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Role of the extracellular matrix in the degradation of connective tissue

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Abstract Cell-matrix interactions have an important impact on regulating connective tissue degradation during physiological and pathological processes, e.g., development, wound healing and tissue remodeling and tumor invasion and metastasis. Connective tissue breakdown is initiated by a specific class of enzymes, the matrix metalloproteinases, which include the type I collagenases, the type IV collagenases/gelatinases and the stromelysins and which vary with respect to their substrate specificities. The activity of the metalloproteinases is regulated by de novo synthesis of the proenzymes, the activation of the zymogens and by the presence of the inhibitors, TIMPs. This tight control is required in order to guarantee normal functioning of connective tissue.

Introduction

The extracellular matrix (ECM) in skin has a complex composition and is composed of many constituents. The metabolism of connective tissue involves both the degradation of extracellular matrix proteins and the coordinated synthesis of new matrix components. Both pathways normally are maintained under tight control to allow physiological function of the tissue. Degradation of connective tissue and basement membranes occurs during inflammatory processes, cell migration, tumor invasion and metastasis. Several types of enzymes that degrade different extracellular matrix components have been found to be involved in this process. They include three groups of proteases that are divided into major families dependent upon their active center: the serine, cysteine and metalloproteinases. These proteases have individual or in part overlapping substrate specificities for matrix proteins. In addition a mutual activation has been demonstrated for some members. Most of these proteases have been found to be membrane-bound, whereas others are secreted into the extracellular space. The serine proteases include the well-characterized plasmin, thrombin, and urokinase and tissue-type-plasminogen activators (u-PA and t-PA). Plasminogen activators convert the zymogen plasminogen into its active form, plasmin, which is capable of degrading a number of proteins present in the ECM, such as fibronectin, laminin and fibrin. It also activates prostromelysins and procollagenases in vitro. In addition, u-PA degrades fibronectin and it also has been found to activate the 72 kDa type IV collagenase [1]. Among the cysteine proteases, cathepsin B may indirectly contribute to connective tissue degradation by activation of latent interstitial collagenase [2].

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The matrix metalloproteinases

The matrix metalloproteinases (MMPs) belong to a family of enzmyes that share several structural and functional properties, but differ in terms of their substrate specificities (Table 1; for review see references 3–5). At present this family includes nine members with activity against most matrix proteins. They can be divided into three subclasses:

1. The interstitial collagenase and PMN-collagenase that can cleave native triple helical collagens into characteristic ¾ (TCA) and ¼ (TCB) fragments that denature and can be further degraded by unspecific proteases as well as metalloproteinases

2. The 72 kDa and 92 kDa type IV collagenases/gelatinases that degrade basement membrane collagens as well as denatured collagens and gelatins [6]

3. The stromelysins and PUMP-1 (matrilysin). Stromelysin-1 and -2 are very similar in size and amino acid sequence, while PUMP-1 is a smaller truncated version of the stromelysins. Recently, a new member of this class has been described in stromal cells of breast carcinoma,

Table 1 The matrix metalloproteinase family (n.d. not determined)

Enzyme	MMP	M_r (kDa)	Substrates
Institital collagenase	MMP-1	57, 54	Collagens I, II, III, VII, VIII, X; gelatin
PMN-collagenase	MMP-8	75	Collagens I, II, III, VII, VIII, X; gelatin
72 kDa type IV collagenase/gelatinase (gelatinase A)	MMP-2	72	Collagens IV, V, VII, X, XI; gelatin; elastin; fibronectin; PG core protein
92 kDa type IV collagenase/gelatinase (gelatinase B)	MMP-9	92	Collagens IV, V; gelatin; elastin
Stromelysin-1 (SL-1)	MMP-3	60, 55	Fibronectin; PG core protein; collagens IV, V, IX, X; laminin; elastin
Stromelysin-2 (SL-2)	MMP-10	60, 55	Fibronectin; PG core protein; collagens IV, V, IX, X; laminin; elastin
Stromelysin-3 (SL-3)	MMP-11	n.d.	n.d.
PUMP-1 (matrilysin)	MMP-7	28	Collagen IV; gelatin; laminin; fibronectin; PG core protein

which, with respect to homology to stromelysin-1 and -2, was named stromelysin-3 [7]. The class of stromelysins has a wider substrate specificity and is active on gelatins, fibronectin, proteoglycan core protein, laminin, elastin and various collagens. These enzymes are secreted as latent zymogens and have a Zn^{2+}-ion in their active center.

Most human MMPs have been cloned and the amino acid sequence has been deduced from the cDNA clones revealing a characteristic composition of different functional domains that share strong homology between the various members and suggest the existence of a common ancestral gene. The largest member of the MMP family, the 92 kDa type IV collagenase (gelatinase B), is composed of seven domains. These include the hydrophobic signal sequence, followed by the propeptide domain which represents the amino-terminal end of the secreted latent enzyme, and the catalytic domain which is separated from the Zn^{2+}-binding domain in both gelatinases by three repeats of a sequence homologous to the gelatin-binding region of fibronectin. In gelatinase B there is an additional domain of unknown function that shares homology with collagen and is named the collagen-like domain. The carboxyterminal domain, which is missing in PUMP-1 but is present in all other MMPs, shares some homology with hemopexin and vitronectin, and is separated from the catalytic domain by a hinge region. Although the C-terminal domain is not required for proteolytic activity, recent results have revealed several important functions, e.g., mediating specific binding to the substrates. In addition, in the case of the 72 kDa type IV collagenase (gelatinase A), this domain also is bound by the specific inhibitor TIMP-2 (tissue-inhibitor of metalloproteinases-2) and is thought to play an important role in the activation of the proenzyme [8–10].

Regulation of metalloproteinase activity

The activity of MMPs is tightly regulated. This is not surprising when one considers the physiological importance of the substrates that active MMPs can degrade. The cellular control of MMPs is exerted at several levels. Regulation occurs at the level of gene expression, as well as extracellularly where the latent forms become activated. Finally the activity of the enzymes is dependent upon the amount of specific inhibitors present in the tissue, the TIMPs. Most cell types normally synthesize very little MMPs in vitro, and also in vivo the MMPs are not stored in most skin cells, except macrophages and neutrophils, but are synthesized as soon as a clear signal arrives. These regulatory signals work almost exclusively at the level of transcription.

De novo synthesis of MMPs can be induced in cell culture by a variety of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) and by growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Several of these effector molecules are synthesized by connective tissue and inflammatory cells as well as by tumor cells. Interstitial collagenase and stromelysin are often coordinately regulated and their genes are located close together on chromosome 11. However, a coordinate regulation is not always the rule. IL-1α and β and TNFα induce both type I collagenase and stromelysin-1 gene expression, but Il-1 is more potent than TNFα in the induction of stromelysin-1. A stronger indication for a dissociable control of metalloproteinase expression has been demonstrated for IFNγ, which can decrease the collagenolytic activity induced by IL-1β in human skin fibroblasts. Thus, stromelysin synthesis is strongly inhibited by IFNγ, whereas the induction of collagenase synthesis by IL-1β is not altered by this cytokine [11, 12]. In addition, although some MMPs have almost identical substrate specificity which is true for the interstitial collagenase and the PMN collagenase, and the 72 kDa type IV collagenase and the 92 kDa type IV collagenase, respectively, they are differently controlled at the transcriptional level. For example, the interstitial collagenase and the 92 kDa type IV collagenase respond to cytokines and growth factors, whereas the 72 kDa type IV collagenase is only moderately induced [13, 14]. Regulation of PMN collagenase mainly occurs on the release of stored enzymes rather than by transcriptional events [15].

Several growth factors and cytokines exert their effect on a common regulatory element of the MMP genes, the

AP-1 binding site. This element constitutes the phorbol ester-responsive element, TRE (TPA responsible element), which is complexed by a heterodimer composed of the transcription factors Fos and Jun [16, 17]. Many growth factors and cytokines are known to induce the expression of the protooncogene products c-Fos and c-Jun. Induction of interstitial collagenase by oncogenes and phorbol esters as well as induction of stromelysin by PDGF involve de novo synthesis of Fos, whereas EGF regulates stromelysin gene expression via a fos-independent mechanism [18]. AP-1 binding sequences have been identified in the promotors of the interstitial collagenase, stromelysin and the 92 kDa type IV collagenase but are missing in the 72 kDa type IV collagenase gene [16, 19]. The AP-1 site is necessary but not sufficient for transcriptional activation of the genes of interstitial collagenase and stromelysin [20–22]. The collagenase promoter contains an additional oncogene-responsive unit (TORU) that is closely located to the AP-1 site and is bound by the transcription factor PEA3. These two elements act synergistically to induce maximal levels of transcriptional activation by TPA and oncogenes [21].

Activation of the MMPs is complex and is still incompletely understood. It can be obtained by several agents and involves the dissociation of a cysteine-Zn^{2+} bond of an unpaired cysteine residue in the propeptide domain to the Zn^{2+}-ion in the active center and replacement by an H_2O molecule that is necessary for the catalytic activity. The break of the cys-Zn^{2+} bond can be achieved in vitro by chemical agents, e.g., organomercurials, chaotropic agents and detergents, which interact directly with the cysteine residue or induce a conformational change of the propeptide. In vitro activation also can be obtained by proteolytic enzymes, e.g., trypsin, plasmin, elastase and kallikrein, which cleave short sequences of the propeptide. As soon as the active center is exposed, these partially active forms can be autolytically cleaved to remove the propeptide and provide permanent activity. Although the knowledge about the susceptibility of MMPs to proteolytic cleavage has increased during recent years, the mechanisms relevant for MMP activation in vivo are still unclear. Activation of MMPs does not occur via a general, unique mechanism. It probably involves the action of various proteases and oxidative pathways, presumably by free radicals and oxidants that act on the unpaired cysteine residue.

Inhibitors of Metalloproteinases

In recent years it has become increasingly clear that the control of degradation of connective tissue is regulated by the amount of MMPs and their inhibitors present in the tissue. The balance of these protein families finally determines the degradation process in the tissue. Like the MMPs, the inhibitors of metalloproteinases (TIMPs) also represent a multigene family which includes a minimum of four members. TIMP-1 and TIMP-2 have related amino acid sequences. They both bind non-covalently to the active forms of MMPs and inhibit their activities. Whereas TIMP-1 binds all active MMPs but preferentially inhibits the interstitial collagenase [23], TIMP-2 is more effective than TIMP-1 against the two gelatinases [8]. In addition TIMP-2 forms a complex with latent 72kDa type IV collagenase by binding to the C-terminal end, and appears to block or retard activation of MMP precursors [10]. The role played by TIMPs in regulating matrix degradation therefore might be exerted not only by inhibiting proteinase activity but also by a perhaps even more potent effect, the blockage of MMP activation.

Role of the extracellular matrix in regulation of connective tissue breakdown

The interactions of cells with components of the ECM, such as fibronectin, laminin, tenascin and collagen, play an important role in morphogenesis, tissue repair and regeneration, and tumor invasion and metastasis [24–26]. During these processes the interaction of cells with the ECM changes continuously since the matrix is remodeled by the cells by local proteolytic degradation and de novo synthesis. In a reciprocal way, the matrix influences several cellular functions including migration, chemotaxis, cell morphology, proliferation and differentiation, as well as the regulation of connective tissue synthesis and degradation. Over recent years studies from a number of different investigators have demonstrated that cells specifically recognize and bind to ECM molecules. These matrix components contain multiple biologically active sites with distinct activities and cell specificities. The integrin multigene family represents a major group of adhesion receptors mediating cell attachment to a variety of ECM proteins, including fibronectin, laminin, collagens, vitronectin, fibrinogen and thrombosponsin (for review see references 27 and 28). The integrins are heterodimeric complexes consisting of one α- and one β-subunit which are covalently linked. Both subunits have a transmembrane segment, a large N-terminal extracellular domain and a small C-terminal cytoplasmic domain connected to the cytoskeleton. Numerous studies have shown that cells attach and spread on their ligands and organize their respective integrin receptor molecules into focal contacts which contain cytoplasmic proteins such as vinculin, talin and α-actinin, and are thought to mediate the interaction between the extracellular ligand with the actin-containing microfilaments on the inside of the cells [29, 30]. The functional diversity of integrins is mediated by the particular composition of two subunits and the specificity of the α-subunit for the individual matrix proteins. However, several integrins with distinct subunit composition recognize the same ligand, which is the case for collagen, laminin and fibronectin.

In order to study fibroblast-matrix interactions, an in vitro model has been established several years ago which is considered to resemble some aspects of the in vivo situation of the skin. Skin fibroblasts which are seeded into a reconstituted three-dimensional type I collagen gel ad-

here to the collagen fibers and contract the gel to a dense connective tissue, a process which is similar to wound contraction [31]. Although the mechanism of contraction is not completely understood, forces generated by the cells during spreading and consecutive aligning of collagen fibers in the direction of the cells as well as migration of fibroblasts seem to be important. The functional integrity of the cytoskeleton and protein synthesis are a requirement for the reorganization of the collagen gel. While fibroblasts interact with the three-dimensional collagenous environment, major changes in cell morphology can be observed. And this is associated with a strict organization of the cytoskeletal proteins with mutual interactions which enable them to generate forces within the cells [32]. The reorganization of the collagen matrix is accompanied by the change of several cellular functions, e.g., a reduced proliferation and the reprogramming of connective tissue metabolism. Synthesis of types I and III collagen is particularly decreased as retraction of the gels progresses and reaches values of about 10% of fibroblasts grown as monolayer cultures, resembling the biosynthetic activity of fibroblasts in normal skin [33]. In parallel, a rapid increase of collagenolytic activity can be observed when fibroblasts are grown in contact with native collagen fibrils. In contrast to monolayer cultures with low levels of interstitial collagenase and most of the enzyme being present as inactive, latent precursor, in three-dimensional collagen gels de novo synthesis of collagenase is strongly upregulated and a major part of the enzyme has been found to become activated [34]. Thus, removal of serum inhibitors leads to a complete breakdown of the collagenous scaffold within several days, liberating the cells. At present, the mechanism for the activation of latent collagenase is not understood. Recent data have shown that synthesis of other members of the metalloproteinase family, such as stromelysin and 72 kDa type IV collagenase, is concomitantly induced to a small extent, but it is not clear whether in this system these enzymes contribute to the activation of the interstitial collagenase or how these enzymes become activated themselves. Elevated collagenase protein is paralleled by a strong induction of de novo synthesis of specific mRNA which is increased after only 9 h of culture and reaches a maximum of about 30-fold induction after 5 days compared with monolayer cultures.

The mechanisms underlying signal transmission from the surrounding ECM into the cell nucleus are poorly understood. Recent investigations, however, have indicated that integrin receptors are involved in transferring the extracellular signal into the cytoplasm. De novo synthesis of the $\alpha_2\beta_1$ (VLA-2) integrin is selectively upregulated in fibroblasts during contraction of the collagen gels and returns to baseline production after contraction is completed [35]. In addition, retraction of collagen gels is inhibitable by antibodies specific for both the α- and β-chains. The important role of the integrin VLA-2 in mediating the reorganization of collagen gels has been substantiated by other investigators by demonstrating that cell lines that display low constitutive expression of VLA-2 and that fail to contract collagen gels gain the capacity after transfection of α_2-cDNA into the cells [36]. So far, it is not clear how integrins may transduce signals from the ECM to the nucleus. Recent reports provide evidence that protein phosphorylation of tyrosine residues might be involved in integrin signaling [37, 38].

Beside the interstitial collagens, other components of the ECM have specific effects on MMP gene expression. Werb et al. [39] have demonstrated that interaction of synovial fibroblasts with fibronectin via the integrin VLA-5, a specific fibronectin receptor, is followed by a strong induction of collagenase and stromelysin synthesis. Whereas native fibronectin itself does not induce MMP, adhesion of cells to fragments as well as to peptides derived from fibronectin induces the accumulation of mRNA for collagenase and stromelysin, suggesting that degradation products of fibronectin are the natural ligands for the fibronectin receptor. This effect is also mimicked by using monoclonal antibodies to the fibronectin receptor that block initial adhesion of fibroblasts to fibronectin [39]. Although other studies have shown that changes of cell shape and reorganization of cytoskeleton proteins induced by TPA and cytoskeleton disrupting agents, are linked to the induction of MMP expression [40, 41], these and other studies suggest that the substrate alone can modulate expression of MMPs.

Role of metalloproteinases in diseases of the skin

MMPs play an important role in physiological conditions such as wound healing and tissue regeneration. They are also involved in pathological conditions such as rheumatoid arthritis and tumor invasion. During the last decade, the MMPs have become of major interest with respect to matrix degradation during tumor invasion and metastasis since increased activity of several of these enzymes has been associated with the metastatic potential of cultured cells and in malignant tissues. Investigations on various tumors in vitro and in vivo have clearly shown a correlation of increased activity of 72 and 92 kDa type IV collagenases with neoplasia [42, 43], and serum levels of the 72 kDa type IV collagenase have been found to be elevated in lung cancer patients when compared with normal sera [44]. By examining various human tumor cell lines for their synthesis of mRNA specific for different MMPs, Sato et al. [45] have observed a clear difference in mRNA expression for the 72 and 92 kDa type IV collagenase between tumor cells originating from mesenchymal and epithelial cells. Whereas all mesenchymal tumor cells express considerable levels of mRNA for the 72 kDa type IV collagenase, only a few epithelial tumor cells synthesize it. Transcripts for the 92 kDa type IV collagenase are also more frequently detected in mesenchymal tumor cells than in epithelial cells [45].

Stromelysin expression has also been shown to correlate with tumor progression. Using a classic two-stage model of carcinogenesis in mouse skin, benign papillomas and squamous cell carcinomas have been induced by

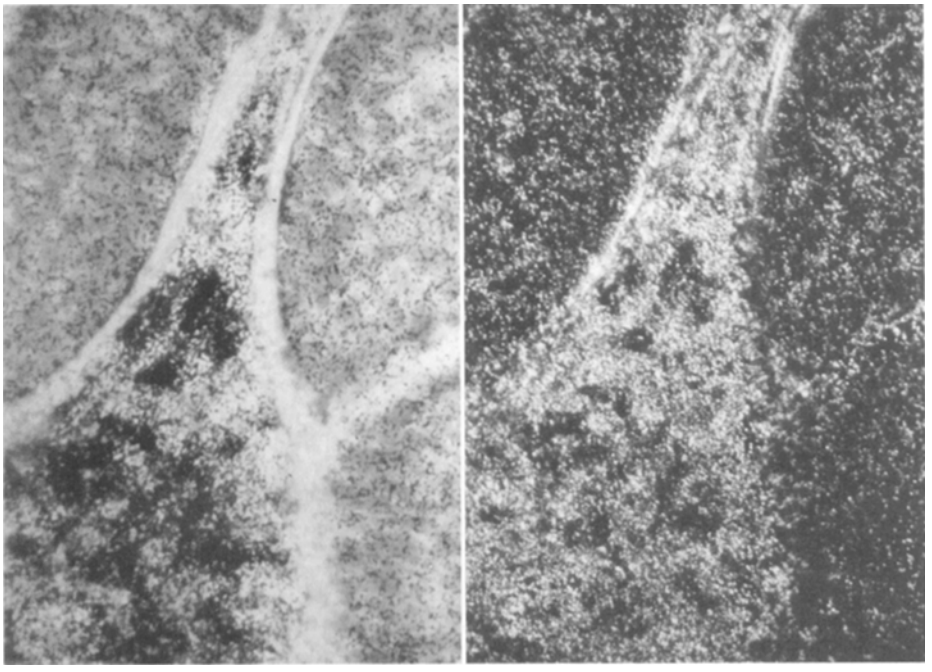

Fig. 1 Localization of mRNA specific for interstitial collagenase in a stromal fibroblast surrounding a basal cell carcinoma by in situ hybridization with ^{35}S-collagenase antisense RNA probe (1.5×10^6 cpm/section). De novo synthesis of collagenase mRNA is reflected by the amount of dark silver grains (bright field *(left)* and dark field *(right)* microscopy; × 250)

the treatment of mouse skin with the carcinogen DMBA (7,12-dimethylbenzanthracene) followed by repeated applications of the phorbol ester tetradecanoyl-phorbol-13-acetate (TPA). When RNA was isolated from these tumors, stromelysin synthesis is frequently detected in mouse skin carcinomas with a high potential to metastasize, but is rarely found in benign papillomas and is not detectable in normal epidermis [46].

Recent immunohistochemical studies have demonstrated that the 72 kDa type IV collagenase is mainly confined to the tumor cells in human breast, colon, ovarian, prostate, gastric and lung cancers suggesting that the tumor cells themselves are the major producers of the enzyme in the tumors in vivo (for review see reference 47). Other investigators have recently demonstrated by in situ hybridization studies on human skin, prostate and ovarian tumors that mRNA specific for 72 kDa type IV collagenase is expressed in stromal cells adjacent to the invading tumor front, but not in the tumor cells themselves [48–51]. For example, in basal cell carcinoma a strong signal for gelatinase A has been demonstrated in the stromal cells while the tumor cells are completely negative. In addition, this group have shown that more aggressive tumors such as the squamous cell carcinoma display mRNA for the 92 kDa type IV collagenase in tumor cells and in tissue macrophages surrounding the malignant epithelium. In contrast, no activity of this enzyme has been detected in the basal cell carcinoma, which suggests that differences exist between these two tumors in their mechanism of invasion.

Involvement of cells in the tumor stroma in the degradation of connective tissue in basal cell carcinoma has previously been suggested by an immunofluorescence study of interstitial collagenase [52]. In this study no specific staining of the carcinoma cells was seen, whereas the surrounding connective tissue showed staining of the interstitial collagenase. These findings have been confirmed by in situ hybridization using a specific antisense probe showing that strong de novo synthesis of collagenase mRNA is localized in stroma cells separating the islands of the tumor (Fig. 1) [53].

More recently, additional evidence for the involvement of stromal cells in the generation of proteolytic activity has been shown for another member of the metalloproteinase family. Immunohistological and in situ hybridization studies of stromelysin-3 in human breast cancer, and basal and squamous cell carcinomas have demonstrated transcripts and protein only in fibroblastic cells and in most stromal areas surrounding the islands of the invasive cancer. The highest levels of expression were found in the fibroblastic cells closest to the malignant cells, while type I collagenase-expressing cells were more randomly distributed throughout the tumors [7, 54, 55]. Interestingly, expression of metalloproteinases restricted to stromal fibroblast in different tumors includes the interstitial collagenase, the 72 kDa type IV collagenase/gelatinase and stromelysin-3, whereas synthesis of other metalloproteinases induced in tumor tissue such as stromelysin-1 and -2, matrilysin and the 92 kDa type IV collagenase is localized in neoplastic cells. In addition, coexpression of several members of the MMP family appears to be a general characterisic of human carcinomas, which is consistent with the concept that degradation of connective tissue during tumor invasion requires the synergistic action of several proteinases produced by tumor cells and/or by stromal cells.

At present, the mechanisms responsible for the induction of MMP synthesis in the surrounding stromal fibroblasts are not clear. Several growth factors and cytokines such as PDGF, EGF, IL-1, IL-6 and TNFα, and

oncogenes are known to induce gene expression of various MMPs in cultured fibroblasts, but the factors responsible for their induction in vivo are unknown. In addition, the tumors may indirectly alter adjacent connective tissue matrix by regulating the function of stromal cells. These alterations of the ECM may play a critical role in the growth and invasiveness of the tumor. For example in the basal cell carcinoma the tissue adjacent to the tumor has an increased number of fibroblasts and increased amounts of hyaluronic acid and extractable interstitial collagenase. It also has been shown that fibroblasts isolated from the surrounding connective tissue produce more collagenase than fibroblasts isolated from normal skin [56]. Other investigators have observed stimulatory activity of supernatants from isolated basal cell carcinoma cells on fibroblast proliferation and collagenase production, suggesting that tumor cell-derived factor(s) are responsible for these effects [57–59]. Biswas [60] and Biswas and Nugent [61] have postulated a membrane protein in melanoma cells that is responsible for inducing collagenase synthesis in fibroblasts. A synergistic effect was observed on synthesis of interstitial collagenase when melanoma cells and fibroblasts were cocultured and compared to the individual cell types grown separately [60, 61]. Interestingly, this group succeeded in isolating a membrane protein from a lung carcinoma cell lane which was found directly to stimulate collagenase synthesis in fibroblasts in a concentration-dependent manner [62].

To date, the best experiments demonstrating a causal role for MMPs in tumor progression and invasion come from experiments with the inhibitors of MMPs, TIMPs. Khokha et al. [63] have demonstrated the importance of TIMP in inhibiting tumor invasion and metastasis using an antisense approach. After transfection of a DNA construct with leads to synthesis of TIMP-specific antisense-RNA, Swiss 3T3 cells, which are normally not invasive by the amnion invasion assay, become invasive as a result of reduced levels of TIMP [63]. Similar results have been obtained for TIMP-2 by a different group using a c-Ha-*ras*-1 transfected cell line characterized by secretion of large amounts of MMPs and by the absence of inhibitors. Secretion of functional TIMP-2 in stably transfected cells results in a marked decrease in MMP activity and is associated with suppressed tissue invasion and metastasis [64]. In other studies, recombinant TIMP has been injected into mice after intraperitoneal administration of B16 murine melanoma cells. Frequent injection of recominant TIMP significantly inhibits metastatic lung colonization by melanoma cells [65]. Recent data using various metastatic and non-metastatic adenocarinoma cell lines indicates that lower levels of TIMP are distinctive for metastatic cells and this leads to increased levels of collagenase activity in these cells [66].

Taken together, the information available at present indicates that regulation of connective tissue degradation during tumor invasion is the result of a concerted action of both the malignant cells and the non-malignant cells in the tumor stroma. Hormones, cytokines and growth factors are likely to be implicated in the induction of enzymes, receptors and inhibitors, and studies of inducing factors will be important topics for investigation in the near future.

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