direct evidence that cathepsin B synthesis is enhanced in murine tumors [39, 41] and in human colon carcinomas [48]. We present here as an example data on two representative murine tumors syngeneic to C57Bl/6J mice and murine liver from the same mice (Fig. 1). The two tumors are Hepa cl 9, a hepatoma that arose spontaneously in the liver of C57Bl/6J mice [49] and therefore a malignant counterpart of liver from these same animals, and the B16 amelanotic melanoma (B16a). Although of low metastatic po-

tential, in our hands the Hepa cl 9 is highly invasive, invading through the peritoneal wall after subcutaneous implantation and spreading throughout the peritoneal cavity. The B16a melanoma is one we have shown to be highly metastatic, both upon tail-vein injection and subcutaneous implantation [21]. Thus the two tumors distinguish between the abilities to invade locally and to metastasize. Based on our previous observations that cathepsin B activity in the plasma membrane/endosomal fractions of metastatic tumors is increased in parallel with metastatic capability [6, 21, 37], we determined the mRNA level, activity and subcellular localization of cathepsin B. Using a human cathepsin B cDNA probe, we detect two hybridizable RNA species of 2.2 kb and 4.1 kb in the murine tissue [39]. The concentrations of the 2.2 kb mRNA in B16a and Hepa cl 9 are 4- and 2-fold greater, respectively, than in liver, whereas the concentration of the 4.1 kb transcript is only greater in B16a than in liver (Fig. 1A). For liver, B16a and Hepa cl 9, respectively, the specific activities of cathepsin B in homogenates are 6, 11 and 10 nmoles/min · mg protein. Thus the homogenate values for cathepsin B activity in liver and hepatoma parallel the levels of the 2.2 kb mRNA transcripts in the two tissues. The apparent discrepancy between the levels of the 2.2 kb and 4.1 kb mRNA transcripts and cathepsin B activity in the B16a melanoma may reflect an enhanced synthesis of inactive, high Mr precursor forms of cathepsin B in this tumor. A strikingly large proportion of the cathepsin B activity in the two tumors is associated with the plasma membrane/endosomal fraction, a finding consistent with previous studies of both rodent and human tumors [6, 21, 37]. The percentages of cathepsin B activity in the plasma membrane/endosomal fractions of liver, B16a and Hepa cl 9 are 4, 59 and 52, respectively (Fig. 1B). Thus the increase in expression of the cathepsin B gene in Hepa cl 9 and B16a is reflected in increased cathepsin B activity in the tumors as well as in an apparent alteration in the intracellular trafficking of cathepsin B in the two tumors, suggesting that alterations in the expression and subcellular distribution of cathepsin B are linked to invasive and metastatic capabilities of malignant tumors.

Only one cathepsin B-specific RNA transcript of 2.3 kb had been previously identified in a series of nine normal rat tissues and one rat tumor using a rat cathepsin B cDNA [50]. However, recently using a murine cathepsin B cDNA Qian *et al.* [41] confirmed our observations of two transcripts (2.1 kb and 4.0 kb) in murine B16 melanomas and reported a third transcript of 5.0 kb, but were unable to detect either of the two larger transcripts in normal murine tissues. Our studies on murine tissues and those of Murnane [48] on human colon (normal mucosa and colorectal carcinoma) suggest that the two smaller transcripts for cathepsin B (i.e., 2.2 kb and 4.1 kb) are present in both normal and tumor tissues. Technical differences (e.g., hybridization and washing conditions, different cDNA probes) may account for the differences observed from one laboratory to another. The multiple transcripts could represent the products of different cathepsin B genes or could result from different polyadenylation sites on one gene product. Based on Southern blot analysis, Fong *et al.* [51] and Qian *et al.* [41] conclude that cathepsin B is transcribed from a single gene in normal tissues and melanomas, respectively. Southern blot analysis did not reveal gross chromosomal rearrangement or amplification of the gene for cathepsin B in B16a melanoma [41]. Recently, Qian *et al.* [52] have reported in abstract form that the 4.0 and 5.0 kb transcripts in B16a melanoma result from different polyadenylation signals and hypothesize that 'the putative molecular machinery governing termination and poly A addition' of many cellular proteins may be modified in malignant tumors. Multiple transcripts for other cysteine proteinases have not been reported. Although Taggert and coworkers [53] found multiple transcripts for the aspartic proteinase cathepsin E in human gastric carcinomas, they have recently found those same transcripts in normal human gastric mucosa (Taggart, personal communication). Further studies will be needed to determine the significance of multiple transcripts for cathepsin B and to confirm an association of multiple transcripts with malignancy.

Cathepsin B: Processing and activation

Post translational processing of cathepsin B could influence the release of this enzyme from tumors as well as its subcellular distribution. In this regard, Pagano *et al.* [54] have demonstrated that cathepsin B precursors found in human malignant ascites fluid possess complex carbohydrates typical of secretory or membrane-bound proteins rather than the mannose-rich carbohydrates typical of lysosomal enzymes. They postulate that the release and membrane association of cathepsin B found in malignant tumors results from a defect in the maturation of the carbohydrate moieties during post translational processing of cathepsin B.

During post translational processing of cathepsin B, the latent precursor forms are activated and converted to single chain and double chain mature forms. Kato and coworkers [18] found that pepstatin, an inhibitor of aspartic proteinases, inhibited the processing of procathepsin B to its single-chain mature form and that cathepsin D could perform this cleavage *in vitro*. In contrast, Hara *et al.* [17] reported that inhibitors of metalloproteinases prevented this step. Thus it is plausible that cathepsin B precursors released from breast carcinomas may be activated *in vivo* by the cathepsin D released from these tumors [55] or that cathepsin B released from other tumors could be activated *in vivo* by the metalloproteinases found extracellularly. A possible explanation, *albeit* entirely hypothetical at this point, for the association of cathepsin B with membrane fractions of malignant tumors is that proteolytic processing of cathepsin B at the carboxy terminus is altered in tumor cells. Murnane (personal communication) has shown that there are regions of sequence homology between G-proteins, including the *ras* protein p21, and cysteine proteinases. These regions include the four amino acid sequence Cys-A-A-X that has been linked to the binding of *ras* proteins to the plasma membrane through a farnesyl isoprenoid (for review, see Ref. [56]). In cysteine proteinases, this Cys-A-A-X box is 8–11 amino acid residues from the carboxy terminus rather than at the carboxy terminus as in *ras* and other G-proteins. However, one might speculate that in tumors, where there is a general increase in proteolytic activity, the carboxy terminus of cathepsin B may be clipped allowing this cysteine to bind to a farnesyl isoprenoid in the plasma membrane.

Cathepsin B: Intracellular trafficking and delivery

Intracellular trafficking and delivery of lysosomal enzymes to the lysosomes occurs through a receptor dependent pathway; in most cells this occurs via the MPR pathway [12]. A defect in phosphorylation of the mannose residues on lysosomal enzymes and their consequent inability to bind to the MPR leads in I-cell disease to the release of lysosomal enzymes [12]. Based on the ability to block uptake of cathepsin B into normal fibroblasts with mannose-6-phosphate and the release of cathepsin B from I-cell fibroblasts, Hanewinkel *et al.* [15] concluded that in human fibroblasts cathepsin B is transported via a MPR pathway. Recently, Chi *et al.* [57] isolated cation dependent and cation independent MPRs from brain and showed that cathepsin B binds to both, but preferentially to the cation dependent MPR. Studies on the MPRs of malignant cells now in progress are needed to further our understanding of the intracellular trafficking of cathepsin B and its possible alteration during progression of normal cells to malignant cells.

In fibroblasts from patients with I-cell disease there is an association of some lysosomal enzymes with small vesicles near the plasma membrane [58]. Our observations that cathepsin B activity is present in small membrane vesicles shed by murine 15091A mammary adenocarcinoma cells [59] and in tumor plasma membrane/endosomal fractions [21, 37–39, 45] parallel the findings in I-cell disease. Our observations now extend to three histologic types of human and rodent tumors from eight tissues. The association of lysosomal enzymes with plasma membrane/endosomal vesicles appears to be selective [21, 58]. For example, we have shown that plasma membrane/endosomal fractions of B16a tumors contain activities of the glycosidase β-hexosaminidase [21] and the cysteine proteinase cathepsin L [60] in addition to cathepsin B, but do not contain the glycosidase β-glucuronidase or the

cysteine proteinase cathepsin H [21]. Thus tumor cells seem to have at least two cysteine proteinases and a glycosidase localized near or at the cell surface. This may be important in dissolution of the basement membrane during tumor cell extravasation as concomitant degradation of carbohydrate has been shown to enhance proteolytic degradation of basement membrane *in vitro* [61]. The presence of cathepsins B and L and β-hexosaminidase at the tumor cell surface could lead to the establishment of a local microenvironment in which focal degradation of the basement membrane is enhanced.

The extensive literature on the release of cathepsin B from both human and animal tumors has been reviewed previously (see [4–9]). Release of cathepsin B occurs primarily in the form of a latent, high M_r precursor of 40 kDa that can be activated with pepsin to a 33 kDa active form [33]. However, Mort and Recklies [33] have also reported the release of an active, high M_r (also 40 kDa) form of cathepsin B from breast tumor cells and lactating mammary glands. The release of this active 40 kDa cathepsin B is greater in malignant tumors than in non-malignant tumors. Similar results have been reported for human colorectal tumors [62] and human liver tumors [63]. One group [41] has reported a correlation between release of latent cathepsin B from B16 melanomas and their metastatic potential, whereas we have not seen a correlation between the amounts of either active or latent cathepsin B released and the metastatic potentials of B16 melanomas [6]. Both Recklies [7] and we (Rozhin and Sloane, unpublished observation) have been unable to demonstrate a correlation between release of active or latent cathepsin B and malignancy of murine mammary tumors. Thus in murine tumors the correlation between release of cathepsin B (active or latent) and malignancy remains unclear, whereas in human tumors there does appear to be a correlation between release of active cathepsin B and malignancy. These disparate results may reflect the difficulties in measuring the small quantities of active enzyme released from murine tumors (Rozhin and Sloane, unpublished observations).

The mechanism(s) resulting in release of cathepsin B by tumors or association of cathepsin B with the plasma membrane-enriched fractions of tumors are not known. Recklies [7] reported that the ratios between levels of intracellular cathepsin B and secreted cathepsin B in murine mammary lines are not constant and concluded therefore that the release of cathepsin B does not simply result from a saturation of the normal MPR pathway for processing and delivery to the lysosomes. Defects in MPR (absence or increased rate of turnover) occur in some tumor cell lines [64, 65]. Recently platelet-derived growth factor has been shown to induce alterations in the functioning of MPR [66]. This is of particular interest due to the known association of growth factors (autocrine and exocrine) with malignancy. We conjecture that in malignant tumors a combination of these factors is responsible for the release of cathepsin B and/or its change in subcellular distribution.

One can also obtain information on the cellular distribution and trafficking of cathepsin B by morphologic techniques. For examples, Erdel *et al.* [67] have recently confirmed our biochemical observations by using the morphologic techniques of immunofluorescence microscopy and confocal laser scanning microscopy to localize cathepsin B to the surface of a human lung adenocarcinoma cell line (derived from a metastasis to the adrenal gland). These studies follow the distribution of cathepsin B protein. Cathepsin B activity can be followed using a fluorescent substrate for cathepsin B. In this case in malignant and transformed breast epithelial cells, a microgranular staining pattern of cathepsin B activity is seen throughout the cytoplasm and in some cytoplasmic projections near the cell surface [68]. In normal breast epithelial cells the staining appears to be more perinuclear than in the malignant and transformed cells. Using this same technique to localize cathepsin B activity in cultured cells of the highly metastatic B16a melanoma, we found evidence that the reaction product was present near the cell surface and in vesicles extending out along the cytoplasmic projections of the cells (Fig. 2A). No reaction product was visible in controls stained for cathepsin B activity in the presence of E-64, an irreversible inhibitor of cysteine proteinases (Fig. 2B). Note that there is an absence of reaction product in the controls even

though the time period for the staining reaction in the controls is twice that used for the experimental samples (Fig. 2). Confocal techniques will be needed to confirm the apparent surface localization of cathepsin B in B16a cells.

Endogenous low Mr cysteine proteinase inhibitors (CPIs)

The cystatin superfamily of cysteine proteinase inhibitors (CPI) consists of three families: 1) stefins, 2) cystatins and 3) kininogens (for review, see Ref. [69]). In terms of inhibition of cathepsin B, the low M_r inhibitors (i.e., the stefin and cystatin families) appear to be of most physiological importance as the K_i's for inhibition of cathepsin B by the low M_r inhibitors range from 10–1000-fold less than the K_i for inhibition of cathepsin B by kininogen, i.e., 600 nM [69]. However, in plasma, kininogen and α_2-macroglobulin are the major CPIs [70].

The stefin family of CPIs is primarily intracellular proteins of about 11 kDa that do not contain disulfide bonds or carbohydrates. Two types of stefins have been identified: stefins A with acidic isoelectric points and stefins B with neutral. Although properties of stefins are similar from tissue to tissue, the distribution differs with stefins A being abundant in epithelial tissues and stefins B being distributed more equably. Of the two, stefin A is most effective against cathepsin B, but both stefins A and B are more effective against cathepsins L and H than against cathepsin B. The cystatin family is primarily extracellular proteins of about 13 kDa with two disulfide loops near the carboxyl terminus. Cystatins have basic isoelectric points.

Endogenous low Mr CPIs and malignancy

Numerous studies have linked cysteine proteinases to tumor malignancy, yet the number of studies linking CPIs to tumor malignancy is still small (for further review, see Ref. [8]). Our studies indicating that cathepsin B is linked to malignant progression use assays for cathepsin B activity in biological fluids, homogenates or culture media that are affected by the presence of endogenous substrates and endogenous inhibitors (see Refs. [8, 44] for a discussion of this problem). Although the use of several concentrations of substrate in our assay procedure improves the quantitation of cathepsin B activity, the pseudoirreversible binding of the endogenous low M_r CPIs means that the cathepsin B activity one measures will reflect the concentration and efficiency of CPIs in the sample. To determine the levels of endogenous CPIs, we dissociate inhibitor-cysteine proteinase complexes by taking advantage of differences in stability of cysteine proteinases and their inhibitors to heating at 100° C for 5 min [71]. We assayed tumor cell subpopulations [100 and 340] isolated by centrifugal elutriation from B16a melanoma and Lewis lung carcinoma (3LL) for activities of both cathepsin B and of heat stable CPIs (Fig. 3). The B16a subpopulations isolated by this technique exhibit a 10-fold differential in lung colonization potential, whereas 3LL subpopulations exhibit no differential. In the B16a subpopulations, plasma membrane/endosomal associated cathepsin B activities also exhibit a 10-fold increase, whereas CPI activities in the plasma membrane/endosomal fractions are decreased 5 fold. In the 3LL subpopulations, neither cathepsin B nor CPI activities change.

Since the binding of cysteine proteinases to the endogenous low M_r CPIs (cystatins) is 1 : 1 [72], we performed an arbitrary calculation of the 'working activity' of cathepsin B by determining the ratio of the activities of cathepsin B and the endogenous inhibitors in the tumor cell subpopulations isolated by centrifugal elutriation [60]. The 'working activities' were normalized to account for the fact that CPI activities were determined against papain, a cysteine proteinase against which inhibition constants are in the picomolar range, rather than against cathepsin B against which inhibition constants are in the nanomolar range [69]. The ratio of cathepsin B/CPI in the plasma membrane/endosomal fractions of elutriated subpopulations of B16a with a high potential for lung colonization [340 subpopulation] is 7, suggesting that the plasma membrane/endosomal fractions of B16a cells may not contain sufficient quantities of CPIs to regulate cathepsin B activity. This indicates again the poten-

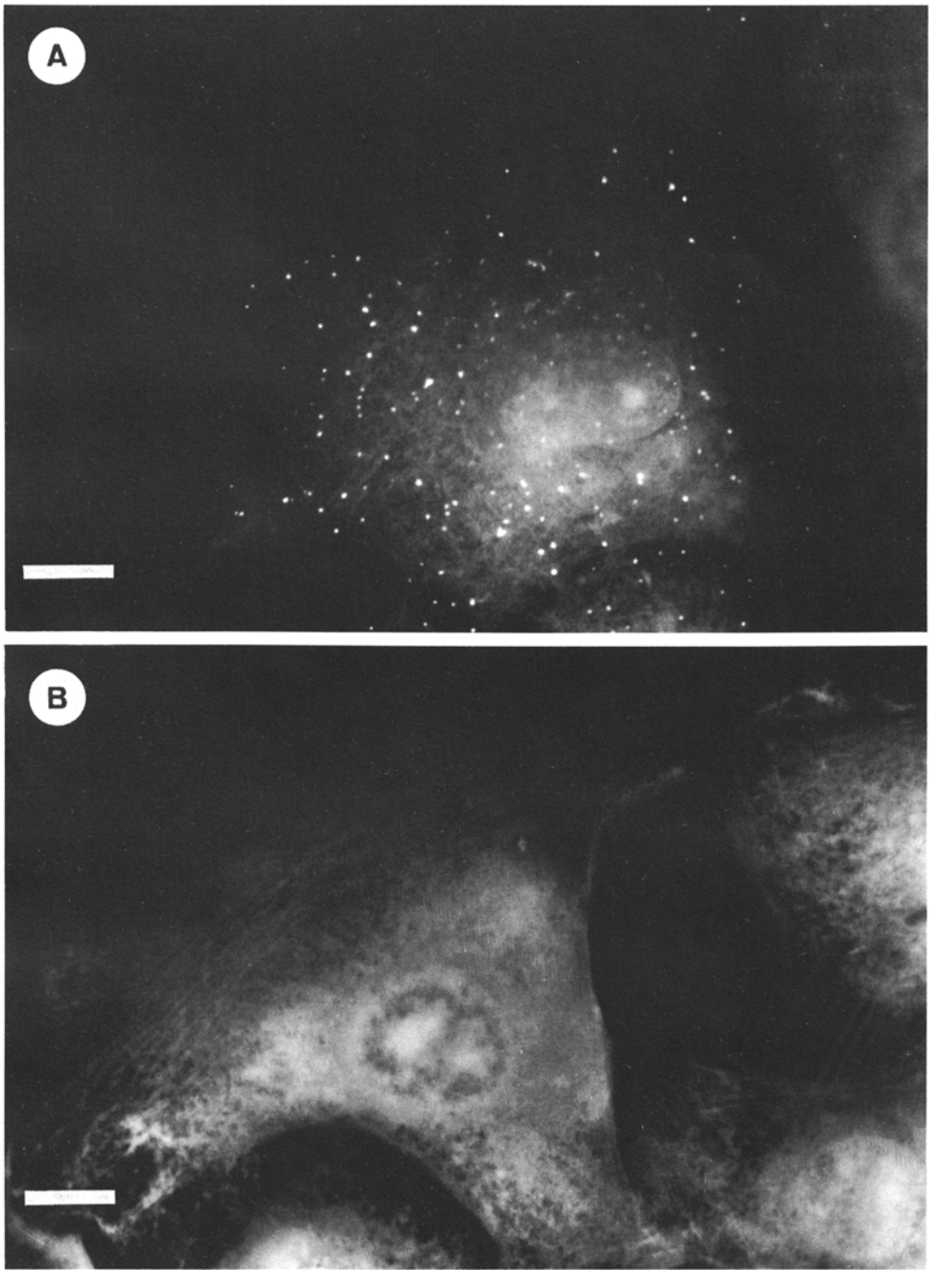

Fig. 2. Fluorescence cytochemical staining for cathepsin B activity in B16 amelanotic melanoma cells. Panel A, reaction product visible after 5 min of incubation with the specific cathepsin B substrate, Z-Arg-Arg-NNapOMe, using published methodologies [68]. Panel B, absence of reaction product after 10 min of incubation when cells were reacted in the presence of 10 μM E-64, a specific inhibitor and active site titrant for cysteine proteinases. Bars = 10 μm.

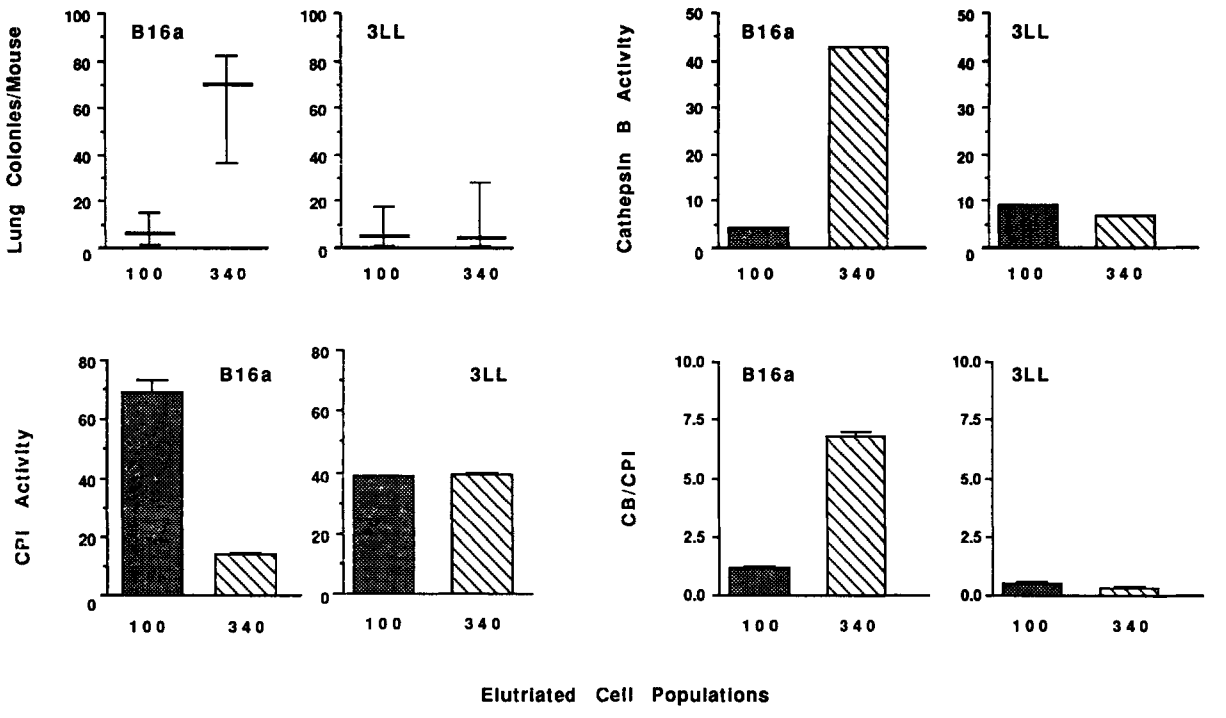

Fig. 3. Correlation of lung colonization potential with activities of cathepsin B, endogenous heat stable cysteine proteinase inhibitors and the ratio of cathepsin B and inhibitory activities in plasma membrane fractions of two B16 amelanotic melanoma (B16a) and Lewis lung carcinoma (3LL) subpopulations (100 and 340) isolated by centrifugal elutriation (Adapted from Ref. [45]). Upper left, lung colony formation; median and range for 12 mice per fraction. The Kruskal-Wallis test for non-parametrically distributed groups indicated that there is a statistically significant difference ($p = 5 \times 10^{-8}$) between the 100 and 340 subpopulations. Upper right, cathepsin B (CB) activity; nmoles/min · mg protein. Lower left, cysteine proteinase inhibitor (CPI) activity; munits/mg protein. Lower right, an arbitrary calculation of the potential 'working' activity of cathepsin B or CB/CPI. Data on additional subpopulations and details on methodologies used for analysis can be found in Ref. [45].

tial importance of this association of cathepsin B with plasma membrane/endosomal fractions of malignant tumor cells.

Stefin A and malignancy

We do not know whether all or any one of the endogenous CPIs are present in reduced amount in the metastatic B16a cells or whether all or one of the CPIs is a less efficient inhibitor in metastatic B16a cells. However, based on the following observations, we speculate that stefin A may be responsible for an alteration in the regulation of cathepsin B activity in B16a cells: 1) Yuspa and coworkers [73] have shown that there is a reduction in stefin A mRNA during the progression of skin papillomas to carcinomas, 2) Rinne and coworkers [74, 75] have demonstrated a reduced staining for stefin A in malignant human tumors, and 3) we have shown that tumor stefin A is significantly less inhibitory than stefin A from normal tissues (Table 2). Stefins B from human liver and sarcoma exhibit comparable inhibition of papain, human liver and tumor cathepsin B and murine tumor cathepsin B [44]. In contrast, the inhibition constants for inhibition of papain, human liver cathepsins B, H and L, and human and murine tumor cathepsin B by human sarcoma stefin A range from 2- to 23-fold higher than for inhibition of the same enzymes by human liver stefin A (Table 2 and Ref. [44]).

These inhibition constants were measured *in vitro* under conditions in which the following are absent: 1) competition among the inhibitors for

binding to cathepsin B, 2) competition among the cysteine proteinases for binding to the inhibitors and 3) competition among physiological substrates and inhibitors for binding to cathepsin B. In addition, in these *in vitro* assays the question of access of the inhibitor to cathepsin B does not arise. Although one might assume that the reduced ability of sarcoma stefin A to inhibit cathepsin B *in vitro* is responsible for the elevated activities of cathepsin B we measure in animal and human tumors, at this point this assumption cannot be tested directly as we do not know the concentrations of CPIs in these tumors, the concentrations of active cysteine proteinases (e.g., cathepsins B, H, L, S), the concentrations of the physiological substrates for the cysteine proteinases, the kinetic constants for interaction of the cysteine proteinases with their physiological substrates, or even the identity of the physiological substrates for the various cysteine proteinases.

The higher inhibition constants for the interaction between sarcoma stefin A and cathepsin B are due to two factors: 1) a decrease in the rate of association of inhibitor and enzyme and 2) an increase in the rate of dissociation of enzyme-inhibitor complexes [44]. One possible explanation for the lesser stability of the complexes between enzyme and sarcoma stefin A is an alteration in the structure of sarcoma stefin A. Due to the limited availability of sarcoma stefin A, we used an indirect method to analyze the structure of stefin A, i.e., the fluorescence spectra of liver and sarcoma stefins A [44]. The spectrum of sarcoma stefin A differs from that of liver stefin A in that the maximum fluorescence peak is broader and shifted to 310 nm. A red-shift and a decrease in fluorescence intensity of the sarcoma stefin A spectrum is consistent with a conformational change in the inhibitor, perhaps due to a difference in primary structure. There is a precedent in which a structural alteration in a CPI, in this case a single point mutation in cystatin C, results in an inhibitor with reduced activity and in turn leads to a pathologic state, amyloidosis [76]. We speculate that stefin A by virtue of its role in regulating cysteine proteinase activity may play an important role in malignant progression.

Cathepsin B trafficking in tumor cells

Liotta and coworkers have popularized a three step theory for tumor cell invasion through capillary walls during the metastatic cascade (see Ref. [77] for a recent discussion of this theory). In the first step, tumor cells attach to the basement membrane underlying the capillary endothelial cells. In the second step, hydrolytic enzymes are released and produce local degradation of the basement membrane. This is followed by the locomotion of the tumor cells into the degraded area. In Fig. 4, we present a hypothetical representation of the intracellular trafficking and release of cathepsin B in a tumor cell adherent to the basement membrane. At this time, much of this model is hypothetical and is derived from the extensive literature on alterations in lysosomal/endosomal trafficking in osteoclasts adherent to bone matrices (see Refs. [78–80] for recent reviews on osteoclasts and bone resorption). Experimental data are incorporated into this model where available.

The trafficking and targeting of lysosomal enzymes have been the subject of numerous studies (for recent reviews, see Refs. [12, 81, 82]). The oligosaccharides of newly synthesized lysosomal enzymes are phosphorylated in the Golgi and bind to MPRs. The enzymes are transported to an acidified compartment (¶-Fig. 4), a compartment often designated as late endosomal or prelysosomal, where they dissociate from the MPRs. The MPRs

Table 2. Inhibition constants for stefins A

Enzyme	Liver stefin A	Sarcoma stefin A
Papain	0.027[a]	0.110
Cathepsin L-liver	5.0	11.0
Cathepsin H-liver	0.4	1.2
Cathepsin B-liver	2.4	25.0
Cathepsin B-tumor	10.9	19.2
Cathepsin B-tumor LYS	1.0	23.0
Cathepsin B-tumor PM	2.0	20.0

[a] Apparent inhibition constants (K_i) in nM were determined from continuous rate assays at 25° C. Inhibitor concentrations were based on their active concentration as determined by titration against papain (itself titrated against E-64). LYS, lysosomal fraction; PM, plasma membrane/endosomal fraction.

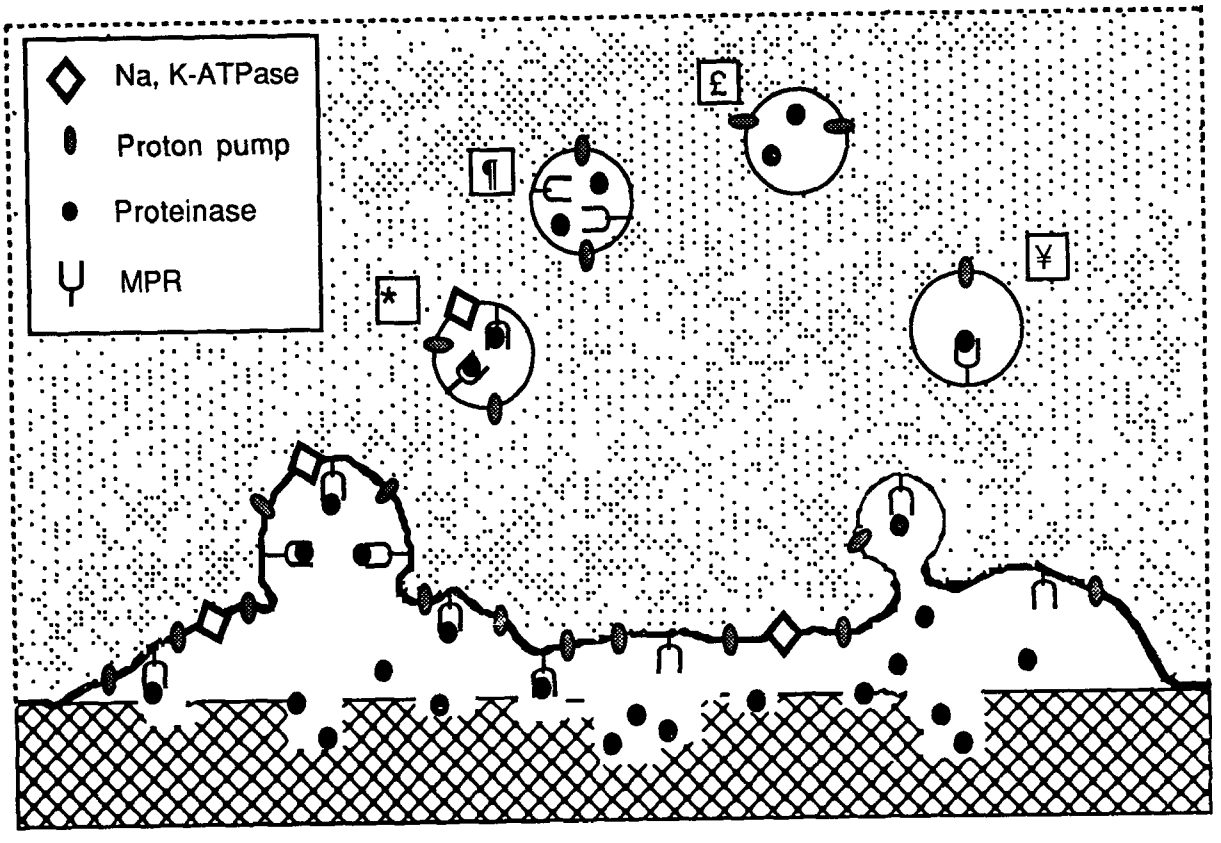

Fig. 4. Hypothetical model for lysosomal/endosomal system in a tumor cell adherent to endothelial basement membrane. See text for discussion.

recycle to the Golgi or to the cell surface. Studies on isolated endosomes by Murphy and coworkers [83, 84] have shown that endosomes can directly mature into lysosomes (£-Fig. 4) *in vitro* concomitant with an increase in density and a decrease in pH. The final processing of the precursor forms of lysosomal enzymes to mature forms takes place in the lysosome. Normally, a minor fraction of the lysosomal enzymes does not bind to the MPRs in the Golgi and is secreted from the cell. If the secreted lysosomal enzymes bind to MPRs on the cell surface, they can be taken up into the cell via endocytosis (early endosome, *-Fig. 4).

The trafficking of lysosomal enzymes in osteoclasts is altered in response to the attachment of the osteoclast to bone (see Refs. [78–80] for recent reviews on osteoclasts and bone resorption). This attachment of osteoclasts to bone is thought to be mediated by integrins as the vitronectin receptor is present in abundance in the osteoclast membrane [78]. The membrane at the interface with the bone is highly folded or ruffled. Transport of coated vesicles containing MPR-bound lysosomal enzymes to the ruffled-border membrane results in polarized secretion of lysosomal enzymes from the osteoclast (¥-Fig. 4). Proton pumps are also transported to the ruffled-border membrane, thereby enabling the osteoclasts to acidify the extracellular compartment between their ruffled-border and the bone matrix. Acidification of the extracellular compartment leads to dissociation of the lysosomal enzymes from the MPRs. Etherington and coworkers [85] have demonstrated that the acid microenvironment generated under cultured osteoclasts *in vitro* is at pH 3.0 or lower, whereas that between osteoclasts and bone *in situ* is pH 4.7. Thus osteoclasts are able to form an 'extracellular lysosome', a microenvironment favorable for dissolution of

bone by lysosomal enzymes. Control of the process of bone resorption is believed to be regulated by systemic hormones through the mediation of local growth factors such as platelet-derived growth factor [80].

Several groups have now shown that tumor cells may attach to basement membrane via integrin receptors [86–88]. The intracellular signals generating movement of integrin receptors to the cell surface and tumor cell attachment to extracellular matrix may lead in turn to polarized secretion of lysosomal enzymes and polarization of proton pumps at the interface between the tumor cell and the underlying substratum. Macrophages, cells that like the metastatic tumor cell are involved in local proteolysis during pathological states, have been shown to be capable of acidifying *in vitro* the attachment zone between the cells and collagen films to quite acid pH, i.e., 3.6–3.7 [85]. Like osteoclasts and malignant tumor cells, activated macrophages release lysosomal enzymes [89]. In addition, activated macrophages like malignant tumor cells seem to have surface-associated lysosomal enzymes including the lysosomal cysteine proteinase, cathepsin L [90]. Thus it is plausible that tumor cells could acidify the attachment zone formed between the tumor cell membrane and the basement membrane.

Tumor cells, particularly metastatic tumor cells, secrete cathepsin B (for review see Ref. [4–9]). If the secretion of cathepsin B from tumor cells paralleled the secretion of lysosomal enzymes from osteoclasts, i.e., secretion of MPR-bound enzyme (¥-Fig. 4), then one would expect that the secreted enzyme would be in a latent, precursor form. In the tumor cell lines we have studied, the cathepsin B secreted is primarily in a latent form [38, 91], therefore suggesting that the cathepsin B secreted from tumor cells did not reach the lysosomal compartment. Some tumor cell lines shed membrane-derived vesicles into their culture media; these vesicles (perhaps those labeled ¥ in Fig. 4) contain cathepsin B [59]. Plasma membrane/endosomal fractions isolated from malignant tumor cells also contain cathepsin B; this cathepsin B is in both latent and active forms (Rozhin and Sloane, unpublished data). The cathepsin B present in these plasma membrane/endosomal fractions cannot be eluted by mannose-6-phosphate and/or acid pH treatment [21], indicating that the cathepsin B is not bound to MPRs or to an undescribed receptor. Our experimental findings with the plasma membrane/endosomal fractions may be interpreted in several alternative ways. 1. The pH in early endosomes (*-Fig. 4) of malignant tumor cells is more acid than in normal cells, thus favoring dissociation of the cathepsin B ligand from its receptor. 2. The pH in secretory vesicles (¥-Fig. 4) of malignant tumor cells is more acid than in normal cells, thus favoring dissociation of the cathepsin B ligand from its receptor. 3. There are alterations in MPRs in malignant cells. As indicated above, some tumor cell lines exhibit an apparent absence of MPRs or an increased rate of turnover of MPRs [64, 65]. The functioning of MPRs may also be altered in tumors by growth factors as platelet-derived growth factor has been shown to affect MPRs [66]. Achkar *et al.* [92] recently proposed that release of cathepsins B and L from transformed fibroblasts is due to an apparent decrease in MPRs in BALB/3T3 fibroblasts transformed by Moloney murine sarcoma virus. This finding seems to be in contrast to those of Sahagian and coworkers [93, 94] in which release of cathepsin L from Kirsten virus-transformed NIH 3T3 fibroblasts appears to be due primarily to the low affinity of cathepsin L for MPRs (see below for further discussion). 4. In malignant tumor cells, the fraction we term plasma membrane/endosomal is enriched in late endosomes/prelysosomes (¶-Fig. 4). However, the high content of Na^+, K^+-ATPase in this fraction [21, 37] does not support this interpretation since Na^+, K^+-ATPase is found only in plasma membrane and early endosomes (*-Fig. 4). 5. In malignant tumor cells, cathepsin B is present in lysosomes (£-Fig. 4) that are shifted in shape and distribution by an alteration in cytoplasmic pH (Fig. 5). Heuser [95] has demonstrated that acidification of the cytoplasm of macrophages and fibroblasts results in a redistribution of lysosomes from the perinuclear region to just under the cell membrane and an alteration in their shape from tubular to vesicular (compare Fig. 5A and Fig. 5B). This phenomenon occurs in regions of the cell that were actively ruffling prior to acidification and thus

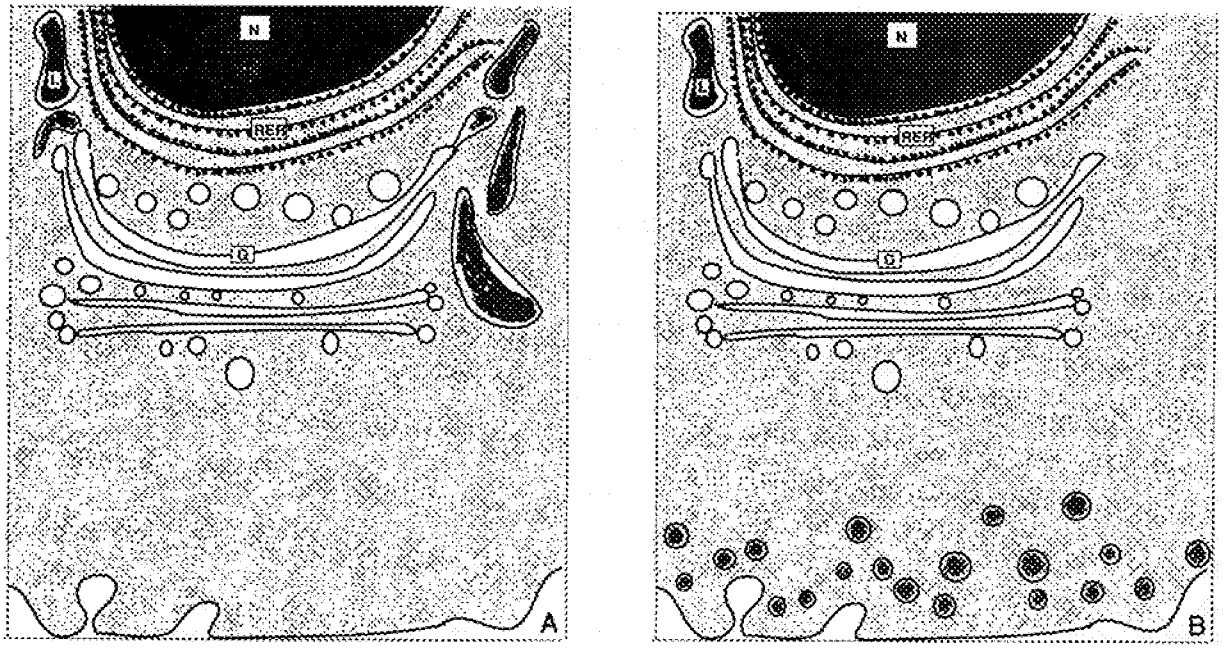

Fig. 5. A two part cartoon depicting possible alterations in the lysosomal/endosomal systems in tumor cells. A. Perinuclear distribution of tubular lysosomes at a neutral cytoplasmic pH. B. Peripheral distribution of vesicular lysosomes at an acidic cytoplasmic pH.

is reminiscent of the redistribution of lysosomal enzymes and proton pumps in osteoclasts (see above). Krepela *et al.* [96] have demonstrated that acidification of the cytoplasm of rat sarcoma cells induces greater release of cathepsin B from the more malignant of two sarcoma cell lines. Release of cathepsin B is not inhibitable by cycloheximide, indicating that the released cathepsin B is not newly synthesized. Nevertheless, the interpretation that a more acid cytoplasmic pH is responsible for the presence of cathepsin B in a plasma membrane/endosomal fraction in malignant tumors is not consistent with the density of the plasma membrane/endosomal fraction isolated on a Percoll gradient in our laboratory, i.e., 1.045 g/ml [37]. This density is indicative that the isolated fraction contains endosomes rather than lysosomes which have a density on Percoll of 1.07 g/ml (see also Ref. [97] for a further discussion of the changes in density and pH which accompany maturation of endosomes to lysosomes). Further studies using well-defined model systems will be needed to delineate the mechanisms responsible for redistribution and release of cathepsin B in malignant cells.

The fact that cathepsin B is released from tumor cells both as an active enzyme and a latent one and is present in membrane fractions as both an active enzyme and a latent one suggests that more than one mechanism is responsible for the redistribution and release of cathepsin B in malignant cells. Studies on release of cathepsin L by untransformed and transformed cells have established a diversity of steps, each of which can be independently regulated, at which the normal transport of this enzyme to lysosomes can be altered. These include enhanced synthesis resulting in saturation of the MPR pathway, low affinity of cathepsin L for the cation-independent MPR and redistribution of MPRs to the cell surface induced by platelet-derived growth factor [66, 93, 94]. Studies to date suggest that the mechanisms identified as responsible for induction of cathepsin L release may not extend to other lysosomal enzymes. Further study specifically on release of cathepsin B will be needed.

During the metastatic cascade tumor cells move through extracellular matrices containing collagens, glycoproteins and proteoglycans. We hypothesize that the presence of lysosomal proteinas-

es like cathepsins B and L and lysosomal glycosidases like β-hexosaminidase adjacent to the tumor cell surface may enable tumor cells to establish a local microenvironment in which focal degradation of connective tissues is enhanced. Both cathepsin B and cathepsin L have been shown to degrade isolated basement membrane components or intact basement membranes [8, 32, 62, 98]. Furthermore, the proteolytic degradation of basement membrane *in vitro* has been shown to be enhanced by concomitant degradation of carbohydrate by glycosidases [61]. Cathepsin B has activity against isolated basement membrane components at both acid and neutral pH [8, 32]. Thus, acidification of the local microenvironment is not necessarily required for cathepsin B to degrade basement membrane. Nevertheless, activity of cathepsin B against basement membrane components is optimal at acid pH. Establishment of an 'extracellular lysosome' may result in exclusion of extracellular CPIs from this microenvironment, thus preventing the inactivation of active cathepsin B by extracellular CPIs. Therefore it is possible that active cathepsin B released from malignant tumor cells that are adherent to the basement membrane may have a significantly longer half-life in this 'extracellular lysosome' than one would predict for active cathepsin B released from non-adherent tumor cells. The release of precursor forms of cathepsin B into this 'extracellular lysosome' will result in a pool or reservoir of stable cathepsin B available for subsequent activation. Timing of release of cathepsin B may be a critical factor in whether cathepsin B plays a role in malignant progression. Gingras *et al.* [99] have recently shown that transient alterations in the expression of proteolytic enzymes including the cysteine proteinase cathepsin L are critical for the selection of transformed tumor cells with an invasive phenotype. In their study, a transient increase in expression occurred after arrest and implantation in the lungs, i.e., after adherence to an extracellular matrix. Thus adherence to matrices may well be the rate-limiting step responsible for altering expression and trafficking of cysteine proteinases in cells capable of local proteolysis, i.e., in osteoclasts, macrophages and malignant tumor cells.

Conclusions

- Regulation of cathepsin B (synthesis, activity, intracellular processing/trafficking) is altered during malignant progression.
- Increased mRNA for cathepsin B correlates with increased malignancy.
- Increased activity of cathepsin B correlates with increased malignancy.
- Increased association of cathepsin B with membrane/endosomal fractions correlates with increased malignancy.
- Increased release of cathepsin B correlates with increased malignancy.
- Tumor stefin A, an endogenous cysteine proteinase inhibitor, has reduced inhibitory capacity (increased K_i's) against cysteine proteinases.
- Stefin A expression (mRNA and protein) is decreased during malignant progression.
- Data to date at both the molecular and protein levels supporting a functional role(s) for cathepsin B and its endogenous inhibitors in cancer progression are only correlative.

Key unanswered questions

- Does cathepsin B participate in any rate-limiting step(s) of malignant progression?
- Does cathepsin B play a causal role in development of the malignant phenotype?
- What are the mechanisms regulating alterations in cathepsin B (synthesis, activity, intracellular processing/trafficking) during malignant progression?
- What are the negative and positive regulatory elements on the cathepsin B gene?
- Does stefin A participate in any rate-limiting step(s) of malignant progression?
- Does stefin A play a causal role in development of the malignant phenotype?
- What are the mechanisms regulating alterations in stefin A (synthesis, activity, intracellular processing/trafficking) during malignant progression?
- What are the negative and positive regulatory elements on the stefin A gene?

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Address for offprints:
B.F. Sloane,
Department of Pharmacology,
Wayne State University,
540 E. Canfield,
Detroit, MI 48201, USA