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FUNCTIONAL IMAGING OF TUMOR PROTEOLYSIS

Bonnie F. Sloane,[1,2] Mansoureh Sameni,[1] Izabela Podgorski,[1,2] Dora Cavallo-Medved,[1,2] and Kamiar Moin[1,2]

[1]Departments of Pharmacology and [2]Barbara Ann Karmanos Cancer Institute, School of Medicine, Wayne State University, Detroit, Michigan 48201; email: bsloane@med.wayne.edu, msameni@med.wayne.edu, ipodgors@med.wayne.edu, dcavallo@med.wayne.edu, kmoin@med.wayne.edu

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■ **Abstract** The roles of proteases in cancer are now known to be much broader than simply degradation of extracellular matrix during tumor invasion and metastasis. Furthermore, proteases from tumor-associated cells (e.g., fibroblasts, inflammatory cells, endothelial cells) as well as tumor cells are recognized to contribute to pathways critical to neoplastic progression. Although elevated expression (transcripts and proteins) of proteases, and in some cases protease inhibitors, has been documented in many tumors, techniques to assess functional roles for proteases require that we measure protease activity and inhibition of that activity rather than levels of proteases, activators, and inhibitors. Novel techniques for functional imaging of protease activity, both in vitro and in vivo, are being developed as are imaging probes that will allow us to determine protease activity and in some cases to discriminate among protease activities. These should be useful clinically as surrogate endpoints for therapies that alter protease activities.

INTRODUCTION

More than one catalytic-type of protease has been implicated in the progression of human tumors (for reviews, see 1–8). Although the preclinical data implicating matrix metalloproteinases (MMPs) in malignant progression were compelling, the clinical trials on MMP inhibitors (MMPIs) did not fulfill the promise of MMPs as therapeutic targets in cancer. Possible explanations for this disconnect between the preclinical and clinical data are discussed in a number of insightful and thought-provoking reviews (7–11).

The initial working hypothesis for those studying proteases in cancer was that invasive processes (both those that occur locally and those that occur during metastatic spread of tumors) required degradation of extracellular matrices by proteases. The roles of proteases in cancer are now known to occur earlier and to

involve substrates other than extracellular matrix (ECM) proteins. These include roles in premalignant lesions, proliferation, angiogenesis, etc. Furthermore, we know that the proteases themselves derive not only from tumor cells but also from the multiple cell types present in the tumor microenvironment, e.g., stromal cells such as fibroblasts, inflammatory cells such as macrophages, mast cells and neutrophils, and blood vessel cells such as endothelial cells. A relative lack of knowledge in regard to the substrates of proteases and, in the case of cancer, the substrates relevant to acquisition and maintenance of a malignant phenotype is impeding our understanding of how proteases function in malignant progression. Indeed, that a purified protease is capable of cleaving a potential ECM substrate in vitro may not indicate that this protease is the single protease responsible for degradation of that substrate in vivo or even one of the proteases responsible for degradation of that substrate in vivo. Efforts to define the in vivo substrates for various proteases are ongoing under the auspices of the Center for Proteolytic Pathways *(*a National Technology Center for Networks and Pathways funded through the NIH Roadmap Initiative, http://cpp.burnham.org/metadot/index.pl) and primarily for MMPs by Overall and colleagues (12–14). Many of the in vivo functions of proteases are being elucidated through the use of transgenic mice deficient in specific proteases. Although such studies are informative, additional techniques will be required owing to the large number of proteases [>500 in the human genome (15, 16)]; some notable differences between human and mouse proteases (15, 16); the complex interplay of proteases with their endogenous inhibitors, activators, and receptors/binding proteins; redundancy and compensation of proteases; and the diversity of their biological roles. We describe here the functional imaging assays and imaging probes that are being developed to assess activities of the tumor/tumor microenvironment degradome, i.e., the complement of proteases produced by the tumor, the tumor microenvironment, and by these two entities in response to each other.

ASSAYS FOR IMAGING PROTEASE ACTIVITY

The recent discordance between the results of preclinical and clinical trials with MMPIs has led us to reevaluate what we know and do not know about the functions of proteases in tumors. The MMPI clinical trials did not include functional assays to determine whether the MMPIs actually reached the sites of action of their target MMPs and, most importantly, reduced the MMP activity at those sites. Thus, those in the cancer protease community are left wondering whether their preclinical studies were inaccurate and perhaps poorly designed or whether the clinical studies did not use efficacious doses or dosing regimens for the MMPIs. Our hope is that imaging protease activity in preclinical models will improve our understanding of how proteases contribute to neoplastic progression. Our intent is to use functional imaging techniques and probes to delineate the roles played at the stages of neoplastic progression by protease classes and ultimately by individual

proteases. This is an obviously ambitious goal that will require collaborative efforts among many investigators. Those presently partners in this effort are the Protease Consortium (17), a Department of Defense Breast Cancer Center of Excellence (http://bccoe.med.wayne.edu), and the Center for Proteolytic Pathways (http://cpp.burnham.org/metadot/index.pl).

There is an extensive literature documenting the association of proteases with cancer. Indeed, a search of PubMed for the two terms proteases and cancer brings up a list of >34,000 papers, including >3700 reviews. Nonetheless, we still have not identified and validated most of the proteases that play causal roles in neoplastic progression, nor have we determined which proteases would be appropriate therapeutic targets in premalignant lesions as compared with end-stage disease or in breast cancer as compared with colon cancer. We designed in partnership with Affymetrix a custom oligonucleotide microarray, the Hu/Mu ProtIn Chip, for use in identifying which proteases are expressed in human cancer (http://bccoe.med.wayne.edu). The Hu/Mu ProtIn chip, in contrast to the ClipChip developed by Overall and colleagues (14), has on a single chip oligonucleotide probes for human and mouse proteases, protease inhibitors, and protease interactors. Thus, using a single platform we can identify the proteases expressed in normal, premalignant, and malignant human specimens and determine whether the patterns of expression are found in mouse transgenic and xenograft models, required to test causality of the proteases and for preclinical studies of therapeutic agents that target proteolytic pathways and protease imaging probes. Although the Hu/Mu ProtIn Chip is proving invaluable in identifying potential protease targets (D. Schwartz, K. Moin, B. Yao, H. Nassar, S. Krawetz & B.F. Sloane, unpublished data), levels of transcripts for proteases, protease inhibitors, and protease interactors do not tell us whether the proteases are active. Thus, we need assays that will assess protease activity.

Individual Proteases or Protease Classes

Early attempts to define the protease activity in tumors and other tissues used histochemical techniques, such as those pioneered by Robert E. Smith, who coauthored the first review in 1975 on probes (substrates) for assaying activities and localization of tissue proteases (18). His protease probes were designed primarily to assess the activities of lysosomal proteases, in particular the enzymes later classified as cysteine cathepsins, and the kallikrein family of serine proteases. Cryostat sections of the tumors were subjected to histochemical analyses employing the substrates in solution or later in a cellulose membrane overlay, a technique first described in 1985 (19) using fluorescent detection after isoelectric focusing. The latter is similar to the primary imaging technique that has been used to evaluate tumor activities of serine proteases and MMPs, i.e., in situ zymography. The first reported use of in situ zymography to determine protease activity in tumors was for the localization of plasminogen activators of the urokinase-type (uPA) and tissue-type (tPA) in tissue sections of skin carcinomas (20). This technique, however, is now known to

be unable to discriminate between the inactive pro form of uPA and active uPA. Use of in situ zymography to detect MMPs was not reported until 1999 when it was used to establish that the gelatinolytic activity of human thyroid carcinomas arose from tumor cell nests rather than the stromal cells (21).

Assessing the activity and localizing that activity for any given protease can help to define a possible function. For example, we had established that the lysosomal cysteine protease cathepsin B becomes associated with the membrane of tumor cells using two separate techniques: (*a*) immunocytochemical techniques to demonstrate that cathepsin B protein associates with the tumor cell membrane (22, 23) and (*b*) subcellular fractionation techniques to demonstrate that cathepsin B activity is present in tumor cell membrane fractions (22, 24) and membrane vesicles shed from tumor cells (25). In human colorectal specimens, we observed that cathepsin B protein localizes to apical vesicles in normal colonic mucosa, but to the basal membrane in late adenomas and early carcinomas (26). Furthermore, high levels of expression of cathepsin B in the colorectal carcinomas correlates with shortened survival. As cathepsin B degrades basement membrane proteins in vitro (27, 28) and cathepsin B activity localizes to the invasive regions of colon tumors that were isolated by microdissection (29), we speculated that basal cathepsin B is involved in degradation of the underlying basement membrane. Van Noorden and colleagues (30) confirmed that the cathepsin B protein, at the basal pole of late adenomas and early carcinomas, is active by using a fluorometric substrate to localize cathepsin B activity. Although not proof that one function of cathepsin B in colon cancer is degradation of the underlying basement membrane, localizing cathepsin B activity to that region is consistent with such a function.

Proteolytic Pathways

Using selective substrates allows us to determine the activity of individual proteases or protease classes but does not allow us to define proteolytic pathways involving more than one class of proteases, pathways known to be important to neoplastic progression (31, 32). Therefore, in the studies with live tumor cells described below, we have chosen to use protein substrates in an effort to delineate which proteases and whether more than one class of proteases are responsible for degrading these proteins.

PROTEOLYSIS IN VITRO BY LIVE TUMOR CELLS

Our laboratory has developed a novel confocal microscopy assay for functional imaging of degradation of quenched-fluorescent (DQ) proteins by live tumor cells grown in two-dimensional (2-D) monolayer (33, 34), three-dimensional (3-D) monotypic (34–38), and 3-D multicellular or organotypic (36) cultures (Figure 1). The live tumor cells exhibit the anticipated pericellular proteolysis and, in addition, an intracellular proteolysis dependent on endocytosis of substrate by the live cells (33, 35). MMPs, serine, and cysteine proteases all contribute to the pericellular

tumor proteolysis, as demonstrated by use of broad spectrum and selective protease inhibitors (33, 36, 37). The fluorescent degradation products that accumulated intracellularly were localized to vesicles that also stained for lysosomal markers such as LysoTracker and the cysteine protease cathepsin B (33, 35, 36). Cathepsin B protein was localized to these vesicles by immunostaining and cathepsin B activity was localized to these vesicles by histochemical staining. Inhibition of endocytosis reduced the accumulation of fluorescent degradation products (33), whereas stimulation of endocytosis increased their accumulation (35). The individual proteases responsible for pericellular and intracellular proteolysis have not been unequivocally identified (33, 35–37). The intracellular degradation appears to be mediated primarily by lysosomal cysteine cathepsins (33, 35–37), as in human breast carcinoma cells the selective, cell–permeable cysteine cathepsin inhibitor, CA074Me (39), reduces intracellular degradation of bovine serum albumin (BSA) by 90% and type IV collagen by 80% (33). Broad-spectrum inhibitors of MMPs and serine and cysteine proteases reduced pericellular degradation of type IV collagen by human prostate carcinoma cells as much as 95% (37). The ability to abrogate either pericellular or intracellular proteolysis seems to reflect the degradome of the tumor cell line as inhibition varied among tumor cell lines, even those of the same tissue origin, and among the protein substrates tested (33, 37). Recent studies by Friedl & Wolf and Sahai (for reviews, see 40, 41) have suggested that proteolysis may not be required for tumor cells to migrate and invade. Those studies are discussed below in Is Proteolysis Associated with Migration and Invasion?

Proteolysis in Two Dimensions

Initially, we grew tumor cells on fluorescein isothiocynate (FITC)-labeled ECM protein matrices and imaged the ability of those cells to degrade the underlying ECM (42), as had others (43). The advantage of such an assay is that it allows one to image the proteolysis of large protein substrates that may be relevant to the ability of tumor cells or other migrating cells such as endothelial cells to move through the ECM in vivo. A limitation is that one is assessing discrete areas in which there is loss of fluorescence in an extensive and high background of the fluorescently tagged proteins. Furthermore, the cells and fluorescently tagged matrices are not observed in real-time but after fixation. Therefore, one is observing proteolysis of a matrix that occurred at an earlier time as a result of cells actively migrating on and invading into the matrix. Indeed, when we imaged proteolysis of FITC-labeled laminin by BT20 human breast carcinoma cells or U87 human glioblastoma cells, we were initially surprised to find that the loss of fluorescence often did not coincide with the location of the fixed tumor cells. Rather, the pattern of fluorescence loss suggested that the tumor cells had degraded the FITC-collagen IV as they migrated on this matrix (42). It was such findings that led us to use quenched fluorescent protein substrates as described below.

We have analyzed the ability of a variety of live cells to degrade DQ-protein substrates when growing in monolayers on those substrates mixed with a

nonfluorescent matrix (gelatin, the reconstituted basement membranes Matrigel or Cultrex, collagen I). To date, monolayers of BT20 and BT549 human breast carcinoma cells (33) have been shown to degrade DQ-BSA; monolayers of BT20 and BT549 human breast carcinoma cells (33), U937 human macrophages (36), U87 human glioblastoma cells (34), and human umbilical vein (D. Cavallo-Medved, D. Rudy & B.F. Sloane, unpublished data) and mammary microvessel endothelial cells (Figure 2) to degrade DQ-collagen IV; and DU145, PC3 and LNCaP human prostate carcinoma cells (37), and human WS-T_i breast and CCD colon fibroblasts (M. Sameni, J. Dosescu & B.F. Sloane, unpublished data) to degrade DQ-collagen I. In early studies, we used DQ-protein substrates mixed with gelatin (33–35). Gelatin, due to being denatured, is not representative of a matrix encountered by migrating or invading cells in vivo. In addition, we were concerned that DQ-protein substrates embedded in a denatured matrix might be more readily endocytosed. If this was the case, their being degraded intracellularly might not represent a normal pathway for degradation or alternatively enhanced endocytosis might increase the ratio of intracellular to pericellular proteolysis. Therefore, we now routinely use either DQ-collagen IV mixed with Matrigel or Cultrex, which contain collagen IV, or DQ-collagen I mixed with collagen I for our studies. Recent studies have ascribed endocytosis and internalization of collagen for intracellular degradation to uPARAP, a protein associated with the cell surface receptor for uPA (for review, see 44) and established that uPARAP-mediated intracellular degradation represents a major pathway of ECM turnover in murine mammary tumors (45). These findings are consistent with our observations of intracellular proteolysis of collagens IV and I by human breast, colon, and prostate carcinoma cells and glioblastoma cells (33, 34, 36–38) and collagen IV by rat fibroblasts (35) and human macrophages (36).

Proteolysis in Three Dimensions

Elegant studies by Bissell, Brugge and coworkers (for reviews, see 46, 47) have established the importance of studying cells in vitro in a 3-D context. Therefore, we have analyzed proteolysis of DQ-collagen IV by cells grown as 3-D spheroids. In some cases, we used preformed spheroids, but we also found that many of the cell lines when plated on Matrigel as single cells migrated into spheroids. The patterns of fluorescent degradation products for preformed spheroids and those formed from migrating cells were comparable. In the case of U87 human glioblastoma cells, we only studied preformed spheroids (34). In general, we have observed both pericellular and intracellular proteolysis with spheroids of all cell lines examined to date: human carcinoma cells of breast (36), colon (36, 38), and prostate (37) origin; human fibroblasts of breast and colon origin (36); human glioblastoma cells (34); and rat fibroblasts (35). The levels of pericellular to intracellular proteolysis vary from one cell line to another irrespective of their tissue of origin, and they presumably reflect the degradome of the particular cell line. BT20 human breast carcinoma cells both grown as monolayers (33) and as spheroids (36) exhibit primarily pericellular degradation of DQ-collagen IV. In contrast, another human

breast carcinoma cell line, BT549, exhibits only intracellular degradation of DQ-collagen IV in monolayer cultures (36), yet both pericellular and intracellular degradation when grown as spheroids (36). The cohesiveness of spheroids also varies from one cell line to another. For example, spheroids formed by the HCT 116 human colon carcinoma cell line are cohesive, whereas those formed by a daughter cell line in which the Ki-ras allele has been deleted are more amorphous (36). Despite their greater cohesiveness, HCT 116 spheroids exhibit greater pericellular and intracellular degradation of DQ-collagen IV (36). Stably downregulating the expression of caveolin-1 in the HCT 116 cells reduces their degradation of DQ-collagen IV in parallel with reducing the expression and localization to caveolae of cathepsin B, uPA, and their cell-surface receptors, p11/S100A10 (48, 49) and uPAR, respectively (38). By using an ECM protein substrate for these studies rather than a substrate selective for an individual protease or protease class, we have been able to identify a potential proteolytic pathway involved in degradation of DQ-collagen IV by live HCT 116 cells. Given the recent findings on uPARAP and intracellular collagen degradation, it will be of interest to see whether uPARAP is involved in this pathway. Intriguingly, Friedl, Sahai, and colleagues (50, 51) both have linked tumor cell invasion to beta1 integrin, a protein which also exhibits reduced localization to caveolae in the HCT 116 cells in which caveolin 1 was stably downregulated (38).

Proteolysis in Four Dimensions

With extended time in culture, the images include spheroids as well as tumor cells that appear to have migrated out of the spheroids. Images of BT20 human breast carcinoma spheroids are consistent with tumor cells migrating out of the spheroids and forming secondary metastatic colonies (Figure 3). Therefore, it seemed important to image proteolysis both in a 3-D context and over time. This is particularly true as the cells that migrate away from spheroids of HCT 116 human colon carcinoma move out of the plane of focus during a 90-min period of observation. Observations to date indicate that neither a broad-spectrum MMPI nor a cell-permeable cysteine protease inhibitor are able to completely block proteolysis of DQ-collagen IV by single HCT 116 cells migrating into spheroids; on the other hand, these inhibitors do dramatically reduce spheroid formation (M. Sameni, J. Dosescu & B.F. Sloane, unpublished data), suggesting that MMPs and cysteine proteases are involved in migration of these cells.

Is Proteolysis Associated with Migration and Invasion?

Friedl, Sahai, and colleagues (40, 52) have suggested that there are two forms of cellular migration, one that requires proteolysis and one that does not. Friedl & Wolf (40) have reported that tumor cells treated with a cocktail of protease inhibitors at high concentrations become amoeboid in shape and thereby remain motile and invasive in the absence of an ability to degrade the ECM. In our hands, cocktails of protease inhibitors at those high concentrations result in cytotoxicity

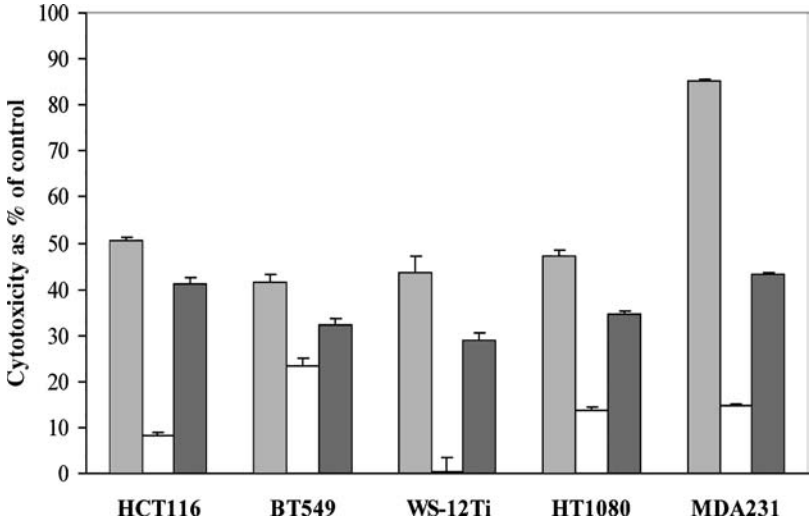

Figure 4 Cytotoxicity resulting from incubation of cells with protease inhibitors overnight. Cells were grown on collagen I matrix as in Wolf et al. (76). Cytotoxicity was assessed by release of lactate dehydrogenase using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Effects of E-64 (*open bar*) and GM6001 (*dark gray bar*) alone were tested at 250 μM and 100 μM concentrations, respectively. The protease cocktail (*light gray bar*) consisted of E-64, 250 μM; GM6001, 100 μM; 2 μM leupeptin; 100 μM pepstatin A; and 2.2 μM aprotinin.

of 40%–50% for most cell lines, although the MDA-MB-231 human breast carcinoma cell line is considerably more sensitive (85% cytotoxicity) (Figure 4). Wolf & Friedl (53) recently described a battery of sophisticated techniques by which to image proteolytic tumor cell invasion through 3-D fibrillar collagen I matrices in vitro, perhaps indicating a modulation of their earlier views on a dissociation of proteolysis and tumor cell invasion. Sahai & Marshall (52) have divided tumor cell motility in 3-D Matrigel into one mediated by Rho/ROCK signaling and one mediated by proteases. If one mode of motility is blocked, then the tumor cells switch to the other. Blocking both prevents the tumor cells from invading.

In light of such studies, we determined whether fibroblasts migrating on DQ-collagen I:collagen I degraded the DQ-substrates as we had observed for tumor cells migrating on DQ-collagen IV:Matrigel (see above). Fluorescent degradation products of DQ-collagen I were observed as a result of WS-12T$_i$ human breast fibroblasts migrating on the collagen I matrix. A single fibroblast moving across the collagen fibrils leaves behind discrete fluorescent spots along the length of fibrils, as depicted in Figure 5. Degradation was reduced by either a broad-spectrum MMPI or a cell-permeable cysteine protease inhibitor, with the MMPI appearing to increase the amount of fluorescent degradation products observed intracellularly.

Once again, this may relate to the recent observations on dual pathways for collagen degradation (44, 45).

PROTEOLYSIS IN VITRO BY LIVE ORGANOTYPIC COCULTURES

The contribution of the tumor microenvironment to neoplastic progression has become increasingly well accepted. This has been appreciated by the cancer protease community for some time, as many of the so-called tumor proteases are known to derive from tumor-associated cells, such as fibroblasts and inflammatory cells, rather than the tumor cells themselves (54–57). There are several reviews that discuss the role of the tumor microenvironment in regulating expression, activation, and localization of tumor proteases and thereby tumor invasion (58–61).

Proteolysis in Three Dimensions

By analyzing proteolysis in 3-D organotypic cocultures of tumor cells with stromal cells, we have demonstrated a role for the stromal cells as important enhancers of colon and breast tumor proteolysis of DQ-collagen IV (36). Coculture of either HCT 116 human colon carcinoma cells with CCD-112CoN human colon fibroblasts or BT20 human breast carcinoma cells with WS-12T_i human breast fibroblasts resulted in an increase in proteolysis of as great as 15-fold. Macrophages themselves increased proteolysis by threefold, but did not further increase the high levels of proteolysis that occurred when fibroblasts were cocultured with the tumor cells. The intensity of the fluorescent degradation products was highest at sites where the stromal fibroblasts are interacting with the tumor cell spheroids, consistent with our finding that cell:cell contact was required for enhancement of proteolysis by the fibroblasts.

Proteolysis in Four Dimensions

When tumor cell:stromal cell cocultures were observed in real-time, fibroblasts could be seen actively migrating into the tumor cell spheroids. The tumor cells sent out processes that appeared to be gathering in the fibroblasts (Figure 6). Furthermore, as the fibroblasts came in contact with the tumor cells, bursts of proteolysis could be observed (Figure 7).

Tumor Proteolysis In Vivo

Weissleder and colleagues have pioneered the development of technologies and near-infrared optical probes by which to image protease activity in tumors (and other biological systems) in vivo (for reviews, see 62, 63). Using one of their protease sensors, they have shown in vivo that hematopoietic cells contribute to the measured protease activity (64). Weissleder and coworkers (64) attribute this

activity to the cysteine protease cathepsin B; however, the selectivity of this probe for cathepsin B has not been tested in transgenic models of tumors arising in wild-type and cathepsin B–deficient mice. Other probes designed for selectivity of MMP-2 over –9, using peptide sequences identified by phage display for cleavage by the recombinant enzymes in vitro (65), are not selective when tested in MMP-2- and –9-deficient mice (K. Moin, L. Coussens, C. Tung & B.F. Sloane, unpublished data). Whether this will prove to be the case for protease imaging probes in general is not clear. Bugge, Leppla and coworkers (66, 67) have shown that when sequences identified as selective for uPA (68, 69) are used to replace the furin cleavage site in anthrax protective antigen and directed to the tumor cell surface (via the fast binding of anthrax protective antigen to its receptor) there is high selectivity for cell surface uPA. They confirmed their results by demonstrating the resistance of uPA- and uPAR-deficient mice to the toxins and by the ability of the inhibitor PAI-1 (plasminogen activator-1), ATF (an amino terminal fragment of uPA that competes with prouPA for binding to uPAR), and R3 (a monoclonal antiuPA antibody that blocks the binding of prouPA to uPAR) to prevent cell killing. Weissleder and colleagues (68), in collaboration with Bugge, have used this sequence information to design a near-infrared optical imaging probe for uPA, and they tested it in vitro (70) and as a substrate for optical zymography (71). Other efforts to develop imaging probes for detecting proteolytic activities in vivo include fluorogenic proteolytic beacons, built on a polyamido amino dendrimer core, for imaging MMP-7 activity (72) and activity-based probes for imaging cysteine cathepsin and caspase activities (for review, see 73). The MMP-7 proteolytic beacon has been tested in a mouse xenograft model in which SW480 human colon carcinoma cells transfected with a control vector or one expressing MMP-7 differentially fluoresced (72). Activity-based probes for cysteine cathepsins have been used for in vivo imaging of pancreatic tumors arising in the RIP1-Tag2 model (74). The activity-based probes are designed on a protease inhibitor scaffold, rather than the substrate scaffold used for most imaging probes, and form covalent bonds with the active-site cysteine of the enzymes; this design may well result in inhibitors of greater selectivity (73, 74a). Although the selectivity of imaging probes to detect protease activity remains an issue, the efforts of Weissleder and colleagues in advancing this technology are to be applauded. It is not yet clear that small-molecule imaging probes will retain their selectivity when used in complex biological systems in vivo; it may well be that probes in which protease-selective sequences are incorporated into a macromolecular context, as was done in generating the cell surface uPA-dependent anthrax toxin (66, 67), will prove to be more selective (for review, see 75).

CONCLUSIONS

An ability to image the activity of proteolytic pathways, and in some cases the activity of individual proteases, is needed to define the biological and pathobiological roles of these enzymes. Most importantly, these efforts will be critical to evaluating therapeutic strategies that target proteolytic pathways, whether those

strategies be ones that target proteases specifically or target upstream signaling pathways and thereby proteases that are downstream of those signaling pathways. Our ultimate goal is to develop and optimize technologies and imaging probes that can be used in vitro as a screening tool for their clinical use. Eventually, we hope to have technologies and imaging probes that would be useful for diagnosis and for following patients during the course of therapies that alter protease activities, perhaps even providing the crucial data needed to alter the course and/or the agents used for those therapies.

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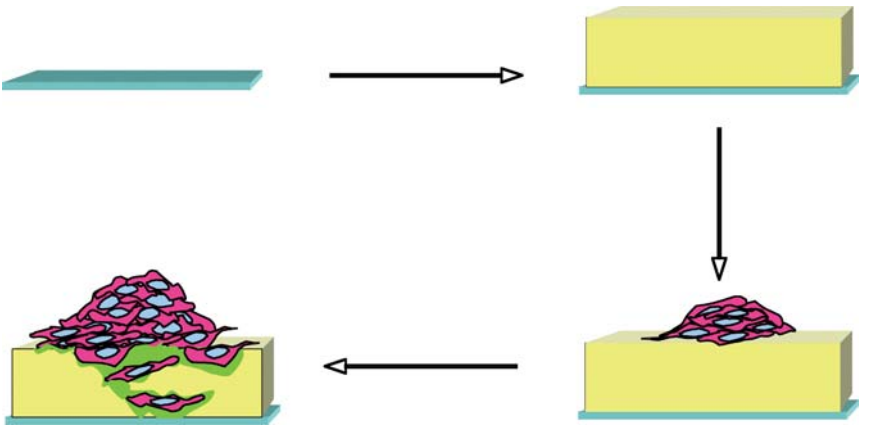

Figure 1 Cartoon illustrating confocal microscopy assay for functional imaging of proteolysis. Glass coverslips are coated with a matrix (gelatin, Matrigel, collagen I) mixed with the DQ-substrate. Cells (single-cell suspensions or preformed spheroids) are plated on top of the matrix. At given time periods after plating, the live cultures are observed on a Zeiss LSM upright microscope, using a dipping lens. Cleavage of the DQ-substrates is visualized as pericellular and intracellular green fluorescence, as described in the text.

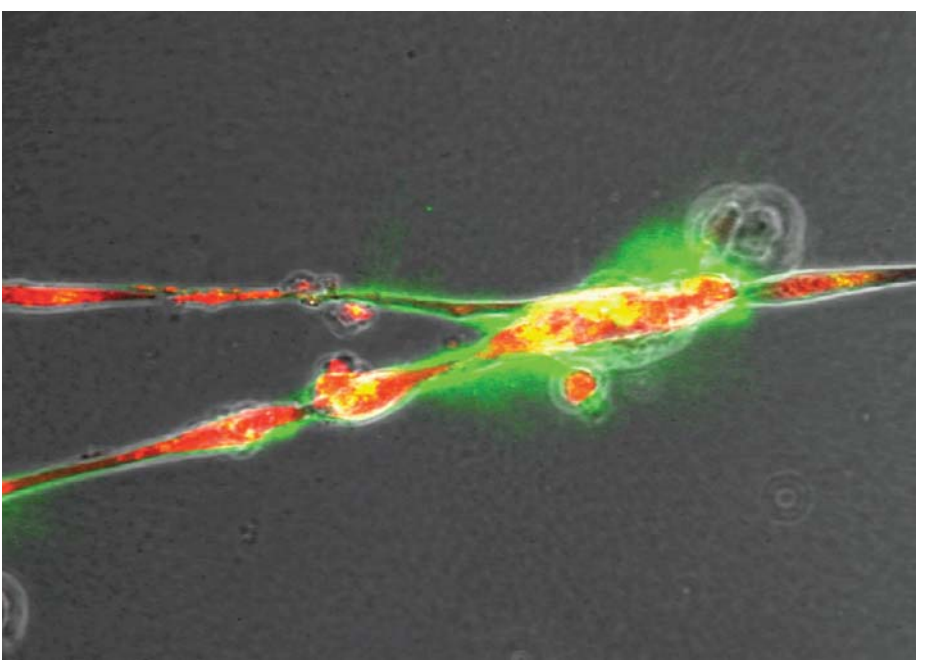

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Figure 2 Human mammary microvessel endothelial cells degrade DQ-collagen IV as they migrate on a Matrigel matrix. Endothelial cells were prelabeled with CellTracker Orange to facilitate visualization of the cells and plated on Matrigel that had been mixed with DQ-collagen IV. The green fluorescence represents degradation products owing to proteolytic cleavage of DQ-collagen IV at the periphery of the endothelial cells.

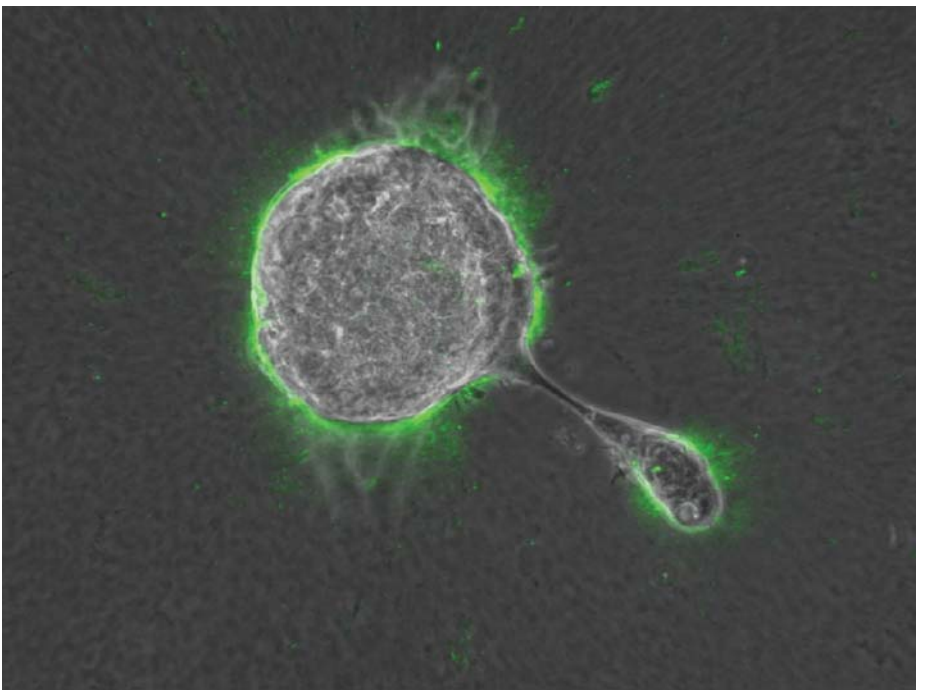

Figure 3 Formation of a secondary colony by tumor cells, which migrated out of a spheroid of BT20 human breast carcinoma cells. Cells were grown on Matrigel containing DQ-collagen IV. The pericellular green fluorescence represents degradation products owing to proteolytic cleavage of DQ-collagen IV at the periphery of the spheroid and the secondary colony.

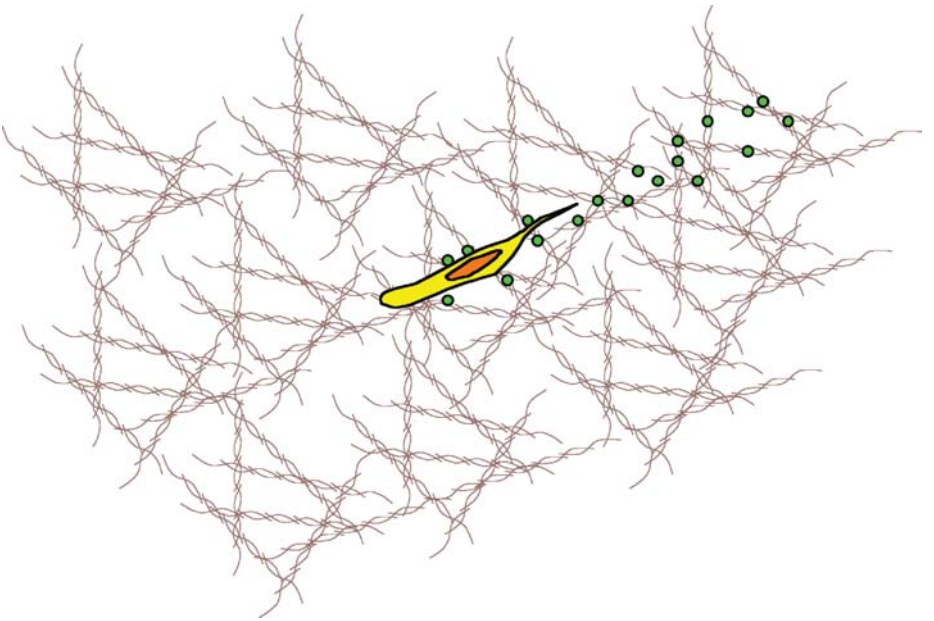

Figure 5 Cartoon depicting discrete degradation (*green spots*) of DQ-collagen I that occurs as a fibroblast migrates along the collagen fibrils.

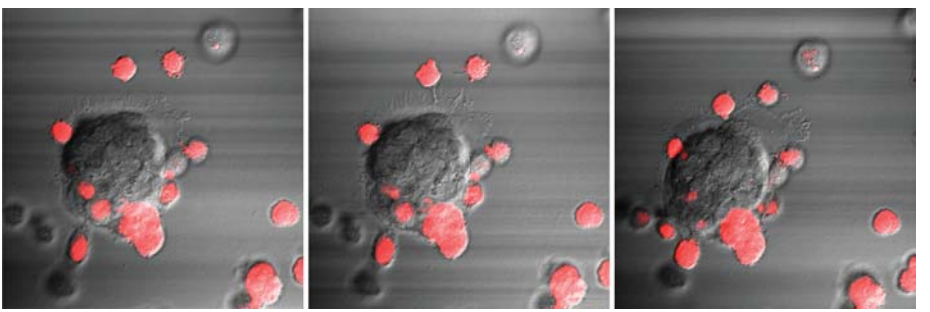

Figure 6 Three successive still images of a BT20 human breast carcinoma cell spheroid extending processes that reach out towards WS-12T_i human breast fibroblasts (prelabeled with CellTracker Orange) and resulting in incorporation of the fibroblasts into the tumor cell spheroid.

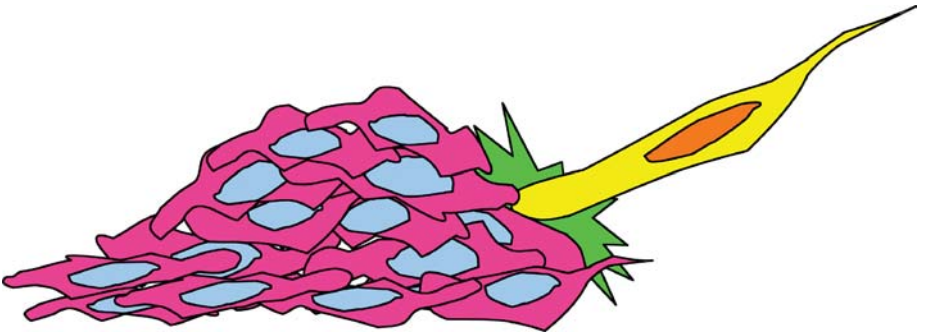

Figure 7 Cartoon depicting the bursts of green fluorescent degradation products resulting from a fibroblast coming into contact with a spheroid of tumor cells.

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ERRATA

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