Proteinase inhibitors in malignancy: therapeutic promise or another white elephant?

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Introduction

Tissue proteinases are important in many physiological and developmental processes including embryogenesis, angiogenesis, cell migration, blood coagulation, fibrinolysis, reproduction, uterine involution and inflammation[1]. Close control of the synthesis and secretion of proteinases is essential to limit their action to the site of proteolysis, to prevent their spread to normal tissues and preclude their aberrant release and activation.

Over the last 10-15 years a considerable body of evidence has accumulated which correlates the aberrant expression of proteinases with a number of pathological states, both malignant and benign. Collagens are the major structural proteins in all tissues and therefore interest has focused on the role of collagenolytic enzymes, secreted by epithelial and stromal cells in the accelerated tissue breakdown which occurs in tumour invasion and degradative diseases.

Metalloproteinases

The metalloproteinases; collagenase, gelatinase and stromelysin (MMP-1, 2 and 3) are especially important in this regard since they specifically degrade connective tissue macromolecules. Overexpression of collagenolytic enzymes has been particularly implicated in the pathogenesis of disorders of connective tissue such as rheumatoid arthritis, periodontal disease, corneal ulceration, certain ulcerative skin conditions, the collagen degradation occurring in atherosclerotic diseases, osteolysis and the pathogenesis, invasion and metastasis of malignant tumours.

Three major metalloproteinases have been identified and their genes cloned[2]. Interstitial (Type I) collagenase specifically degrades the fibrillar collagens (types I, II and III), whilst type IV collagenase (gelatinase) degrades type IV and V collagen which are found predominantly in basement membranes. Stromelysin is a non-specific metalloproteinase and degrades many matrix components including proteoglycans, laminin and the non-helical regions of collagens[2].

Metalloproteinases are secreted by epithelial cells, endothelial cells, stromal fibroblasts and some types of leukocytes. Primary human mammary tumours and cell lines have been demonstrated to secrete interstitial[3] and type IV[4] collagenases. To date, stromelysin has been detected in primary human squamous tumours, but not breast carcinomas. In rat mammary tumours, which contain mixtures of epithelial and myoepithelial cells, collagenases appear to be produced exclusively by epithelial cells[5].

Interstitial collagenase, stromelysin and type IV collagenase (gelatinase) are secreted in a proenzyme form and activated extracellularly. All three enzymes can be activated *in vitro* by treatment with organomercurial compounds. Interstitial collagenase and stromelysin can also be activated by limited proteolysis with trypsin, whereas type IV collagenase is a poor substrate for trypsin activation. Catalytic amounts of activated stromelysin can also convert procollagenase into a fully active enzyme[4]. Activation of collagenases also occurs through a urokinase plasminogen activator-dependent (uPA) pathway. The uPA converts plasminogen to plasmin which is capable of activating all three collagenolytic enzymes. Plasmin-dependent activation of procollagenase generates an enzyme species identical to that generated by limited proteolysis with trypsin or by treatment with organomercurial compounds. There is evidence that this proteolytic cascade for collagenase activation originally described in cultures of normal fibroblasts and endothelial cells is also involved in degradation of basement membranes by tumour cells. However, tumour cells have also been demonstrated to be capable of secreting collagenases which are already activated.

Role of proteolytic enzymes in malignancy

It can be seen that the cellular control of the synthesis, expression and release of proteinases is complex and partly dependent on a proteolytic cascade system (Figure 1). A wide variety of tumour and host cells are capable of expressing such enzymes and exerting paracrine or autocrine effects on surrounding cells to secondarily influence proteinase activity (Figure 2).

Numerous experimental observations have led to the hypothesis that the over expression of collagenolytic enzymes enables tumours to penetrate and invade normal tissue. The evidence for this is discussed.

Many workers have demonstrated that tumours contain higher levels of proteinases than the normal tissue from which they are derived. Measurement of type 1 (interstitial) collagenase in a series of colorectal tumours demonstrated that there were significantly higher concentrations of this enzyme in tumours compared to control epithelium. There was also a strong correlation between the level of enzyme

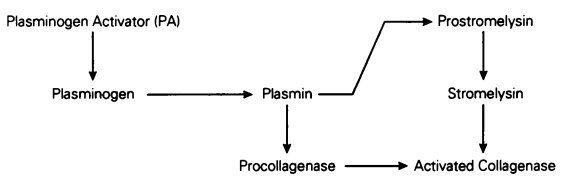

Figure 1. Proteolytic cascade involved in the production of activated collagenase

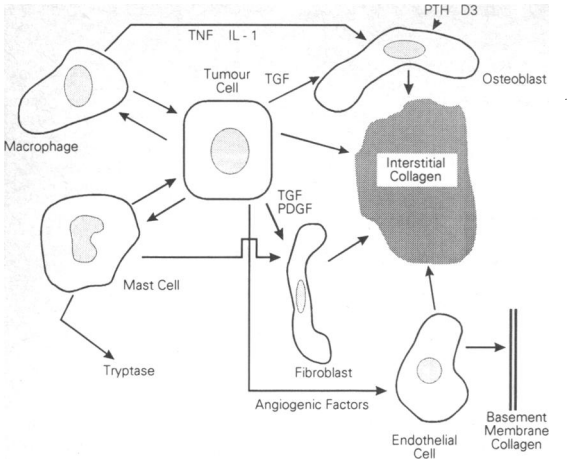

Figure 2. Factors which may control collagenase production by various cell types. TNF=tumour necrosis factor (β); TGF=transforming growth factor (α); *IL-1=interleukin-1; PDGF=platelet derived growth factor; PTH=parathyroid hormone; D3=activated vitamin D*

activity and the grade of histological differentiation: poorly differentiated tumours exhibiting the highest level of collagenolytic activity.

Similar correlations between proteinase activity and malignancy are provided by study of cultured cell lines. The neoplastic transformation of fibroblasts by X-rays, herpes simplex virus and the H-ras oncogene caused marked increases in the synthesis of type IV collagenase (gelatinase). Similar increases in the synthesis of stromelysin were seen when fetal rat cells were transformed by polyoma virus, rous sarcoma virus or H-ras[6].

Numerous studies have demonstrated that transfection of the H-ras oncogene into suitable non-malignant recipient cells yielded tumour clones which were fully metastatic[7]. Multiple changes occur in ras-transfected cell lines, ranging from increased production of degradative enzymes such as type IV collagenase and increased production of, or response to, motility factors[8]. Experimentally transformed cells, such as those transfected with H-ras, can degrade and pass through basement membranes whereas their normal counterparts cannot. This invasion may be prevented by naturally occurring or synthetic inhibitors of proteinases.

Tumours and malignant cell lines also produce a variety of growth factors and cytokines including transforming growth factor alpha, and parathyroid hormone related peptide (PTHrP) which have been shown to promote the synthesis of procollagenase in host cells. Thus, it has been postulated that tumours may promote the synthesis and release of collagenases and other proteinases from host cells as well as producing such enzymes themselves (see Figure 2).

The process of metastasis in animal models has also been studied. Alvarez *et al.* demonstrated a negative correlation between the amount of anti-collagenolytic activity of a recombinant human tissue inhibitor of metalloproteinase (rTIMP) added to cultures of a highly metastatic rat embryo cell line (transfected by the H-ras oncogene) and the ability of these cells to colonize the lungs after intravenous injection[9].

Nakajima *et al.* demonstrated that retinoic acid caused specific inhibition of type IV collagenase synthesis in a metastatic rat mammary carcinoma cell line and a corresponding decrease in their ability to invade through a reconstituted basement membrane (Matrigel-coated filter)[10]. Similar results were reported in four invasive human melanoma cell lines by Wood *et al.* These results are of interest as retinoic acid derivatives are currently under investigation as possible antitumour agents in humans acting as differentiation inducers[11].

Further support for the role of proteinases in tumour invasion is derived from the results of immunolocalization studies. Woolley and colleagues developed an antibody to activated type I collagenase. Active enzyme was identified at the periphery of a number of human gastric tumours and human melanomas. No enzyme was seen in normal tissues, but within tumours the enzyme was only expressed in small regions of the periphery and many tumours did not exhibit collagenase activity[12,13].

However, the most compelling experimental evidence to date for the causal role of proteinase expression in an animal model of metastasis is derived from an elegant series of experiments recently reported by Yu and Schultz[14]. They used two melanoma cell lines in two bioassays of metastasis. One cell line was of low metastatic 'efficiency' but could be rendered highly metastatic by transfection with the gene for pre-pro-urokinase-type plasminogen activator, whereas the other cell line of high natural metastatic 'efficiency' expressed high levels of this proteinase but was rendered non-metastatic by transfection with the antisense mRNA for this enzyme[14].

Certain clinical studies also provide evidence for the relationship between the potential for extracellular protein degradation and aggressive malignancy. Janicke *et al.* studied the relationship between total urokinase-type plasminogen activator (uPA) activity in breast cancer homogenates and disease free survival in these patients after primary surgery. At a median follow up of 12.5 months, patients with a uPA content greater than 2.6 ng/mg had a significantly higher frequency of relapse ($P<0.0002$) compared to patients with a lower level of uPA activity. Multivariate analysis revealed that the level of uPA had the strongest impact on relapse rate and was a more powerful prognostic indicator in this study than hormone receptor status or axillary lymph node involvement[15]. Similar findings, correlating high uPA activity with extensive local breast cancer, were reported by Duffy *et al.* These results are particularly significant because of the key role of plasminogen activator in the proteolytic cascade system (see Figure 1).

Guthrie *et al.* treated a patient with refractory advanced cervix cancer with intraperitoneal infusions of the protease inhibitor aprotinin (Trasylol, Bayer) an agent more commonly used (although of uncertain value) for the treatment of acute pancreatitis. There was evidence of a tumour response and the patient remained well for 9 years after treatment and was then lost to follow up. However, although further studies were planned by the investigators the reports of these were not forthcoming and there are no other reported clinical studies. Thus, it may be seen that although existing studies lend support to the general hypothesis that proteinases play a role in tumour invasion and metastases, absolute proof that proteinase activity is an obligate requirement for the progression, invasion and metastasis of human cancer remains elusive. One approach to the examination of this hypothesis is the use of specific inhibitors of these enzymes and this will now be discussed.

Physiological inhibitors

Inhibitors of collagenases may be conveniently classified into those occurring naturally and those created synthetically. Of the naturally occurring metalloproteinase inhibitors, TIMP (tissue inhibitor of metalloproteinases) is the most important and has been the subject of much investigation. TIMP is a glycoprotein with a molecular mass of 28 500 Da. It is secreted by several mesenchymal cells such as fibroblasts, vascular smooth muscle cells, endothelial cells and chrondrocytes. Because of its ubiquitous nature and its ability to form a tight complex with numerous metalloproteinases, including interstitial (type I) collagenase, gelatinase (type IV collagenase) and stromelysin in a one to one molar ratio, TIMP is likely to play a key role in the normal turnover of the extracellular matrix. Recent experimental evidence has suggested that this specific natural inhibitor may control the activity of metalloproteinases *in vivo* and also that many pathological conditions associated with excess collagen degradation or deposition may be due to an imbalance between the activities of proteinases and proteinase inhibitors[16].

With regard to the possible role of this inhibitor in tumour invasion and metastasis it has been shown that TIMP can block the invasion of human amniotic membranes by B16 melanoma cells and also lung colonization by these cells. Alvarez *et al.* used a recombinant human tissue inhibitor of metalloproteinase (rTIMP) in a tumour invasion model using a highly metastatic rat embryo cell line transfected by the H-ras oncogene. The proteolytic activity of these cells was completely inhibited by rTIMP and their ability to colonize the lungs after intravenous injection into nude mice was inhibited by 83% when the inhibitor was given concurrently by repeated intraperitoneal injection[9]. Gavrilovic *et al.* demonstrated that rabbit VX-2 tumour cells in culture produce collagenolytic activity, shown to be immunologically identical to interstitial collagenase from rabbit articular chondrocytes and bone. These cells degraded type I collagen films spontaneously and produced no detectable levels of TIMP. The degradation of type I collagen films by the VX-2 cells was significantly inhibited by a specific antibody to rabbit collagenase and by purified TIMP demonstrating unequivocally the role of collagenase in this model system.

It has also been suggested that the resistance of hyaline cartilage to tumour cell invasion is attributable to a particularly high level of TIMP within this tissue. More recently, further physiological inhibitors have been identified. Goldberg *et al.* identified a 24 kDa TIMP-like inhibitor which interacted with type IV collagenase, however the physiological role of this inhibitor is unknown.

The natural inhibitors of the cysteine proteinases are the cystatins which are found in three main forms (A, B and C). They are present in numerous tissues including squamous epithelium, lymph nodes, spleen, liver and neutrophils. Additionally, cystatins have been found in a number of breast cancers. A study by Kolar *et al.* demonstrated that the expression of all three cystatins in MCF-7 and ZR-75-1 breast cancer cell lines were inhibited by oestrogens, suggesting that the increased cellular invasiveness seen after oestrogen administration may be explained, at least in part, by a decreased level of cystatin expression and therefore an unapposed action of cysteine proteinases.

Non-physiological inhibitors

Naturally occurring inhibitors

Leupeptin and antipain are low molecular weight proteinase inhibitors of microbial origin which are resistant to metabolism in man. Aprotinin (Trasylol, Bayer) is a large molecular weight peptide (6100 Da) which is derived from bovine tissues. All three of these naturally occurring products have been evaluated in tumour, or tumour cell, models. The formation of lung metastases in rats injected with Yoshida ascites hepatoma cells was inhibited by leupeptin given twice daily by intraperitoneal injection. However, no effect was seen on the induction of bladder tumours in mice treated with the carcinogen N-butyl-N-(4-hydroxybutyl) nitrosamine. These results indicate that proteinase activity is more important in metastasis than tumour promotion in this model.

Antipain has been demonstrated to prevent the radiation-induced transformation of certain cell lines in culture and carcinogen or radiation-induced chromosomal damage. There are however no reported studies on the use of antipain *in vivo*.

Aprotinin reduces tumour growth and invasion when administered to hamsters with highly invasive fibrosarcomas or mice with mammary carcinoma. However, in other experiments, this proteinase inhibitor did not prolong survival in mice with transplantable ascites tumours.

In models of metastasis, similarly conflicting results have been reported. The number of lung metastases was reduced by 45% in aprotinin-treated hamsters injected with melanoma cells and a much greater reduction (94%) occurred if the cells were preincubated with aprotinin prior to injection.

In the Lewis lung carcinoma of the mouse, aprotinin was demonstrated to inhibit the formation of lung metastases by 50%, but in similar experiments an increase in the number of lung metastases was reported. Similar increases in the numbers of metastatic lesions have also been reported in other animal models.

Synthetic inhibitors

These products have been designed to specifically inhibit proteinase activity but also to have desirable pharmacological properties, for example solubility, high oral absorption and ease of manufacture.

The anti-fibrinolytic agents tranexamic acid and aminocaproate competitively inhibit the generation of plasmin from plasminogen. They are commonly administered for the treatment of various conditions characterized by excessive bleeding. Recently, aminocaproic acid was shown to cause a dose dependent inhibition of cellular migration through a synthetic basement membrane in an *in vitro* model of cellular invasion[4].

As zinc-requiring enzymes, collagenase, stromelysin and gelatinase are non-specifically inhibited by chelating agents such as EDTA and 1,10-phenanthroline.

Peptide derivatives based on natural substrates, with a metal binding group positioned in place of the cleaved peptide bond interact with the active site of the enzyme and are reported to inhibit collagenase. Thiol based tripeptides have been synthesized which have an IC_{50} (the concentration of inhibitor required to inhibit half of the activity of the enzyme) of 10 to 20 nM in short term collagenase assays but, auto-oxidation means that their degree of inhibition decays to an IC_{50} of 1 to 3 μM after 2 h to 3 h.

Nevertheless, one such inhibitor of this class has been shown to inhibit corneal ulceration and neural cell migration *in vivo*.

A tripeptide derivative synthesized to mimic the binding structure of Cystatin C, a human cysteine proteinase inhibitor present in extracellular fluid, has been shown to inhibit the cysteine proteinases produced by Group A streptococci. Experimental animals injected with lethal doses of such bacteria were rescued by intravenous injection of this derivative and similar bacteriocidal activity was seen *in vitro*. There are no published data concerning the action of this inhibitor in tumour invasion and metastasis.

Another peptide derivative {N-[3-N-(benzyloxycarbonyl) amino-1-(R)-carboxy propyl]-L-leucyl-O-methyl-L-tyrosine N-methylamide} has been used to determine the role of interstitial collagenase in bone resorption. Complete inhibition of collagen matrix degradation in resorbing embryonic calvarial bones was seen at a concentration of 50 μM.

A number of stable derivatives of hydroxamic acid have recently been synthesized which were designed to mimic the helical topography of type I collagen in the region of the sequences flanking the collagenase cleavage site and to form a coordinate complex with zinc when bound to the active site.

Hydroxamic acid derivatives are inhibitors of mammalian collagenases types I and IV. The most active members of this group have activity against fibroblast and neutrophil collagenases in the 1 to 10 nM range. Less potent but significant inhibition has also been reported for stromelysin and gelatinase.

There is limited published experimental data with this class of compound, but inhibition of tumour cell invasiveness has been reported in an *in vitro* amnion model[4].

The hydroxamic acid-based inhibitors represent an important new class of synthetic collagenase inhibitors which are stable and active at low nanomolar concentrations. Such concentrations are easily achievable *in vivo*. These compounds are likely to represent powerful tools to elucidate the contribution of metalloproteinases expressed by cancer cells or cancer-associated host cells in tumour development, invasion and metastasis.

Discussion

Should these agents be developed as potential anticancer drugs? The basis for rational pharmaceutical development is an understanding of pathophysiological processes. On that score it may be argued that current antineoplastic drugs are only of limited effectiveness because of our poor understanding of the cancer process. There is currently interest in the pharmaceutical development of these agents to inhibit tumour progression and metastasis and sufficient scientific and clinical data has accumulated to justify careful clinical trials. The potential consequences of widespread proteinase inhibition must be considered in the design of such studies. Many of the newer tissue proteinase inhibitors have a suitable profile of toxicity and efficacy to rationalize such studies.

Regretfully, only the iron test of careful clinical trials can really tell us whether these agents will become a disappointment along with the great majority of novel antineoplastic agents or whether they may represent any sort of advance in the struggle against cancer. Little is certain except that nihilism can serve only failure.

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