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Short Communication

92-kd Gelatinase Is Actively Expressed by Eosinophils and Stored by Neutrophils in Squamous Cell Carcinoma

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Tumor invasion and metastasis are assisted by multiple proteinases that degrade basement membrane and stromal matrix components. We used in situ hybridization with ^{35}S-labeled RNA probes and immunohistochemistry to localize cellular sites of 92-kd gelatinase production in sections of invasive squamous cell carcinoma. Signal for enzyme messenger RNA was detected only in numerous eosinophils that surrounded the tumor nodules, and immunohistochemical staining verified the presence of enzyme protein in these granulocytes and also revealed strong reactivity in neutrophils. No resident or other migratory cell type was positive for gelatinase messenger RNA or protein, and no signal was detected by either assay in samples of healthy skin. These data indicate that eosinophils have the capacity to synthesize actively 92-kd gelatinase, whereas neutrophils store and probably release the enzyme on demand. Because of the capacity of 92-kd gelatinase to degrade both basement membrane and interstitial extracellular matrix molecules, the expression, delivery, and secretion of this metalloproteinase by granulocytes may be critical for tumor invasion. (Am J Pathol 1993, 142:995–1000)

During the processes of tumor growth and metastasis, proteases degrade the extracellular matrix, allowing for cell migration and tissue remodeling. However, the cell source of degradative enzymes and the proteases expressed are not well understood, and the pattern of protease production may vary among cancers. Among the various proteases associated with metastatic potential, metalloproteinases and their inhibitors are thought to play an important role in tumor growth.[1,2] Metalloenzymes are a structurally related family of zinc-dependent enzymes that are secreted as inactive proenzymes and are proteolytically activated in the extracellular space. With the exception of interstitial and neutrophil collagenases, which have the unique ability to cleave fibrillar collagen types I, II, and III, most metalloenzymes have a broad substrate specificity and can degrade multiple and spatially distinct matrix proteins.[3,4] Consequently, as a group these enzymes can degrade most connective tissue proteins that would be encountered by an invasive tumor.

Various metalloproteinases have been associated with tumor growth, and expression of these enzymes has been attributed to neoplastic, stromal, and migratory cells. A potentially important metalloproteinase for tumor growth is 92-kd type IV collagenase or gelatinase, and, in fact, activity of this gelatinase correlates with *in vitro* invasive potential.[5,6] In its active form, 92-kd gelatinase has the ability to degrade components of both the basement membrane (type IV collagen) and dermal stroma (elastin, type V and type VII collagens, and collagenase-cleaved fibrillar collagen or gelatin).[7–10] In addition, the 92-kd en-

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zyme is thought to be primarily a product of migratory cells, and, thus, its degradative activity may be involved in various conditions associated with inflammation.[11,12]

We have recently reported that 92-kd gelatinase messenger (m)RNA is expressed by eosinophils associated with nodular basal cell carcinoma.[13] Although the number of mRNA-positive eosinophils was low, these cells may still contribute to tissue remodeling in basal cell carcinoma. Because squamous cell carcinomas are typically more aggressive and because they are often associated with an influx of eosinophils,[14] we examined the expression of 92-kd gelatinase in this cancer. We report here that eosinophils are the sole source of active production of the 92-kd enzyme in squamous cell carcinoma but that additional enzyme is contributed, although not actively made, by neutrophils.

Materials and Methods

In Situ *Hybridization*

Formalin-fixed, paraffin-embedded archival specimens of invasive squamous cell carcinomas were obtained from the Department of Dermatology, Karolinska Hospital. Sections were hybridized with ^{35}S-labeled anti-sense or sense RNA probes and washed under stringent conditions, including treatment with RNAse-A, as described.[13,15] After autoradiography, slides were stained with hematoxylin and eosin (H & E) or Giemsa. The design and specificity of the probes for 92-kd gelatinase mRNA has been described.[13]

Antibody Purification

Polyclonal anti-human 92-kd gelatinase antiserum and enzyme protein purified from activated U937 cells were supplied by Dr. Howard G. Welgus (Jewish Hospital, St. Louis, MO), and characterization of these reagents has been reported.[16,17] To enhance the specificity of the immunohistochemical assay, we affinity-purified anti-92-kd enzyme antibodies. Highly purified gelatinase protein was coupled to CNBr Sepharose 4B (Pharmacia, Uppsala, Sweden), and specific antibodies were adsorbed and eluted as described.[18] As determined by enzyme-linked immunosorbent assay, nearly equal immunoreactivity was recovered by sequential elution with 0.1 mol/L glycine, pH 2.5, and with 0.1 mol/L 3-[cyclohexylamino]-1-propane-sulfonic acid, pH 11.5.

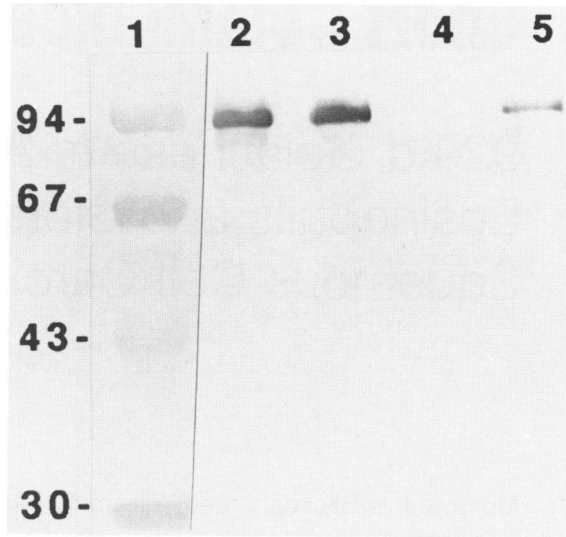

Figure 1. *Specificity of affinity-purified anti-92-kd gelatinase antibody. Anti-human 92-kd gelatinase antibody was affinity-purified as described under Methods, and the specificity of the purified product, diluted 1:500 from the original serum volume, was assessed by immunoblotting.* **Lane 1:** *Molecular mass markers transferred to the blotting membrane were stained with Coomassie blue.* **Lane 2:** *0.85 μg of purified human 92-kd gelatinase.* **Lane 3:** *Medium (100 μl) conditioned by phorbol ester-stimulated human fibroblasts was lyophilized and dissolved in 15 μl of sodium dodecyl sulfate sample buffer containing 0.85 μg of purified human 92-kd gelatinase.* **Lane 4:** *Concentrated fibroblast-conditioned medium alone.* **Lane 5:** *Serum-free conditioned medium (25 μl) from U937 cells.*

Antibody specificity was assessed by immunoblotting to purified 92-kd enzyme, serum-free medium conditioned by phorbol ester-stimulated fibroblasts, which secrete interstitial collagenase, stromelysin-1, and 72-kd gelatinase but no 92-kd enzyme,[16] this same medium spiked with purified 92-kd protein, and serum-free conditioned medium from phorbol ester-stimulated U937 cells. These samples were resolved by denaturing electrophoresis through a 10% polyacrylamide–sodium dodecyl sulfate gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and immunoblotting was done as described.[19] Stimulated U937 cells secrete high levels of 92-kd gelatinase.[20] The

Figure 2. *Detection of 92-kd gelatinase in eosinophils and neutrophils.* **A, B:** *Paired bright-field and dark-field photomicrographs of a section of squamous cell carcinoma hybridized with an ^{35}S-labeled anti-sense RNA for 92-kd gelatinase mRNA and stained with H&E. The field-of-view shows portion of the invasive tumor nodules (N) deep within the dermis. Surrounding the advancing edge of the carcinoma are numerous cells (arrows) positive for 92-kd enzyme mRNA. Autoradiography was for 3 days (×125).* **C, D:** *Numerous eosinophils (arrows) with autoradiographic signal for 92-kd gelatinase mRNA are seen surrounding a blood vessel within the stroma near the advancing carcinoma. Autoradiography was for 3 days, and the section was stained with Giemsa (×420).* **E:** *High-power photomicrograph showing signal confined to eosinophils identified by granular cytoplasm, bilobar nucleus, and Giemsa staining (×3300).* **F:** *Giemsa-stained eosinophils (small arrows) within the lumen of a blood vessel are*

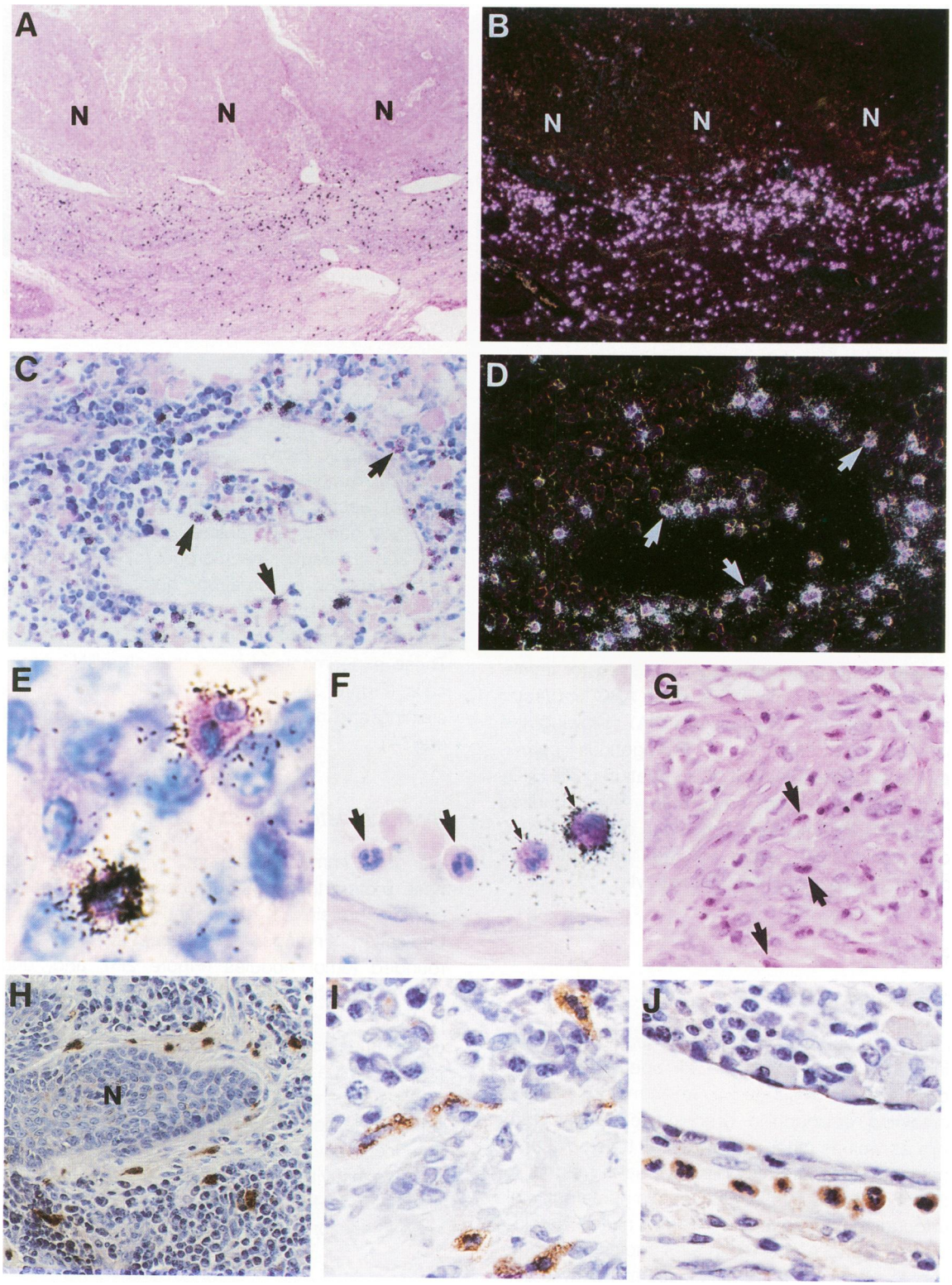

Figure 2. *Continued*

positive for 92-kd gelatinase mRNA, but neutrophils (large arrows) have no signal (×3300). **G:** *No autoradiographic signal was detected in eosinophils (arrows) in a section of squamous cell carcinoma hybridized with an ^{35}S-labeled sense RNA probe transcribed from 92-kd gelatinase complementary DNA. Autoradiography was extended for 10 days, and the section was stained with H&E (×670).* **H:** *Immunostaining showed granulocytic cells with immunoreactivity (brown precipitate) for 92-kd enzyme protein surrounding a tumor nodule (N). The section was counterstained with Harris hematoxylin (×625).* **I:** *Immunoreactivity was seen in eosinophils within the inflammatory infiltrate adjacent to the advancing carcinoma (×1000).* **J:** *Neutrophils within the lumen of a blood vessel had strong immunoreactivity for 92-kd gelatinase (×1300).*

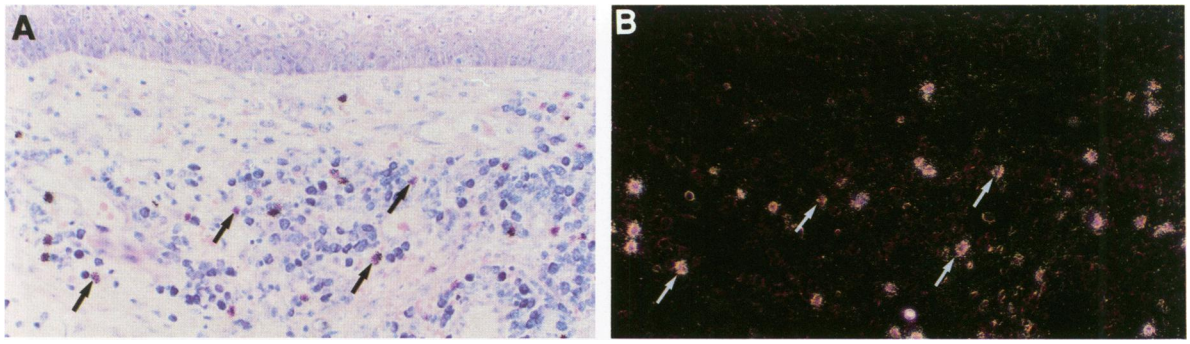

Figure 3. *Signal for 92-kd gelatinase mRNA is confined to eosinophils. Sections of squamous cell carcinoma hybridized with an ^{35}S-labeled antisense RNA for 92-kd gelatinase were exposed to photographic emulsion for 18 hours and were differentially stained with Giemsa.* **A:** *Bright-field photomicrograph showing numerous eosinophils within an infiltrate adjacent to the carcinoma, which is seen along the top edge of the figure.* **B:** *With dark-field illumination, autoradiographic signal for 92-kd gelatinase mRNA is seen only in and in all eosinophils. Arrows indicate a few representative cells (×850).*

affinity-purified antibody reacted only with the purified protein (Figure 1, lanes 2 and 3) or with enzyme protein secreted by U937 cells (Figure 1, lane 5). No immunoreactivity was detected in the fibroblast medium without exogenous 92-kd enzyme (Figure 1, lane 4), indicating no cross reactivity with other metalloenzymes.

Immunohistochemistry

Deparaffinized sections were processed for immunohistochemistry using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's instructions. Endogenous peroxidase activity was blocked by incubation 0.3% H_2O_2 for 30 minutes at room temperature. Affinity-purified anti-92-kd enzyme antibody was diluted 1:2000 from the original serum volume. Sections were counterstained with Harris hematoxylin.

Results

In Situ *Hybridization*

A total of 8 samples of invasive squamous cell carcinoma were examined. In all specimens, tumor nodules were surrounded by inflammatory infiltrates containing eosinophilic granulocytes and neutrophils as well as other migratory cell types. As demonstrated by in situ hybridization, strong signal for 92-kd gelatinase mRNA was seen in numerous cells surrounding the advancing edges of the carcinomas (Figure 2, A and B). In all samples, autoradiographic signal for 92-kd gelatinase mRNA was confined to eosinophils that were identified by Giemsa staining and by their characteristic bilobar nucleus (Figure 2, E and F). Eosinophils were also seen around and within blood vessels (Figure 2, C, D, and F). All identifiable eosinophils within a section were positive for 92-kd enzyme mRNA, and no autoradiographic signal was seen in any resident or other migratory cell type including neutrophils (Figure 2F). Sections hybridized with sense RNA probe had only background autoradiographic signal (Figure 2G). Because the morphology of some cells was concealed by the strong autoradiographic signal, we exposed sections for a shorter period to better demonstrate signal specificity for eosinophils. These experiments showed that all mRNA-positive cells (Figure 3B) were differentiated with Giemsa staining and had an eosinophilic morphology (Figure 3A).

Immunohistochemistry

We used immunohistochemistry for 92-kd gelatinase protein to verify that eosinophils synthesized enzyme protein. In agreement with the *in situ* hybridization findings, immunoreactive cells surrounded tumor nodules (Figure 2H), and, under high power, these cells had a morphology consistent with that of eosinophils (Figure 2I). In addition, strong immunostaining was detected in neutrophils seen in tissue infiltrates and in the lumen of blood vessels (Figure 2J). Control sections processed with preimmune serum had no staining, indicating that immunoreactivity was not due to endogenous peroxidase activity (data not shown). No detectable signal for 92-kd gelatinase mRNA or protein was seen in sections of healthy skin (data not shown).

Discussion

Our findings demonstrate that 92-kd gelatinase is actively expressed by eosinophils associated with

squamous cell carcinoma and that neutrophils contain the enzyme but do not synthesize the protein. These conclusions are based on *in situ* hybridization observations, which revealed the presence of 92-kd enzyme mRNA only in eosinophils, and by immunohistochemical staining, which demonstrated enzyme protein in both eosinophils and neutrophils. Our observations suggest that tissue eosinophils are the sole source for active production of 92-kd gelatinase in dermal neoplasms. Because 92-kd gelatinolytic activity was first isolated from neutrophils,[21] it is not surprising that these cells had a strong immunohistochemical signal for enzyme protein. The abundance of eosinophils and neutrophils seen in our studies suggest that 92-kd gelatinase secreted from these granulocytes contributes significantly to matrix remodeling associated with invasive squamous cell carcinoma. Furthermore, the presence of mRNA-positive and immunoreactive cells within the dermal stroma and around advancing tumor nodules and proliferating blood vessels indicates that 92-kd gelatinase participates in tissue restructuring within various matrix compartments.

In contrast to our results, Pyke et al reported that 92-kd gelatinase mRNA is expressed by malignant cells and by a subpopulation of tissue macrophages associated with squamous cell carcinoma.[22] We, however, detected no mRNA for 92-kd enzyme in malignant cells or macrophages. It is possible that 92-kd gelatinase expression by malignant squamous cells is a rare event rather than a characteristic property of the cancer, and, indeed, in the studies by Pyke et al, enzyme mRNA was seen in occasional cells of a few tumors. In their studies, macrophages, identified by immunostaining of separate sections with an anti-macrophage antibody, were present in the areas where signal for 92-kd gelatinase mRNA was detected, but cellular colocalization of macrophage antigen and enzyme mRNA was not demonstrated. Thus, it is unclear from their results if the cells positive for 92-kd enzyme mRNA are indeed macrophages.

Consistent with our observation, neutrophils are considered to be terminally differentiated cells with minimal capacity for protein synthesis. Eosinophils, on the other hand, produce various cell products, such as transforming growth factor-α[23] and platelet-activating factor.[24] Findings presented here (Figure 2F) indicate that eosinophils within blood vessels produce 92-kd gelatinase in patients with squamous cell carcinoma. The expression of 92-kd gelatinase may be influenced by the interaction of eosinophils with endothelial adhesion molecules at sites of inflammation, and, indeed, eosinophils bind selectively to VCAM-1 on the surface of activated endothelial cells.[25] Our future studies will be directed at delineating the mechanisms controlling this gelatinase induction in eosinophils.

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