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Type IV collagenases in invasive tumors

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Summary

The matrix metalloproteinases appear to be elevated in tumors with metastatic potential, and may well be involved in penetration of the basement membrane and degradation of extracellular proteins including type IV collagen. An imbalance between the 72 kDa and 92 kDa type IV collagenases and the associated tissue inhibitors of these metalloproteinases (TIMPs) may therefore have a role in the invasive phenotype. Cultured tumor cells with invasive potential secrete both type IV collagenases, though in tumors there is some evidence that the 72 kDa form at least may be produced by stromal cells at the invading tumor front rather than primarily by the tumor cells themselves, while the 92 kDa form may be synthesized in macrophages near the front. These collagenases are elevated in invasive as compared with *in situ* tumor components, but their specific roles and prognostic significance are not yet established.

Introduction

The mammalian matrix metalloproteinases belong to a family of enzymes which are the products of related genes [1]. This enzyme family contains several members, all of which share several structural and functional properties, and all of which exhibit the capacity to degrade one or more of the molecules that constitute the extracellular matrix. The matrix metalloproteinases presumably play an important role in embryonic development and other physiological conditions which involve tissue remodeling, such as wound healing. Matrix metalloproteinases are also involved in several pathological conditions like rheumatoid arthritis. Furthermore, experimental systems using cultured cells and studies on tumor tissues have demonstrated that these enzymes are involved in the degradation of the extracellular matrix during cancer invasion. The metalloproteinases have been of great interest with respect to matrix destruction in malignancies because increased activity of several of these enzymes has been associated with the metastatic potential of cultured tumor cells and in malignant tissues [2,3]. During the process of tumor invasion and formation of metastases, the first barrier to be penetrated by the tumor cells is usually the basement membrane, after which the cells migrate into the interstitial stroma which is also degraded. This process involves the degradation of extracellular proteins such as type IV collagen, the major

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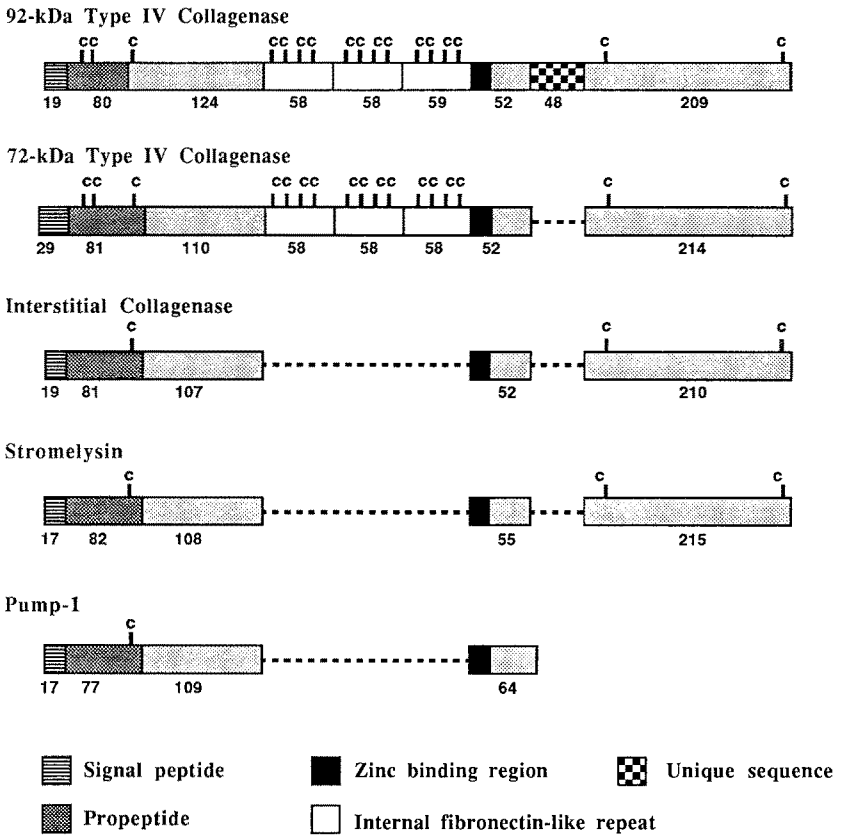

Figure 1. Schematic comparison of the structural domains of mammalian metalloproteinases. The domains are indicated by boxes. Domains missing from the protein are depicted by a dotted line. The number of amino acids of a domain is shown below the boxes. Location of cysteine residues is shown as (c).

structural component of basement membranes, the basement membrane specific laminin and proteoglycans, as well as interstitial collagens, fibronectin, and proteoglycans of the stroma. The different collagenases specifically cleave the collagenous components, which are quite resistant to other proteolytic enzymes. The proteolysis of the extracellular matrix by metalloproteinases is controlled by specific tissue inhibitors of metalloproteinases (TIMPs), so that the normal remodeling of the extracellular matrix requires a finely controlled interplay of proteinases and their inhibitors. In malignant tumor invasion there appears to be an imbalance of this interplay. In this review the properties of the metalloproteinase family and TIMPs, as well as the association of type IV collagenases with tumor invasion, are discussed.

The metalloproteinase family

To date, at least eight genetically distinct matrix metalloproteinases have been identified and characterized. All these enzymes are similar in that they are secreted as latent enzymes, they have a Zn^{2+}-binding active site, and they can be inhibited by the TIMPs. The primary structure and close interrelationships of the metalloproteinases characterized to date are depicted in Figure 1.

The two type IV collagenases with molecular

weights of 72 kDa (gelatinase A) and 92 kDa (gelatinase B) respectively are the largest members of the family, and they resemble each other closely with respect to both primary structure and substrate specificity [4-6]. Both enzymes have a similar size propeptide, an amino terminal domain, a fibronectin-like collagen-binding domain with three homologous internal cysteine-rich repeats, a Zn^{2+}-binding domain, and a carboxyl terminal hemopexin-like domain. The 92 kDa enzyme contains a 48-residue sequence which has no counterpart in the 72 kDa type IV collagenase or in any of the other metalloproteinases [5,7]. The function of this domain is unknown. The fibronectin-like domain is believed to facilitate the binding of these two enzymes to their collagen and gelatin substrates. Both type IV collagenases have been shown to cleave triple-helical type IV collagen molecules at a single site [8,9]. These enzymes have not been shown to degrade fibrillar collagens, but both have high activity against denatured collagen (gelatin) [1].

The two interstitial collagenases, the 57 kDa fibroblast procollagenase [10] and the 85 kDa neutrophil procollagenase [11,12], which degrade the fibrillar collagens of types I, II, and III, differ from type IV collagenases in that they lack the three internal fibronectin-like domains. These enzymes cleave the triple-helical molecules of fibrillar collagens specifically at a single site into 1/4 and 3/4 size fragments [1]. These proteolytic fragments are denatured at body temperature, thus becoming gelatin which can serve as a substrate for the type IV collagenases (gelatinases) and other enzymes.

Three types of stromelysin have been identified, stromelysin-1, -2, and -3, which have molecular weights about 53-57 kDa [1,13-16]. The stromelysins can degrade type IV collagen, and presumably other collagen types which have interrupted triple-helices. Additionally, they can degrade the noncollagenous components of the extracellular matrix, such as laminin and different proteoglycans. The most recently identified member of this family, stromelysin-3, was identified by molecular cloning and shown to be expressed in breast cancer mainly by the stromal cells [16].

Pump-1 is a small proteinase which lacks the hemopexin-like domain at its carboxyl terminal end [17,18]. It is particularly expressed in the involuting uterus, but its specific substrates are not known.

All metalloproteinases are secreted in a latent form and need activation to obtain catalytic activity. *In vitro* studies imply that the activation occurs by cleavage of the amino terminus (about 80 amino acids) which has one unpaired cysteine residue which possibly forms a coordination bond with the Zn^{2+} atom at the active site, thus keeping the enzyme in its latent form. The activation process *in vivo* is not well understood, but *in vitro* these enzymes can be activated by proteases which cleave off the propeptide or by organomercurials which bind the unpaired cysteine residue, after which cleavage of the amino terminus follows through an autoproteolytic mechanism [19,20]. *In vitro* studies indicate that the stromelysins and pump-1 can activate procollagenases by cleavage of the propeptide [1].

Tissue inhibitors of metalloproteinases (TIMPs)

The first specific inhibitor for collagenases, termed β1-anticollagenase, was reported as a serum protein with a molecular weight about 30 kDa [21]. The same year another specific inhibitor for collagenase with a molecular weight about 45 kDa was identified in rabbit V2 carcinoma cultures [22]. A year later bovine gingival cell cultures were shown to produce a collagenase bound to an inhibitor with a molecular weight about 20 kDa [23]. However, it was not until 1985 that the primary structure of a specific tissue inhibitor of metalloproteinase (TIMP) was elucidated by molecular cloning. TIMP had been purified and characterized from several sources, mainly by Reynolds and his collaborators, and

was shown to have a molecular weight of 28 kDa and to be identical to a protein with erythroid-potentiating activity [24,25]. Another member of the family, TIMP-2, reported by Stetler-Stevenson and his collaborators [26] with a molecular weight of 21 kDa, was shown to specifically inhibit the 72 kDa and 92 kDa enzymes. Both TIMPs share a close homology and inhibit in a 1:1 stoichiometric manner the activity of different metalloproteinases, and also prevent the autoproteolytic activation.

Elevated levels of TIMP-1 in human colon tumors have been reported compared to paired samples of adjacent normal mucosa when measured with a TIMP-1 specific ELISA [27]. Also, mRNA levels for TIMP-1 in colorectal carcinoma have been reported to be elevated, whereas TIMP-2 levels had no change when compared to matched normal tissue [28]. On the other hand, targeted disruption of the TIMP-1 gene increases the invasive behavior of primitive mesenchymal cells derived from embryonic stem cells *in vitro*, which can be reversed by adding exogenous TIMP-1 [29]. Recombinant TIMP-2 was able to inhibit the invasion of smooth muscle cell layers by invasive tumor cell lines [30]. Similarly, down-regulation of metalloproteinase activity and inhibition of invasion was achieved by transfection of invasive and metastatic rat cells with the cDNA for TIMP-2 [31]. These and other studies point out the importance of the balance between the secretion of matrix metalloproteinases and their specific inhibitors (TIMPs) in order to maintain the normal physiological function of these proteins. Any change to this balance may lead to severe pathological conditions and allow tissue invasion and metastasis formation by tumor cells.

Expression of type IV collagenases in physiological states and in neoplasia

The actual physiological mode of action of the two 72 kDa and 92 kDa type IV collagenases is, as yet, not known in detail. *In vitro* studies have shown that both enzymes cleave native triple-helical type IV collagen molecules specifically at a single site into 1/4 and 3/4 size fragments [8,9], and they also degrade gelatin (denatured collagen). These enzymes do not cleave collagen types I, II, and III, which make up the collagen fibrils of interstitial stroma and cartilaginous tissues. The specificity for type IV collagen has implied a crucial role of these enzymes in basement membrane turnover, since this collagen type makes up the highly cross-linked structural framework of these structures. Recent *in situ* hybridization studies with anti-sense RNA probes for the mouse 72 kDa type IV collagenase have shown that it is expressed in mouse embryos mainly in mesenchymal cells [32]. In contrast, ectodermal and endodermal tissues, with the exception of salivary gland, showed mostly weak signals or were negative. Expression was seen in a large variety of tissues such as lung, heart, kidney, and psoas muscle, whereas only weak signals or no signals were observed in liver, spleen, and brain. The high expression of the 72 kDa type IV collagenase in mesenchymal tissues, particularly close to epithelial tissues, indicates that this enzyme has a general role in the remodeling of extracellular matrix, not only for the turnover of the basement membrane specific type IV collagen, but possibly also for the removal of denatured proteolytic fragments of fibrillar collagen molecules (gelatin) through the gelatinase activity. Although there is still little knowledge about the physiological role and general tissue expression of the 92 K type IV collagenase, it is known that it is secreted by lung alveolar macrophages, polymorphonuclear leukocytes, and keratinocytes [1]. It is possible that the macrophages and leukocytes use the enzyme for penetration through the extracellular matrix during their migration through different tissue compartments in the body. However, this needs to be substantiated by further studies.

A large body of data has demonstrated the association of the 72 kDa and 92 kDa type IV collagenases with neoplasia, both in cultured cells and tumor tissues. A number of cultured cell lines exhibiting invasive capacity both in *in vitro* invasion assays and *in vivo* when injected into experimental animals have been shown to secrete these enzymes in varying amounts [2,3,28]. This has created great interest among researchers in the role of these enzymes in the process whereby differentiated cells acquire invasive properties when converted to the malignant phenotype. For example, cultured breast tumor cell lines have been shown to secrete both the 72 kDa and the 92 kDa type IV collagenases into the culture media, and the secretion of these enzymes depends on the culture conditions [33]. A rat mammary adenocarcinoma cell line (13762NF) and clones from that cell line with different metastatic potential were tested using type IV collagen containing lung subendothelial matrix and purified mouse type IV collagen as substrates. The metastatic potential of the cell lines was based on their ability to form lung metastases after s.c. and i.v. injections. The conclusion of the study was that the more metastatic clones also had higher type IV collagenase activity, and that the degradation of type IV collagen is important during lung metastasis formation by mammary adenocarcinoma cell lines [33]. Another study using rat mammary carcinoma cell line BC1 showed that this cell line produced the 92 kDa enzyme [34].

In vitro studies with different types of cultured cells often give rise to conflicting results, and their relevance to the *in vivo* situation is not always clear. Therefore, examination of tissue samples with specific antibodies or DNA probes is important for showing the actual presence of antigens or localization of expression at tissue and cell levels *in vivo*. Recent studies with antibodies have demonstrated the presence of the 72 kDa type IV collagenase in cells of different tumors. These studies have shown that the antigen is mainly confined to the tumor cells in desmoplastic basal cell carcinoma [35] and in human breast [36-38], colon [38,39], ovarian [40], gastric [38], hepatocellular [41], and lung [42,43] cancer. An example of immunostaining of human breast cancer tissues is shown in Figure 2. These findings have suggested that the tumor cells themselves are the major producers of 72 kDa type IV collagenase in malignant tumors *in vivo*. Interestingly, we recently showed by *in situ* hybridization studies on human skin tumors that 72 kDa type IV collagenase mRNA is actually expressed in stromal cells adjacent to the invading tumor front, but not in the tumor cells themselves [44]. For example, in basal cell carcinoma strong signals were shown with a 72 kDa enzyme anti-

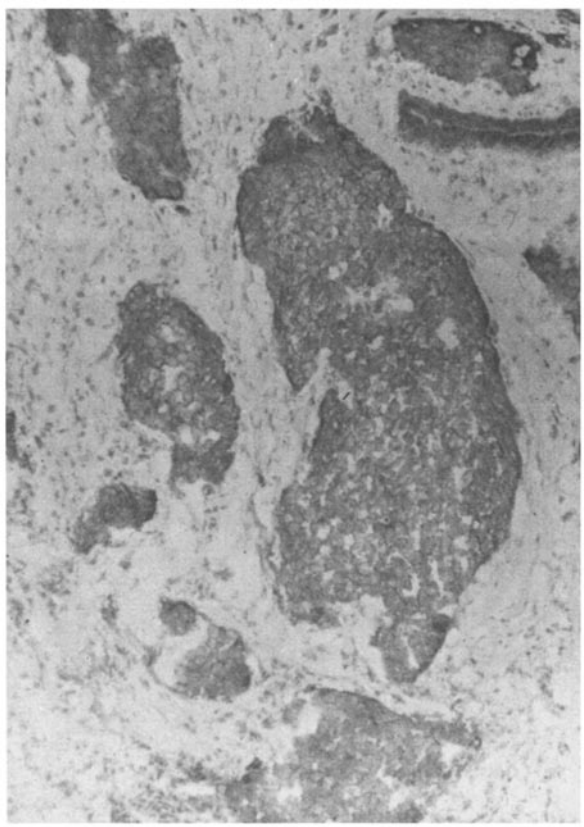

Figure 2. Immunostaining showing the localization of 72 kDa type IV collagenase in the tumor cells of human breast cancer tissue using a monoclonal antibody. Little if any staining is observed in stroma cells.

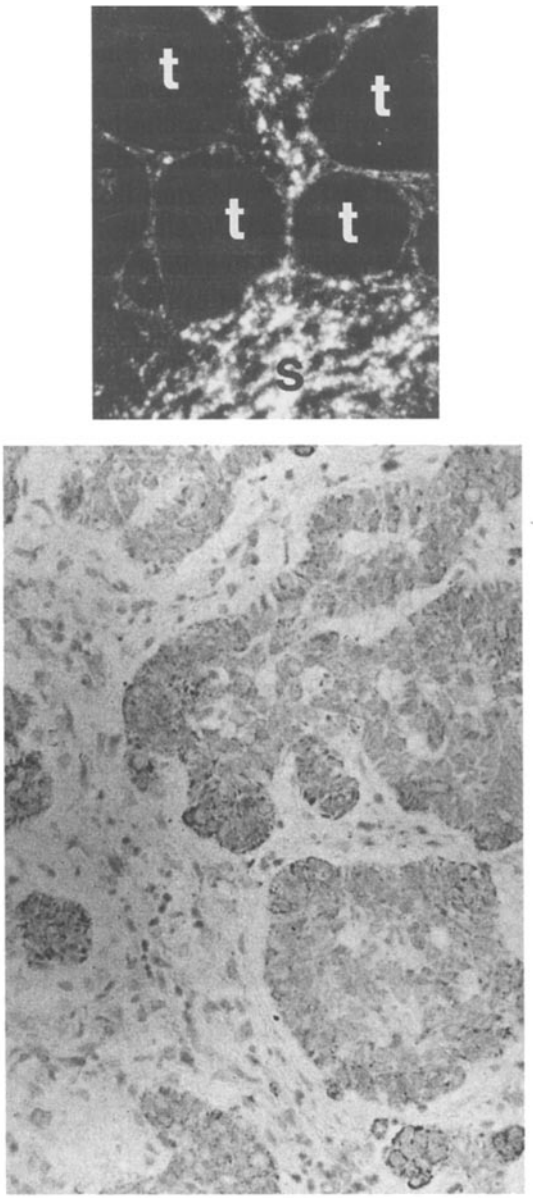

Figure 3. Localization of 72 kDa type IV collagenase antigen and expression in human basal cell carcinoma. (a) A darkfield image micrograph of *in situ* hybridization with an antisense probe shows intensive signals in numerous fibroblasts surrounding tumor nodules. The basal carcinoma cells themselves are negative. T, tumor nodule; S, stroma. (Reproduced with kind permission from the American Association for Cancer Research.) (b) Immunostaining with a specific monoclonal antibody localizes the enzyme to basal carcinoma cells at the front of the invading tumor (courtesy Haruo Ohtani).

sense probe in the stroma cells, while the cancer cells were completely negative (Figure 3a), and a similar result was obtained for squamous cell carcinoma tissue (not shown). In contrast, immunostaining localized the enzyme to the tumor cells at the invading front (Figure 3b). These findings have been confirmed by us and others for other types of tumors such as colon [45,46], and ovarian [40] cancer. The apparent discrepancy between the immunostaining and *in situ* hybridization studies is puzzling and may be due to binding and/or internalization in the cancer cells of the 72 kDa type IV collagenase produced by the fibroblasts, or to a lack of direct relationship between mRNA expression and protein synthesis.

Immunohistochemical studies with antibodies against 72 kDa type IV collagenase have not shown a clear correlation between intensity of staining and degree of malignancy. Antibodies against the 72 kDa enzyme showed strong cytoplasmic staining in myoepithelial cells in both normal and hyperplastic breast tissue, but discontinuous staining in intraductal carcinomas [36]. Epithelial cells were positive for 72 kDa type IV collagenase in 87% of intraductal carcinomas, 90% of invasive carcinomas, and 83% of metastases in lymph nodes, which supports previous findings on the role of the enzyme in tumor invasion and metastasis, as well as in the basement membrane remodeling of normal breast. In another study with 187 node-negative breast cancer cases, however, high levels of 72 kDa collagenase type IV failed to influence relapse-free or overall survival, indicating the complexity of invasion and metastasis formation in breast cancer and a possible requirement for the collaboration of several matrix metalloproteinases during the process [37]. Thirty cases of breast neoplasia were compared with 30 adjacent samples of normal duct epithelium. Invasive ductal and lobular carcinomas had a significantly higher percentage of immunoreactivity for 72 kDa type IV collagenase compared with corresponding *in*

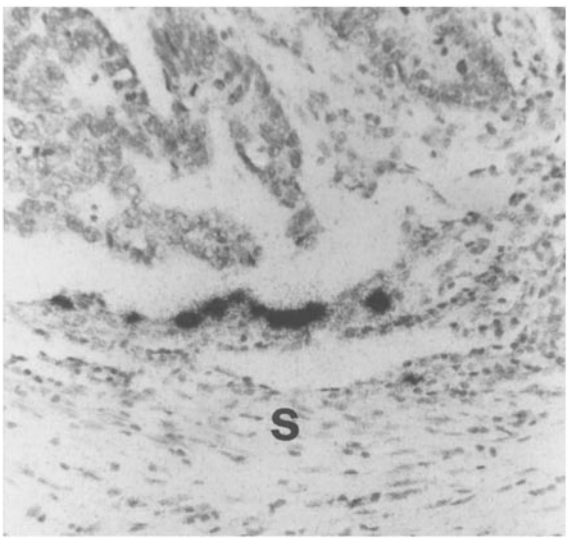

Figure 4. *In situ* hybridization for 92 kDa type IV collagenase in colon adenocarcinoma. Strong signals are observed in a number of macrophages located at the interface between the tumor cell front and stromal tissue (S).

situ lesions and normal epithelium, supporting the role of the 72 kDa enzyme in breast cancer progression [38].

We have examined the expression of the 92 kDa type IV collagenase in human skin [44] and colon [46] cancers using *in situ* hybridization. These studies showed that in six out of nine squamous cell carcinomas, tumor cells located at the tumor/stroma border expressed the enzyme. In contrast, cancer cells in five out of five basal cell carcinomas showed no signal. In both cases cells identified as macrophages located in the stroma adjacent to the tumor front were positive for 92 kDa type IV collagenase expression. No expression of enzyme was observed in any cells of normal skin. In colon cancer strong expression of 92 kDa type IV collagenase was observed specifically in macrophages of all tissue samples studied (Figure 4). Immunostaining of breast cancer tissues with antibodies specific for the human 92 kDa type IV collagenase has also shown the antigen to be located in macrophages

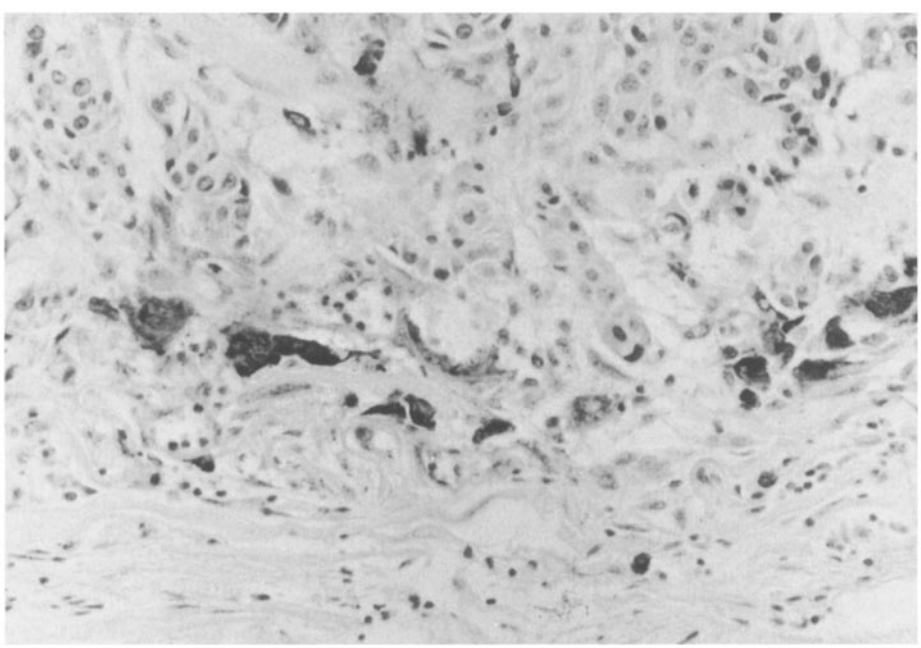

Figure 5. Immunolocalization of 92 kDa type IV collagenase in human breast carcinoma using a monoclonal antibody. The antigen is entirely confined to macrophage-like cells located adjacent to the invading tumor front. Tumor cells and fibroblasts are negative.

around the invading tumor cells (Figure 5). These results emphasize the previous observations showing the macrophage origin of the 92 kDa enzyme, but also indicate that in certain types of cancers, the cancer cells themselves can be activated to express the gene.

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