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Short Communication

Interstitial Collagenase Gene Expression in Colonic Neoplasia

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Tumor invasion and metastasis are complex phenomena believed to be facilitated by the disruption of collagen and elastin fibers in the extracellular matrix. Interstitial collagenase gene expression was studied in colonic adenocarcinoma and adenoma using* in situ *hybridization. The data indicated that three cell types within the tumor stroma expressed collagenase transcripts; they were eosinophils, fibroblasts, and vascular endothelium. In all 12 adenocarcinomas, a high to moderate level of expression was seen in 1 to 5% of eosinophils and in occasional fibroblasts, whereas these cell types in non-neoplastic mucosa adjacent to tumor showed no detectable expression. Two adenocarcinomas showed expression in hyperplastic endothelium in vascularized granulation tissue. Two out of three adenomas showed expression in eosinophils and fibroblasts at a reduced level. Tissue inhibitor of metalloproteinase-1 gene expression was, however, negligible in all tissue examined. These results suggest that interstitial collagenase gene activation in the tumor stroma, especially eosinophils, may have an important role in tumor invasion and metastasis. (Am J Pathol 1993, 143:663–671)

Colorectal cancer is the second leading cause of cancer-related death in men and the third in women, in Western society.[1] The majority of colonic carcinomas arise in preexisting adenomas. The resulting adenocarcinoma is a malignant growth capable of infiltrating surrounding tissues and giving rise to metastases.[2] As in other cancers, this is a complex phenomenon thought to be facilitated by the disruption of collagen and elastin fibers in the extracellular matrix. Whereas genetic alterations during colorectal neoplasia have been detected,[3] the exact mechanism of extracellular matrix degradation has not been identified. Several investigators have suggested a role for metalloproteinases.[4,5]

Although much attention has focused on type IV collagenase,[6,7] which is believed to degrade specifically the basement membrane, the role of other members of the metalloproteinase family[8] in neoplasia remains unclear. Whereas no direct association between the secretion of interstitial collagenase and metastatic potential has been established, cultured fibroblasts from basal cell carcinomas were found to secrete more collagenase activity than fibroblasts cultured from non-neoplastic skin.[9] In other systems, normal cells in either the tumor complex or associated stroma are believed to be induced by tumor cells to produce increased amounts of matrix-degrading metalloproteinases.[10]

Evidence that tumor cells can directly influence normal cell collagenase production is based on cell culture explants using rat lung fibroblasts that normally secrete latent collagenase but, when incubated with plasminogen activator derived from mammary adenocarcinoma, secrete an active collagenase.[11] This suggests tumor cell plasminogen activator recruits normal cells to degrade tissue by activating collagenase in them. Furthermore, tumor cells have

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been shown to secrete a factor that induces the expression of collagenase type I in fibroblasts.[12–14] As a precedent for the apparent induction of a metalloproteinase in stromal cells by a tumor, however, Basset et al[15] demonstrated the expression of a new member of the metalloproteinase gene family, stromelysin-3. Expression seems to be restricted to the stromal cells immediately surrounding neoplastic cells of the invasive but not the *in situ* component of breast carcinomas. Stromelysin-3 expression has recently been detected within basal cell carcinoma-associated stroma.[16]

The control of metalloproteinase is complex. In addition to requiring conversion to an active form, this form can subsequently be inactivated by tissue inhibitors of metalloproteinase (TIMPs).[17–20] Apart from serum α_2-macroglobulin (M_r 780 kd), TIMPs are the only known collagenase inhibitor. Due to their smaller size, it is assumed to be more ubiquitous than α_2-macroglobulin and may play an important role in controlling metalloproteinases produced by tissues. Two types of TIMPs have been reported, ie TIMP-1 (M_r 21 kd) and TIMP-2 (M_r 28.5 kd).[17] The former seems to inhibit most of the interstitial collagenases[18] and 92-kd type IV collagenase,[19] whereas the latter inhibits the 72-kd type IV collagenase.[20] These data suggest that TIMPs also play an important role in modulating the contribution of metalloproteinases to invasion by tumor cells.

Complete understanding of the role of proteases in invasion and metastasis is impractical unless the cellular origin, location, and inhibitors of these enzymes are identified. In our previous study, we observed high levels of interstitial collagenase gene expression in fibroblasts of the tumor associated stroma in oral squamous cell carcinomas.[21] It was of interest to us to determine if a similar pattern of expression could be detected in other forms of carcinoma. In this preliminary investigation, the location and relative levels of interstitial collagenase and TIMP-1 gene expression in colonic tumors and non-neoplastic tissue were assessed. The purpose was to identify cell types responsible for interstitial collagenase and TIMP-1 production and whether these correlated with tumor invasion and eventual metastasis.

Materials and Methods

Tissue

Formalin-fixed, paraffin-embedded blocks from 12 well- to moderately differentiated colonic adenocarcinomas and three adenomas were studied, which included two Dukes' A tumors, five Dukes' B, five Dukes' C, one tubular adenoma, and two tubulovillous adenomas. Seven uninvolved resection margins from colectomy specimens were also selected. Five-μ-thick sections were mounted on 2% aminopropyltriethoxysilane- (Sigma, Chemical Co., St. Louis, MO) coated slides.

Plasmids

Plasmid pGbColl11 contained a 1.58-kb Xba-1 fragment that was subcloned from the 1.97-kb complementary DNA fragment of pCol 185.2, a gift from Dr. A. Eisen.[22] Our previous Northern blot analysis of total RNA extracts from human skin fibroblasts, cultured with phorbol myristate acetate,[23] confirmed hybridization of pGbColl11 with a single RNA species of approximately 2.5 kb. This was consistent with previously reported data,[24] suggesting pGbColl11 had appropriate specificity for *in situ* hybridization studies. Plasmid pGEMHuTIMP[25] (TIMP-1) was a gift from Dr. B. Williams. The 700-bp *Eco*RV-*Bal*l fragment from β-actin complementary DNA[26] was cloned into pGEMINI-3 to check the integrity of cytoplasmic messenger (m)RNA.

RNA Probes and in Situ Hybridization

Preparation of ^{35}S-labeled RNA sense and antisense probes, methods for *in situ* hybridization, and autoradiography were described previously.[21] Hybridization signal was evaluated independently by two investigators. Cells containing more than seven silver grains were recorded as positive.

Results

A and B of Figure 1 represent tissue sections hybridized with sense and anti-sense collagenase probes, respectively, in which a boundary area between adenocarcinoma and non-neoplastic colonic mucosa is shown. The finding indicates that hybridization is specific because only tissue with antisense probes shows signal, whereas sections reacted with sense probes showed no silver grains. The result also showed that almost no interstitial collagenase gene expression was detected in non-neoplastic colonic mucosa, whereas the stroma immediately adjacent to tumor contained localized distinct signal (Figure 1B), suggesting that the activation of interstitial collagenase gene was a tumor-specific event. In all 12 adenocarcinomas examined, a varied level of collagenase transcripts were

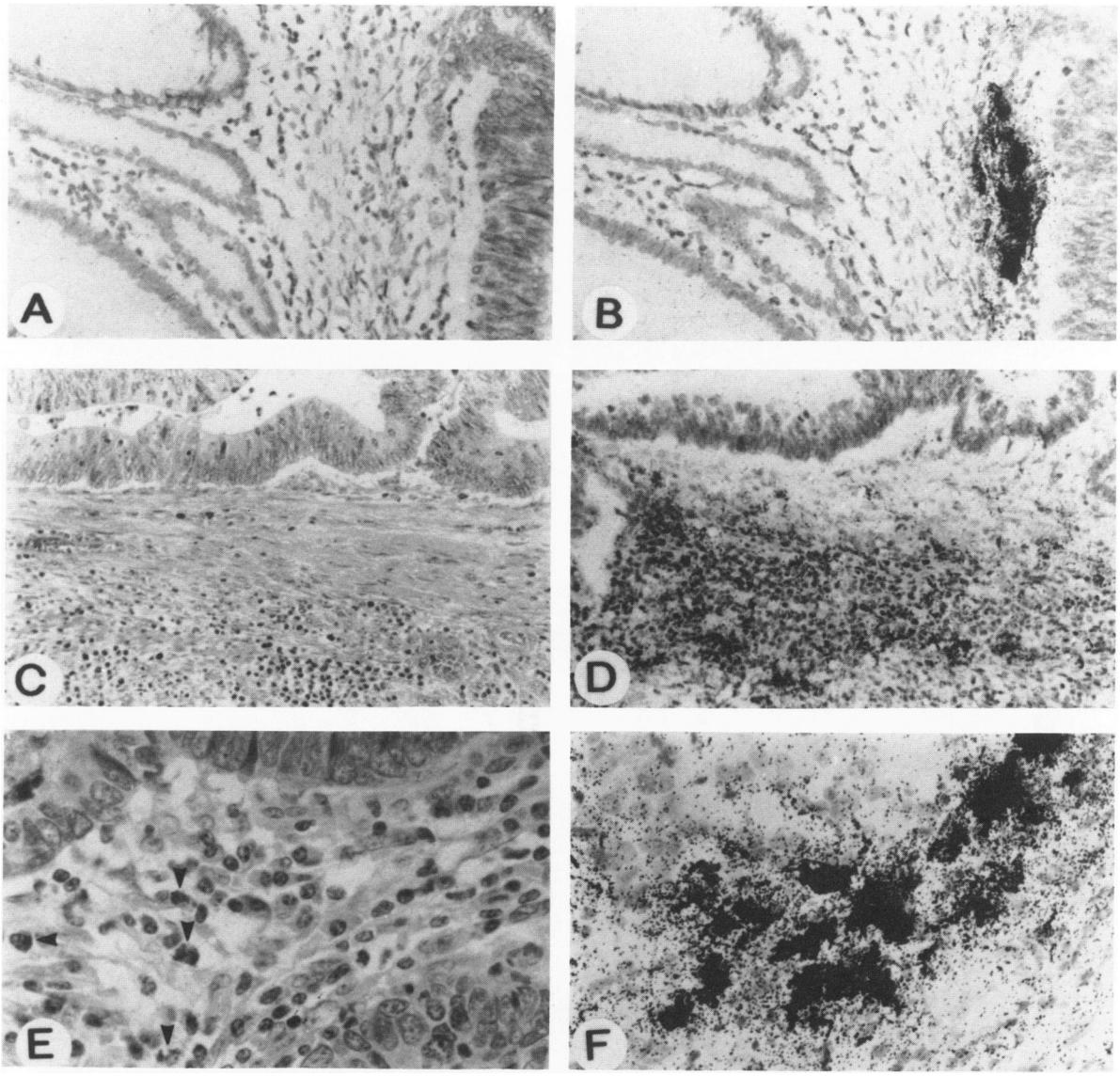

Figure 1. **A** *and* **B**: *Sections show a boundary area between colonic adenocarcinoma and non-neoplastic mucosa hybridized with sense* (**A**) *and anti-sense* (**B**) *interstitial collagenase probes. Signal is detected within the stroma adjacent to neoplastic glands, whereas non-neoplastic mucosa shows no signal* (*hematoxylin, original magnification 250×*). **C** *and* **D**: *Comparison between simple H&E-stained* (**C**) *and hybridized sections* (**D**) *demonstrates that signal is associated with inflammatory infiltrates in the stroma* (**C**: *H&E;* **D**: *hematoxylin; original magnification 200×*). **E** *and* **F**: *At higher magnification, the tumor stroma is consisted of inflammatory infiltrates and fibrous tissue. Only eosinophils* (*arrowheads*) *express a high level of collagenase transcripts* (**E**: *H&E,* **F**: *hematoxylin; original magnification 450×*).

present in the stroma, but not in the malignant epithelium. Because the hybridization signal was often strong enough to mask cell morphology, careful comparison between hybridized sections and adjacent hematoxylin and eosin- (H&E) stained sections was necessary. C and D of Figure 1 represent H&E-stained and hybridized sections, respectively, which demonstrate that collagenase signal seems to be associated with inflammatory infiltrates. E and F of Figure 1 represent higher magnified photomicrographs of H&E-stained and hybridized sections, respectively, which reveal that a high level of transcripts seemed to be localized in eosinophils. To identify cell type(s) more specifically, serial sections were hybridized with sense and anti-sense probes, and stained with H&E, which should allow differentiation of eosinophils from other cell types by their characteristics cytoplasmic eosinophilic granules. A and B of Figure 2 represent serial tissue sections hybridized with sense and anti-sense probes, respectively, which demonstrate clearly collagenase mRNA expression in occasional eosinophils adjacent to tumor cells. No signal was evident in other inflammatory cell types, such as lymphocytes,

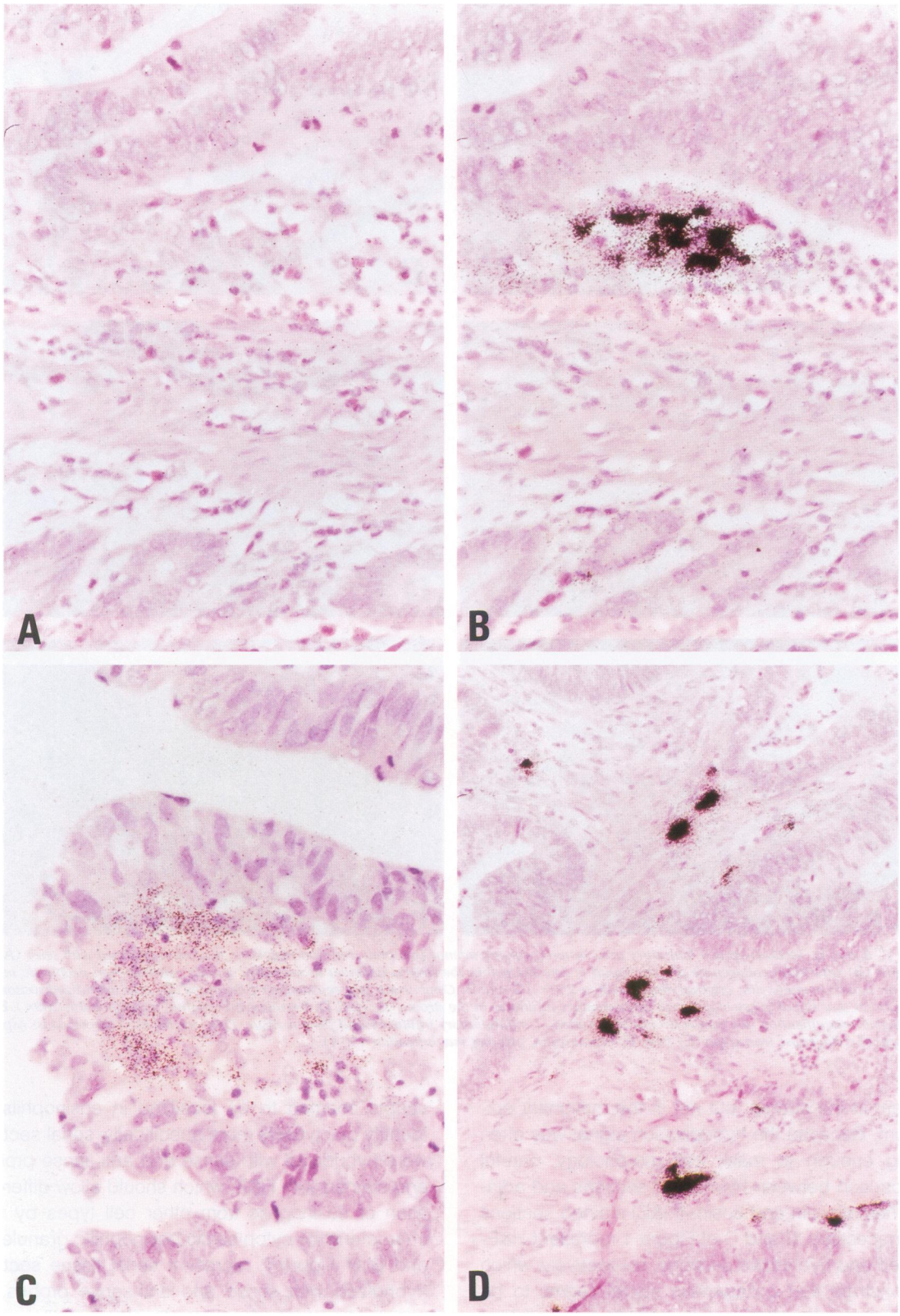

Figure 2. A *and* B: *Serial sections show a boundary area between colonic adenocarcinoma and non-neoplastic mucosa hybridized with sense* (A) *and anti-sense* (B) *interstitial collagenase probes. Some eosinophils contiguous with tumor cells contain a high level of transcripts.* C: *Superficial fibroblasts beneath tumor cells contain significant collagenase expression.* D: *Occasional fibroblasts in the deeper stroma show high expression.* (A, B, *and* D: *H&E; original magnification 470×;* C: *650×).*

plasma cells, macrophages, or neutrophils. Collagenase expression in eosinophils was observed in all 12 adenocarcinomas at varying levels. However, not all eosinophils expressed collagenase as seen in Figure 2, A, and B. By counting 10 high-power fields of H&E and hybridized sections, respectively, it was estimated that 1 to 5% of eosinophils in the tumor stroma expressed a substantial level of transcripts. The level of expression seemed to be correlated with the degree of inflammatory infiltration and showed no obvious correlation with the depth of tumor invasion or the tumor stage.

Other areas containing collagenase expression included the stroma just beneath tumor cells at the luminal surface (Figure 3, A and B). Signal was often seen as a linear deposition of silver grains and often almost parallel to the lining tumor cells. This feature was observed in all cases of adenocarcinoma at varying levels of expression. At a high-power magnification (Figure 2C), fibroblasts seemed to be responsible for signal. Although collagenase mRNA expression by superficial fibroblasts was dominant, occasional fibroblasts in the deeper stroma contained distinct signal as well (Figure 2D). These features were not seen in fibroblasts in non-neoplastic tissue.

Two adenocarcinomas contained occasional submucosal foci of highly vascularized granulation tissue over which diffuse silver grains were seen (Figure 3, C and D). This signal pattern was different from that observed over eosinophils that showed granular deposition of silver grains and from surface stromal fibroblasts that showed linear deposition. The granulation tissue contained almost no inflammatory infiltrates, except for many erythrocytes. The cell type responsible for expression here seemed to be vascular endothelium. This finding was not seen in vascular endothelial cells of non-granulated tumor stroma or non-neoplastic mucosa.

Two out of three adenomas were positive for collagenase transcripts and showed similar patterns of labeling, albeit at reduced levels. However, no collagenase expression was detected in endothelial cells (data not shown). In contrast, TIMP-1 gene expression was low, with grains scattered throughout the fibrous connective tissue of all non-neoplastic epithelia examined, as it was in all the neoplastic tissue investigated (data not shown).

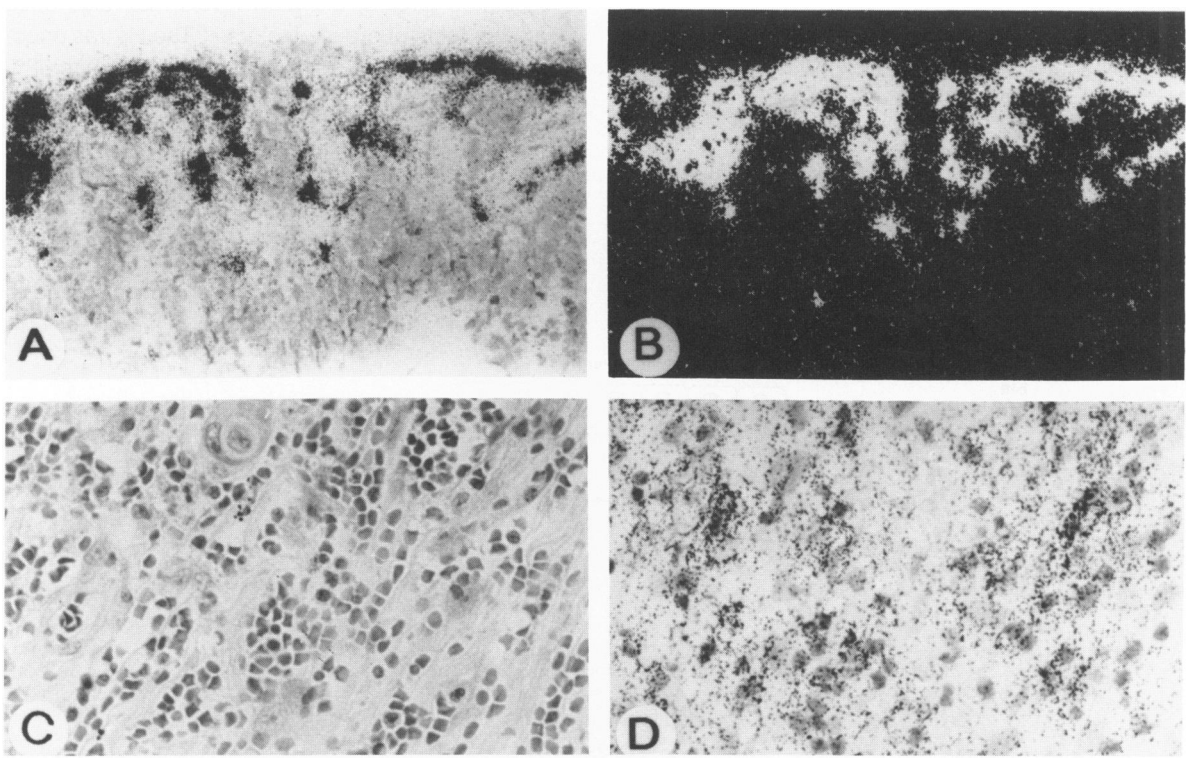

Figure 3. A *and* B: *Linear localization of collagenase expression is seen over fibroblasts in the lamina propria just beneath the surface tumor cells* (A: *hematoxylin;* B: *dark-field image; original magnification 125×*). C *and* D: *Hyperplastic endothelial cells in vascularized granulation tissue contain a modest level of collagenase transcripts* (C: *H&E;* D: *hematoxylin; original magnification 400×*).

Hybridization signal observed in this study was considered to be specific, because 1) in all cases, control *in situ* hybridization performed with sense probes (Figures 1A and 2A) gave very low and uniform grain distribution in both the cases of collagenase and TIMP-1; 2) in several adenocarcinomas hybridized with the β-actin anti-sense probe, the signal was the strongest in neoplastic epithelia and moderate in the tumor stroma (data not shown); 3) not all but 1 to 5% of eosinophils in the tumor stroma showed collagenase expression; and 4) eosinophils present in non-neoplastic tissue did not show significant collagenase expression.

Discussion

In agreement with Irimura et al,[27] this study has detected low levels of interstitial collagenase gene expression within adenocarcinomas of the colon. However, three cell types, namely, fibroblasts, eosinophils, and endothelial cells, present within the stroma contiguous with several adenocarcinomas expressed varying levels of transcription. Of these, eosinophils were the predominant producer cell. This is consistent with reports of type-specific collagen degradation by eosinophils[28,29] and hints at a role for eosinophils in matrix remodeling. Eosinophils have recently been found to express the 92-kd type IV collagenase mRNA in basal cell carcinoma,[30] suggesting that eosinophils may be capable of producing multiple metalloproteinases. Eosinophils arise from bone marrow, emigrate to peripheral tissues, and aggregate near mucosal surfaces such as those of the gastrointestinal tract.[31] The specific functions of eosinophils in association with malignancies such as adenocarcinoma are unknown. However, human colonic adenocarcinomas and oral carcinomas are frequently associated with eosinophil-rich inflammatory infiltrates.[32,33] This phenomenon may be due to an eosinophilotactic factor secreted by tumor tissue.[34] The degree of eosinophil infiltration may be important because eosinophilia associated with colonic carcinoma has been linked with favorable outcome.[35,36]

The most intriguing questions concerning proteinases and tumor invasion currently are what turns on the expression of certain proteinase genes and could interstitial collagenase play a role? Several mechanisms (depicted schematically in Figure 4) may account for the increased collagenase gene expression observed in fibroblasts, eosinophils, and endothelium within the stroma contiguous with adenocarcinomas.

Collagenase expression within fibroblasts near the tumor surface or in the deeper stroma may result from tensile forces generated by tumor expansion, by mechanical stimulation during the passage of gut contents, or by factors released by tumor tissue. Studies on the regulation of metalloproteinase gene expression suggest that growth factors and oncogenes may control their transcription. Matrisian points out that not only may elevated levels of metalloproteinases be a consequence of an activated oncogene within a tumor but, in the tumor-associated stroma, elevated levels may also arise via the effects of growth factors.[17] The Ha-*ras* oncogene, of which expression is increased in 50 to 62% of colon cancer,[37,38] is a potent inducer of stromelysin, collagenase IV/gelatinase, and interstitial collagenase. Tumor necrosis factor-α, secreted by macrophages in response to cancer,[39] is now known to induce c-*fos* and c-*jun* proto-oncogene expression in target cells such as fibroblasts. The protein products of these genes specifically bind to

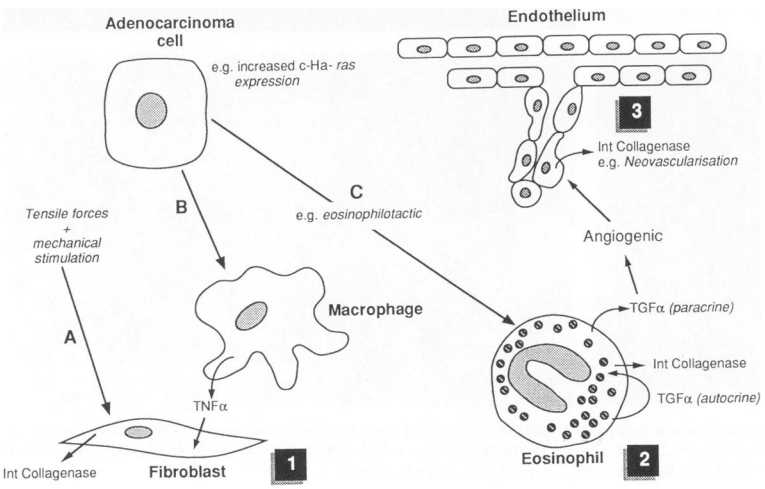

Figure 4. *Schematic depiction of interstitial collagenase induction within three cell types; 1) fibroblasts, 2) eosinophils, and 3) endothelium, in colonic neoplasia. Arrows* **A**, **B**, *and* **C** *represent some potential pathways in interstitial collagenase activation.*

the TRE/AP-1 DNA sequence,[40] located in the upstream control region of the human collagenase genes, which may result in prolonged activation of collagenase gene expression[41] (Figure 4, cell type 1).

Transforming growth factor α (TGF-α) has also been implicated. This multifunctional cytokine, expressed in a variety of neoplasms, particularly carcinomas, as well as several normal tissues, is believed to elicit its effects by autocrine/paracrine mechanisms,[42] via the epidermal growth factor receptor.[43] Yoshida et al demonstrated induction of interstitial collagenase and stromelysin genes, in addition to c-*fos*, c-*myc*, and c-*erb* B-2 oncogenes and TGF-α mRNA levels, by TGF-α treatment of the human gastric adenocarcinoma cell line MKN-28.[44] TGF-α has been also shown to induce multiple species of matrix metalloproteinases, including interstitial collagenase *in vivo*[45] and *in vitro*.[46] The finding that approximately 90% of eosinophils adjacent to colonic adenocarcinomas express high levels of TGF-α gene activity,[47] combined with the results of this and previous[30] studies, suggests the presence of autocrine/paracrine control of interstitial collagenase activation within tumor-associated eosinophils. Such mechanisms may account for the eosinophil interstitial collagenase expression observed here (Figure 4, cell type 2).

A further potential mode of interstitial collagenase modulation involving this cytokine may be relevant. Schreiber et al, using the hamster cheek pouch model of oral carcinogenesis, demonstrated that TGF-α from eosinophils potentially induces angiogenesis, suggesting a role for TGF-α in malignancy-associated neovascularization.[48] Authors observed that significant numbers of TGF-α-positive eosinophils are in close proximity to tumor microvasculature. Furthermore, TGF-α can directly stimulate proliferation of cultured vasculature endothelial cells.[49] The finding of interstitial collagenase expression within endothelial cells in granulation tissues observed in this study, may reflect angiogenic stimulation of endothelial cells by tumor- and host cell-derived factors, including perhaps, TGF-α from tumor-associated eosinophils (Figure 4, cell type 3). Eosinophils may thus be a previously overlooked normal host cell population, recruited by tumor cells to not only aid neovascularization but augment dissolution of the extracellular matrix, allowing tumor invasion and metastasis.

It now seems interstitial collagenase may function in concert with the 72-kd type IV collagenase in colorectal neoplasia.[50] The authors also demonstrated high TIMP-2 gene expression, whereas, in the present study, there was no detectable TIMP-1 gene expression. Identification of the precise interplay between these and other metalloproteinases along with TIMPs will be crucial for a thorough understanding of tumor biology and tissue remodeling in this common human neoplasm.

Acknowledgments

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