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Unraveling the role of proteases in cancer

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Abstract

Investigators have been studying the expression and activity of proteases in the final steps of tumor progression, invasion and metastasis, for the past 30 years. Recent studies, however, indicate that proteases are involved earlier in progression, e.g., in tumor growth both at the primary and metastatic sites. Extracellular proteases may co-operatively influence matrix degradation and tumor cell invasion through proteolytic cascades, with individual proteases having distinct roles in tumor growth, invasion, migration and angiogenesis. In this review, we use cathepsin B as an example to examine the involvement of proteases in tumor progression and metastasis. We discuss the effect of interactions among tumor cells, stromal cells, and the extracellular matrix on the regulation of protease expression. Further elucidation of the role of proteases in cancer will allow us to design more effective inhibitors and novel protease-based drugs for clinical use.

Keywords: Cathepsin B; Cancer; Proteases; Tumor progression; Tumor invasion; Tumor-stromal interactions

1. Introduction

Tumor progression is a step-wise process: multiple alterations in a normal cell can occur which lead to a localized tumor, and finally to one that has the ability to invade and metastasize. Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis and migration of

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tumor cells through the proteolytically modified region [1]. Local proteolysis is facilitated by proteases outside the tumor cell, perhaps bound to the cell surface and/or secreted from the tumor cell. Recent data suggest that proteases inside the tumor cell also participate in local proteolysis by digesting phagocytosed extracellular matrix. In order for a cell to metastasize, it must be able to move into the vasculature (intravasate), survive in the circulation, arrest, move out of the vasculature (extravasate), invade into the surrounding tissue, and grow (see Fig. 1). Data accumulated over the past 15 years indicate that all of these steps involve interactions among tumor cells, stromal cells, invading lymphocytic cells, endothelial cells and the extracellular matrix. Proteases, which are expressed in these cells (Table 1), are believed to participate in many of these steps (see bold numbers in Fig. 1) [21–23]. To date, most investigators have focused on the role of proteases in the final steps of tumor progression: invasion and metastasis. Studies are now emerging which indicate that proteases are involved in tumor growth both at the primary and metastatic sites (Fig. 1, number 1) [24–27]. The dedication of a recent APMIS volume (vol. 107) to the

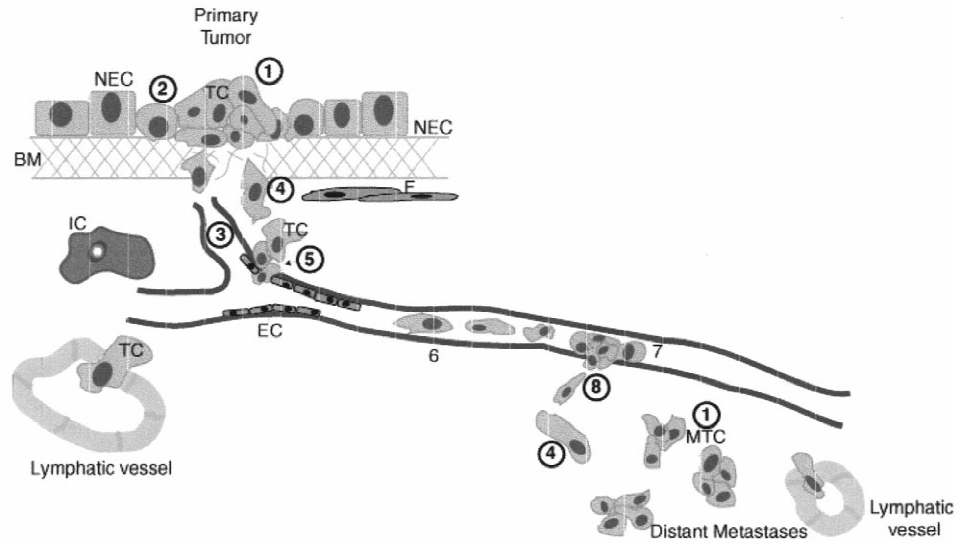

Fig. 1. The role of proteases in the metastatic process. Epigenetic changes that occur in normal epithelial cells (NEC) lead to tumor formation and growth **(1)**. Tumor cells (TC) also undergo epithelial-mesenchymal transformation **(2)** during this time. Formation of neovessels (angiogenesis) is stimulated where endothelial cells (EC) proliferate and invade towards the tumor site **(3)**. TC also invade the connective tissue **(4)** and then intravasate **(5)**. The TC must survive in circulation **(6)**, arrest (7), extravasate **(8)**, invade the local environment **(4)**, and grow (1) to set up distant metastases (MTC: metastatic tumor cell). These metastatic steps occur through the interaction of TC, EC, fibroblasts (F), invading inflammatory cells (IC), such as macrophages, and the extracellular matrix. Steps where proteases are believed to participate in this process are shown in bold.

Table 1
Expression of proteases and protease receptors in human breast and colon tumors

Cell types	Breast		Colon	
	mRNA	Protein	mRNA	Protein
Tumor cells	cathepsin D [2], uPA [2], uPAR [3]	cathepsin B [4], cathepsin D [4], stromelysin-1 [5], interstitial collagenase [5], MT1-MMP [6], uPA [7], uPAR [3]	Matrilysin [8], uPAR [9], MT1-MMP [10]	cathepsin B [11], cathepsin D [12], matrilysin [8], MT1-MMP [9], gelatinase-A [13], uPA [14], uPAR [15]
Fibroblast-like cells	uPA [16], stromelysin-1 [17], stromelysin-3 [17], gelatinase-A [17], interstitial collagenase [17], MT1-MMP [17]	cathepsin B [4], stromelysin-1 [5], interstitial collagenase [5], uPA [7], uPAR [3], annexin II [18]	gelatinase-A [13], uPA [9], MT1-MMP [10]	MT1-MMP [10], uPA [15,19], uPAR [10]
Inflammatory cells	gelatinase-B [17]	cathepsin B [4], uPA [7], cathepsin D [20], uPAR [3,7]	gelatinase-B [13], uPAR [9]	cathepsin B [11], MT1-MMP [10], uPAR [10]
Endothelial cells	gelatinase-B [17], uPAR [3]	cathepsin B[a], uPAR [3]	gelatinase-A [13], MT1-MMP [9]	uPA [19], MT1-MMP [10], uPAR [10]

[a] Lah T, unpublished data.

subject of proteases and protease inhibitors in cancer is indicative of the wide interest in this field of study.

Endopeptidases are categorized into five major classes: cysteine (e.g., cathepsins B, L, S, K, Q; caspases; bleomycin hydrolase), aspartic (e.g., cathepsins D and E), serine (e.g., urokinase-type plasminogen activator, plasmin, chymase), metallo- (e.g., gelatinases A and B, meprin) and threonine (e.g., proteasome) proteases. We will not review the literature on all these proteases, since there are several recent reviews (e.g. [21,22,28]). Cathepsin B will be used as an example in this review to address the following questions: Are these proteases active participants in tumor progression and metastasis? If so, when? Which cells are involved? How do interactions among different cell types and between cells and the extracellular matrix regulate tumor protease expression? Are we using the right model systems to study these proteases? All of these questions need to be considered in order to understand how proteases are involved in tumor progression and metastasis.

2. Cathepsin B and malignancy

Cathepsin B was the first lysosomal protease to be associated with breast carcinoma [29]. Subsequently, increased expression and activity and changes in localization including altered subcellular distribution, surface localization, and secretion of cathepsin B have been observed in many different tumors. Increased

expression and/or activity of cathepsin B is seen in breast [4,29], colorectal [11], gastric [30], lung [31], and prostate [32] carcinomas, gliomas [33], melanomas [34], and osteoclastomas [35], suggesting that this protease might be involved in the development, invasion and metastasis of more than one type of tumor. The increased expression of cathepsin B at the message level is associated with gene amplification (see below), increased transcription and stability of the message [36], and the possible use of multiple promoters and alternatively spliced transcripts [37]. An amplicon at 8p22, the locus of the cathepsin B gene [38], has recently been identified and associated with amplification and overexpression of the cathepsin B gene in esophageal adenocarcinoma [39]; amplification of the cathepsin B gene also has been found in transformed rat ovarian cells [40]. High cathepsin B levels have been associated with significantly shorter overall survival of colon cancer patients, implying a causal role in progression of colorectal tumors [11].

Interestingly, there is a change in cathepsin B localization in colon carcinomas [11]. The normal localization of lysosomes is at one pole of the nucleus in the region of the microtubule organizing center; therefore, cathepsin B staining would be predicted to be in this region. In normal colonic epithelium and early adenomas, cathepsin B staining is in the apical region, but during progression of colon cancer (late adenomas/early carcinomas) cathepsin B staining undergoes a redistribution to the inner surface of the basal plasma membrane. This staining is immediately adjacent to the underlying basement membrane. Changes in localization of cathepsin B seem to precede the increase in levels of cathepsin B protein, suggesting that alterations in trafficking of cathepsin B are independent of increased expression. A basal localization of cathepsin B has also been observed in thyroid cancer [41], and in vitro in several types of tumor cells [33,42,43].

Perhaps the most interesting alteration in the localization of cathepsin B is its association with the external cell surface [44,45]. Using a yeast two hybrid screen, our laboratory has identified a number of putative binding proteins for cathepsin B. One of these is the light chain of the annexin II heterotetramer [144]. The light chain of the annexin II heterotetramer also binds plasminogen [46], whereas the heavy chain binds tissue plasminogen activator (tPA) [47]. Annexin II has been localized to caveolae isolated from endothelial cells [48], although Stan et al. dispute this [49]. The urokinase (uPA) receptor has also been localized to caveolae of human melanoma cells [50]. Such a common locus could be of functional importance as cathepsin B has been shown to activate soluble or receptor bound pro-uPA [51,52] (see Fig. 2 and below). In ovarian cancer cells, inhibition of cell surface cathepsin B prevents activation of pro-uPA and thereby the invasion of the carcinoma cells through Matrigel [52]. A similar interaction may occur in colon carcinomas as we have identified a morphological and clinically aggressive subset of infiltrating nests of poorly differentiated

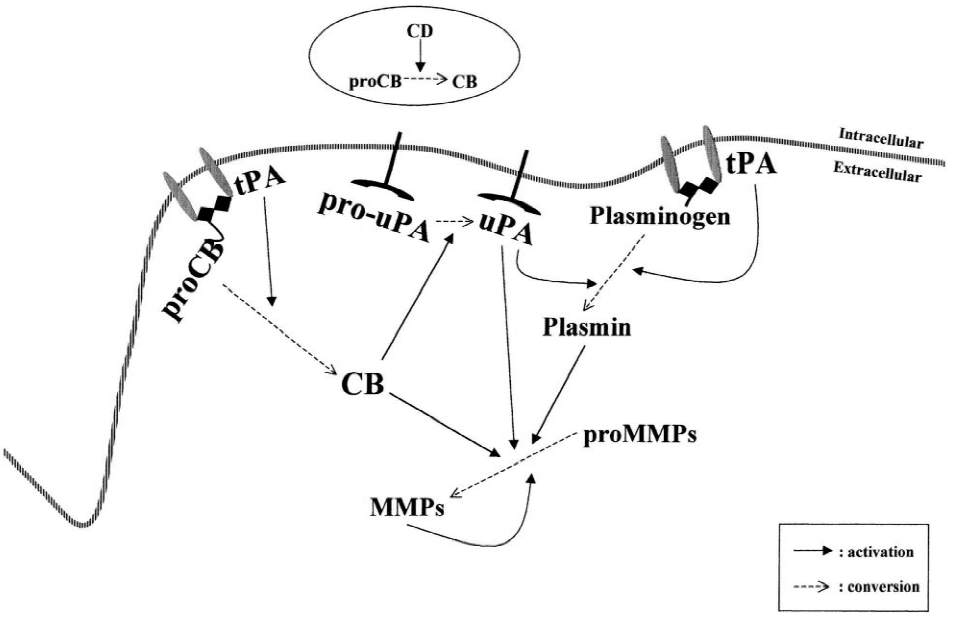

Fig. 2. Cascades of proteolytic activation. Procathepsin B (proCB) may be activated by tPA initiating a proteolytic cascade which results in the activation of plasmin and MMPs (see text for more detail). Collectively, active proteases can degrade all components of the extracellular matrix.

colon tumor cells that stain for cathepsin B and associated stromal cells that stain for uPA [53]. Eighty-three percent of this subset of colorectal tumors recur as compared to 51% of tumors expressing only cathepsin B or urokinase. These results also suggest that interactions between tumor cells and stromal cells can be important for the invasive phenotype of tumors.

3. Proteolytic cascades: regulating activation of proteases

What is the location of the proteases involved in cancer and how are they activated? Proteases are normally synthesized as latent forms, known as zymogens, which must be converted to mature, active enzymes. Other enzymes may convert the zymogen to active enzyme or this process may occur autocatalytically. Fig. 2 illustrates how proteases may interact, resulting in the activation of other proteases that collectively can degrade all extracellular matrix components. Procathepsin B can be activated by cathepsin D [54], tPA, uPA, cathepsin G and elastases [55,56]. Once activated, cathepsin B may activate pro-uPA [51,52], which then in turn can convert plasminogen to plasmin [57]. Plasmin is capable of degrading several components of the extracellular matrix (e.g., fibrin, fibronectin, proteoglycans, and laminin) and may activate MMPs

such as interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), gelatinase B (MMP-9), metalloelastase (MMP-12), and collagenase-3 (MMP-13) [58–62]. These MMPs can degrade many different components of the extracellular matrix, including collagen I and IV, gelatins and proteoglycans, and can activate other MMPs [22]. Cathepsin B, in addition to indirectly activating MMPs via the plaminogen activator/plasmin cascade, may also directly activate MMPs, e.g., interstitial collagenase and stromelysin-1 [63,64]. By activating stromelysin-1, cathepsin B could initiate a cascade of activation of interstitial collagenase and gelatinase A (MMP-2) [65,66]. Cathepsin B, therefore, may well be an important upstream regulator in the activation of pro-uPA/plasminogen and proMMPs. Taken together, the in vitro data suggest that there may be complex interactions between these proteases in vivo resulting in their activation. Overexpression and activation of one protease may provide a proteolytically active environment surrounding the tumor. In vitro assays, however, do not replicate the complex tumor environment and thus we are not sure if these interactions between proteases actually occur in vivo.

There is evidence which strengthens the hypothesis that these proteases interact in situ. In our studies on matrix degradation by living cells, we found comparable inhibition by a cathepsin B inhibitor and a uPA inhibitor [145]. Use of both did not further reduce degradation suggesting that one of the proteases is acting upstream of the other. Carmeliet et al. [62] utilized tPA-and uPA-deficient mice to demonstrate that aneurysm formation is dependent on uPA, but not on tPA. This in vivo data showed that decreased aneurysm formation in uPA-deficient mice is associated with decreased elastin degradation. Degradation of elastin is significantly increased by supplementation of plasminogen to the culture medium of macrophages from wild-type mice and tPA-deficient mice but not from uPA-deficient mice. Furthermore, metalloelastase-deficient macrophages could not digest elastin even when supplemented with plasminogen, although stromelysin-1- and gelatinase B-deficient macrophages could. These results strongly suggest that, in vivo, uPA-mediated activation of metalloelastase is necessary for elastin degradation, and thus, aneurysm formation. Since cathepsin B is an upstream regulator of uPA in vitro [51,52], it would be interesting to see if inhibition of cathepsin B would cause decreased aneurysm formation. Utilization of transgenic mice to study the role of proteolytic cascades in tumor development and invasion could greatly enhance our knowledge of in vivo interactions among these proteases.

4. Tumor–stromal interactions: regulating expression of proteases

The involvement of the stroma in malignancy was thought to be passive, but data has accumulated in the past 15 years suggesting that the stroma is an active

participant [67–69]. Numerous histological studies of tumor tissue indicate stromal cell involvement in the malignancy process. Table 1 uses breast and colon carcinoma to illustrate that proteases and their receptors are expressed in more than one cell type in these tumors. This is a 'table in progress' as the mRNA and protein levels of some proteases have yet to be examined in the cells comprising some of these tumors. The cellular location of proteases has been identified by detecting: message but not protein, protein but not message, or both message and protein. In order to understand the role of proteases in vivo, we need to determine the cellular location of message and protein in the same tumor tissues. Where protein and message have been examined, their cellular location may be the same or different. Gelatinase A message [13] is found in fibroblasts but the protein [13] is found in tumor cells, which also contain the receptor, MT1-MMP [9]. These results indicate that complex interactions between these cells may exist in vivo.

Direct and indirect interactions between tumor-cells and stromal cells can upregulate the expression of proteases in vitro. Expression of gelatinases A [70] and B [71] is increased in fibroblasts upon direct contact with tumor cells. The increased expression of gelatinase B in fibroblasts could not be induced by conditioned media from tumor cells [71], indicating that paracrine factors were not involved. This suggests that as tumor cells invade the basement membrane and migrate into the stroma, they may make direct contact with fibroblasts and increase protease expression in the fibroblasts. These fibroblasts may then assist in further matrix degradation. Other evidence indicates that the regulation of protease expression by epithelial–stromal cell interactions may be indirect and involve the action of diffusable factors [72–74]. Expression of uPA and MMPs such as collagenase-3, gelatinases A and B, and stromelysin-3 can be regulated by cytokines and growth factors, e.g., interleukin-1α and -1β, basic fibroblast growth factor (bFGF), epithelial growth factor (EGF), and plasma derived growth factor (PDGF) [22,74–77]. Cathepsin B and D expression can also be regulated by diffusable factors. Growth factors such as bFGF, EFG, insulin growth factor-1 (IGF-1) and insulin increase expression of cathepsin D in MCF-7 human breast carcinoma cells [78]. Lympho-cytokine granulocyte-macrophage colony stimulating factor increases the expression of cathepsin B in human U937 promonocytic cell line [79]. Thus, paracrine factors secreted from tumor cells and stromal cells may regulate expression of proteases in stromal cells and tumor cells, respectively. These in vitro studies indicate that increased expression of proteases in the tumor environment may involve indirect i.e., paracrine factors and/or direct interactions between tumor and stromal cells in vivo.

There is in vitro and in vivo data suggesting that cathepsin B expression is regulated by tumor–stromal cell interactions. Using immunofluorescence staining and enzyme histochemistry, Graf et al., found that cathepsin B is localized in

fibroblasts, leukocytes, and extracellular matrix at the invasive front of rabbit V2 carcinoma [80]. These investigators later found that an unidentified cytokine produced by rabbit V2 carcinoma increases cathepsin B synthesis and secretion by rabbit skin fibroblasts [81,82]. Increased expression of cathepsin B is also seen at the invasive edge of several different human tumors. Increases of cathepsin B mRNA, protein and/or activity are observed in tumor cells at the invasive edges of bladder [83], colon [53,84], gastric [Ren WP, Sloane BF, unpublished data] and prostate [32,85] carcinomas and in infiltrating glioblastoma cells [33]. The increase in cathepsin B expression at the invasive edge is not limited to the tumor cells, but is also seen in fibroblasts and invading macrophages at the invasive edge in breast [4], colon [11] and gastric [Ren WP, Sloane BF, unpublished data] carcinomas. The increase in cathepsin B expression in macrophages at the invasive edge may be due to diffusable factors as macrophage activators such as lipopolysaccharide and interferon increase cathepsin B expression [86]. Cathepsin B secretion also can be increased by tumor necrosis factor-α and interleukin I [87]. The markedly increased expression and activity of cathepsin B at the tumor-stromal interface suggests that interactions between the tumor and surrounding stroma may upregulate cathepsin B expression.

The interaction of extracellular matrix with epithelial, endothelial, and fibroblast cells regulates various functions including: apoptosis, cell proliferation, polarity, differentiation, adhesion, migration, and tumorigenicity [88–92]. Recent studies have found that extracellular matrix components, e.g., vitronectin, fibronectin, laminin and collagen I also regulate the expression and activation of several proteases including uPA and MMPs in epithelial cells, tumor cells, fibroblasts and macrophages [93–97]. Similar observations have been made for cathepsin B. Singhal et al. [98] found that expression of cathepsin B increases when mesangial cells are grown on Matrigel. Our laboratory is currently examining the role that collagen I plays in regulation of cathepsin B expression. Human breast fibroblasts isolated from tissue adjacent to breast carcinomas were grown on collagen I gels. Although there were morphological difference between fibroblasts grown on collagen I gels and those grown on plastic, the subcellular localization and intracellular levels of cathepsin B were not changed. In contrast to the unaltered intracellular levels of cathepsin B, increased cathepsin B secretion was seen from fibroblasts grown on collagen I, but not from those grown on plastic [Koblinski JE, Sloane BF, unpublished data]. Thus, the interaction of breast fibroblasts with collagen I preferentially increases expression of cathepsin B. We are currently investigating whether interaction of integrins with collagen I mediates this increased expression/secretion of cathepsin B.

There is indirect evidence that changes in integrin expression can result in secretion of cathepsin B. 12-(S)-HETE, which can upregulate surface expression

of integrins [99], induces secretion of cathepsin B from transformed cells and tumor cells [100]. Expression of other proteases can be regulated by interaction of integrins with extracellular matrix components. Interaction of $\alpha_2\beta_1$ with collagen I mediates signals through activation of protein kinase C-ζ, resulting in increased expression of interstitial collagenase in fibroblasts [94,101,102]. The role that the extracellular matrix plays in regulating the expression of proteases in vivo needs further study. By examining interactions of tumor cells, fibroblasts, and macrophages with extracellular matrix components, we can come closer to mimicking the tumor environment in situ.

5. Matrix degradation: intracellular and extracellular

A common belief is that proteases degrade extracellular matrix during cell invasion. Cathepsin B can degrade components of the extracellular matrix (laminin, fibronectin and collagen IV) [103,104] and intact basement membrane in vitro. Whether these components are degraded outside the cell by cathepsin B at neutral pH or are taken up into the cell and then degraded by this protease has yet to be determined in vivo. In vivo studies have found that there is an inverse correlation between cathepsin B staining and basement membrane (type IV collagen or laminin) staining in bladder ([83]; Visscher DW and Sloane BF, unpublished data), gastric [105], lung [31] and colon [106] carcinomas. This would be consistent with a functional role for cathepsin B in degrading these extracellular matrix components in vivo.

Cathepsin B is active against large substrate components such as laminin and fibronectin at physiological conditions of pH (neutral pH) and temperature (37°C) [103], and consequently could degrade extracellular matrix components outside the cell. Additionally, the extracellular pH around tumors is generally more acidic than in corresponding normal tissue [107]. Montcourrier et al. have also found that breast cancer cells can liberate protons in the extracellular milieu through lactic acid production and a functional H^+/ATPase pump at the plasma membrane level reducing the extracellular pH to 5.5 [108]. Tumor cells may therefore acidify the microenvironment around the tumor resulting in activation of secreted proforms of cathepsin B as found in osteoclasts during bone resorption (for review see [109]).

Intracellular degradation of extracellular matrix also seems to be important for tumor cell invasion. Coopman et al. have shown a correlation between the invasive ability of different cancer cell lines and their ability to phagocytosis the extracellular matrix [110]. Another study found that large acidic vesicles, identified as heterophagosomes [111], are present more frequently in breast cancer cells that have migrated through Matrigel [112]. These large acidic vesicles can phagocytose extracellular matrix and digest this material within the

heterophagosome. Heterophagosomes contain mature cathepsin D and have been seen in human breast carcinoma cells in vivo [111–113]. We also have seen very large vesicles that stain for cathepsins B and D in breast carcinoma cell lines [Koblinski JE, Sameni M, Sloane BF, unpublished data].

A novel assay has recently been developed in our laboratory that allows us to visualize matrix proteolysis by living cells during invasion through a three-dimensional gelatin matrix [146]. In this assay, cells are grown on a gelatin matrix containing a quenched fluorescence substrate (DQ-substrate). This substrate fluoresces when cleaved by proteases. Using this assay, we have observed that transfection of Rat1 fibroblasts with an activated form of the GTP-binding protein Rac1 increases the intracellular accumulation of degraded DQ-BSA [145]. This accumulation is significantly reduced by treatment of cells with CA074Me, a highly selective intracellular inhibitor of cathepsin B, thus indicating that DQ-BSA is degraded by intracellular cathepsin B. Increased endocytosis, namely phagocytosis and macropinocytosis is responsible for the increased accumulation of degraded DQ-BSA in Rac1 transfectants. Proteolytically-active cells may facilitate invasion by other cells as invasive cells are attracted into 'cleared' tracks in a 3-dimensional laminin matrix [114]. The track in this case is formed by other invasive cell(s), acting as plows to move the extracellular matrix aside. Perhaps, degradation of extracellular matrix components by cathepsins B and D in endocytic vesicles clears the way for other migrating cells that do not express these proteases.

6. Protease involvement in premalignant lesions

Is it possible that proteases play a role in cancer other than degradation of the extracellular matrix? Several well known examples suggest that MMPs are involved in early alterations leading to tumor formation. Mice deficient in stromelysin-3 exhibit lower tumor incidence and tumor size after carcinogen treatment [115]. Overexpression of stromelysin-1 in mice is sufficient to generate preneoplastic and malignant mammary gland lesions [25]. Matrilysin also plays a role in tumor development [26,116]. The Min mouse model of familial adenomatous polyposis carries a mutation in the Apc gene that results in the development of spontaneous intestinal polyps [117]. When matrilysin-deficient mice are crossed with Min mice, the number and size of tumors are significantly reduced [26]. Collectively, these results suggest that MMPs participate in stages of tumor progression prior to invasion and metastasis. As mentioned earlier amplification and overexpression of cathepsin B is found in esophageal adenocarcinoma [41]. Amplification is present in stage I adenocarcinomas and in 5% of Barrett's esophagus, a premalignant lesion. The increased expression of cathepsin B in Barrett's esophagus suggests that this

enzyme, like matrilysin and stromelysin, may have a role in the transition of premalignant lesions to malignant tumors.

In general, alterations in cathepsin B levels and localization are more dramatic in malignant than pre-malignant lesions; however, they have been reported in several pre-malignant tumors including those of colon, thyroid, glioma, liver, breast, prostate, and ovarian (Table 2). These alterations include overexpression of mRNA and protein, redistribution from the perinuclear region to the cell periphery and plasma membrane, and increased secretion [11,33,39,42,106,118–124].

Perhaps cathepsin B increases cell proliferation and thereby tumorigenesis. The lysosomal cysteine protease inhibitor 9-fluorenylmethyloxycarbonyl-tyrosylalanyl-diazomethane, which targets intracellular cathepsin B and an unidentified 39 kDa-protein, inhibits the growth of SK-Br-3 and MCF-7 breast cancer cell lines [125]. Increased cathepsin B secretion parallels increases in proliferation of urinary bladder epithelial cells and endometrial cells [126], and

Table 2
Cathepsin B in pre-malignant lesions

System	Observation	Reference
Colon	Increased activity in sera of human benign tumors[a]	[118]
	Increased immunostaining and altered localization in human late adenoma	[11]
	Increased immunostaining in adenomas with high-grade dysplasia as compared to adenomas[a]	[106]
	Elevation of activity in DMH-induced rat colon tumors[a]	[119]
Thyroid	Increased activity in human benign tumors	[120]
Glioma	Increased mRNA, immunostaining, and activity in pre-invasive human tumors[b]	[33]
	Increasing peripheral immunostaining during tumor cell progression	
Liver	Elevated protein levels in sera of human pre-malignant diseases[a]	[121]
Breast	Increased cathepsin B activity in human fibroadenomas[b]	[122]
	Increased protein and membrane association and peripheral redistribution in pre-malignant human Ha-ras-transfected MCF-10A cells	[42]
Ovarian	Elevated activity in sera of human benign cysts[a]	[123]
Esophagus	Amplification of cathepsin B gene and increased mRNA levels in Barrett's esophagus	[39]
Prostate	Elevation of immunostaining in human pre-malignant prostatic intraepithelial neoplasia (PIN)	[124]
Pancreas	Elevated protein levels in sera of human pre-malignant diseases[a]	[121]

[a] Significant.
[b] No statistical analyses were performed.

leupeptin, a cysteine protease inhibitor, reduces intracellular cathepsin B activity, DNA synthesis and cell number in neoplastic cervical epithelial cells. Leupeptin also reduces the size of rat colon tumors [119]. High cathepsin B activity had been shown to correlate with the larger sized rat colon tumors. Van Noorden et al. [127] recently showed that experimental liver metastases of colon cancer cells can be reduced with a selective inhibitor of extracellular cathepsin B. Treatment of these colon cancer cells, which have high surface cathepsin B activity, with Mu-Phe-homoPhe-fluoromethylketone resulted in a 60% reduction in the number of tumors and a 80% reduction in their volume. These studies suggest that cathepsin B may play an important role in tumor growth.

One way cathepsin B may increase cell proliferation is by activating growth factors or liberating them from the extracellular matrix where they are sequestered. Growth factors such as bFGF, EGF, IGF, transforming growth factor-β (TGF-β) and vascular endothelial growth factor are bound to extracellular matrix and can be released upon proteolysis of extracellular matrix components [128,129]. For example, the ability of fibroblasts to promote tumorigenicity of MCF7 cells requires Matrigel containing low-molecular-weight factors [130,131], and MMP inhibitors abolish the tumor-promoting effects of the fibroblasts [131], suggesting that MMPs from the fibroblasts release growth factors from the Matrigel. Similarly, cathepsin B could release growth factors from the extracellular matrix or directly activate growth factors. Oursler et al. [132] found that treatment of normal human osteoblast-like cells with dexamethasone increases cathepsin B secretion in parallel with increased activation of TGF-β. These investigators also showed that cathepsin B can activate TGF-β in fetal calf serum or conditioned media of the osteoblast-like cells. Cathepsin B may directly activate TGF-β or it could be involved in releasing TGF-β from the extracellular matrix so that it can then act on cells. TGF-β can bind to several matrix proteins including fibronectin and type IV collagen [133,134], both of which can be degraded by cathepsin B [103]. Whether cathepsin B can release or activate other growth factors has not been studied.

Neovascularization or angiogenesis, a process enhanced by proteases, is necessary for tumors to grow beyond 1–2 mm [135]. Intense staining for cathepsin B is present in endothelial cells of neovessels but not in pre-existing microvasculature in human breast [Lah T, unpublished data] and prostate [136] carcinomas and human gliomas [137]. In situ hybridization studies have also demonstrated cathepsin B expression in endothelial cells of neovessels in gastric tumors [Ren WP and Sloane BF, unpublished data]. When grown on Matrigel, rat endothelial cells differentiate into a cord-like structure that stains intensely for vesicular cathepsin B [138]. Whether cathepsin B participates in or is only a marker for neovessel formation has yet to be elucidated. One belief is that cathepsin B may help degrade the extracellular matrix in order for endothelial

cells to migrate. Cathepsin B could also regulate the bioavailability of angiogenic factors such as TGF-β (see above), which has been shown to be critical for proper development of the vascular structure [139].

7. Novel use of protease overexpression in the tumor environment

Protease inhibitors are already being tested as anti-tumor agents in the clinic. Some investigators are targeting the overexpression of proteases in tumors by developing novel clinical prodrugs which are inactive until activated by a protease. For example, normal cytotoxic agents such as adriamycin can be rendered inactive by peptide chains that are substrates for cysteine proteases, e.g., cathepsin B. In vitro studies show that cathepsin B associated on the surface tumor cells can cleave this peptide thus releasing adriamycin and killing the tumor cells [Linebaugh B, Firestone R, Sloane BF, unpublished data]. Another example of a drug that can be converted to its active form by cathepsin B is α-hemolysin. Panchal et al. [140] engineered α-hemolysin so that a cassette containing cathepsin B recognition sites is flanked by a peptide that inactivates α-hemolysin. This engineered prolysin can kill tumor cells once activated. As predicted, it is effective against tumor cells that express high levels of cathepsin B. Similar strategies could be used to design prodrugs from other tumor proteases. The hope is that such drugs will only be activated at the tumor site where protease activity is increased, increasing tumor cell kill and reducing the toxic side effects of these drugs.

Weisssleder et al. [141] have utilized the knowledge that tumor cells have increased proteolytic activity and endocytosis to develop a novel in vivo imaging system for tumors. These investigators used an optically quenched near-infrared fluorescence (NIRF) imaging probe that will generate a strong NIRF signal after enzyme activation. The NIRF probe consists of a graft copolymer with 44 unmodified lysines on the backbone and, therefore, has sites for cleavage by enzymes with lysine–lysine specificity. Intracellular NIRF can be detected in tumor cells in culture. Fluorescence is generated by cleavage of the lysine backbone by lysosomal cysteine and serine proteases. Cysteine protease inhibitors such as E64, trypsin inhibitors, and trypsin-like serine protease inhibitors can inhibit NIRF generation, whereas pepstatin, a cathepsin D inhibitor, cannot. Implanted breast tumors in mice are detected within 24 h after the NIRF probe is injected. Even microscopic tumors less than 300 μm in diameter can be detected. This represents a new strategy for applying the knowledge that protease expression and endocytosis are increased in tumor cells. This imaging system could be modified for other applications. Specific peptides could be added to the carrier backbone which would then allow only a certain protease to cleave the peptide and release the fluorescence. Once these NIRF

probes are made they could be used in vivo to determine which proteases are key to tumor growth and invasion, arthritis, etc. In addition, imaging techniques like this could provide the much-needed clinical verification that protease inhibitors are reaching and reducing the activity of their intended target in situ.

8. Consortium to study protease involvement

Proteases have been widely studied for their roles in tumor growth, invasion and metastasis; however, we still have not answered key questions relating to the use of protease inhibitors for therapeutic intervention. To advance the understanding of proteolytic enzymes as therapeutic targets for cancer, Drs. Lynn Matrisian, Thomas Bugge, James Quigley, and Bonnie Sloane have formed a protease consortium. The goal of the consortium is to utilize the expertise of the consortium members to look at several different proteases in a few well-defined tumor model systems. These investigators will try to answer the following questions: Is one protease or one class of proteases more important in specific tumors and at specific steps in tumor progression than another? Do extracellular proteases co-operatively influence matrix degradation and tumor cell invasion through proteolytic cascades, or do individual proteases have distinct influences on tumor growth, invasion, migration and angiogenesis? If proteolytic cascades are important, can an upstream protease be targeted so only one protease inhibitor is needed? Will selective inhibitors be able to decrease tumor growth and metastasis, possibly decreasing the side effects from broad spectrum protease inhibitors? Currently synthetic inhibitors of MMPs are being tested in clinical trials [142]. Side effects are arising, in part due to the broad specificity of these MMP inhibitors. The lack of MMP inhibitors that are highly selective for individual MMPs makes it difficult to use these inhibitors to investigate the role of individual MMPs. This is an area of intense research development and recently, Koivunen et al. [143] reported the use of libraries of random peptides to isolate a cyclic decapeptide that selectively inhibits gelatinases A and B. This peptide suppresses migration of both tumor cells and endothelial cells in vitro, homes to tumor vasculature in vivo and prevents the growth and invasion of tumors in mice.

One of the problems in sorting out the role that proteases play in tumor growth and invasion is that many different model systems are used with little overlap between investigators. The consortium members determined that an optimal assortment of model systems would be needed and evaluated model systems based on the following criteria: (1) multiple stages of tumor progression can be examined, (2) tumor cells are in an orthotopic environment, (3) tumors grow at organ sites of most prevalent human cancers, and (4) multiple classes of proteases are expressed. The first model systems to be studied will be breast, colon and lung. The consortium members hope to translate the information

accrued into clinical trials of protease inhibitors with the ultimate purpose of benefiting the cancer patient. Consortiums bringing investigators together from different areas of expertise to analyze the same model systems should enhance our knowledge of the function of proteolytic enzymes in cancer.

9. Conclusions

Currently we know that increased expression, increased activity and altered localization of many proteases are associated with tumor progression. Cathepsin B is just one of these proteases. The redistribution of cathepsin B within tumor cells as well as the increased expression in tumor cells adjacent to the extracellular matrix suggest that proteases can be mobilized to regions of tumor cell invasion. Traditionally it was thought that only proteases outside the tumor cell were important in tumor cell invasion, but recent data suggest that intracellular proteolysis is also important. In vitro and in vivo studies have shown that protease inhibitors can reduce the invasive and metastatic capabilities of tumor cells. The effect of protease inhibitors on tumor invasion could be direct due to inhibition of extracellular matrix proteolysis or indirect due to inhibition of activation of a proteolytic cascade. Proteases may also play a role early in malignant conversion, perhaps by increasing tumor growth through activation of growth factors and/or by facilitating angiogenesis. The use of transgenic animals is helping to elucidate the in vivo role of proteases. We need to remember that tumor cells are only one part of the tumor environment, extracellular matrix components and stromal cells are important contributors to the proteolytic activity of tumors. Collaborations like the protease consortium that will analyze multiple proteases in a single tumor should further our understanding of how proteases are involved in tumor progression as well as help us to design better inhibitors and novel protease-based drugs for clinical use.

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