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Perspective

Cysteine Cathepsins and the Cutting Edge of Cancer Invasion

Vasilena Gocheva[1,2]

Johanna A. Joyce[1,*]

[1]Cancer Biology and Genetics Program; Memorial Sloan Kettering Cancer Center; New York, New York USA

[2]Weill Graduate School of Medical Sciences; Cornell University; New York, New York USA

*Correspondence to: Johanna A. Joyce; Cancer Biology and Genetics Program; Memorial Sloan Kettering Cancer Center; Box 372, New York, New York 10021 USA; Tel.: 646.888.2048; Fax: 646.422.0231; Email: joycej@mskcc.org

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ABSTRACT

Cysteine cathepsins are a family of lysosomal proteases that are often upregulated in various human cancers, and have been implicated in distinct tumorigenic processes such as angiogenesis, proliferation, apoptosis and invasion. During cancer progression, cathepsins are often translocated to the cell surface of tumor cells or are secreted into the extracellular milieu, where they can promote tumor invasion through several possible mechanisms. First, they can directly cleave components of the extracellular matrix and basement membrane, essentially clearing a path for the migration of tumor cells away from the primary tumor mass. Second, at the cell membrane, cathepsins can direct a proteolytic cascade in which they activate other proteases such as matrix metalloproteinases and urokinase plasminogen activator, which in turn promote invasion. Finally, cleavage of the cell adhesion protein, E-cadherin, at the cell surface can disrupt adherens junctions and thus facilitate cancer cell migration and invasion. Therefore, cathepsins are now emerging as major players in tumor progression, making them potential drug targets for a wide range of human cancers.

Metastatic progression still remains the biggest challenge facing the development of effective therapies for the treatment of cancer today. In fact, the majority of patients with solid tumors die not because of their primary tumor burden, but due to the formation of metastases at distant sites. Metastasis is a complex process consisting of a series of discrete steps, leading to the spread of malignant cells to new locations, typically far from where the primary cancerous mass formed. The first step in the process is invasion of tumor cells into the surrounding tissue, resulting from alterations in cell-cell adherence, changes in anchorage to the stroma and modifications to the extracellular matrix (ECM). Proteolysis plays a crucial role in this initial step of invasion through the cleavage of proteins involved in mediating adherence to neighboring cells, such as E-cadherin, thereby promoting the ability of solitary cancer cells to break away from the bulk of the tumor and migrate. Additionally, protein degradation can promote invasion when it is directed toward components of the ECM itself, thus creating a path for the invading tumor cells and facilitating their access to the circulation. Local invasion is then followed by limited degradation of the basement membrane (BM), a specialized sheet of ECM underlying all epithelial cells, and intravasation into the blood or lymphatic vessels. Again, proteases play important roles in allowing the migrating cells to traverse the vessel walls and enter the circulation, where they are transported until the metastatic destination is reached. Tumor cells that are endowed with the ability to survive in the circulation and extravasate at the secondary location may survive to establish a metastatic lesion. Proteolysis can help the cancer cells at this step as well by remodeling the tissue at the metastatic site through cleavage of ECM components, which not only facilitates expansion of the tumor mass, but also stimulates tumor growth, invasion and angiogenesis through the release of growth factors embedded in the surrounding matrix.

Given the critical importance of proteolysis at multiple stages in the metastatic cascade, identifying the precise mechanisms and key players that contribute to this process should further the development of novel therapeutic agents and treatment regimens. Several protease families have been implicated in invasion due to their ability to directly degrade components of both the basement membrane and the surrounding ECM, thereby allowing cancer cells to escape the primary tumor mass. Until recently, most of the attention in the field of proteolysis and cancer was focused on the matrix metalloproteinases (MMPs)—a family of 23 endopeptidases that have been implicated in many aspects of tumor progression including invasion and metastasis.[1,2] The serine protease urokinase plasminogen activator (uPA) has also been associated with cancer spread through the activation of a

proteolytic cascade, ultimately resulting in ECM degradation.[3] By comparison, relatively little is known about the contribution of cysteine proteases, such as cathepsins, to cancer. In recent years there have been several reports describing their involvement in distinct tumorigenic processes in vivo that require proteolysis; namely angiogenesis, invasion and metastasis,[4-8] and it is now becoming clear that cysteine cathepsins play pivotal roles in cancer progression.

The human cysteine cathepsin family comprises 11 members (Cat B, C, F, H, L, K, O, S, V, W, X/Z), all of which share a conserved active site that is formed by cysteine and histidine residues.[9] Some of the proteases are endopeptidases; others are exopeptidases, while some possess both enzymatic activities,[10] resulting in potentially distinct substrate preferences for individual cathepsins. All cathepsins are synthesized as inactive precursors, which are typically activated in the acidic environment of the lysosomes. Historically, the major function of cathepsins was thought to be restricted to terminal protein degradation in the lysosomes, which is not an exclusive property of any one cathepsin. However, the results from generation of knockout mice have revealed additional physiological roles for specific cathepsins. For example, cathepsin S was shown to be important for MHC class II-associated antigen processing and presentation.[11] On the other hand, mice deficient for *cathepsin L* have epidermal hyperplasia, periodic hair loss and develop cardiomyopathy with age, indicating the importance of that particular protease in skin morphogenesis, hair cell cycle and heart function.[12,13] Cathepsin C is known to be a processing enzyme involved in the activation of several serine proteases such as granzyme A and B; thus mice in which this cathepsin is deleted have defects in cytotoxic T cell-induced apoptosis.[14] The phenotype of *cathepsin B* null mice is very subtle and these animals can only be distinguished from their littermates when subjected to experimental pancreatitis and liver injury, to which they are resistant.[15,16] Although all of the individual *cathepsin* knockout mice engineered to date are viable and fertile, when mice are mutant for both *cathepsin B* and *L*, they die shortly after birth with severe brain atrophy associated with selective neuronal loss in the cerebral cortex and in the cerebellar Purkinje and granule cell layers,[17] implicating redundant actions of both enzymes in the maturity and integrity of the postnatal central nervous system.

In addition to their involvement in normal physiological processes, several cathepsins have also been implicated in cancer progression. Many studies have reported the increased expression, activity and mis-localization of individual cathepsins in human cancers. Cathepsin upregulation has been reported in cancers of both epithelial and mesenchymal origin, including breast, brain, lung, gastrointestinal, colorectal cancer and melanoma among others (reviewed in refs. 18 and 19). Moreover, increased expression of certain cathepsins has diagnostic and prognostic value in several types of cancer. For example, elevated cathepsin B expression correlates with poor prognosis in lung, breast, ovarian, brain, head and neck cancer and melanoma (reviewed in ref. 19). Similarly, increased cathepsin L activity has been observed in multiple tumor types and can be used as a prognostic indicator of shorter survival rates in patients with breast, colorectal and head and neck cancers.[18] Recently, we found that both cathepsin B and L are also implicated in human pancreatic endocrine tumors.[6] Using a tissue microarray composed of 80 lesions, including primary pancreatic endocrine neoplasms and associated metastases, we demonstrated that these two proteases are progressively upregulated as pancreatic cancers become more malignant. Cathepsin B expression was particularly intense in invasive cancer cells as well as in pancreatic tumor cells that had metastasized to the lymph nodes, suggesting a role for this protease in the metastatic cascade. Cathepsin B has been observed at the invasive tumor margin in samples from patients with other cancers as well,[20,21] indicating that members of the cysteine cathepsin family are not innocent bystanders in the tumorigenic process, but are actively involved in promoting cancer cell invasion and metastasis.

Following these initial observations, numerous studies have established the importance of some members of the cathepsin family in mediating tumor invasion using in vitro assays. Most reports have focused on cathepsins B and L since these are also the two members of the family most commonly found to be upregulated in human cancers. Downregulation of cathepsin B expression using siRNA in several different cancer cell lines resulted in decreased motility and invasion, as assessed by Matrigel assays.[22,23] Similarly, inhibition of cathepsin activity using broad spectrum or cathepsin B-specific inhibitors also diminished invasion in vitro.[24,25] Conversely, overexpression of cathepsin B in a weakly metastatic melanoma cell line increased invasion through reconstituted Matrigel ECM at least three-fold.[23] Analogous studies have been performed for cathepsin L and yielded similar results. Human melanoma cells stably transfected with a single chain variable segment from an anti-cathepsin L monoclonal antibody showed impaired invasiveness in Matrigel as well as diminished metastatic properties in an experimental lung metastasis assay.[26] Downregulation of cathepsin L by RNAi in a glioma cell line also decreased invasion in transwell Matrigel assays.[27] These studies have collectively confirmed the clinical correlation between elevated expression of certain cathepsins and advanced malignancy in multiple cancers.

While such reports have consistently documented a role for cathepsins B and L in invasion in vitro, functional analyses of cathepsin involvement in tumorigenesis in animal models have been limited. Several recent studies using mouse models of cancer have now provided critical in vivo evidence supporting the role of cathepsin family members in promoting cancer progression and invasion. Cathepsins B, C, H, L, S and X were shown to be upregulated in the RIP1-Tag2 mouse model of pancreatic islet cell cancer using microarray expression profiling.[4] Furthermore, using a fluorescently tagged probe to detect the active form of the proteases, it was determined that there was an increase in the activity of cathepsins in vivo, particularly at the invasive tumor margins. Treatment of mice with a broad-spectrum cathepsin inhibitor led to a significantly reduced tumor burden and a decrease in the invasiveness of the resulting lesions. This study was extended to a mouse model of human cervical cancer, in which the human papillomavirus 16 oncogenes are expressed under the keratin 14 promoter, where a comparable increase in cathepsin activity was visualized in vivo.[4] Similarly, in a conditional K-ras^{G12D} mouse lung adenocarcinoma model, upregulation of cathepsin B, H and L was observed at the protein level in the tumors, while expression in normal tissues was below the level of detection.[28] These reports have demonstrated that the upregulation observed in human cancer samples is replicated in mouse models, thus warranting the use of such experimental systems to investigate cathepsin involvement in tumorigenesis.

While the above studies showed the general importance of the cathepsin family in cancer progression in animal models, an essential question that remained was which of the eleven cathepsins are the key players in tumorigenesis. In particular, the individual importance of these proteases in key processes such as tumor cell proliferation, apoptosis, angiogenesis and invasion was unclear. We proposed a model that integrated the stage-specificity of increased expression

Table 1 **Summary of the effects of *cathepsin* deletion on RT2 tumorigenesis**

	Cat B$^{-/-}$ RT2	Cat C$^{-/-}$ RT2	Cat L$^{-/-}$ RT2	Cat S$^{-/-}$ RT2
Angiogenic switching	24% decrease	No change	No change	24% decrease
Tumor volume	72% decrease	No change	88% decrease	47% decrease
Tumor microvascular density	56% decrease	No change	No change	48% decrease
Apoptosis	229% increase	No change	337% increase	164% increase
Cell proliferation	44% decrease	No change	58% decrease	No change
Tumor invasion	Significant reduction	No change	Significant reduction	Significant reduction

of individual cathepsins, with the tumorigenic processes required at these different steps in cancer progression. This allowed us to make a number of predictions regarding the possible function(s) of individual cathepsins, based on their temporal and spatial expression.[5] We recently tested this model using a genetic approach to determine the individual functions, if any, of cathepsin B, C, L and S in cancer.[6] We crossed mice null for each of the four cathepsins to RIP1-Tag2 (RT2) mice to determine the role of each protease in pancreatic islet cell cancer. We showed that RT2 mice null for *cathepsin B*, *L* or *S* had a significantly reduced tumor burden, as summarized in (Table 1). We found that deletion of *cathepsin B* or *S* reduced tumor angiogenesis, while *cathepsin B* or *L* knockouts had decreased tumor cell proliferation. Critically, deletion of any one of the three cathepsins resulted in decreased tumor invasion with a shift towards more benign lesions.[6] The importance of cathepsin B in tumor growth and metastasis has also been established in the polyoma middle T antigen (PyMT) mouse model of mammary cancer.[7] It was determined that deleting one or both alleles of *cathepsin B* in MMTV-PyMT mice delayed mammary tumor growth and decreased the formation of lung metastasis. Interestingly, cathepsin X, another member of the cathepsin family, was found to partially compensate for the absence of cathepsin B, and its inhibition by a neutralizing antibody caused an even further reduction in invasion of *cathepsin B* null PyMT cells in culture. Therefore, while individual cathepsins clearly contribute to tumor growth and metastasis, collectively inhibiting several pro-tumorigenic family members might prove to be a more successful therapeutic strategy in potentially treating human cancers. However, more research is needed to determine the general applicability of these findings, for example by investigating a wider range of preclinical models, before the administration of broad-spectrum or specific inhibitors in a clinical setting.

The contribution of cathepsins to invasion in both human and murine cancers is well documented, although the precise mechanisms through which cathepsins are exerting their effects is still under active investigation. In normal cells, cathepsins are typically localized to lysosomes in the perinuclear region, whereas during cancer development they are often translocated to the cell surface or secreted (reviewed in Refs. 29,30). At the cell surface, cathepsins can activate other proteases, thereby affecting invasion indirectly by participating in proteolytic cascades (Fig. 1A). For example, cathepsin B has been shown to directly activate MMP-1 and MMP-3,[31] which in turn can cleave components of the ECM such as collagens, gelatin and tenascin, thus facilitating the migration of tumor cells through the extracellular space. Cathepsin B and L can also convert the precursor form of urokinase-type plasminogen activator (uPA) to the active enzyme, which once activated catalyzes the cleavage of plasminogen into plasmin.[32,33] Plasmin, a broad-spectrum serine protease, directly degrades ECM components and activates MMPs thereby promoting cell invasion and cancer spread.[34] Therefore it is not surprising that collective inhibition of several of these enzyme classes would be more effective than blocking a single protease. Indeed, RNAi-mediated inhibition of both cathepsin B and MMP-9, or cathepsin B and the uPA receptor, reduced in vitro invasion of a glioblastoma cell line.[35,36]

Besides their participation in proteolytic cascades, it has been known for some time that cathepsins can also directly cleave components of the BM/ ECM such as laminin,[37] fibronectin,[38,39] tenascin-C[40] and type IV collagen,[38,41] which leads to limited proteolysis of the ECM (Fig. 1B). Most cathepsins require slightly acidic pH for optimal activity,[42] but since the extracellular microenvironment of tumors is generally acidic from the excessive production of cellular acid, primarily in response to hypoxia,[43] these proteases can still function even when secreted into the extracellular space. Indeed, reduced pericellular pH has been shown to cause the redistribution of vesicles containing cathepsin B toward the cell periphery in macrophages and fibroblasts, leading to increased secretion of the active form of the enzyme.[44] Once at the cell surface or the extracellular space, cathepsins have easy access to their ECM substrates, although some studies have demonstrated that degradation of ECM components can also occur intracellularly, thus not requiring translocation of cathepsins to the outer membrane. For example, type IV collagen cleavage by cathepsin B was visualized intracellularly in human breast cancer cell lines as well as human glioma cells by using a quenched fluorescent type IV collagen matrix (DQ-collagen IV).[45,46] Moreover, treatment of the cells with a membrane-permeant cathepsin B specific inhibitor decreased intracellular collagen degradation by nearly 90%, showing that ECM components could also be degraded within cancer cells.[45] The importance of intracellular cathepsin activity has been confirmed by other reports, where using membrane permeable inhibitors was most effective in decreasing the invasion of human melanoma and prostate carcinoma cell lines.[23] Whether cathepsins are acting primarily inside or outside of the cell to promote invasion and metastasis in human cancers still remains unclear.

A novel cathepsin substrate we recently identified is E-cadherin, which is cleaved by cathepsin B, L and S, thus demonstrating another mechanism by which invasion could be achieved[6] (Fig. 1C). E-cadherin is the main component of adherens junctions and mediates cell-cell adhesion in epithelial cells.[47] Its importance in cancer has been well established as most epithelial cancers lose E-cadherin function when they become malignant, which abrogates its adhesive properties, thus promoting cell invasiveness and metastasis. Indeed, in the RIP1-Tag2 model it was demonstrated that loss of E-cadherin mediates the transition from adenoma to carcinoma and promotes an invasive phenotype.[48] Common mechanisms of E-cadherin downregulation include transcriptional repression, gene mutations, epigenetic silencing[47] as well as proteolytic cleavage of the ectodomain by proteases such as MMP-3 and -7 or ADAM10 among others.[49,50] We found that tumors from *cathepsin B*, *L* or *S* null RT2 mice displayed a consistent maintenance of E-cadherin levels, as opposed to the typical protein downregulation observed in invasive tumors from wild-type or *cathepsin C* null RT2 mice.[6] To confirm

that these three cathepsins could cleave E-cadherin directly, the in vivo observations were followed by in vitro cleavage assays using recombinant proteins.[6] Incubation of cathepsin B, L or S with the adherens junction component resulted in E-cadherin cleavage in the extracellular portion of the enzyme, which we hypothesize would lead to loss of cell-cell adhesion and therefore enhance tumor invasion (Fig. 1C). The exact cellular mechanism, and the interaction between tumor and host cells in this process (see below) are currently under investigation.

As discussed above, cathepsins can promote invasion via several different mechanisms, which are in no way mutually exclusive. In the complex tumor microenvironment it is most likely that tumor cells exploit all possible means to facilitate their expansion and migration. However, cathepsins are not always expressed solely in the tumor cells. In fact, numerous reports have shown cathepsin expression in other cell types within the tumor microenvironment (reviewed in ref. 51). Many normal host cells, such as endothelial cells, fibroblasts and macrophages are recruited to the lesion and can be coopted by tumor cells to execute pro-tumorigenic functions.[52-54] Certain proteolytic enzymes can be supplied to the tumor by recruited stromal cells and promote invasion. For example, in the RT2 model it was found that while tumor cells preferentially expressed cathepsin L, infiltrating immune cells had high cathepsin B and S expression.[4,6] Similarly, in the MMTV-PyMT model it was determined that cathepsin B is supplied to the tumor by both cancer cells and infiltrating macrophages.[7] In culture, macrophages have been shown to secrete active cathepsins B, L and S, which can then participate in ECM remodeling in pathophysiological conditions.[55] In fact, coculturing a colon carcinoma cell line with monocytes led to a significant increase in cathepsin B expression in both the tumor as well as the nonmalignant cells.[56] Consequently, the presence of monocytes increased the invasive abilities of the tumor cells five-fold, and this effect was ablated upon treatment with a siRNA construct targeting cathepsin B. Thus there could be an active interplay between the different cell types of the microenvironment contributing to tumor invasion, among other processes.

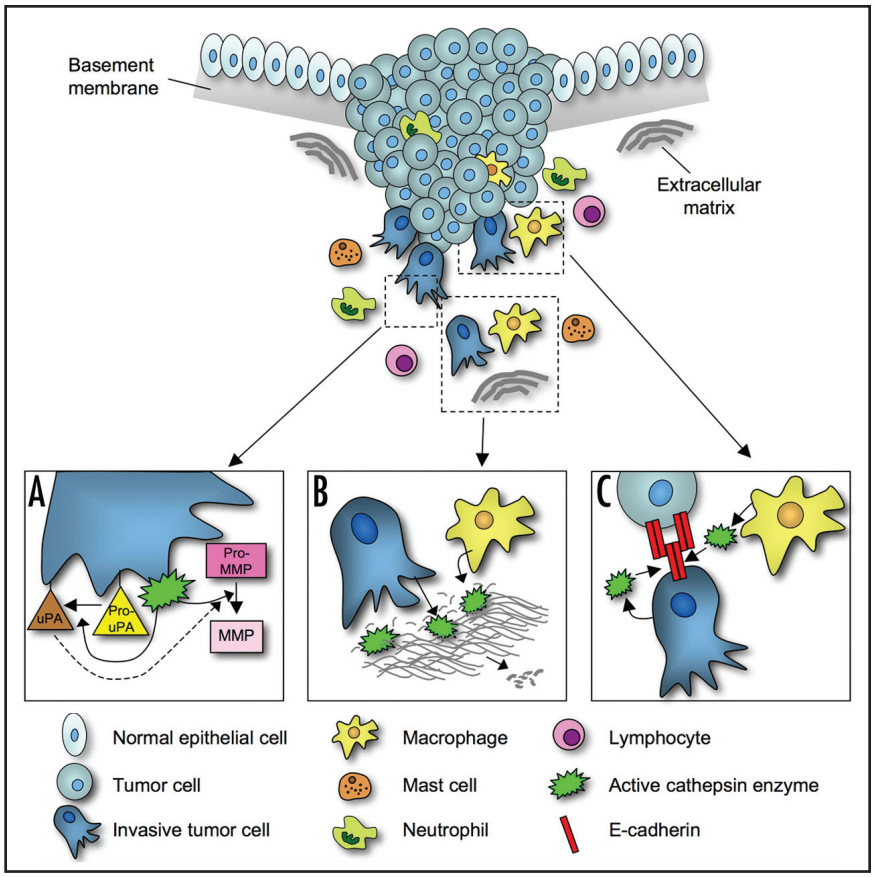

Figure 1. Potential mechanisms through which cathepsins promote tumor invasion. (A) Proteolytic cascade: Cathepsins expressed at the surface of tumor cells can activate other proteases such as pro-MMPs or pro-uPA. MMPs can then directly cleave ECM components thereby facilitating cancer cell migration. uPA cleaves plasminogen into plasmin, which can activate MMPs or cleave ECM. (B) Degradation of the extracellular matrix and/ or basement membrane: Secreted cathepsins from the tumor cells or from stromal cells, such as macrophages, recruited to the invasive tumor edge can directly cleave ECM/ BM components. These proteins include laminin, fibronectin, tenascin-C and type IV collagen among others. (C) Inactivation of cell adhesion proteins: Cathepsins can cleave the cell surface protein E-cadherin, which is the principal component of adherens junctions. E-cadherin cleavage abrogates its cell-cell adhesion function and promotes tumor cell invasion.

While our understanding of the roles of the cathepsins in tumor cells as well as in the host microenvironment has improved, there are many outstanding questions. The identification of some cathepsin targets has provided clues towards delineating the possible mechanisms through which they exert their pro-tumorigenic effects, although discovering novel substrates would allow us to gain a more complete understanding of the involvement of cathepsins in cancer development and progression. Through genetic and pharmacological approaches we have been able to identify some of the key tumor-promoting cathepsins, and have begun to uncover the molecular mechanisms by which they elicit their functions in pancreatic cancer. Future studies in both preclinical models and clinical samples should now allow us determine whether these enzymes have similar or unique roles in different tumor microenvironments. This knowledge will ultimately help guide consideration of clinical applications to target cathepsins, alone or in combination with other anti-tumor agents.

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