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Imaging and quantifying the dynamics of tumor-associated proteolysis

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Abstract

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The roles of proteases in cancer are dynamic. Furthermore, the roles or functions of any one protease may differ from one stage of cancer to another. Proteases from tumor-associated cells (e.g., fibroblasts, inflammatory cells, endothelial cells) as well as from tumor cells make important contributions to 'tumor proteolysis'. Many tumors exhibit increases in expression of proteases at the level of transcripts and protein; however, whether those proteases play causal roles in malignant progression is known for only a handful of proteases. What the critical substrate or substrates that are cleaved in vivo by any given protease is also known for only a few proteases. Therefore, the recent development of techniques and reagents for live cell imaging of protease activity, in conjunction with informed knowledge of critical natural substrates, should help to define protease functions. Here we describe live cell assays for imaging proteolysis, protocols for quantifying proteolysis and the use of such assays to follow the dynamics of proteolysis by tumor cells alone and tumor cells interacting with other cells found in the tumor microenvironment. In addition, we describe an in vitro model that recapitulates the architecture of the mammary gland, a model designed to determine the effects of dynamic interactions with the surrounding microenvironment on 'tumor proteolysis' and the respective contributions of various cell types to 'tumor proteolysis'. The assays and models described here could serve as screening platforms for the identification of proteolytic pathways that are potential therapeutic targets and for further development of technologies and imaging probes for in vivo use.

Keywords

Imaging probes; Invasion; Malignant progression; Proteolytic pathways; Tumor microenvironment

Introduction

The roles of proteases in cancer are dynamic as is described in a recent book dedicated to this area of investigation [1]. The myriad roles include degradation of extracellular matrices as tumors invade into adjacent tissues or endothelial cells form neovessels, activation of growth factors, inactivation of protease inhibitors, apoptosis, etc. The roles of any one protease or protease class may vary from one type of cancer to another and within a single type of cancer from one stage of malignant progression to another. Furthermore, multiple classes of proteases and thus proteolytic pathways appear to be involved. The cellular source of tumor proteases may be tumor-associated cells (e.g., fibroblasts, inflammatory cells, endothelial cells) as well as tumor cells. Expression of proteases may also be affected by the interactions of tumor cells with other cells in the surrounding stroma (e.g., see [2-5]). Proteases are generally thought of as promoting malignant progression. Thus, it is of interest that there are more examples of proteases that suppress progression, with many of those proteases originating in tumor-associated cells [6]. In order to define the various functions of proteases in tumors and the relevance of those functions to malignant progression, we need to be able to assess protease activity and to determine what are the critical substrates cleaved by the proteases [7,8]. The recent development of functional imaging techniques has provided investigators with probes and methodologies to both measure and localize protease activity [9-11]. By using selective probes or protease inhibitors, one can discriminate the activity of one class of protease from another. In this review, we discuss assays for imaging the proteolysis associated with tumor cells and tumor cells in the context of their microenvironment. We describe an in vitro assay that our laboratory has developed for imaging and quantifying proteolysis by live cells and model systems that we have designed in an effort to recapitulate the architecture of the tumor and its microenvironment. These in vitro assays and models can serve as screening platforms for: (1) the identification of proteases and proteolytic pathways that are potential therapeutic targets, (2) the testing of

reagents such as inhibitors that abrogate protease activity, and (3) further development of imaging probes for in vivo use.

Live cell imaging of proteolysis or protease activity

Functional roles for proteases in cancer, other disease processes and normal development can only be defined if we have methods by which to analyze protease activity in live cells and tissues (for review, see [12]). Smith and van Frank [13] were pioneers in optimizing histochemical techniques for the measurement of protease activities against small synthetic substrates. Such substrates while allowing one to assess the activity of a single protease do not indicate whether protein substrates that are critical for cancer progression are cleaved by this protease. Thus, many investigators have used fluorescently-tagged protein substrates to assess protease activity, using primarily proteins that might be degraded by migrating or invading tumor cells such as fibronectin, laminin and various collagens including denatured collagen (gelatin). Indeed, the identification of invadopodia, a protease-rich structure involved in invasion of tumor cells, is based on their ability to degrade fluorescently (FITC)-labeled fibronectin or gelatin in distinct spots beneath the cells (for a recent review, see [14]). The spots of degradation are somewhat difficult to visualize as they are small areas of reduced fluorescence in a field of bright fluorescence. Kindelskii et al. [15] have also used fluorescently-labeled proteins, i.e., FITC-casein and Bodipy FL-bovine serum albumin, to assess proteolytic activity associated with single cells, in this case human neutrophils, HT1080 human fibrosarcoma cells, T47D human breast carcinoma cells and mouse macrophages. FITC-casein and Bodipy FL-bovine serum albumin, in contrast to FITC-fibronectin, FITC-laminin or FITC-gelatin, are weakly fluorescent and become strongly fluorescent upon cleavage. This property allowed Kindelskii et al. [15] to: (1) image sequential areas of proteolysis beneath single live cells as they migrated on labeled matrices, (2) show that pericellular proteolytic activity is not uniformly distributed along the cell surface, and (3) demonstrate an association of uPA with the pericellular proteolysis of casein and albumin. Others, including ourselves, have used quenched fluorescent (DQ)-protein substrates for imaging substrate degradation or proteolysis. DQ-protein substrates, which are commercially available from Invitrogen, do not fluoresce until they are cleaved by a protease at which point they fluoresce green. As a result, proteolytic activity is seen as an increase in fluorescence on a background that is not fluorescent. This is much easier to visualize than the loss in fluorescence discussed above. As an example, one can image pericellular proteolysis of either FITC-collagen IV or DQ-collagen IV, but one cannot image an intracellular loss of fluorescence due to proteolysis of FITC-collagen IV. Our laboratory has used a number of the DQ-protein substrates in a confocal microscopy assay for imaging of proteolysis by live cells grown in monolayer or 3D monocultures or organotypic cocultures [8], as will be described in more detail below. Wolf et al. [16] have used DQ-collagen I to localize the sites of degradation of fibrillar collagen by single HT1080 human fibrosarcoma cells migrating in 3D collagen lattices. Interestingly, the cells have an anterior degradation-free region that coincides with the pseudopods. Such an observation was only made possible through use of the DQ-substrate that allows one to localize proteolytic activity produced by live cells. DQ-substrates, however, have been used to localize proteolytic activity in tissue sections. For example, Mook et al. [17] used DQ-gelatin as a substrate for in situ zymography of unfixed cryostat sections of colon cancer metastases in rat liver. They observed that the areas of degradation of DQ-gelatin corresponded to regions of staining for MMP-2 and collagen IV.

An advantage of a live cell proteolysis assay with DQ-substrates is that one can localize proteolytic activity while cellular processes are ongoing. In the case of cell migration, this allowed Wolf et al. [16] to identify regions on the cell surface where proteolysis of collagen I occurs. In our laboratory, we have found that the ability of live cells to endocytose

substrates in conjunction with the use of DQ-substrates has allowed us to image cleavage fragments of the DQ-substrates intracellularly as well as extracellularly [18]. There had been static images showing collagen fragments within the lysosomes of cells, but this had been presumed to be the result of phagocytosis [19]. By using a live cell proteolysis assay and a DQ-substrate, we have demonstrated that some cells endocytose undigested DQ-substrates and degrade those substrates in the endolysosomal compartment, whereas other cells endocytose partially degraded DQ-substrates and degrade them further in the endolysosomal compartment [18,20] (Cavallo-Medved and Sloane, unpublished data). We have distinguished between these two pathways with cell-permeable inhibitors that target lysosomal cysteine cathepsins or cell-impermeable inhibitors of cysteine cathepsins, serine proteases and MMPs [18,21]. We confirmed that the cysteine cathepsins degrade the DQ-substrate in vitro in a 'test tube' fluorometric assay, in which we used the DQ-substrates in place of the conventional synthetic substrate [18].

Components of the live cell proteolysis assays as performed in our laboratory are illustrated in panels a and b of Fig. 1. We do not use the DQ-substrates alone, but rather incorporate them into an ECM. For example, we mix DQ-collagen IV into rBM, DQ-collagen I into collagen I, or DQ-gelatin into gelatin. We then use these mixtures to coat coverslips or culture dishes. For collagen I, we use plastic to improve the adherence of the matrices to the coverslips or dishes. We then plate either single cells, cellular spheroids or organotypic co-culture models on top of the ECM mixture and cover the cells with culture media appropriate for the cells being studied. The exceptions are: (1) rBM overlay cultures such as those described by Brugge and colleagues [22,23] in which the media covering the cells includes 2% rBM and (2) analyses of stromal cells such as fibroblasts in which the cells are embedded in type I collagen containing DQ-collagen I. In all cases, fluorescent degradation fragments are generated and can be imaged over time, either sequentially at various intervals over a period of days or continuously.

In earlier proof-of-principle studies for this live cell proteolysis assay, we incorporated DQ-protein substrates into gelatin [18,24]. The processes of degradation, migration and invasion observed with a gelatin matrix are comparable to those observed with either rBM or type I collagen, but occur over a shorter timescale due to the ease of digestion of gelatin. Nonetheless, a gelatin matrix does not reflect the in vivo situation so we only employ this matrix for comparison with rBM in regard to cord formation by endothelial cells [25]. Our goal is to develop models for analysis of proteolysis in vitro, models that mimic in vivo architecture such as the tripartite MAME model (shown in the cartoon of Fig. 1b and described below) and models that use matrices and substrates that are relevant to ECM degradation in vivo.

Imaging dynamics of proteolysis by tumor cells and by tumor cells interacting with cells found in the tumor microenvironment

We have previously shown that interactions of stromal cells with breast and colon tumor cells enhance degradation of ECM proteins [21]. To further study these interactions and the associated proteolysis, we used confocal/multiphoton microscopy to analyze in 4D (3D + time) degradation of DQ-collagen IV in rBM under live cell conditions. The cells used for these studies were BT20 and BT549 human breast carcinoma cells and HCT 116 human colon carcinoma cells alone or in co-culture with WS-12Ti human breast fibroblasts and CCD-112CON human colon fibroblasts, respectively, in the presence and absence of protease inhibitors. Fibroblasts were prestained with Cell Tracker Orange or Cell Tracker Far Red to distinguish them from tumor cells. We first assessed proteolysis by the tumor cells in the absence of stromal cells. Live cells were imaged at intervals over a 24-h period by confocal microscopy. Initially, HCT 116 and BT549 cells were motile and migrated

together to form spheroids/aggregates; fluorescent cleavage products of DQ-collagen IV were present intracellularly (Fig. 2a1 and b1, respectively). By 24 h, fluorescent cleavage products were found pericellularly, particularly around the BT549 aggregates (Fig. 2b2). In contrast, the cleavage products associated with BT20 cells were pericellular at all timepoints (Fig. 2c1 and c2). Degradation of DQ-collagen IV was increased when tumor cells were cocultured with fibroblasts (Fig. 2a3, b3 and c3). Quantification of the proteolysis per cell in a three-dimensional stack of images of the cultures was performed using methodology described in detail in Current Protocols in Cell Biology [26]. This revealed a 4-fold increase in HCT 116/CCD-112CON cocultures [21], 3-fold in BT549/ WS12Ti cocultures (Fig. 3) and 15-fold in BT20/WS12Ti cocultures [21]. Inhibitors of three classes of proteases reduced the proteolysis to levels below that in cultures of the tumor cell alone, as illustrated here for the BT549/ WS12Ti cocultures (Fig. 3). In the BT549/WS-12Ti co-cultures, degradation was reduced with a broad-spectrum MMP inhibitor (25 μ M GM6001), cathepsin B-selective cysteine protease inhibitors (10 μ M CA074 + 10 μ M CA074Me) or a broad-spectrum serine protease inhibitor (2 μ M aprotinin) (Fig. 3). These findings support the contention that stromal cells contribute significantly to overall tumor proteolysis. Furthermore, several catalytic-types of proteases participate, suggesting that targeting multiple classes of proteases may be a more effective strategy than targeting a single class such as the MMPs. Whether this contributed to the failure of MMP inhibitors in clinical trials is an open question as are other possibilities for the failure of these clinical trials [27-30].

Imaging dynamics of proteolysis by migrating endothelial cells

Angiogenesis is critical to tumor growth and metastasis and therefore it is not surprising that angiogenesis inhibitors are proving to be efficacious in the clinic [31,32]. Pericellular proteases and degradation of ECM are required for angiogenesis [33,34] so we were interested in seeing whether proteolysis could be imaged as endothelial cells form cord-like structures in vitro (Fig. 4a). We plated HUVECs on rBM containing DQ-collagen IV and imaged cultures maintained on a Zeiss LSM 510 microscope in a controlled environmental chamber. Images were acquired every 20 min over a 16 h period (Fig. 4b, c). Pericellular degradation products were observed as early as 2 h, i.e., at a time when the cells first began to form multi-cellular clusters and to align into small cord-like structures (Fig. 4b), and were extensive at 6 h (Fig. 4c). The extent of degradation products was reduced by 14 h when the cells had coalesced into larger cord-like structures (Fig. 4d). Degradation products also were observed around individual HUVECs migrating toward and becoming incorporated into the structures (not illustrated). These observations are consistent with ECM degradation being required for formation of cord-like structures by endothelial cells (for review, see [35,36]).

MAME: an in vitro model designed to facilitate studies on how dynamic interactions with the surrounding microenvironment impact tumor proteolysis

Epithelial/carcinoma cells in 3D laminin-rich, rBM: We have extended an established model for analyzing morphogenesis and oncogenic transformation of human breast epithelial cells, i.e., 10A cells, in a 3D laminin-rich, rBM overlay culture system [22,23] to include isogenic premalignant (AT1, DCIS1) and malignant (CA1d) variants of the pseudodiploid, parental MCF-10A cells, hereafter referred to as 10A [37-42]. The 10A cells were obtained from a reduction mammoplasty of a woman diagnosed with fibrocystic breast disease and have previously been characterized in 3D rBM overlay culture as a model for normal mammary epithelia. This model recapitulates apicobasal polarity, cell-cell junctions, strict control of cell proliferation and apoptosis, and formation of functional glandular structures [22,23,43].

When single 10A cells are grown in 3D rBM overlay cultures, they proliferate and form spheroids that over time develop into polarized acini with lumens, as illustrated in Fig. 5a by staining for the basal polarity marker, $\alpha 6$ integrin (red). In contrast, when the isogenic 10A variants are grown in 3D rBM overlay culture [44], they form structures that exhibit abnormalities as compared to normal acini such that they model the stages of atypical hyperplasia, dysplasia and carcinoma (Fig. 5b). Indeed, the morphologies of the isogenic variants in 3D rBM overlay cultures are comparable to those observed when these cells are grown as xenografts in immunodeficient mice [37,40]. Staining for cleaved caspase 3 (Fig. 5b, green) reveals that apoptotic cells are common in the central region of 10A structures, increasingly scattered in AT1 and DCIS1 structures and rare in CA1d structures. The CA1d cells invade into the rBM, forming large invasive structures in which staining for $\alpha 6$ integrin is absent (Fig. 5b, red). Thus, we now have in hand an isogenic breast model system that represents stages in the transition from non-invasive through pre-invasive to invasive and that can be used to delineate causal roles of proteases in the progression to invasive carcinomas.

Cells of tumor microenvironment in a collagenous stroma: There is a body of literature [45-52], including a series of recent reviews in Breast Cancer Research [53-59] on the role of the tumor microenvironment in promoting breast tumor progression. There is also striking evidence from mouse transgenic models of mammary carcinoma that the tumor microenvironment, including cellular constituents that are present in normal mammary tissue or that infiltrate as a consequence of angiogenic or inflammatory processes, contributes to progression of these tumors (e.g., see [60-66]). Although increases in expression of proteases have long been associated with tumors, many of those proteases originate from cells that infiltrate the tumors rather than the tumor cells themselves (e.g., see [66,67]). Using the live cell imaging assay described above [12,18,21,26,68], we have shown that co-culturing fibroblasts (see above) and/or macrophages with breast carcinoma cells [21] increases degradation of the basement membrane protein type IV collagen. Polyak and colleagues [69] have found dramatic changes in gene expression in the myoepithelial cells/[myo]fibroblasts of DCIS lesions, suggesting that the microenvironment affects preneoplastic progression as well as later stages of breast cancer progression. In the normal breast, the breast fibroblasts are present in a stroma consisting of type I collagen. We had previously established that breast fibroblasts degrade DQ-collagen I [21] (Fig. 6 and Supplemental Fig. 6 movie). To provide a better matrix context for the cells, we developed the MAME 3D triple-layer or tripartite co-culture system that is depicted in the cartoon of Fig. 1b. The bottom layer is type I collagen, containing DQ-collagen I, in which we embed breast fibroblasts. The middle and top layers are the two layers of the 3D laminin-rich, rBM overlay cultures using either the 10A isogenic variants described above or a variety of human breast cancer cell lines. In these tripartite MAME cultures, we have observed that the fibroblasts migrate toward the carcinoma cells, eventually infiltrating into the carcinoma structure over a period of 7 days (data not shown). The breast carcinoma cells migrate towards the lower layer of fibroblasts, but more slowly over a period of 3 weeks, and in so doing generate fluorescent degradation products pericellularly (data not shown). In contrast, extensive diffuse fluorescence is associated with the bottom layer of fibroblasts. To date, we have maintained the tripartite MAME cultures for as long as 24 days and imaged live cultures at intervals over that period. Although we ourselves are primarily interested in using MAME cultures for studying proteolysis, the tripartite and other modifications of the MAME cultures provide an experimental system in which one can test other aspects of the tumor microenvironment as it affects the progression of human breast cancer.

Quantifying tumor-associated proteolysis

The live cell proteolysis assay can be used in MAME cultures of the 10A isogenic variants (described above) to identify proteases that are causal in the transition from pre-invasive to invasive. As such proteases might be therapeutic targets, it is critical that we be able to quantify the proteolysis in order to accurately monitor the efficacy of drugs/inhibitors. With this in mind, we developed methods for quantifying the fluorescent degradation products on a per cell basis, as illustrated in Fig. 7 and also in Fig. 3. Detailed protocols for quantifying the fluorescent degradation products per cell in three-dimensional stacks of images have been described [26]. We illustrate in Fig. 7 and also in Fig. 3 the quantification of total proteolysis and in Fig. 7 the quantification of both intracellular and pericellular proteolysis based on fluorescent intensity. By pre-labeling the cells with a cytoplasmic dye or by expression of mRFP, we can define the entire volume of the cell and/or 3D cellular structure under study. This information is used to mask the intracellular proteolysis so that only pericellular proteolysis is considered in the initial determination. Once pericellular proteolysis is measured, the value obtained can be subtracted from the total proteolysis to determine the value for intracellular proteolysis. This allows us to directly compare the intracellular and pericellular proteolysis. Note that in Fig. 7 single confocal sections at the equatorial plane are illustrated for reference. The actual quantification is, however, performed on the entire Z-stack and thus proteolysis of a 3D structure is determined.

Summary

Having assays that allow one to image protease activity in the context of proteolytic pathways is important for defining the roles of proteases in biological and pathological processes. We anticipate that assays to image protease activity may serve as surrogate markers for therapies that target signaling pathways upstream of proteases. As with any assay, however, there are advantages and disadvantages. Major advantages of the live cell imaging assay and the tripartite MAME model described here are: (1) the dynamic nature due to the use of live cells; (2) the increase in signal or 'gain-of-function' that allows one to localize discrete areas of proteolysis; (3) the ability to quantify the proteolytic products generated on a per cell basis; (4) the system can be engineered to include different cell types, matrices and substrates; (5) the cells can be engineered to reduce or increase expression of a protease or protease inhibitor of interest; (6) proteolysis can be monitored in 'real-time' over time periods up to weeks; (7) the components of the assay can be fixed and processed so that the fluorescent degradation products can be colocalized with markers for subcellular compartments and membrane microdomains; and (8) other selective protease probes can be employed at the same time to identify and localize active proteases. We have established that DQ-substrates and activity-based probes for cysteine cathepsins [70] can be used simultaneously (Jedezsko and Sloane, unpublished data). Perhaps the most important advantage is that in this system one can assess proteolysis in an all-human system, e.g., human tumor cells, human stromal cells, human endothelial cells, human inflammatory cells and human ECMs, or for that matter in an all-mouse system that would allow the use of cells isolated from mice deficient in given proteases or protease inhibitors. There are also disadvantages such as: (1) the system is in vitro so any roles for in vivo processes such as blood flow cannot be evaluated; (2) the DQ-substrates used may be less resistant to proteolysis as a result of labeling; and (3) at present only FITC is used for labeling the commercial DQ-substrates, yet FITC is labile at acidic pHs such as found in endolysosomal compartments. This means that we may be underestimating intracellular proteolysis. Despite some limitations, the live cell imaging assay offers a unique system for dynamic imaging of proteolysis. Furthermore, the tripartite MAME model, which was designed to mimic the architecture of the human breast, provides a system that, in conjunction with the live cell imaging assay, can be used to assess the contribution of various breast cells and cell:cell

interactions to proteolysis. Comparable models could be developed for the study of proteolysis in other tissues of interest.

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Abbreviations

10A	MCF-10A human breast epithelial cell line
3D	Three dimensions
4D	Three dimensions + time
AT1	MCF10.AT1 atypical hyperplastic cell line
BSA	Bovine serum albumin
CA1d	MCF10.CA1d breast carcinoma cell line
DQ	Dye-quenched
DCIS1	MCF10.DCIS breast ductal carcinoma in situ cell line
ECM	Extracellular matrix
FITC	Fluorescein isothiocyanate
HUVEC	Human umbilical vein endothelial cells
MAME	Mammary architecture and microenvironment engineering
MMP	Matrix metalloproteinase
neoT	MCF-10AneoT ras-transfected breast epithelial cell line
rBM	Reconstituted basement membrane
uPA	Urokinase plasminogen activator

Fig. 1.

Diagrammatic representation of the components of a 3D rBM 'overlay culture' grown on rBM containing a DQ-substrate (**a**) and of the tripartite MAME model (**b**). *Green*, *red* and *dark blue* represent the fluorescent cleavage fragments of the DQ-substrate, cells labeled with a cytoplasmic dye and nuclei stained with a DNA binding dye, respectively. The invasion of cells into the underlying ECM and pericellular and intracellular (*green spots* adjacent to nuclei) proteolysis are depicted. Imaging in our laboratory is primarily done on an upright microscope using a dipping lens as represented here. The tripartite MAME model (**b**) includes a lower stromal layer, which consists of fluorescently pre-labeled fibroblasts embedded in a collagen I matrix containing DQ-collagen I. Note the use of a plastic coverslip in order to promote adherence of the collagen I matrix. A 3D rBM overlay culture of breast cells (e.g., isogenic 10A variants) is layered on top of the stromal layer

Fig. 2.

Tumor cell degradation of ECM is increased when the tumor cells are co-cultured with fibroblasts. Images illustrate degradation fragments of DQ-collagen IV (*green fluorescence*) in live 3D cultures of HCT 116 human colon carcinoma cells (**a1–3**), BT549 human breast carcinoma cells (**b1–3**) and BT20 human breast carcinoma cells (**c1–3**). Pericellular degradation of DQ-collagen IV is increased when HCT 116 cells are co-cultured with CCD-112CON normal colon fibroblasts (**a3**) and BT549 or BT20 cells are cocultured with WS-12Ti breast carcinoma-associated fibroblasts (**b3** and **c3**, respectively). (Collagen IV degradation by monocultures of the two fibroblast lines can be observed in Fig. 5 of [14].) Tumor cells were seeded alone or with fibroblasts that had been prelabeled with Cell Tracker Orange (Invitrogen). Starting at 1 h after seeding, live cells were imaged by confocal microscopy over a total time period of 24 h and representative confocal sections at the equatorial plane are shown at 2 h (**a1**, **b1** and **c1**) and 24 h (**a2**, **b2**, **c2**, **a3**, **b3** and **c3**). Bar, 20 μ m

Fig. 3. Inhibitors of MMPs (GM6001), cysteine cathepsins (CA074/CA0Me) and serine proteases (aprotinin) reduce degradation of DQcollagen IV (*green fluorescence*) in co-cultures of BT549 breast carcinoma cells and WS-12Ti fibroblasts to levels below that in cultures of the tumor cells alone. Images illustrate top views of cultures from which optical sections were acquired. Fluorescent intensity per cell, represented as *bar graphs* above each image, was quantified with Metamorph 6.3 software (Molecular Devices) in Zstacks of optical sections; nuclei were stained with Hoechst (*blue fluorescence*) at the time of imaging and counted in Z-stacks reconstructed in 3D, using Volocity 3.6.1 software (Improvision) [62]. WS-12Ti fibroblasts were prelabeled with Cell Tracker Far Red (Invitrogen). Bar, 10 μ m

Fig. 4. DQ-collagen IV is degraded as HUVECs migrate in vitro into cord-like structures. HUVEC were grown for 18 h on glass coverslips coated with rBM containing 25 $\mu\text{g/ml}$ DQ-collagen IV. Panel **a** illustrates a DIC image of the HUVEC cord-like structures at 18 h after plating. Panels **b**, **c** and **d** are confocal images of live HUVECs at 2, 6 and 14 h, respectively, after plating. DQ-collagen IV degradation products (*green*) are seen pericellularly adjacent to the cells. Bars, 100 μm

Fig. 5.

Isogenic MCF-10A variants model premalignant progression when grown in 3D rBM overlay cultures. **a** Polarized 10A acinus; bar, 10 µm. **b** Stages of normal, atypical hyperplasia, dysplasia and carcinoma; bar, 50 µm. At 12 days, the structures were fixed and processed. Images represent a single confocal section at the equatorial plane. Structures were stained for: $\alpha 6$ integrin, as a marker of basal polarity (*red*); DAPI, as a marker of nuclei (*blue*); and cleaved caspase-3, as a marker of apoptosis (*green*)

Fig. 6. 2D imaging in real-time of breast fibroblasts degrading DQ-collagen I (*green fluorescence*) as they migrate on a matrix of collagen I containing DQ-collagen I. Note trail of fluorescent cleavage products (denoted in panel **b** and the inset by *arrows*) left behind by migrating fibroblast. Round cells represent cells that are in process of detaching prior to migrating. Confocal images were taken over a 90 min period starting 2 h after plating (panel **a**, 0 min; panel **b**, 90 min). Bar, 10 μ m

Fig. 7.

Quantification of DQ-collagen IV degradation products, including extracellular and intracellular products, generated by live HCT 116 human colon carcinoma cells in 3D rBM culture. Total proteolysis of DQ-collagen IV in all optical sections throughout the volume of the structure was determined as normalized integrated intensity based on cell number. Total fluorescence was separated into pericellular and intracellular degradation using image arithmetic in Metamorph 6.3 software. Optical sections were reconstructed in 3D Z-stacks of confocal sections with Volocity 3.6.1 software. **a** Single optical section taken at equatorial plane showing fluorescence from cleaved DQ-collagen IV (*green*), cells pre-labeled with Cell Tracker orange (*pseudocolored red*) and nuclei stained with Hoechst (*blue*) at the time of imaging. **b** Cleavage products of DQ-collagen IV from same optical plane, representing both intracellular and pericellular cleavage products of DQ-collagen IV. **c** Pericellular cleavage products of DQ-collagen IV obtained by masking image from panel **b** with a binarized image of the cells used to designate x, y coordinates of intracellular areas within the same optical section. This eliminates all of the signal from cytoplasmic areas. **d** Image of intracellular cleavage products of DQ-collagen IV obtained by subtracting the image in panel **c** from that in panel **b**. A binarized image of nuclei was used to create a 3D reconstruction for counting nuclei, thus allowing us to determine the number of cells within the field of view. Bar, 10 μ m