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# Short Communication

## Interstitial Collagenase Gene Expression in Oral Squamous Cell Carcinoma

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***In this study,* in situ *hybridization techniques were used to determine the location of interstitial collagenase and tissue inhibitor of metalloproteinase (TIMP) gene expression in samples from 11 squamous cell carcinomas of the head and neck (particularly the oral cavity) and from non-neoplastic mucosa of the same region. Ten of the 11 carcinomas examined showed abundant levels of collagenase gene expression in stromal fibroblasts within connective tissues immediately adjacent to tumor masses. Lower levels were detected in basaloid tumor cells located at the periphery of several tumor masses. Interstitial collagenase expression was consistently low in all normal, hyperplastic, and dysplastic epithelial sections. TIMP gene expression was negligible in all tissues examined. These results support the view that stromal interstitial collagenase production may play a key role in assisting invasiveness of squamous cell carcinoma of the head and neck. (Am J Pathol 1992, 141:301–306)***

Squamous cell carcinoma of the head and neck has a high potential for invasiveness.[1,4] As in other cancers, this is a complex phenomenon believed to be facilitated by the disruption of collagen and elastin fibers in the extracellular matrix (ECM). While the exact mechanism of ECM degradation has not been identified, several investigators have suggested a role for metalloproteinases.[1–5]

Although much attention has focused on the metalloproteinase collagenase IV, which is believed to degrade specifically the basement membrane,[2,3] other members of the metalloproteinase family[6] have not been thoroughly studied for potential correlation with malignant conversion. While 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.[7] 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.[8]

Evidence that tumor cells can directly influence normal cell collagenase production is based on cell culture explants using rat lung fibroblasts, which normally secrete latent collagenase, but when incubated with plasminogen activator (PA) derived from mammary adenocarcinoma, secrete an active collagenase.[9] This suggests tumor cell PA recruits normal cells to degrade tissue by activating collagenase in them. Furthermore, tumor cells have been shown to secrete a factor that induces the expression of collagenase type I in fibroblasts.[10–12] As a precedent for the apparent induction of a metalloproteinase in stromal cells by a tumor, however, Basset et al,[13] demonstrated the expression of a new member of the metalloproteinase gene family, stromelysin-3. Expression appears restricted to the stromal cells immediately surrounding neoplastic cells of the invasive but not the *in situ* component of breast carcinomas.

The control of metalloproteinases is complex. In addition to requiring conversion to an active form, this form can subsequently be inactivated by tissue inhibitor of metalloproteinase (TIMP).[14] Apart from serum $\alpha_2$-macroglobulin ($M_r$ 780,000), TIMP is the only known collagenase inhibitor. Due to its smaller size ($M_r$ 28,000), it is assumed to be more ubiquitous than $\alpha_2$-macroglobulin and may play an important role in controlling metalloproteinases produced by tissues. TIMP could also play an

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important role in modulating the contribution of metalloproteinases to invasion by tumor cells.[15,16]

Complete understanding of the role of proteases in invasiveness is impractical unless the cellular origin, location, and inhibitors of these enzymes are identified. In investigating this issue, squamous cell carcinoma of the head and neck is an appropriate cancer to study. It has a high capacity for infiltrating and breaking down connective tissues and has also been reported as having levels of collagenase activity higher than those of normal tissues of the same type.[1,4] In this preliminary investigation, the location and relative levels of fibroblast collagenase gene expression and TIMP gene expression in nonneoplastic tissue and malignant epithelial tumors of the head and neck was assessed. The purpose was to determine if interstitial collagenase expression or TIMP expression correlated with invasion and eventual metastasis.

## Materials and Methods

Gemini vectors containing genes for human TIMP and human fibroblast collagenase were linearized to create "run off" radiolabeled sense and antisense RNA probes for *in situ* hybridization experiments. To detect gene expression, that is mRNA, in a given tissue, thin sections of normal, hyperplastic, dysplastic, and neoplastic tissues were divided in two. One half was hybridized with the antisense probe, the other with the sense probe (negative control). Evaluation of results involved comparison of detectable signal in each half after hybridization.

### Tissues

Thin sections were prepared from paraffin-embedded blocks of normal, hyperplastic, and dysplastic oral mucosa and head and neck carcinomas (particularly of the oral cavity). All tissues were fixed in 10% buffered formalin. A total of 11 squamous cell carcinomas (Table 1), 23 hyperplastic, 21 dysplastic, and 12 normal oral mucosal tissues were investigated. All tissues were examined independently by two pathologists. Duplicate samples of a normal, hyperplastic, and dysplastic mucosa were analyzed alongside each tumor tissue.

### Plasmids

Plasmid pGEMHuTIMP[17] was a gift from Dr. B. Williams. Plasmid pGbColl11 was subcloned in this laboratory by Y. Kuys and contains a 1.58 Kb *Xba* 1 fragment from the 1.97 Kb cDNA fragment of pCol 185.2, provided by Dr. A. Eisen.[18] Previous Northern blot analysis of total RNA extracts from OT5 human skin fibroblasts, cultured with phorbol myristate acetate (PMA),[19] confirmed hybridization of pGbColl11 with a single RNA species of approximately 2.5 kb. This was consistent with previously reported data,[20] suggesting pGBColl11 had appropriate specificity for *in situ* hybridization studies.

### Preparation of Radioactive RNA Probes

Probes were synthesized as recommended by the manufacturers of the RiboProbe kit (Promega Biotec, Auckland). Transcription reactions carried out in 20-μl volumes contained 40 mmol/l Tris-HCl (pH 7.5), 6 mmol/l $MgCl_2$, 2 mmol/l spermidine, 10 mmol/l NaCl, and dithiothreitol (DTT), 20 U RNasin™ ribonuclease inhibitor, 2.5 mmol/l adenosine triphosphate (ATP), guanosine triphosphate (GTP), and cytosine triphosphate (CTP), 50 mCi[α-$^{35}$S]-UTP, 200 ng template DNA, and 5–10 U of either SP6 RNA polymerase (sense probes) or T7 RNA poly-

**Table 1.** *Squamous Cell Carcinomas of the Head and Neck*

| Case | Age/Sex | Site | Histologic grade[31] | Node metastasis | Survival (months) |
|---|---|---|---|---|---|
| A | 74/M | Maxillary and ethmoid sinus | I | ++ | 11* |
| B | 33/M | Ventral tongue | I | NM | ND |
| C | 64/F | Lateral tongue | II | NM | 36 |
| D | 83/F | Lip | II | NM | 34 |
| E | 67/F | Buccal mucosa | II | NM | 28 |
| F | 43/M | Lip and floor of mouth | II | ++ | 8* |
| G | 61/M | Tonsil and palate | II | NM | 58 |
| H | 78/M | Buccal mucosa and floor of mouth | I | + | 33* |
| I | 41/F | Retromolar mucosa | II | + | ND |
| J | 44/M | Lip | I | NM | 39 |
| K | 72/F | Retromolar mucosa and floor of mouth | I | NM | 35 |

* Deceased.
NM = no lymph node metatasis, ND = no data, + = cervical lymph nodes, ++ = distant lymph nodes.

merase (antisense probes). Probe synthesis at 40°C was stopped when TCA precipitable radioactivity reached 70–80% incorporation (specific activity ~ $10^7$ dpm/mg RNA). Labeled RNA was recovered by precipitation with 2 μl of 3 mol/l Na acetate and 44 μl of ethanol then resuspended in Tris-EDTA buffer to give 4 × $10^6$ dpm/μl.

### In Situ *Hybridization*

Formalin-fixed, paraffin-embedded sections were applied to gelatin-coated slides. These were cleared by 3 × 10 minute washes in xylene, then 3 × 5 minute washes in absolute ethanol. Sections were dried, placed in 0.2 N HCl at room temperature for 15 minutes, and rinsed with 2 × SSC (0.3 mol/l NaCl, 0.3 mol/l $Na_3$ citrate). Slides were then treated with 2 μg/ml proteinase K (Promega Biotec, Auckland) in buffer (0.2 mol/l Tris pH 7.2, 2 mmol/l $CaCl_2$) at 37°C for 15 minutes and rinsed in 2 × SSC. After dehydration in alcohol washes, rubber cement was applied, using syringe and needle, to create a well about each section for incubations with probes. Prehybridization was performed using 75 μl/$cm^2$ of hybridization buffer (4 × SSC, 50% formamide, 0.5% SDS, 5 × Denhardt's solution, 1 mg/ml denatured herring sperm DNA, 0.25 mg/ml yeast tRNA) to each well and incubating slides at 50°C in a 50% formamide/2 × SSC humidified chamber. After 1 hour, the prehybridization buffer was pipetted off and 25 μl/$cm^2$ of hybridization buffer containing 5 × $10^4$cpm/μl of probe was applied to each well and incubated as before for 16 hours. Slides were then rinsed for 2 × 15 minutes in 2 × SSC/50% formamide/10 mmol/l β-mercapto-ethanol at 50°C, incubated at 37°C for 30 minutes with 100 mg/ml RNAse A and 1 mg/ml RNAse T1 (Sigma, Auckland) in 2 × SSC, rinsed twice in 2 × SSC, then dehydrated in ethanol and dried.

### *Emulsion Autoradiography*

Slides were smeared with NTB-2 emulsion (Eastman Kodak, Rochester, NY) at 42°C under safelight, dried, then stored in light-tight boxes with dessicant at 4°C. After 10–14 days of exposure, slides were developed in D-19 developer (Coburg, Vic., Australia) for 1 minute, rinsed in 1% acetic acid stop solution for 30 seconds, and fixed in (Ilford, Mobberley, Cheshire, England) Ilofix fixative for 5 minutes, then rinsed in tap water.

### *Staining*

Slides were stained with hematoxylin and eosin (H&E), then mounted routinely for light microscopy.

## *Results*

Little or no interstitial collagenase gene expression was detected in normal, hyperplastic, or dysplastic tissues (Figure 1A, B). However, in ten different sections each containing an invasive squamous cell carcinoma, modest collagenase expression was noted within the constituent cells of the tumor whereas increased levels of expression were observed in the fibrous connective tissue immediately adjacent to each tumor mass (Figure 1C, E, G, I, J) compared with normal tissues and negative controls (Figure 1H). The tumor cells found expressing collagenase gene activity were located at the extremity of the tumor mass and were typically basaloid in form (Figure 1I, J). The elongate distribution of silver grains found above constituent cells in the fibrous connective tissue suggested that fibroblast-like cells were responsible for the collagenase expression there. Furthermore, serial sections of the same tissues stained with H&E, revealed the presence of fibroblasts with plump nuclei (Figure 1D, F) coincident with areas in the tissues exhibiting the highest levels of collagenase gene expression. Individual tumor cells were observed within the connective tissue immediately adjacent to the main tumor mass in some cases. An inflammatory cell infiltrate, typically consisting of plasma cells, lymphocytes, and some macrophages intermingled with fibroblasts and collagen bundles, was also present in most of the malignant samples examined. In contrast, TIMP 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 tissues investigated (data not shown).

In all cases, control *in situ* hybridizations performed with sense probes gave low and uniform grain distribution in both the case of collagenase and TIMP.

## *Discussion*

Consistent with previous reports of elevated levels of collagenase activity in squamous cell carcinoma of the head and neck,[1–5] interstitial collagenase gene expression has been found to be elevated in constituent cells of tumor tissue but, even more so, in activated mature fibroblast-like cells in the fibrous connective tissue adjacent to invasive oral squamous cell carcinomas. These results suggest that production of the bulk of collagenolytic factors in these tumors is carried out in the tumor-associated stroma rather than in the neoplastic cells themselves. Although hybridization of the interstitial collagenase probe was observed in malignant epithelial cells of invasively growing tumor margins in some sites, it is not clear whether this represents detection of interstitial collagenase gene expression alone in these cells or crosshy-

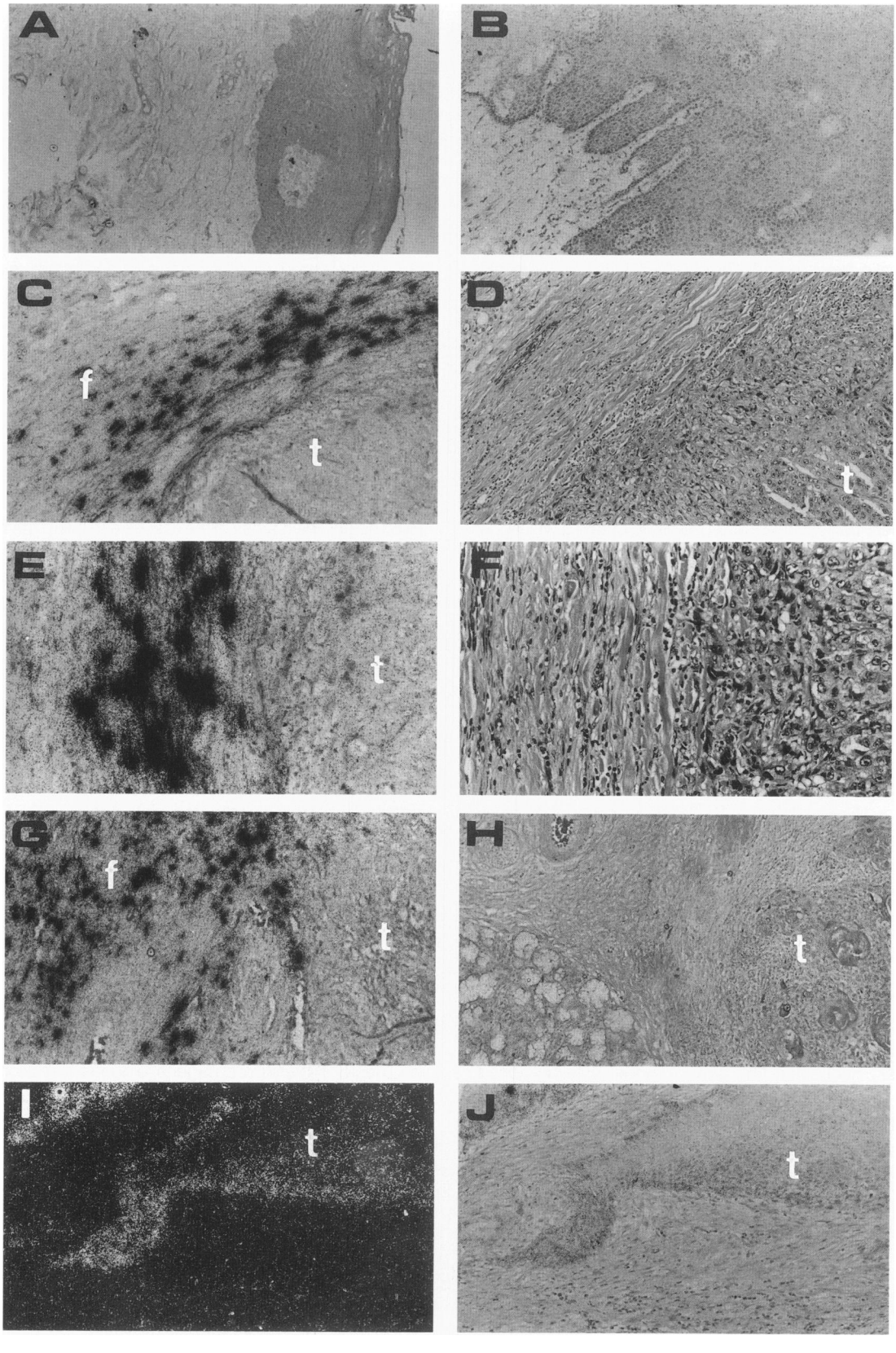
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**Figure 1.** *Interstitial collagenase gene expression detected after hybridization with a $^{35}$S-labeled antisense RNA probe for human fibroblast (interstitial) collagenase; negligible signal was seen in normal* (A) *and hyperplastic* (B) *oral mucosa but, abundant signal was observed in the stroma adjacent to invading tumor masses as seen in two different carcinomas* (C,G); (E) *is a higher power view of the result shown in* (C). *The cellular details of* (C) *and* (E) *are depicted in* (D) *and* (F), *respectively. In contrast, no signal is seen in the same sections hybridized in neighboring regions with a $^{35}$S-labeled sense RNA (negative control) probe* (H). *At some sites several carcinomas exhibited localization of signal within basaloid-like cells found at their leading invasive edges, as seen in a third carcinoma* (J); (I) *represents a dark field view of the tissues seen in* (J). *All sections H & E stained. t = tumor, f = fibrous connective tissue. Magnifications: ×25,* A *and* B; *×33,* C,D,G,H,I *and* J; *×60,* E *and* F.

bridization with other members of the collagenase gene family, such as type IV collagenase, as well. The finding of some individual tumor-like cells in the stroma adjacent to some tumor masses examined here may represent the seeding cells of future metastases.

Although an *in vitro* cascade pathway for the activation of human interstitial collagenase has been elucidated,[21] the *in vivo* pathway of activation of the metalloproteinases remains a matter of conjecture. Direct proof of the causal role of collagenases during *in vivo* malignant conversion awaits transfection of collagenase antisense mRNA into suitable recipient cells. Since collagenase does contribute to the processes of inflammation, wound healing, and tissue remodeling,[22] it would be believed to facilitate the progression of malignant tumors.

Although more studies detailing the ultrastructural localization and quantities of proteinases and their inhibitors are still required, 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? At least three mechanisms may account for increased collagenase gene expression. Fibroblasts may secrete elevated levels of collagenase in response to an increase in tensile forces generated by the continued expansion of a growing tumor mass, or as a consequence of factors released by the local inflammatory response, or factors released by tumor cells themselves.[10–12]

Studies on the regulation of metalloproteinase gene expression suggest that growth factors and oncogenes may control their transcription. Matrisian[23] 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 arise via the effects of growth factors. The Ha-*ras* oncogene is a potent inducer of stromelysin, collagenase IV/gelatinase, and interstitial collagenase. Tumor necrosis factor-α (TNF-α), secreted by macrophages in response to cancer,[24] however, 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 the TRE/AP-1 DNA sequence[25] located in the upstream control region of the human collagenase genes. The resulting prolonged activation of collagenase gene expression is apparently mediated through the TRE/AP-1 binding site via modulation by the c-*jun* protein.[26–28] Mechanisms such as these may account for the high levels of collagenase expression found in fibroblast-like cells of the stroma adjacent to oral squamous cell carcinomas observed in this study.

The results with TIMP gene expression are inconclusive and may simply reflect the finding of Flenniken and Williams[29] that *in situ* hybridization is not always sufficiently sensitive to detect low levels of expression. The TIMP protein can be detected in non-malignant skin if immunohistochemical methods[30] are used, albeit at decreased levels (associated with the extracellular matrix in the dermis). Around basal cell carcinomas, much larger quantities of TIMP protein have been detected surrounding dermal islands. Enhanced TIMP levels may have some kind of "anti-metastatic" or "anti-invasive" role. However, the present data suggests that this is not the case in oral squamous cell carcinomas given that the *in situ* sensitivity would have been sufficient to detect levels of enhancement of TIMP expression seen by Wegener Childers et al.[30]

Negative controls used in this study were consistently clear of detectable hybridization signal, suggesting that regulation of both collagenase and TIMP gene expression does not involve the synthesis of antisense RNA in these non-neoplastic and malignant epithelial tissues.

Elucidation of the regulatory mechanisms of these genes remains a major goal in metastasis research.

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