

Theme Issue Article

Plasminogen activation and cancer

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Summary

Breakdown of the extracellular matrix is crucial for cancer invasion and metastasis. It is accomplished by the concerted action of several proteases, including the serine protease plasmin and a number of matrix metalloproteases. The activity of each of these proteases is regulated by an array of activators, inhibitors and cellular receptors. Thus, the generation of plasmin involves the pro-enzyme plasminogen, the urokinase type plasminogen activator uPA and its pro-enzyme pro-uPA, the uPA inhibitor PAI-1, the cell surface uPA receptor uPAR, and the plasmin inhibitor α_2 -antiplasmin. Furthermore, the regulation of extracellular proteolysis in cancer involves a complex interplay between cancer cells and non-malignant stromal cells in the expression of the molecular components involved. For some types of cancer, this cellular interplay mimics that observed in the tissue of ori-

gin during non-neoplastic tissue remodelling processes. We propose that cancer invasion can be considered as uncontrolled tissue remodelling. Inhibition of extracellular proteases is an attractive approach to cancer therapy. Because proteases have many different functions in the normal organism, efficient inhibition will have toxic side effects. In cancer invasion, like in normal tissue remodelling processes, there appears to be a functional overlap between different extracellular proteases. This redundancy means that combinations of protease inhibitors must be used. Such combination therapy, however, is also likely to increase toxicity. Therefore for each type of cancer, a combination of protease inhibitors that is optimised with respect to both maximal therapeutic effect and minimal toxic side effects need to be identified.

Keywords

Cancer invasion, plasminogen activation, matrix degradation, tissue remodelling, cancer therapy

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Introduction

Today we have a clear, although complicated, general picture of the mechanisms behind proteolytic degradation of the extracellular matrix in cancer. Matrix breakdown, which appears to be crucial for cancer invasion and metastasis, is accomplished by the concerted action of several proteases, notably including the serine protease plasmin and a number of matrix metalloproteases (MMPs). Plasmin activity is the result of the proteolytic cascade reactions of the plasminogen activation (PA) system, which is the focus of the present review. This system appears to be active in virtually all types of cancer, while various MMPs appear to be active more selectively in different types of cancer (1, 2).

The activity of each of the matrix-degrading proteases is regulated by an array of activators, inhibitors and cellular receptors. Thus, the PA system involves the pro-enzyme plasminogen, the urokinase type plasminogen activator uPA and its pro-enzyme pro-uPA, the uPA inhibitor PAI-1, the cell surface uPA receptor uPAR, and the plasmin inhibitor α_2 -antiplasmin (3–5).

Furthermore, it has become evident that the regulation of extracellular proteolysis in cancer involves a complex interplay between cancer cells and non-malignant stromal cells in the expression of the molecular components involved (1). For some types of cancer, this cellular interplay appears to mimic that observed in the tissue of origin during non-neoplastic tissue remodelling processes (6). In several tissue remodelling processes there is a functional overlap between the uPA-system and certain MMPs and also mutually between MMPs. A similar redundancy phenomenon is likely to occur during cancer invasion, and therefore must be taken into account in the design of new anti-proteolytic therapies (1, 7).

Research in this field has actually reached a stage where it presents important implications for the clinical management of cancer patients. Several molecular components involved in matrix degradation now have clinical value as strong prognostic markers in several types of cancer, and inhibition of extracellular proteolysis is recognized as a valid approach to cancer therapy. This is particularly attractive because all the targets are extracel-

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lular and therefore readily accessible. However, two main problems remain. First, the proteases have many functions in the normal organism. It is therefore inevitable that efficient inhibition will have toxic side effects. Second, the functional overlap between different protease systems means that combinations of inhibitors must be used in order to obtain efficient anti-invasive therapy. These combinations, however, are also likely to increase toxicity because similar overlaps in function exist in the normal organism. Therefore for each type of cancer, a combination of protease inhibitors that is optimised with respect to both maximal therapeutic effect and minimal toxic side effects needs to be identified. Much work in basic, preclinical and clinical research is still required to provide a rational basis for such an optimal clinical use of inhibitors of protease systems.

In this short review, we will focus on selected aspects of the PA system. We find these aspects to be particularly important for an understanding of the PA system's role in cancer and therefore for the application of this knowledge as basis for therapeutic approaches.

The plasminogen activation system

The zymogen plasminogen is mainly produced by the liver and is present in the plasma (approx 2 μM) as well as extravascularly in the interstitial fluids. It is converted locally to the active serine protease plasmin by proteolytic processing mediated by either of two plasminogen activators, the serine proteases uPA and tPA. It was early proposed that uPA primarily is involved in tissue degradation and tPA in thrombolysis (8). This overall picture is still valid. The currently established functions of uPA-dependent plasminogen activation are mainly within physiological and pathological tissue remodelling processes, including cancer invasion (7, 9) whereas those of tPA are within thrombolysis and neurobiology (10, 11). Despite this divergence in their basic biological functions it is however noteworthy that uPA and tPA can serve as mutual, functional substitutes to some extent, as has been observed in gene deficient mice (12, 13). In addition to uPA and tPA, a few other serine proteases are capable of activating plasminogen in test tubes (8). However, the physiological impacts of such activators *in vivo* have not been demonstrated until recent studies compared the process of mammary gland adipogenesis in mice deficient in plasminogen with that in mice double deficient in uPA and tPA. These studies indicated the involvement of a third plasminogen activator, which was tentatively identified as plasma kallikrein (14). We have since obtained similar evidence in wound healing experiments (L.R. Lund and J. Romer, unpublished results).

uPA-mediated proteolysis is regulated at several levels. Inhibitors of this system include specific serpins, notably the plasminogen activator inhibitor-1 (PAI-1) for uPA and α_2 -antiplasmin for plasmin (3, 15). uPA is secreted as a single polypeptide chain pro-enzyme, pro-uPA, which has only a very low intrinsic proteolytic activity and which is activated efficiently by plasmin (16, 17). Pro-uPA and uPA bind with high affinity to a cell surface uPA receptor, uPAR, a glycolipid-anchored three-finger fold protein (18–20). Studies in cell cultures show that receptor binding of pro-uPA, combined with binding of plasminogen to C-terminal lysine residues in plasma membrane proteins,

strongly enhances plasminogen activation, due to an increased efficiency of the reciprocal activation of the two proenzymes (4, 5, 21). In cell cultures uPAR thus both confines and enhances uPA-catalyzed plasminogen activation at the cell surface. Whether uPAR has a similar role in the intact organism is an important question. Unlike uPA-deficient mice that develop extravascular fibrin deposits (12), no spontaneous abnormal phenotype has been detected in uPAR deficient mice (22). However, studies of mice double deficient in uPAR and tPA have indeed demonstrated a role of uPAR in extravascular fibrin degradation and a functional overlap between uPAR and tPA in this process (13). Further indication for uPAR enhancement of cell surface uPA activity *in vivo* comes from a dramatic synergy between uPA and uPAR found in transgenic mice overexpressing uPA and uPAR in keratinocytes (23, 24) and from studies discussed below, showing that the toxicity of a urokinase-activated engineered anthrax toxin is uPAR dependent (25).

Based on cell culture studies, uPAR has also been reported to have a variety of biological functions independent of its role in uPA-mediated proteolysis (26, 27). Some of these non-proteolytic functions have been suggested to have importance *in vivo* after various challenges, but additional studies are required to strictly define a molecular mechanism in these cases (28–30). An evaluation of these reports is outside the scope of the present review.

Role of stromal cells

The initial studies on the role of plasminogen activation and other extracellular protease systems in cancer were done with cultured cells transformed by oncogenic viruses or established cancer cell lines (31, 32). It was taken for granted that the proteases in tumor tissue were produced by the cancer cells, a view that was corroborated by studies on transplanted tumors (8, 33). However, findings in human colon cancer tissue strongly challenged this picture. uPA immunoreactivity and mRNA in colon adenocarcinomas were confined to stromal cells (fibroblast like cells and endothelial cells) whereas no uPA expression was detected in cancer cells (34, 35). This finding suggested that colon cancer cells recruit stromal cells to produce the uPA that is involved in the tumor's degradation of the extracellular matrix and thereby promoting invasive growth (36).

Stromal cell expression of components of the uPA system as well as other matrix degrading protease systems has since been demonstrated in many types of human cancer and is now well established (1, 2, 6, 9). Precisely which cell type synthesizes each of these molecules is different in different types of human cancer. In ductal breast cancer, as in colon cancer, uPA is primarily expressed by fibroblasts (37, 38), and in prostate cancer by macrophages (39). In contrast, uPA is expressed by the cancer cells in skin squamous cell carcinomas (40). The expression pattern of uPAR also differs between different cancers. uPAR is expressed by both cancer cells and macrophages in colon cancer (35, 41), primarily by macrophages in ductal breast cancer (41), by cancer cells in skin squamous cell carcinomas (42), and by macrophages and neutrophils in prostate cancer (39).

Prognostic studies support the assumption that production of uPA and uPAR, and by extension other components of matrix degrading protease systems, by tumor-associated stromal cells pro-

motes invasion and thereby progression of human cancer. Elevated uPA and uPAR protein levels in tumor tissue and blood samples are associated with poor prognosis in many types of cancer (3, 43–47). Strikingly, this prognostic association seems independent of the cell type in which the molecules are produced. In breast cancer tissue for example, high levels of uPA, mainly derived from myofibroblasts, and uPAR, mainly derived from macrophages, are each strongly associated with decreased survival (43, 44), and in colon cancer high blood levels of uPAR, expressed by both cancer cells and macrophages, are also associated with poor prognosis (45).

Only few experimental studies on the functional role of stromal cell expression of components of proteolytic systems in cancer have been reported, probably because this phenomenon is generally poorly mimicked by transplanted tumors and there are few good animal models of spontaneous cancer available for study (48). However, genetically induced breast cancer in transgenic mice with the polyoma middle T antigen under control of the mouse mammary tumor virus long terminal repeat (MMTV – PyMT) (49) does resemble human ductal breast cancer in this respect, including expression of uPA and several MMPs by tumor-associated stromal cells (7, 50). In this model, absence of uPA leads to a pronounced decrease in lung and lymph node metastasis, indicating an important role of stromally derived uPA in these processes (7).

The involvement of stromal cells in the generation of extracellular proteolysis argues that cancer invasion is the result of an interaction between cancer cells and stromal cells. It is not only the cancer cells that invade but a mixed cell population. The cancer cells are the initiators and probably the organizers, but each cell type contributes in a distinct way to the overall process. Stromal cell involvement in the invasive processes represents a new paradigm with profound consequences for the understanding of both carcinogenesis and establishment of metastasis (6, 9, 36). Thus, for successful development of a cancer it is not sufficient that a given cell has acquired the characteristics of a cancer cell by a number of mutations. It is also necessary that it succeeds in recruiting the right combination of stromal cells and inducing their cooperative activities. Likewise, it is not sufficient for the establishment of a metastasis that a cancer cell settles in a new tissue. The cancer cell must recruit and cooperate with the right combination of stromal cells in the new environment in order to develop into a metastasis. It is likely that the recruitment of stromal cells can be a limiting factor in both carcinogenesis and metastasis. This suggests completely new approaches for prevention and treatment of cancer.

Cancer invasion and non-neoplastic tissue remodelling

Cancer invasion can be viewed as a tissue remodelling process in which the normal tissue is replaced by the invading cancer tissue. In some types of cancer the protease expression pattern during invasion appears to mimic the pattern in certain non-neoplastic remodelling processes within the same tissues (1, 6, 9, 36). Examples of such mimicking of expression are found in ductal breast cancer, squamous cell skin cancer and colon adenocarci-

nomas. In both ductal breast cancer and post-lactational mammary gland involution uPA, MMP-2, MMP-3 and MMP-11 are all expressed by subsets of fibroblasts (37, 51–54). In both skin squamous cell carcinomas and wound healing, epithelial cells (cancer cells and keratinocytes) express uPA, uPAR, MMP-9 and MMP-13, while MMP-2 and MMP-11 in both cases are expressed by fibroblasts (40, 42, 55–60). In colon cancer the expression of uPAR in cancer cells and of uPA in fibroblasts is reminiscent of the expression of uPAR in luminal epithelial cells in the normal gastrointestinal tract and of uPA in adjacent fibroblasts, which may be involved in the continuous epithelial cell shedding into the lumen (34, 35, 61, 62).

We propose that cancer invasion can be considered as non-neoplastic tissue remodelling processes that have gotten out of control (6). This is in analogy with the concept of neoplastic growth being considered as normal growth that has lost control mechanisms.

Redundancy

The similarity between cancer invasion and non-neoplastic tissue remodelling with respect to expression patterns implies that functional studies of proteolysis in non-neoplastic tissue remodelling may predict the role of the same proteases in cancer invasion. Such studies of skin wound healing and placental development in protease-deficient gene targeted mice have revealed a functional overlap between the PA system and other protease systems.

uPA is strongly upregulated in keratinocytes in human and mouse skin wounds (40, 55), and there is a pronounced delay in wound healing in mice deficient in plasminogen (63). In these mice there is a decreased rate of migration of keratinocytes from the wound edge during the re-epithelialization process and an accumulation of fibrin in front of the leading-edge keratinocytes, suggesting that the delay is due to a diminished ability of these cells to proteolytically dissect their way through the extracellular matrix. This interpretation is supported by the observation that skin wound healing is restored in mice deficient in both plasminogen and fibrin (64). Although delayed, complete wound healing is eventually achieved in all plasminogen deficient mice, suggesting that there is a functional overlap between plasmin and other proteases. Several MMPs, including MMP-3, MMP-9 and MMP-13 are expressed in the leading-edge keratinocytes. Treatment of wild-type mice with a broad spectrum MMP inhibitor, Galardin, which inhibits all three of these MMPs, causes a delay in wound healing time. Also in the Galardin-treated wild-type mice all wounds do eventually heal. However, when plasminogen-deficient mice are treated with Galardin, healing is completely arrested, demonstrating that protease activity is essential for wound healing. Thus, in a functional overlap between the two classes of matrix-degrading proteases, each class alone is capable of maintaining limited keratinocyte migration, albeit at decreased speed, but this function is normally performed more rapidly by both classes acting in parallel (57).

A similar functional overlap between plasminogen and MMPs is found in placental development. Treatment of pregnant plasminogen-deficient mice with Galardin leads to pronounced delays in placental decidualization and vascularization and a

high rate of embryonic lethality, while plasminogen deficiency alone or Galardin treatment alone has virtually no effect on decidualization and lethality (65).

A delay of postlactational mammary gland involution is seen in plasminogen-deficient mice, which demonstrates a role for plasminogen in this process (66). Like uPA, proteases such as MMP-2 and MMP-3 are upregulated in fibroblasts during mammary gland involution and are likely candidates for overlapping functions with the uPA/plasminogen system (54).

Functional overlaps also exist mutually between individual MMPs. A well-documented example is the finding of postnatal lethality of mice double-deficient in MMP-2 and MMP-14, which is in contrast to the lack of postnatal lethality in mice with single deficiency in one of these MMPs (67).

In the transgenic MMTV-PyMT mouse breast cancer model discussed above, deficiency in either uPA or plasminogen does not significantly influence occurrence or growth of the primary breast tumor but strongly decreases lung metastasis, indicating a role for plasminogen activation catalysed by uPA in this process. Lung metastases do however eventually occur in virtually all these gene-deficient mice, suggesting a functional redundancy between plasmin and other proteases (7, 68).

Therapeutic targeting

In contrast to the strong advances in experimental research, the clinical results of attempts to use inhibitors of extracellular proteases in cancer therapy have so far been disappointing. Either no, or only a borderline therapeutic effect has been obtained with tolerable doses of MMP inhibitors (69).

These clinical trials have all been done with non-specific inhibitors that interfere with a broad, and most often not well defined, spectrum of MMPs (69, 70). With the current understanding that each of the many MMPs is likely to have several normal physiological functions, including functions that are not related to matrix degradation (2, 71), it now becomes evident that in order to minimize toxicity, only inhibitors of proteases that are functionally involved in the progression of a certain type of cancer should be used.

The redundancy between individual extracellular proteases must have a crucial impact on the design of future strategies for clinical targeting of proteases. Clearly, the targeting of just a single protease, that has a functional overlap with one or more other proteases in a particular type of cancer, will lead to either no effect or only a transient effect, until the point when a selection or induction of cells that use overlapping proteases has occurred. The pattern of functional overlaps therefore needs to be established for each type of cancer and combinations of specific inhibitors of each of the overlapping proteases should then be used for therapy. Because overlaps also exist between the normal functions of proteases, such combination therapy may in some cases be expected to increase the spectrum or severity of side effects. A large research effort is therefore needed to identify the combination of protease inhibitors, which gives the optimal ratio between therapeutic effect and toxicity for each type of cancer.

Because all the target proteases are extracellular this work may be facilitated by the use of humanized monoclonal antibodies, each of which specifically inhibits one protease. A seri-

ous problem, however, is the shortage of experimental model systems that mimic human cancer with respect to extracellular proteolysis. The extensive stromal cell involvement in the generation and regulation of proteolysis seen in human cancer is most often not found in transplanted tumors. Some genetically induced mouse tumors appear in this respect to be closer to the human situation, examples being the MMTV-PyMT breast cancer model (49) discussed above and K14-HPV16 induced skin cancer (72, 73). Nevertheless there is a strong need for additional transgenic animal cancer models suited for preclinical testing of protease inhibitors, while valuable information on the mechanisms involved in extracellular proteolysis may also come from studies of non-neoplastic tissue remodelling processes.

The consistent expression of uPA and uPAR in human cancer, as well as the many studies consistently showing their prognostic impact in many types of cancer, makes uPA-mediated proteolysis a clinically attractive target for protease inhibition as a cancer therapy. uPA has experimentally, in addition to the finding of reduced metastasis of MMTV-PyMT induced breast cancer in mice deficient in plasminogen or uPA, been implicated in the growth and metastasis of transplanted tumors in a number of studies using uPA overexpression (74), anti-uPA antibodies (75), uPA-deficient mice (76, 77) or agents that block uPA-uPAR interaction (78, 79). The interpretation of such studies of transplanted tumors does however have several inherent problems, including the possible immune response directed at a uPA-producing transplant when gene-deficient mice are used.

An indication of the toxicity that can be expected to result from a complete and specific inhibition of the uPA system at different levels (plasmin or uPA activity or uPA-uPAR interaction) comes from experience with genetic deficiencies in man and mouse. The inherited human disease *ligneous conjunctivitis* is due to plasminogen gene deficiency and accompanied by severe symptoms (80), while human genetic deficiency of uPA or uPAR has not been reported. In mice, uPA deficiency causes distinct symptoms although these are less pronounced than those produced by plasminogen deficiency (12, 81). No spontaneous phenotype is observed in uPAR deficient mice (22). The real toxicity challenge can however be expected when inhibition of the uPA-system is combined with inhibition of MMPs or other extracellular proteases.

A completely different approach to therapeutic exploitation of extracellular proteolysis in cancer also has promising perspectives. This strategy includes the use of prodrugs that need proteolytic activation to generate local cytotoxic activity in tumor tissue. It was recently successfully applied to experimental tumors using a genetically engineered variant of anthrax toxin that is activated by cell surface bound uPA (25). The native form of the toxin is activated by the widespread serine protease furin and has a very high general toxicity. The engineered form is much less toxic and studies in gene-deficient mice show that its activation is not only dependent on plasminogen and uPA, but also on uPAR. These findings thus demonstrate the crucial role of both uPA and uPAR for the generation of cell surface uPA activity *in vivo*. The engineered toxins have, at tolerable doses, a potent cytotoxic effect on several transplanted tumors (25). The targeted cytotoxin-approach may be extended to other proteases highly expressed in cancer tissues, including MMPs (82).

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