Jpn. J. Cancer Res. 86, 304–309, March 1995

Immunoelectron Microscopic Localization of Gelatinase A in Human Gastrointestinal and Skin Carcinomas: Difference between Cancer Cells and Fibroblasts

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Previous reports revealed a discrepancy in gelatinase A localization in human cancers; i.e., protein localization in cancer cells and mRNA localization in stromal fibroblastic cells. To clarify this, we conducted immunoelectron microscopic study of gelatinase A in cancer and stromal cells in human gastrointestinal and skin carcinomas. Although both carcinoma cells and fibroblasts were positive for gelatinase A, the subcellular localizations were different. On immunoelectron microscopy, fibroblasts showed immunoreactivity in the lumen of the rough endoplasmic reticulum (rER) or in the cytosol on the surface of rER, demonstrating synthesis of the protein. Carcinoma cells showed diffuse deposition of gelatinase A in the cytosol, suggesting the accumulation of the antigen both in adenocarcinoma and squamous cell carcinoma. Immunoreactivity along the cell membrane was demonstrated in one case of skin carcinoma. Macrophages showed also diffuse deposition of gelatinase A in the cytosol. In conclusion, we found a qualitative difference of gelatinase A localization between carcinoma cells and fibroblasts, and concluded that carcinoma cells may not be important in the secretion of gelatinase A.

Key words: Gelatinase A — Type IV collagenase — Colon carcinoma — Immunoelectron microscopy — Cancer invasion

Carcinoma tissues are composed of both carcinoma cells and the stromal reactions which are induced by carcinoma cell growth. The stromal reactions include desmoplasia, angiogenesis, and immune reactions. Based on the similarity to wound repair processes, carcinomas can be regarded as "wounds that do not heal."[1] Macrophages (tumor-associated macrophages) and lymphocytes (tumor-infiltrating lymphocytes) are also increased in number in cancer stroma.[2] Therefore, studies on the stromal cells are becoming an important field in cancer biology.

The degradation of extracellular matrix proteins, particularly the basal lamina of epithelial cells and vascular endothelial cells is prerequisite for the invasion and metastasis of malignant cells,[3,4] and gelatinases are generally believed to be important for this process. Gelatinases A and B (72 kDa and 92 kDa type IV collagenases) degrade gelatin (denatured, soluble collagen), type IV collagen and type V collagen, but not type I collagen, proteoglycan, or laminin.[5,6] The activity of gelatinase A corresponded well to the metastatic potential in mouse melanoma cells and in 3T3 cells transfected with cancer cell DNA.[7] The enzymatic activity of gelatinase A is regulated by tissue inhibitor of metalloproteinase (TIMP)-2.[8] Previous immunohistochemical studies on gelatinase A showed positive staining in human carcinoma cells.[9–12] Its staining frequency was correlated with the tumor stage or with a worse prognosis.[12] Its mRNA was detected, however, in the stromal fibroblastic cells, not in cancer cells in skin, colon, lung and breast carcinomas.[13–17] Although a recent paper described immunolocalization of gelatinase A in stromal fibroblastic cells,[17] no clear explanation was proposed for this difference. To clarify this, we adopted the immunoelectron microscopy technique in the present study, because this technique can not only elucidate the subcellular localization of substances, but also provide clues to the intracellular pathway of the synthesized substances.

MATERIALS AND METHODS

Monoclonal antibodies Monoclonal antibodies used were two anti-gelatinase A antibodies, i.e., clone CA406, which recognizes both the active and latent forms, and clone CA-4001, which recognizes only the latent form (applied at 40 and 15 μg/ml, respectively). Their specificity was described previously.[18]

Tissues Specimens used for immunoelectron microscopy were 14 cases of colon adenocarcinomas, four cases of gastric carcinomas, four cases of squamous cell carcinoma of the skin, and five cases of granulation tissues (three chronic gastric ulcers, one chronic cholecystitis, and one chronic appendicitis), which were obtained at Tohoku Rosai Hospital and Tohoku University Hospital. All patients were Japanese. All gastrointestinal carcinoma cases showed invasive growth beyond the muscularis propria. After resection, specimens were immediately fixed in periodate-lysine-paraformaldehyde (PLP)

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solution for 6 h to overnight. They were washed in phosphate-buffered saline (PBS) containing 10%, 15% and 20% sucrose, embedded in O.C.T. compound (Miles, Elkhart, IN) and rapidly frozen. For the control, normal-appearing tissues were obtained from the resection margin.

Immunoelectron microscopy The indirect immunoperoxidase method was applied to frozen sections, 6 μm in thickness, as described previously, at both the light and electron microscopic level.[19] For the second antibody, we used $F(ab)_2$ fragment of IgG conjugated with horseradish peroxidase (Amersham, UK; diluted at 1:100). 3,3′-Diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto) was used as the chromogen. For the negative control, the primary antibodies were replaced by PBS or irrelevant mouse monoclonal antibodies.

RESULTS

The two monoclonal antibodies against gelatinase A gave essentially the same results in both adenocarcinoma (stomach and colon) and squamous carcinoma (skin). Carcinoma cells, stromal macrophages and fibroblastic cells were positive for gelatinase A to various degrees (Fig. 1). Immunoreactivity for gelatinase A was also observed in fibroblasts in fibrous tissue, 0.5–5 mm in width, which surrounds the interface between the tumor and the host in gastrointestinal carcinomas (Fig. 2). We have named this zone the "reactive fibrosis zone." This zone was situated outside the area of dense inflammatory infiltration, which was usually observed in the innermost zone of the interface between tumor and host. Other positive cells were Schwann cells, vascular endothelial cells and smooth muscle cells. Normal tissues revealed sporadic reactivity for gelatinase A in epithelial cells of stomach, colon and skin. No remarkable reactivity was observed in the stromal fibroblastic cells.

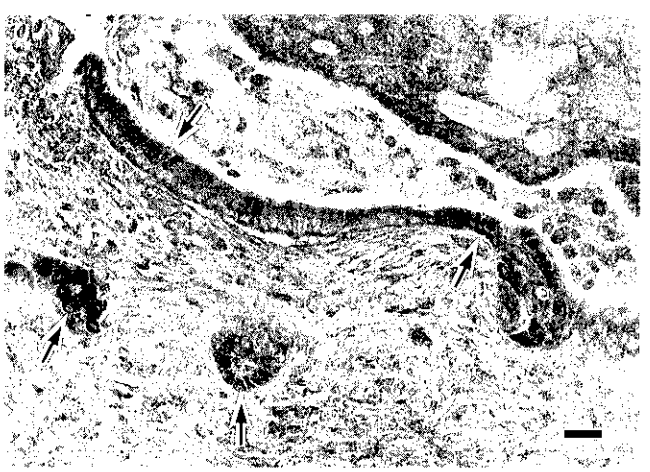

Fig. 1. Immunohistochemistry for gelatinase A in colon carcinoma. Immunoreactivity was observed in both carcinoma cells (arrows) and stromal cells. ×180. Bar; 25 μm.

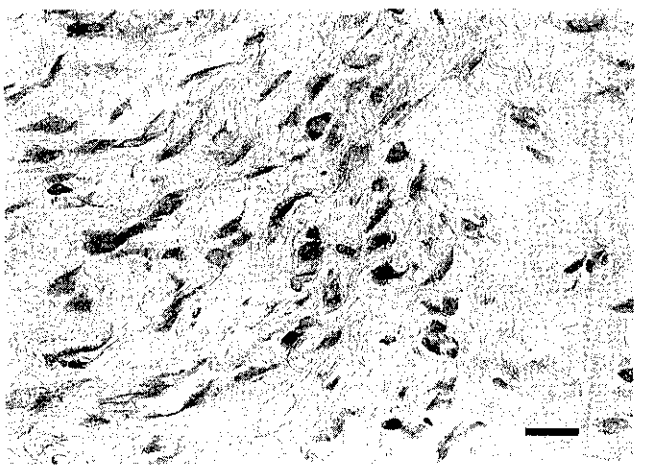

Fig. 2. "The reactive fibrosis zone" in colon carcinoma which surrounds the interface between tumor and host. Note the immunoreactivity for gelatinase A in spindle-shaped fibroblastic cells. ×280. Bar; 25 μm.

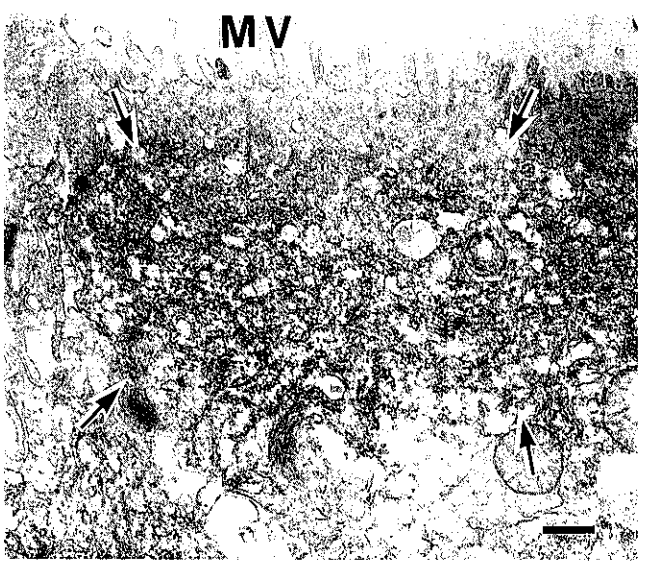

Fig. 3. Immunoelectron microscopy for gelatinase A in colon carcinoma cell. Immunoreactive material is seen as black reaction products (osmificated diaminobenzidine). Immunoreactive gelatinase A was diffusely deposited in the cytosol (cytoplasmic matrix). No cell organelles were labeled here. MV; microvilli. ×14,000. Bar; 0.5 μm.

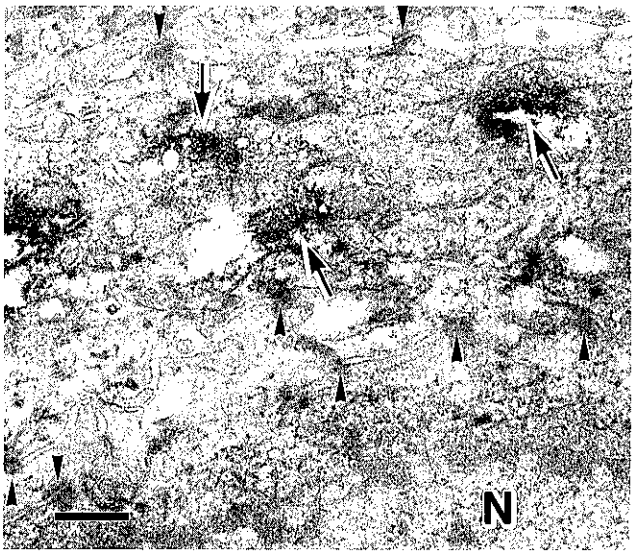

Fig. 4. Squamous cell carcinoma of the skin. Immunoreactivity for gelatinase A is localized in the cytosol as amorphous aggregates (arrows). Arrowheads indicate desmosomes. N; nucleus. ×17,000. Bar; 0.5 μm.

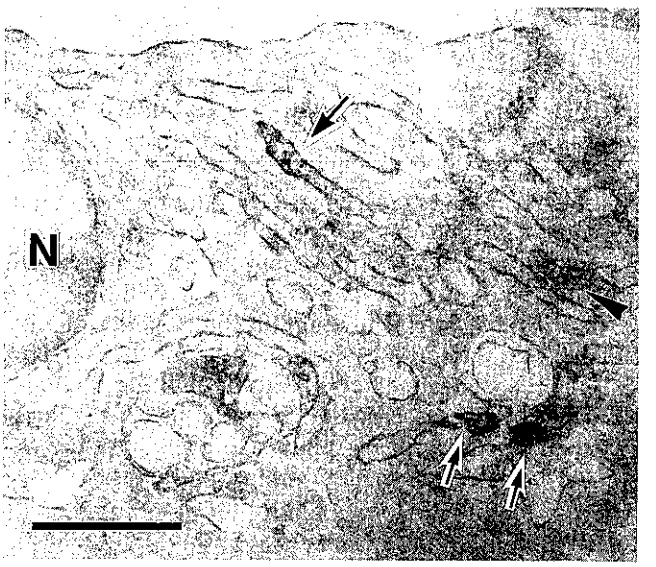

Fig. 6. Fibroblasts in the stroma of colon carcinoma were labeled in the lumen of rough endoplasmic reticulum (rER) (arrows) and on the surface of rER (arrowhead). N; nucleus. ×40,000. Bar; 0.5 μm.

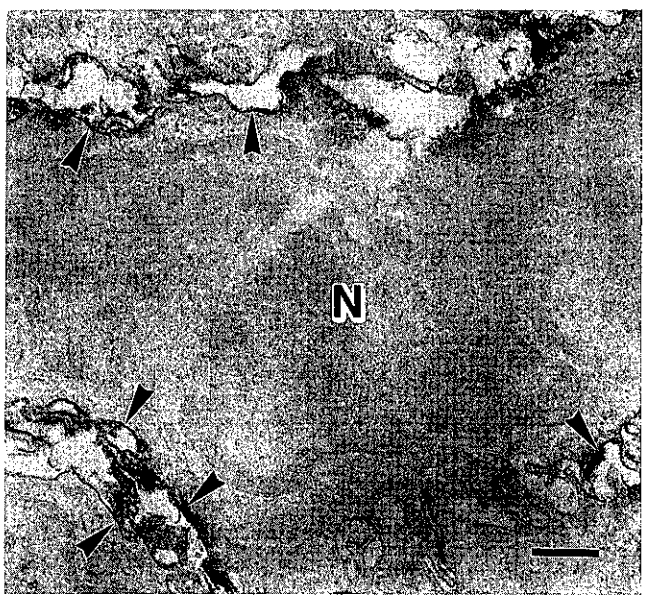

Fig. 5. Immunoreactivity for gelatinase A was detected along the cell membrane of skin squamous carcinoma cells (arrowheads). This was observed in one case. N; nucleus. ×17,000. Bar; 0.5 μm.

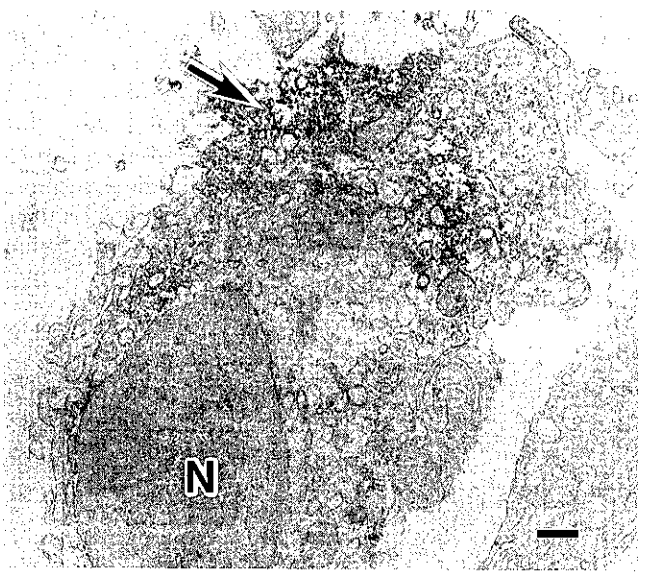

Fig. 7. Macrophage in the stroma of colon carcinoma shows the deposition of immunoreactive gelatinase A in the cytosol (arrow). N; nucleus. ×10,000. Bar; 0.5 μm.

Ultrastructural localization was also the same between the two antibodies to gelatinase A. Carcinoma cells showed deposition of immunoreactive gelatinase A in the cytosol as amorphous aggregates (Figs. 3 and 4). Adenocarcinoma cells and squamous carcinoma cells were characterized by microvilli along the lumen (Fig. 3) and multiple desmosomes (Fig. 4), respectively. There was no significant difference between adeno- and squamous cell

Table I. Summary of the Immunoelectron Microscopic Results

	Carcinoma cells			Fibroblasts	Macrophages	
	cytosol	rER	membrane	rER	cytosol	membrane
Colon carcinoma[a)]						
1. Dukes B	(+)	(+, rare)	(−)	(+)	(+)	(−)
2. Dukes B	(−)	(−)	(−)	(+)	ND	
3. Dukes B	(+)	(−)	(−)	(−)	ND	
4. Dukes B	(+)	(−)	(−)	(+)	(+)	(−)
5. Dukes B	(−)	(−)	(−)	(+)	(−)	(+)
6. Dukes B	(+)	(−)	(−)	(−)	ND	
7. Dukes B	(+)	(+, rare)	(−)	(+)	ND	
8. Dukes B	(+)	(−)	(−)	(+)	(−)	(+)
9. Dukes B	(+)	(−)	(+, rare)	(+)	(+)	(+)
10. Dukes C	(+)	(+, rare)	(−)	(−)	ND	
11. Dukes C	(+)	(−)	(−)	(+)	(+)	(−)
12. Dukes C	(+)	(−)	(−)	(+)	ND	
13. Dukes D	(−)	(−)	(−)	(+)	(−)	(−)
14. Dukes D	(−)	(−)	(−)	(+)	(+)	(−)
Gastric carcinoma[b)]						
15. Intestinal, n(+)	(+)	(−)	(−)	(+)	ND	
16. Intestinal, n(+)	(+)	(−)	(−)	(−)	ND	
17. Diffuse, n(+)	(−)	(−)	(−)	(+)	(+)	(−)
18. Diffuse, n(−)	(+)	(−)	(−)	(+)	(+)	(−)
Skin squamous cell carcinoma						
19.	(+)	(+)	(−)	(+)	rER(+)	(−)
20.	(+)	(−)	(−)	(−)	(−)	(−)
21.	(−)	(−)	(−)	(−)	ND	
22.	(+)	(+, rare)	(+)	(−)	ND	
Non-neoplastic granulation tissue						
23. Chronic appendicitis				(−)	(+)	(−)
24. Chronic cholecystitis				(+)	ND	
25. Gastric ulcer				(+)	ND	
26. Gastric ulcer				(+)	ND	
27. Gastric ulcer				(+)	ND	

a) Dukes B; colon carcinoma invading beyond the muscularis propria without metastasis. Dukes C; colon carcinoma with lymph node metastasis. Dukes D; colon carcinoma with liver metastasis.
b) Gastric carcinomas are divided into intestinal and diffuse types.[25)] n(+), lymph node metastasis present; n(−), negative.
(+), positive; (−), negative, ND; cells not detected.

carcinoma in this localization pattern. Gelatinase A was localized in the lumen of rough endoplasmic reticulum (rER) frequently in one case of skin cancer, and rarely in two cases of colon carcinoma. Immunoreactivity along the plasma membrane of carcinoma cells was definitely observed in one case of skin cancer (Fig. 5), and detected sporadically in one case of colon carcinoma. Pinocytosis of immunoreactive material was not observed in any case. In contrast, fibroblasts in the stroma of gastrointestinal and skin carcinomas and in the reactive fibrosis zone showed gelatinase A localization in the lumen of rER or in the cytosol along the surface of rER (Fig. 6). Fibroblasts were identified by their spindle-shape, well developed rER, and paucity of cytoplasmic projections. We had confirmed this cell identification by immunoelectron microscopy for type I collagen.[20)] Fibroblasts in non-neoplastic fibrous granulation tissue gave the same results (Table I). Macrophages in the carcinoma stroma showed diffuse deposition of immunoreactive substances in the cytosol (Fig. 7). They were morphologically characterized by abundant vacuoles, cytoplasmic projections, and relative paucity of rER. We confirmed this cell identification by immunoelectron microscopy for CD68 and CD11c (LeuM5) (our unpublished data). Immunoreactivity along the plasma membrane in macrophages was detected in three of the carcinoma cases, being more common than in carcinoma cells (data not shown).

DISCUSSION

The present study has revealed the heterogeneity of subcellular localization of gelatinase A between carci-

noma cells and fibroblasts. Fibroblasts showed localization in the lumen and on the surface of rER. Localization in the lumen of rER should theoretically indicate the synthesis (translation of mRNA) of proteins bearing a signal peptide. We have already demonstrated this by *in situ* hybridization and immunoelectron microscopy with type I procollagen.[20] Therefore, the present data are consistent with studies by *in situ* hybridization in colon and skin cancers, indicating that fibroblasts are the major source of synthesis of gelatinase A.[13, 14] Carcinoma cells, which were positive for the protein of gelatinase A, revealed diffuse deposition of gelatinase A in the cytosol. Immunoreactivity in the lumen of rER was only observed in one case of skin squamous cell carcinoma. This localization pattern is different from that of synthesis. Considering that cancer cells were negative for gelatinase A mRNA in colon and skin cancers[13, 14] (and our unpublished data), we suspect that cancer cells produce gelatinase A at a lower level, so that its mRNA is below the detection limit by *in situ* hybridization. Intracellular gelatinase A is thought to be the proform, and cytosolic localization may represent the storage form. However, its exact physiologic significance has not been clarified in the present study. The uptake process of gelatinase A was not demonstrated in our study. This result was similar to that in the case of transforming growth factor (TGF)-β in human stomach carcinoma. TGF-β is localized in the lumen of rER in fibroblasts and macrophages, and diffusely deposited in carcinoma cells.[21]

Recently, membrane-type metalloproteinase was discovered as an activator of gelatinase A on the cancer cell membrane.[22] It was proposed that cancer cells have the potential to activate progelatinase A, which is secreted from stromal fibroblasts, on their plasma membrane to facilitate cancer cell invasion. Our observation of gelatinase A on the plasma membrane may correspond to progelatinase A attached to the plasma membrane to be activated. However, the frequency of the membrane localization of gelatinase A was higher in macrophages than in cancer cells. Therefore, macrophages may also express molecule(s) which bind gelatinase A. Expression of TIMP-2 is also important for clarification of the function of gelatinase A. In colon cancer, TIMP-2 is expressed in stromal cells and partly on the cell membrane of cancer cells.[23]

The present study further revealed the expression of gelatinase A in fibroblasts in the reactive fibrosis zone of gastrointestinal carcinoma and in non-neoplastic granulation tissue. These two localization patterns may suggest another function of gelatinase A; it may be related to the turnover of extracellular matrix proteins. In fact, gelatinase A is expressed in human granulation tissue.[24] We also observed co-localization of mRNAs of gelatinase A and type I procollagen in carcinoma stroma and granulation tissue (unpublished data). Further studies will be required to establish the pathophysiological significance of gelatinases in cancer and granulation tissue.

ACKNOWLEDGMENTS

We are grateful to Dr. Karl Tryggvason and Dr. Matti Hoyhtya for the generous supply of anti-gelatinase antibodies. We also thank Fumiko Date and Katsuhiko Ono for their technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture.

(Received September 16, 1994/Accepted December 16, 1994)

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