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Stromal Expression of 72 kda Type IV Collagenase (MMP-2) and TIMP-2 mRNAs in Colorectal Neoplasia

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We undertook an in situ hybridization study to localize the mRNAs for the 72 kda type IV collagenase (MMP-2) and its specific inhibitor (TIMP-2) in 12 colorectal carcinomas, 3 adenomas, and 4 uninvolved resection margins to see how their distributions correlated with that of the reported distribution of MMP-2 protein. Labeling for MMP-2 and TIMP-2 mRNAs was detectable in 10 of 12 carcinomas and in 2 of 3 adenomas. Unexpectedly, we found much stronger signals for MMP-2 and TIMP-2 mRNAs within the mesenchymal cells in the desmoplastic stroma, of endothelial and/or (myo)fibroblastic nature, rather than in tumor epithelial cells in which localization of MMP-2 was anticipated. Our data indicate that stromal cells may have the ability to synthesize a metalloproteinase that degrades basement membrane, and may together with the neoplastic epithelial cells participate actively in the tissue remodeling and disruption of the basement membrane integrity which is characteristic of invasive tumors. (Am J Pathol 1992, 141:389–396)

Malignant epithelial cells are characterized by their ability to invade the adjacent fibroconnective tissue and subsequently metastasize to distant organs.[1] This is a multistage process in which the degradation of the extracellular matrix (ECM) surrounding the tumor is an essential step in allowing invasion of neoplastic cells.[2] Loss of basement membrane integrity in colorectal and breast carcinomas is associated with increased probability of distant metastasis and poor prognosis.[3–5]

The matrix metalloproteinases comprise a family of enzymes that degrade ECMs, and their functional activity is controlled by tissue inhibitors (TIMP-1 and TIMP-2).[6] The cellular source of the ECM-degrading enzymes has important implications for our understanding of tumor biology and tissue remodeling.[7] Interstitial collagenases degrade types I, II, and III collagens, and are largely a product of stimulated fibroblasts[8] and some tumor cell lines.[9–12] Stromelysin 1 and 92 kda Type IV collagenase degrade basement membrane components and interstitial collagens, although they do not appear to be specifically upregulated in malignant tumors.[13,14] On the other hand, stromelysin 3 appears to be expressed specifically in the stromal cells of malignant breast tumors.[15] Similarly, the expression of the 72 kda type IV collagenase (gelatinase/MMP-2) is associated closely with the metastatic phenotype of malignant cells *in vitro* and *in vivo*.[16,17] MMP-2 cleaves basement membrane type IV collagen as well as degrading gelatins, fibronectin, type V, and type VII collagens.[18] MMP-2 is secreted by human epithelial cell lines[2,19] as well as fibroblasts,[20,21] endothelial cells,[22] and macrophages[23] in a latent pro-form that can be activated by loss of 80 amino acids from the amino terminus.[24] TIMP-2 binds noncovalently to the pro-form of the 72 kda type IV collagenase and inhibits its enzymatic activity,[25] whereas TIMP-1 appears to inhibit most of the interstitial collagenases[26] and the 92 kda type IV collagenase.[27] TIMP-2 is synthesized by endothelial cells and some tumor cell lines[25] which are also known to secrete MMP-2, but the full range of tissue expression is not yet defined.

A large body of experimental evidence supports the

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hypothesis that the expression of metalloproteinases correlates with tumor invasion, and that metastases arise from neoplastic cell subpopulations with an enhanced ability to migrate through tissues.[1,27] Highly invasive tumor cell lines secrete both the 72 kda type IV collagenase (MMP-2) and its inhibitor (TIMP-2),[16,17] and transfected cell lines displaying augmented MMP-2 activity are associated with a metastatic phenotype.[28,29] Levy et al recently used Northern blotting analysis to demonstrate abnormally increased steady-state levels of MMP-2 mRNA in tissue extracts from 72% of colorectal adenocarcinomas.[30] Immunohistochemical staining, using a rabbit affinity-purified MMP-2 anti-peptide antibody, localized the enzyme at the protein level predominantly to tumor cells, rather than stromal cells.[30] In contrast, functional interstitial collagenase and MMP-2 activity was demonstrable in only 4 of 29 colorectal carcinomas,[31] a finding that is difficult to account for. Normal mucosa and adenomas contain little extractable mRNA for either MMP-2 or TIMP-2,[30] although elevated levels of TIMP-1 transcripts have been reported in human colorectal tumor tissues.[25] In mammary carcinomas, antibodies to MMP-2 showed reactivity in invasive tumor cells and to a lesser extent in *in situ* carcinoma and some normal lobular cell populations,[32] with a few immunopositive (myo)fibroblasts noted in the stroma of invasive carcinomas together with smooth muscle and endothelial cells. These findings in breast support *in vitro* data indicating that neoplastic epithelial cells are a significant source of MMP-2 and TIMP-2 in carcinomas.

We undertook an *in situ* hybridization study to localize the mRNAs for MMP-2 and TIMP-2 in colorectal carcinomas to see if the expression pattern differed from that of the predicted protein distributions, since the dynamics of MMP-2 and TIMP-2 secretion, or uptake of potentially immunoreactive postactivation fragments are not yet defined. A further aim was to see if there were identifiable cellular subpopulations in the tumor with increased amounts of the mRNAs for the enzyme or its specific inhibitor.

Materials and Methods

Formalin-fixed, paraffin-embedded blocks from 12 colorectal carcinomas, 3 adenomas, and 4 uninvolved resection margins from colectomy specimens were selected from the archive files at Hammersmith Hospital, and 4 μm sections were taken under 'RNAse limited' conditions and mounted on 3-triethoxysilylpropylamine (Sigma, UK) TESPA-coated slides. Access to this material satisfied the requirements of the Hammersmith Hospital Ethical Committee.

In Situ *Hybridization*

The distributions of specific mRNAs encoding MMP-2, TIMP-2, AUA-1, and β-actin were established by hybridization *in situ* with antisense riboprobes synthesized with SP6 RNA polymerase, labeled with ^{35}S UTP alone or together with ^{35}S CTP (each at ~800 Ci/mmol; Amersham, UK). The methods we have established to pretreat, hybridize, wash and dip the sections for autoradiography[33] were based on those of Senior et al,[34] for formalin-fixed paraffin-embedded tissue. Autoradiography was at 4°C for 11 to 35 days, after which time sections were developed in Kodak D19, then counterstained by Giemsa's method.

The template used for making the MMP-2 riboprobe was *Bam*HI linearized pIV-16[30]; this produced approximately 1 kb of probe complimentary to ~190 bases of the C-terminal coding region and the entire 3′-untranslated region.

The template for TIMP-2 riboprobe was *Eco*RI-linearized pSS38,[25] which produced approximately 0.8 kb of probe complimentary to the 3′-untranslated region and extending as far as base 334 in the human TIMP-2 cDNA sequence (HSTIMP-2).

*Sma*I linearized pAUA 1-12 produced the riboprobe to detect mRNA for the epithelial glycoprotein, recognized by the monoclonal antibody AUA 1, which is an exclusively epithelial product.[35] This construct in pGEM 3Z (Promega, UK) contains a 198 bp *Bam*HI fragment of coding region, avoiding the entactin/nidogen-like domain. The monoclonal antibody AUA1 was used in a standard indirect avidin-biotin complex method[33] with diaminobenzidine substrate, to correlate the protein and mRNA distributions.

As a further control for the presence of mRNA in all compartments of the tissue, adjacent sections were hybridized with a riboprobe to detect β-actin mRNA generated with SP6 RNA polymerase and Dra I linearized phβA-10, prepared by subcloning the ~450bp *Eco*RI-*Rsa*I 3′-untranslated region of human β-actin present in βHF3′-ut[36] into the *Eco*RI-*Eco*RV sites of pSP73 (Promega).

The regions of sequence used to produce riboprobes were selected carefully to avoid stretches of sequence that might crosshybridize. The program ALIGN[37] within the Intelligenetics package was used to assess the similarity of the chosen probe region of MMP-2 with sequences that received high scores in a 'fastdb' search for related sequences, namely; collagenase (MMP-1, HSCOLLR); 92 kda collagenase (HS4COLA; macrophage or lung fibroblast collagenase); PUMP-1 (MMP-7, HSPUMP1); fibroblast collagenase (HSCN2); synovial cell collagenases (HSCCASA, HSCN25); neutrophil

collagenase (HSCLGNA); the three stromelysins (HSSTROMR, HSSTROM2, and the sequence proposed for stromelysin 3[15] and IMP dehydrogenase (HSIMPH). The longest stretch of identical sequence was of 11 bases for the neutrophil collagenase; thus no crosshybridization from the above mRNAs would be expected. Similarly, no particular crosshybridizing regions were found when the sequence of the TIMP-2 probe region was aligned with that of TIMP-1 (HSTIMPR) or the fibroblast/granulosa cell collagenase inhibitor (HSFCI, HUMOGCA).

Further evidence for the specificity of the hybridization signals obtained is provided by the marked differences in the intensity of labeling above different tissue compartments by the four riboprobes.

Immunohistochemistry

Where enough relevant tissue remained after *in situ* hybridization, sections from two adenomas, eight carcinomas, and two normal resection margins were used to stain for MMP-2, and the endothelial markers QBend-10 and Factor VIII-related antigen (vWF) (46). The antibody to MMP-2 was a mouse monoclonal antibody raised against a synthetic peptide corresponding to amino acids 1-16 of the proMMP-2 molecule, producing identical immunohistochemical localization to the polyclonal antipeptide antibodies previously described[30,32] when used at a concentration of 1–2 μg/ml, followed by a standard ABC immunoperoxidase technique on 4 μm sections (Vectastain kit, Vector Laboratories, Burlingame, CA).

Anti vWF(M 616, Dakopatts, UK) and the endothelial marker QBend-10 (Unipath Ltd., Bedford, UK) were demonstrated on corresponding sections using each monoclonal antibody (at dilutions of 1 in 10 and 1 in 100, respectively) at room temperature for 1 hour, followed by a standard ABC technique (reagents from Dakopatts, UK), development with 0.03% DAB (Sigma, UK) and counterstaining with 1% Mayer's hemalum.

Results

Type IV Collagenase (MMP-2)

Labeling for MMP-2 mRNA was seen in 10 of the 12 carcinomas, and in 2 of the adenomas. Signals were much stronger within the stroma of the tumors rather than the anticipated localization in tumor epithelial cells. The labeling did not correspond to the distribution of lymphoid cells or macrophages, and the labeled cells were clearly spindle shaped on lightly labeled areas and by comparison with conventionally stained serial sections. Labeling was heaviest in the more cellular, immature stromal proliferations deep in the tumor, especially where it penetrated the muscle coat, and was usually more discrete for MMP-2 than for TIMP-2, aggregating over the majority, but not all of the fibroblastic cells and possibly immature edothelial cells within the desmoplastic stroma (Figure 1a, b). Interestingly, the heaviest labeling was seen at the lateral aspects of deeply penetrating glands rather than the deepest margin of the tumors (Figure 2), a consistent finding in our series. However, the intensity of the labeling was variable in different tumors, only sparse cells being labeled in three of ten carcinomas, but was moderate or heavy in areas of the remaining seven. The neoplastic cells showed sparse labeling for MMP-2, compared with the labeling seen in the stroma (Figure 3a), although in five of ten cases, epithelial labeling was not convincingly above the 'background' level of grains.

Labeling for MMP-2 mRNA was seen also on discrete individual cells within the normal lamina propria, and occasionally in the submucosa. This labeling was more evident in the noninvolved mucosa adjacent to the carcinomas (Figure 4) than in the mucosa 1 cm or more from the tumors and in the blocks from the resection margins. A few nonendothelial cells within the muscularis propria were also labeled, again more pronounced in the vicinity of the neoplasm, and sparse labeling of neural cells in the intramuscular plexi was noticed. The lamina propria of the adenomas showed a similar pattern of labeling: the glands were unlabeled; the nonneoplastic mucosa labeled sparsely although labeled cells were seen more frequently. Smooth muscle cells in the muscularis mucosae and muscularis propria also showed light labeling in four carcinomas in which the desmoplastic stroma was also heavily labeled, but around the malignant glands invading the muscularis propria signals were confined generally to nonmuscle mesenchymal cells.

In contrast, immunostaining for MMP-2 showed stronger localization to neoplastic epithelial cells, and to a lesser extent to non-neoplastic cryptal epithelial cells and had a rather granular pattern (Figures 1b, 5b). Intensity of staining varied between different cases and in different areas of the same tumor, but there was no clear relationship of strongest areas of epithelial immunostaining to those with highest stromal labeling by *in situ* hybridization (ISH) (for instance, on the margins of the invasive tumor). Both cases studied, which had shown weak labeling over epithelial cells, were moderately positive in the corresponding areas. Smooth muscle cells in blood vessels and muscularis propria were weak or negative by immunohistochemistry for MMP-2. In some areas, endothelial cells lining patent capillaries were weakly to moderately immunopositive (Figure 1b). Although the majority of

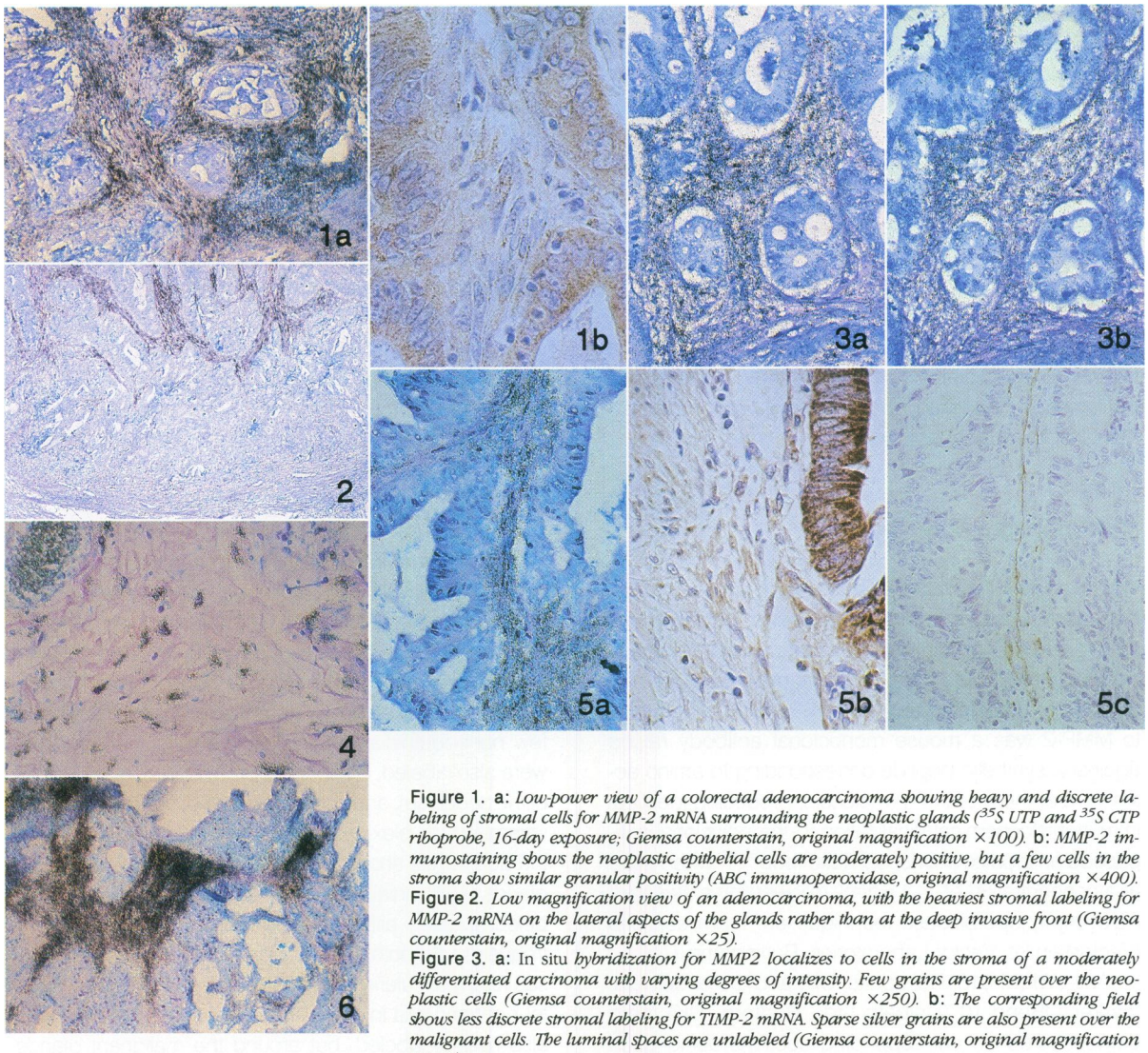

Figure 1. a: *Low-power view of a colorectal adenocarcinoma showing heavy and discrete labeling of stromal cells for MMP-2 mRNA surrounding the neoplastic glands (^{35}S UTP and ^{35}S CTP riboprobe, 16-day exposure; Giemsa counterstain, original magnification ×100).* b: *MMP-2 immunostaining shows the neoplastic epithelial cells are moderately positive, but a few cells in the stroma show similar granular positivity (ABC immunoperoxidase, original magnification ×400).*
Figure 2. *Low magnification view of an adenocarcinoma, with the heaviest stromal labeling for MMP-2 mRNA on the lateral aspects of the glands rather than at the deep invasive front (Giemsa counterstain, original magnification ×25).*
Figure 3. a: In situ *hybridization for MMP2 localizes to cells in the stroma of a moderately differentiated carcinoma with varying degrees of intensity. Few grains are present over the neoplastic cells (Giemsa counterstain, original magnification ×250).* b: *The corresponding field shows less discrete stromal labeling for TIMP-2 mRNA. Sparse silver grains are also present over the malignant cells. The luminal spaces are unlabeled (Giemsa counterstain, original magnification ×250).*
Figure 4. *Submucosa adjacent to an invasive carcinoma with scattered discrete cells labeled for MMP-2 mRNA (Giemsa counterstain, original magnification ×160).*
Figure 5. a: *Focal moderately heavy labeling for MMP-2 mRNA in the stroma of a well differentiated adenocarcinoma. There is sparse labeling of the epithelial cells but not of glandular and capillary spaces (Giemsa counterstain, original magnification ×250).* b: *MMP-2 protein is present in tumor cells, obvious endothelial cells and also in spindle cells in the stroma (ABC immunoperoxidase, original magnification ×400).* c: *Q Bend10 immunostaining shows the distribution of endothelial cells in this stromal area (ABC immunoperoxidase, original magnification ×250).*
Figure 6. *Unusually heavy labeling for TIMP-2 mRNA in another tumor, in areas corresponding to MMP-2 mRNA distribution (Giemsa counterstain, original magnification ×160).*

spindle cells in the desmoplastic stroma appeared negatively stained, some were positive, especially in the areas of strong mRNA labeling but were sometimes inconspicuous because of the delicate nature of their cell processes (Figure 5b).

Staining for vWF and QBend-10 (Figure 5c) showed that there was an increase in the numbers of immunoreactive capillaries (microvessels) in the desmoplastic stroma, but there was no demonstrable excess in those areas showing the highest radiolabeling. The immunoreactive endothelial cells were in a minority in such areas, but occasional positive spindle cells not associated with any lumen were seen. In the surrounding mucosa, the cells scattered in the connective tissue (Figure 4) did not express vWF or QBend-10.

Thus it appears that many of the desmoplastic stromal cells labeling for MMP2 (and TIMP2) mRNAs comprised a population that did not form microvessels or express

vWF/QBend-10 but, using the light microscope, we are unable to say which of these cells represent tangentially sectioned endothelial cells or endothelial precursors and which are (myo)fibroblasts.

TIMP-2

The pattern of labeling with TIMP-2 probe followed broadly that of MMP-2 in terms of distribution, although in all but two cases it was less intense (Figures 3b, 6). In 1 case in which weak MMP-2 labeling was seen there was none for TIMP-2 in a nearby section. The TIMP-2 probe tended to produce a more diffuse scattering of silver grains than the aggregated pattern of MMP-2 probe (Figure 3b). This may be due to a more widespread distribution of cells containing TIMP-2 mRNA. A few individual cells labeled in peritumoral non-neoplastic mucosa and submucosa, but not in the muscularis mucosae. Endothelial cells in capillaries showed light labeling, more easily detected in the vicinity of the tumor or near areas of ulceration.

β-*Actin*

Eleven of the 12 colorectal carcinomas produced detectable labeling for β-actin mRNA to varying degrees, but invariably greatest on the malignant epithelial cells, in keeping with our previous observations (Naylor et al, Experimental Cell Biology, in press), and indicated that mRNA was not degraded totally in a section. In addition, a wide variety of cell types in each section labeled with the β-actin probe to consistent degrees, both within each section and relative to the intensity of labeling within each batch of repeated hybridization runs (i.e., when β-actin labeling was strongest the other probes showed correspondingly greater labeling). The pattern of labeling provided a valuable contrast in pattern to the other probes and indicated that labeling was specific. One of 12 tumors gave no signals with any of the probes, and we assume that all of the mRNA was degraded in that tissue; another showed weak labeling for β-actin mRNA and was uninformative with the other three probes.

AUA1

Malignant epithelial cells in 8 of the 12 colorectal carcinomas labeled with this probe, although the intensity was usually considerably less than for TIMP-2, MMP-2, and β-actin probes. AUA1 mRNA was localized to cells that were demonstrably positive with the specific antibody to the AUA1 membrane antigen. However, the stromal cells were invariably negative for AUA1 mRNA and protein, providing a useful contrast to the TIMP-2 and MMP-2 probes, and further demonstrating the specificity of the stromal cell labeling.

Discussion

This study compared the relative distribution of MMP-2 and TIMP-2 mRNAs in colorectal adenocarcinoma by *in situ* hybridization, anticipating that the highest expression of each mRNA would localize to the epithelial component. Unexpectedly however, we found the strongest signals for MMP-2 and TIMP-2 mRNAs in the desmoplastic stromal cells rather than the neoplastic epithelial cells. Our data indicate that peritumoral stromal cells have the ability to synthesize a metalloproteinase that degrades basement membrane and, therefore, may participate actively together with the neoplastic epithelial cells in breaching the basement membranes integrity. These observations may help to explain why the progression of malignant epithelial tumors in breast depends at least in part on their interaction with the stromal component,[38] and may be particularly relevant in tumors characterized by a florid desmoplastic reaction, such as breast and colorectal carcinomas.

To date, much emphasis has been placed on the neoplastic cell being the principal source of enzymes to degrade the barriers to tissue invasion, thus permitting spreading and distant metastasis.[1,2] The cellular source of extracellular matrix-degrading enzymes is of primary importance in the understanding of neoplastic cell behavior; recent evidence has indicated that the desmoplastic stroma may play an important and complementary role to the neoplastic cell in tissue remodeling in carcinomas. Basset et al (1990) identified a metalloproteinase gene, stromelysin-3, that is expressed, at the mRNA level at least, specifically in stromal cells surrounding invasive breast carcinomas.[15] The substrate specificity of ST-3 is not yet known but stromelysins 1 and 2 are able to degrade interstitial collagens as well as basement membrane components, including type IV collagen.[13,39] It is well known that interstitial collagenase (which degrades collagens types I and III) is a product principally of fibroblasts, and is upregulated in tumor stromal cells, perhaps stimulated by neoplastic cell-derived cytokines.[14,21,40,41] On the other hand, MMP-2 has been demonstrated at the protein level in a number of epithelial cell lines, and its expression appears to correlate well with invasive and metastatic behavior of experimental models *in vitro* and *in vivo*.[29,30] MMP-2 has also been demonstrated in fibroblasts from fetal lung, cornea, synovium[14] and peritubular cells of the testis.[42] The latter

showed increased synthesis of MMP-2 when cocultured with Sertoli cells; this may have implications for the *in vivo* neoplastic situation. Specific mRNA for MMP-2 can be extracted from carcinoma tissue that obviously contains a mixed cell population comprising of neoplastic epithelial cell, as well as inflammatory and mesenchymal cells.[30] However, the assumption was that the MMP-2 mRNA was largely epithelial-derived, supported by the observation that polyclonal antisera raised against the N-terminal 17 amino acid residues of type IV procollagenase, or a 16 amino acid peptide corresponding to the metal binding domain of MMP-2 localized almost exclusively to the neoplastic epithelial cells of colorectal[30] and mammary carcinomas.[32] MMP-2 is secreted in the latent pro-form, which is activated by the loss of 80 amino acids from the N-terminal end, so the detection of the pro-fragment in neoplastic cells using antisera H1 putatively identifies the synthetic cell,[43] or possibly the location of enzyme activation or uptake.

There are arguments that could be proposed against the hypothesis that stromal cells make any significant contribution to basement membrane remodeling in carcinomas, and we were only able to demonstrate MMP-2 protein in a minority of the mesenchymal cells. We have not demonstrated the protein product of MMP-2 or TIMP-2 in stromal cells. However, it seems unlikely that the cells labeled by *in situ* hybridization would maintain increased steady-state levels of these mRNAs without significant translational activity. And if so what purpose would this serve in functionally active non-neoplastic cells? Also, it could be proposed that some of the immunoreactivity seen in neoplastic cells is a result of the uptake of postdigestion fragments, or a stromal fibroblast secreting the enzyme rapidly thus diminishing cellular immunoreactivity. Although there is some evidence that exogenous MMP-2 may be processed by transformed cells *in vitro,*[44] there is no evidence that tumor cells selectively take up MMP-2 fragments and this would seem an unlikely mechanism. Furthermore, the immunohistochemical studies of MMP-2 distribution in human tumors have been performed with several polyclonal, monospecific, antipeptide antibodies against different domains of the enzyme, and all give identical localization of MMP-2 protein within the neoplastic epithelial cells. Thus, the disparity in the distribution of MMP-2 between *in situ* hybridization studies and immunolocalization studies may be explained by differences in the rates of mRNA translation and capacity for intracellular storage of the protein. It could also be proposed that the threshhold of detection in the epithelial compartment for the mRNAs of MMP-2 and TIMP-2 in this system may not be reached, since we anticipate that a proportion of the mRNAs would have degraded in formalin-fixed paraffin-embedded archival material, so only the more abundant mRNAs are demonstrable. Nevertheless, the unequivocal stromal localization demands an explanation since we are convinced this is not an artifact.

It is known that endothelial cells can synthesize both TIMP-2 and MMP-2.[22] The immunohistochemical staining for vWF demonstrates there are increased numbers of formed microvessels in these stromal areas, in keeping with previous studies of angiogenesis in the stroma of breast carcinomas.[45] Immature endothelial cells, which may not have formed lumina or express vWF/QBend-10, could secrete increased amounts relative to the differentiated endothelial cells, but even so it is unlikely that they account for all of the labeled cells in the stroma, so the latter may well be fibroblastic or perhaps myofibroblastic in nature.

The extensive search made of the sequence databases indicated that there is no significant homology to known collagenases or other genes for the probes used in this study, and therefore that the hybridization is specific for the mRNAs of TIMP-2 and MMP-2 as far as can be ascertained. Unrelated probes synthesized concurrently (AUA-1 and β-actin) showed differing and appropriate localizations, indicating yet again that the results were not an artifact of the *in situ* hybridization procedure. In our experience, at the stringency used, small homologies of less than 20 bases in probes of the lengths used in this study would not produce any significant labeling, and certainly not to the degree seen in many of the carcinomas. The fact that there was colocalization of TIMP-2 and MMP-2 further confirms the specificity of the labeling.

In summary, we find by *in situ* hybridization that the mRNAs for the MMP-2 and TIMP-2 are localized overwhelmingly to the stroma of colorectal adenocarcinomas. Thus, a variety of metalloproteinases that degrade basement membrane components seem to be synthesized by stromal cells, as well as MMP-2 by neoplastic epithelial cells as previously described; presumably both sources contribute to the epithelial-mesenchymal interactions, which are fundamental in controlling growth and differentiation. Our results emphasize further that the growth, differentiation, and behavior of colorectal adenocarcinomas are affected by changes in gene expression in both the neoplastic, epithelial, and desmoplastic mesenchymal compartments.

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