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Production of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Human Breast Carcinomas

Hiroji Iwata,[1,4] Shunzo Kobayashi,[1] Hirotaka Iwase,[1] Akira Masaoka,[1] Noboru Fujimoto[2] and Yasunori Okada[3]

[1]The Second Department of Surgery, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467, [2]Biopharmaceutical Department, Fuji Chemical Industries Ltd., 530 Tyoukeiji, Takaoka, Toyama 933 and [3]Department of Molecular Immunology and Pathology, Cancer Research Institute, Kanazawa University, 13-1 Takara-cho, Kanazawa, Ishikawa 920

We examined production and tissue localization of matrix metalloproteinase (MMP)-1 (tissue collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin-1), MMP-9 (gelatinase B), tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in human breast carcinomas. In more than half of the cases, MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 were immunolocalized in carcinoma cells and MMP-2 was on the carcinoma cell membranes as well, whereas MMP-3 was positively stained in less than 15% of the cases. MMP-1 staining in carcinoma cells was significantly higher in scirrhous carcinoma than in other types of carcinoma. MMP-9 expression was remarkably higher in the carcinoma cases with lymphnode metastasis than in the non-metastatic cases. MMP-3 was mainly expressed in T-lymphocytes infiltrated in the tumor stroma. Stromal fibroblasts were positive for all these MMPs except for MMP-3. The TIMP-1 levels released into the culture media by carcinoma tissues were significantly lower than those by fibroadenoma tissues, although there were no significant differences in the levels of MMP-1, MMP-2, MMP-9 and TIMP-2. Gelatin zymographical analyses showed that the activation rate of the zymogen of MMP-2 (proMMP-2) is significantly higher in the more advanced carcinoma group with lymphnode metastasis than in the metastasis-negative and fibroadenoma groups. These data indicate that MMP-1, MMP-2 and MMP-9 are highly expressed in human breast carcinoma tissue and suggest that activation of proMMP-2 may be an indicator of lymphnode metastasis of the breast carcinoma.

Key words: Matrix metalloproteinase — Tissue inhibitor of metalloproteinase — Cancer invasion — Metastasis — Breast cancer

Tumor cells degrade extracellular matrix (ECM) components to invade the surrounding stroma and form metastatic colonies at distant sites.[1] All classes of enzymes have been reported to be implicated in this degradation.[2,3] Matrix metalloproteinases (MMPs) are a gene family of Zn^{2+} metalloproteinases which are composed of at least eleven different gene products and expressed by various malignant tumor cells.[2,4] Among the 11 different MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been the focus of attention in connection with cancer metastasis because of their ability to degrade type IV collagen, a major constituent of vascular basement membranes.[5–8] It has been reported that breast carcinoma cells in culture express MMP-1 (tissue collagenase), MMP-2, MMP-9 and MMP-10 (stromelysin-2), but not MMP-3 (stromelysin-1).[9] MMP-2 production in breast carcinoma and fibroadenoma tissues has been confirmed immunohistochemically.[10,11] In addition, MMP-11 (stromelysin-3) expression in the stromal cells of the breast cancers has been reported to correlate with tumor progression.[12,13] However, information about expression of other MMPs such as MMP-1 and MMP-9 in breast carcinomas is limited.

Quantitative analyses of MMP-2 and MMP-9 by using zymography and enzyme immunoassay (EIA) have been made in brain tumors,[14] prostatic carcinomas,[15] lung cancers[16] and colorectal carcinomas.[17] Zucker *et al.*[17] have shown that plasma MMP-9 levels are significantly increased in patients with breast cancer. However, detailed analytical studies on multiple MMPs in breast carcinomas are not available.

The activities of MMPs *in vivo* are thought to be strictly regulated by tissue inhibitors of metalloproteinases (TIMPs), i.e., TIMP-1, TIMP-2 and TIMP-3.[2,18] Studies using *in vitro* invasion assays have demonstrated that TIMPs added to the assay mixtures inhibit the invasion of reconstituted basement membrane by tumor cell lines.[19] It is also known that overexpression of TIMPs in the tumor cells suppresses metastasis in experimental animal models.[20,21] These results suggest the possibility that TIMPs produced at the local tissues determine the invasion and metastatic behavior of the cancer cells. Lokeshwar *et al.*[15] have reported that prostatic

[4] To whom correspondence and requests for reprints should be addressed.

cancer tissue produces less TIMP activity than the control. However, no studies describing the levels of TIMPs and MMPs in breast carcinomas are available.

In the present studies we examined the production of four different MMPs (MMP-1, 2, 3 and 9) and two TIMPs (TIMP-1 and TIMP-2) in human breast benign and malignant tumors by immunohistochemistry, sandwich immunoassay and gelatin zymography. The data for breast carcinomas were compared with those for fibroadenomas. The results indicate that not only MMP-2, but also MMP-1 and MMP-9 are highly expressed in human breast carcinomas and suggest that activation of the zymogen of MMP-2 (proMMP-2) may be involved in the lymphnode metastasis of breast carcinomas.

MATERIALS AND METHODS

Histology Forty-seven human breast cancer and nine fibroadenoma tissues were obtained at mastectomy or surgical biopsy in the Nagoya City University Medical School Hospital, and Aichi Cancer Center, Nagoya, Japan. Blocks of the tissue were excised from the margin of the carcinomas and the fibroadenomas. Each sample was fixed with a periodate-lysine-paraformaldehyde (PLP) fixative for 18–24 h at 4°C after treatment with 2 μM monensin (Wako Inc., Tokyo) in RPMI medium (Life Technologies, Inc., Grand Island, NY) at 37°C for 3 h as described previously,[22] and then snap-frozen in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN) by dipping in liquid nitrogen. Frozen sections of monensin-treated and untreated samples were made with a cryostat (Miles Inc.). Paraffin sections of the surgical specimens fixed in formalin were stained with hematoxylin and eosin, and examined according to the General Rules for the Breast Cancer Study.[23]

Immunohistochemistry Monoclonal antibodies against human MMP-1 (41-1E5), MMP-2 (75-7F7), MMP-3 (55-2A4), MMP-9 (56-2A4), TIMP-1 (50-2F6), and TIMP-2 (67-4H11) have been characterized and previously used for immunolocalization studies.[24-29] For immunohistochemistry, the frozen sections were reacted with primary antibodies or non-immune mouse IgG for 15–18 h at 4°C after blocking of endogenous peroxidase by treatment with 1% H_2O_2 and nonspecific binding with 10% normal rabbit serum. Concentrations of the primary antibodies used for immunostaining were 0.5 μg/ml for MMP-1, 2.5 μg/ml for MMP-2, 5 μg/ml for MMP-3, 2 μg/ml for MMP-9, 25 μg/ml for TIMP-1, 2 μg/ml for TIMP-2 and 10 μg/ml for non-immune mouse IgG. After having been rinsed with phosphate-buffered saline (PBS) the sections were incubated with biotinylated rabbit antibodies to mouse IgG (SAB-PO Kit, Nichirei Co., Inc., Tokyo) for 10 min and then with a streptoavidin-peroxidase complex (SAB-PO Kit, Nichirei Co., Inc.) for 5 min at room temperature. Color was developed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl buffer, pH 7.6, containing 0.006% H_2O_2. Counterstaining was performed with 1% methyl green.

Immunostaining was scored by measuring the ratio (%) of immunoreactive cells to total carcinoma cells. The scale used was 0–4, where 0 is no staining, 1 is $<25\%$, 2 is 25–50%, 3 is 50–75% and 4 is $>75\%$.

Tissue cultures Tissue samples of the carcinomas and fibroadenomas were excised from the samples obtained for immunostaining. They were rinsed in sterile PBS, cut into small blocks ($\sim 1\times1\times1$ mm) and cultured for 24 h in serum-free RPMI medium containing 0.2% lactalbumin hydrolysate (Life Technologies, Inc.). The culture media were stored at -80°C until used for assays and zymography, and the tissue blocks were weighed after lyophilization.

Sandwich enzyme immunoassays Concentrations of MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in the culture media were measured in the sandwich enzyme immunoassays for these MMPs and TIMPs as described previously.[24, 25, 27, 29, 30] The EIA system for MMP-1 measures both precursor and active forms of MMP-1, but those for MMP-2 and MMP-9 detect their latent forms.[24, 25, 27] The EIA system for TIMP-1 determines the whole amount of TIMP-1, including free TIMP-1 and its complexed forms with MMPs and proMMP-9.[30] On the other hand, the EIA for TIMP-2 detects free TIMP-2 and TIMP-2 complexed with active MMPs, but not the complex with proMMP-2.[29] The values were expressed as nmol/g dry weight tissue. The following molecular weights were used for the calculation: 51,929 for MMP-1, 70,952 for MMP-2, 78,426 for MMP-9, 20,685 for TIMP-1 and 21,755 for TIMP-2 (see Ref. 31).

Enzyme assays Gelatinolytic activities in the culture media were measured in an assay using [^{14}C]acetylated type I gelatin[32] in the presence and absence of 1 mM p-aminophenylmercuric acetate (APMA). Collagenolytic and carboxymethylated transferrin (Cm-Tf)-degrading activities in the media were also measured by using [^{14}C]type I collagen[33] and [^{3}H]Cm-Tf,[34] respectively. The samples were incubated with 1.5 mM APMA at 37°C for 18 h to activate the zymogen of MMP-1 (proMMP-1) and proMMP-3 prior to the assays. All these assays were performed in the presence of 2 mM phenylmethanesulfonyl fluoride and 5 mM N-ethylmaleimide to inhibit serine and cysteine proteinases. One unit of the activities is defined as the amount of 1 μg substrate degraded/min at 37°C.

Gelatin zymography Zymography in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.2% gelatin was performed according to the methods of Hibbs *et al.*[35] The samples were incubated at

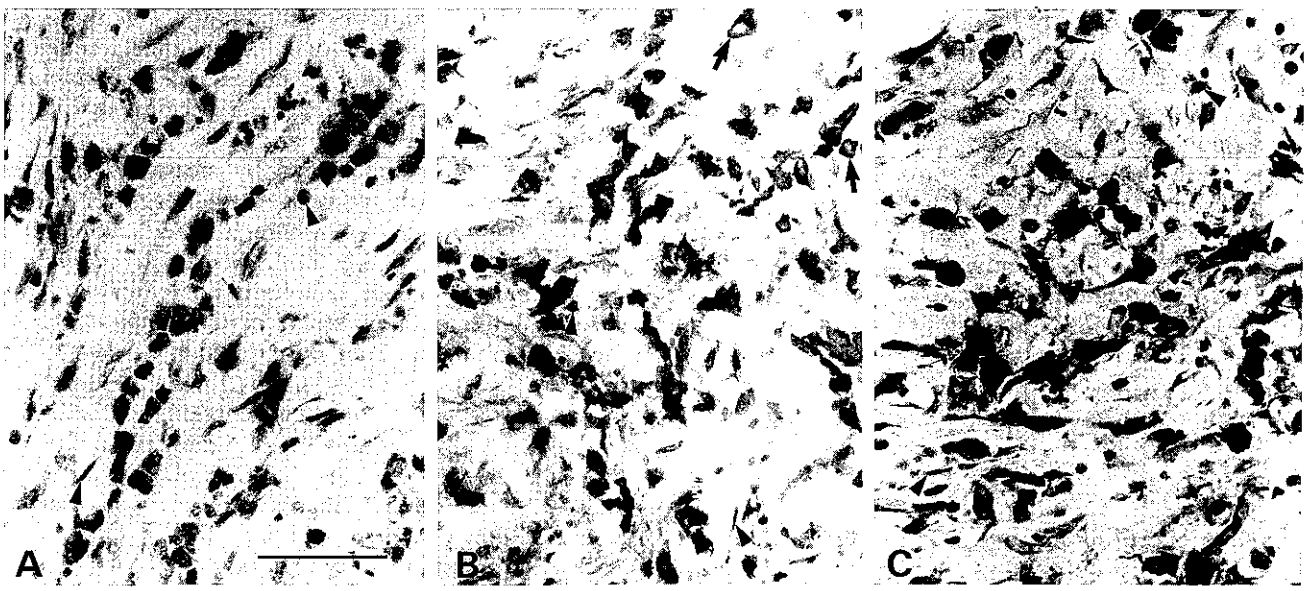

Fig. 1. Immunolocalization of MMP-1, MMP-2 and MMP-9 in human breast carcinoma tissues. Immunostaining was performed with mouse monoclonal antibodies against MMP-1 (A), MMP-2 (B) and MMP-9 (C) as described in "Materials and Methods." Note that all these MMPs are immunostained in the carcinoma cells. In addition, MMP-2 is also localized on the carcinoma cell membranes (arrows). Arrowheads indicate positively stained stromal fibroblasts and macrophages. Methyl green counterstain. Scale bar, 50 μm.

37°C for 20 min in SDS sample buffer without reducing agent and then electrophoresed on 8% polyacrylamide gels at 4°C. The volume of the media loaded was adjusted according to the weight of the tissue used for the culture. After electrophoresis, the gels were washed in 2.5% Triton-X 100 to remove SDS, incubated for 16 h at 37°C in 50 m*M* Tris-HCl, pH 7.4, 10 m*M* $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3 and then stained with 0.1% Coomassie Brilliant Blue R250. Computer-assisted image analyses of the gels were performed according to the methods described by Davies *et al.*[36]

Statistical analyses Correlations between immunohistochemical scores of MMPs and TIMPs and clinicopathological factors were analyzed by use of the Kruskal-Wallis test. Correlations of MMPs and TIMPs production between breast carcinomas and fibroadenomas were statistically examined by use of the Mann-Whitney U test. Furthermore, the correlation between activation rate of proMMP-2 and lymphnode metastases was statistically examined by use of the *t* test.

RESULTS

Histology and immunohistochemistry Immunolocalization of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 was examined in carcinoma samples from 47 cases. Carcinoma cells showed intracytoplasmic staining for MMP-1 (Fig. 1A), MMP-2 (Fig. 1B), MMP-9 (Fig. 1C), TIMP-1 (Fig. 2A) and TIMP-2 (Fig. 2B), and the percentages of positive cases were 87.2, 85.1, 72.3, 59.6 and 46.8%, respectively (Table I). In most cases, cell membranes of some carcinoma cells were also immunostained with the antibody against MMP-2 (Fig. 1B). The immunostaining of MMP-2, MMP-9 and TIMP-2 in carcinoma cells tended to be stronger at the periphery of tumor cell nests, while no such staining patterns were seen with MMP-1 and TIMP-1 localization. Although MMP-3 immunostaining was very weak or negative in carcinoma cells, it was positive in the lymphoid cells infiltrated in the tumors (Fig. 3A). Immunohistochemistry of MMP-3, CD4 and CD20 in the serial sections of the specimens demonstrated that MMP-3-positive cells are composed mainly of T lymphocytes, since they were CD4- (Fig. 3B) and CD8- (Fig. 3C) positive, but CD20- (Fig. 3D) negative. Stromal fibroblasts, vascular endothelial cells and macrophages in the tumor tissues were also positive for MMP-1, MMP-2 and MMP-9 (Fig. 1, A, B and C). In most cases, fibroblasts were also immunostained for TIMP-1 and TIMP-2 (Fig. 2, A and B).

The immunostaining of MMP-1, MMP-2 and MMP-9 was positive in hyperplastic ducts of fibroadenomas. On the other hand, the epithelial cells in sclerosing ducts were negative for either MMPs or TIMPs (Table II). Immunohistochemistry with non-immune mouse IgG

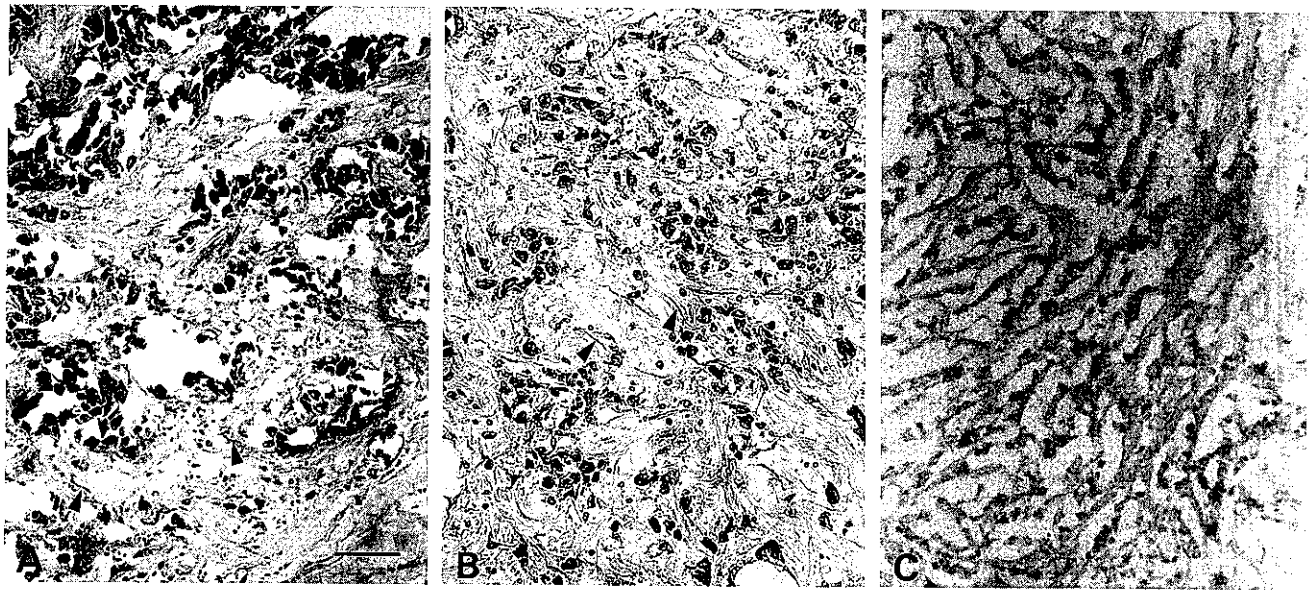

Fig. 2. Immunolocalization of TIMP-1 and TIMP-2 in human breast carcinoma tissues. The tissues were immunostained with antibodies against TIMP-1 (A), TIMP-2 (B) and non-immune mouse IgG (C) as described in "Materials and Methods." TIMP-1 and TIMP-2 are immunostained in the carcinoma cells and stromal fibroblasts (arrowheads), while no staining is observed with non-immune IgG. Methyl green counterstain. Scale bar, 50 μm.

Table I. Immunostaining of MMPs and TIMPs in Human Breast Carcinoma Cells

	MMP-1	MMP-2	MMP-3	MMP-9	TIMP-1	TIMP-2
Positive case rate (%)	87.2	85.1	12.8	72.3	59.6	46.8
Mean score	3.1±1.4	2.6±1.5	0.2±0.5	1.5±1.3	1.2±1.3	0.9±1.2

resulted in no staining in either tumor or stromal cells (Fig. 2C).

When the mean positive scores of MMPs and TIMPs in carcinoma cells were compared with clinicopathological factors, the score of MMP-1 in scirrhous carcinoma was significantly higher than that in papillotubular and solid-tubular carcinomas ($P=0.046$), and the MMP-9 score correlated directly with n-stage ($P=0.035$) (Table III). However, there was no correlation between the immunostaining of MMPs and TIMPs and menopausal status, estrogen receptor (ER) status, t-stage or tnm-stage (Table III).

Sandwich EIA To measure the amounts of MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 produced by the carcinoma tissues, the culture media from 34 breast carcinomas and 9 fibroadenomas were examined with the corresponding sandwich EIA systems. MMP-3 was not measured because it was not immunostained in carcinoma cells in most cases. All the samples contained detectable amounts of MMP-1, MMP-2, MMP-9 and TIMP-1. However, TIMP-2 was detected only in 8 carcinoma and 1 fibroadenoma samples.

The mean levels of MMP-1 and MMP-2 were 2- to 3-fold higher than that of MMP-9 in both carcinoma and fibroadenoma samples. The MMP-9 level tended to be higher in carcinoma samples than in fibroadenoma ($P=0.099$), but MMP-1 and MMP-2 levels showed no difference between carcinoma and fibroadenoma samples (Fig. 4). On the other hand, the mean TIMP-1 level was significantly lower in carcinomas than fibroadenomas ($P=0.00079$), while no difference was seen in the TIMP-2 level (Fig. 4).

Gelatin zymography The gelatinolytic activities were analyzed by gelatin zymography in 34 carcinoma and 9 fibroadenoma samples. ProMMP-9 of 92 kDa, proMMP-2 of 68 kDa and active MMP-2 of 62 kDa were detected in all carcinoma and fibroadenoma samples (Fig. 5), but the processed form of MMP-9 of 83 kDa was present only in 3 carcinoma samples. The intensity of the proteolytic band of proMMP-9 was correlated with the

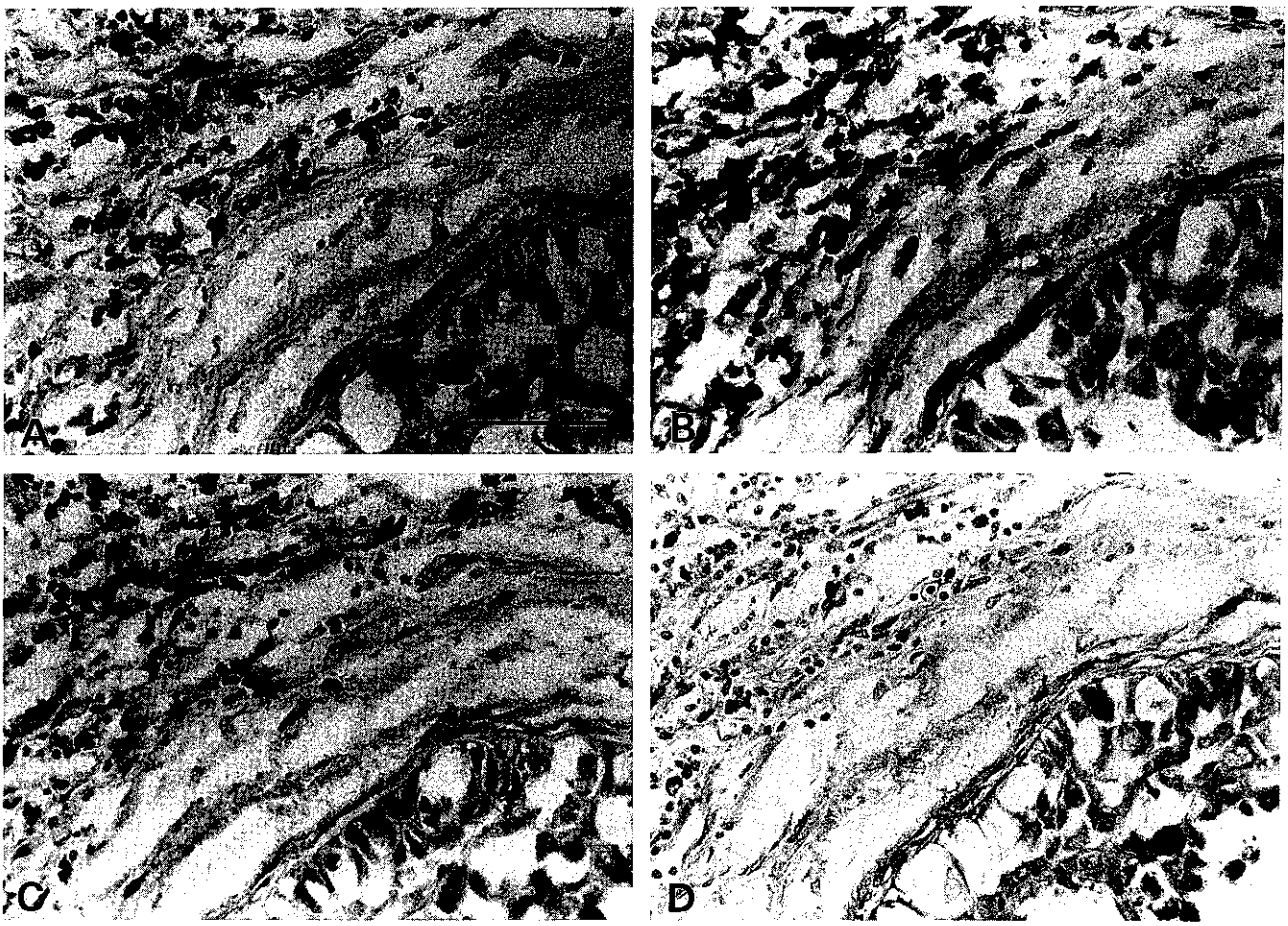

Fig. 3. Immunolocalization of MMP-3, CD4, CD8 and CD20 in human breast carcinoma tissues. Immunostaining was performed with mouse monoclonal antibodies against MMP-3 (A), CD4 (B), CD8 (C) and CD20 (D) in serial specimens as described in "Materials and Methods." Note that lymphocytes infiltrating the cancer tissue (left upper corner in each figure) are positively stained for MMP-3, CD4 and CD8, but not for CD20. Methyl green counterstain. Scale bar, 50 μm.

amount of MMP-9 measured by EIA in all carcinoma and fibroadenoma tissue samples, confirming that carcinoma tissues produce larger amounts of proMMP-9 than fibroadenoma tissues. In addition, the intensity of the proMMP-2 band appeared to be correlated with the amount of proMMP-2 measured by EIA in fibroadenoma samples, and the proteolytic band of active MMP-2 was more intense in the carcinomas than in the fibroadenomas (Fig. 5). Densitometric analyses showed that the activation ratio of proMMP-2 (the ratio of the 62 kDa active form to proMMP-2 plus active MMP-2) was significantly higher in carcinoma samples ($18.3\pm15.2\%$) than in fibroadenoma samples ($4.2\pm1.8\%$) ($P<0.01$) and in the carcinoma groups with lymphnode metastasis ($27.9\pm16.7\%$) than in the metastasis-negative group ($11.6\pm9.7\%$) ($P<0.01$).

Table II. Immunostaining of MMPs and TIMPs in Fibroadenoma Tissues

	Ductal epithelial cells		Fibroblasts
	Hyperplastic duct	Sclerosing duct	
MMP-1	+[a]	—	—
MMP-2	+[a]	—	+[b]
MMP-3	—	—	—
MMP-9	+[a]	—	+[b]
TIMP-1	—	—	+[c]
TIMP-2	—	—	+[b]

a) Positive in luminal cystic cells at piled duct epithelium.
b) All positive.
c) Positive in circumferential normal tissues.

Table III. Relationships between Positive Scores of MMP and TIMP Expression and Clinicopathological Factors in Breast Cancer

Factor	Case	MMP-1 (mean) 3.09		MMP-2 (mean) 2.60		MMP-9 (mean) 1.53		TIMP-1 (mean) 1.21		TIMP-2 (mean) 0.94	
Menopausal	pre (n=18)	2.83	n.s.	2.56	n.s.	1.50	n.s.	1.17	n.s.	0.83	n.s.
	post (n=29)	3.24		2.62		1.55		1.24		1.00	
ER	<13 ng/ml (n=10)	3.11	n.s.	2.33	n.s.	1.44	n.s.	0.89	n.s.	0.78	n.s.
	≧13 ng/ml (n=37)	3.11		2.83		1.63		1.31		1.03	
Histology	papillot[a] (n=16)	2.50		2.75		1.44		1.06		0.75	
	solidt[b] (n=8)	2.75	*	2.63	n.s.	1.00	n.s.	1.00	n.s.	0.63	n.s.
	sci[c] (n=18)	3.56		2.39		1.56		1.17		1.06	
t-factor	t=1 (n=13)	3.08	n.s.	2.69	n.s.	1.31	n.s.	1.00	n.s.	1.00	n.s.
	t=2, 3, 4 (n=34)	3.09		2.56		1.62		1.29		0.91	
n-factor	n=0 (n=25)	3.12		2.56		1.04		1.36		0.72	
	n=1α, 1β (n=17)	2.94	n.s.	2.06	n.s.	2.06	*	0.76	n.s.	1.00	n.s.
	n=2, 3 (n=5)	3.40		4.00		2.60		1.80		1.80	
Clinical stage	stage 1, 2 (n=37)	3.05	n.s.	2.41	n.s.	1.22	n.s.	1.05	n.s.	0.84	n.s.
	stage 3, 4 (n=10)	3.20		3.30		1.80		1.80		1.30	

a) Papillotubular carcinoma.
b) Solid-tubular carcinoma.
c) Scirrhous carcinoma.
* $P<0.05$. n.s.: not significant.

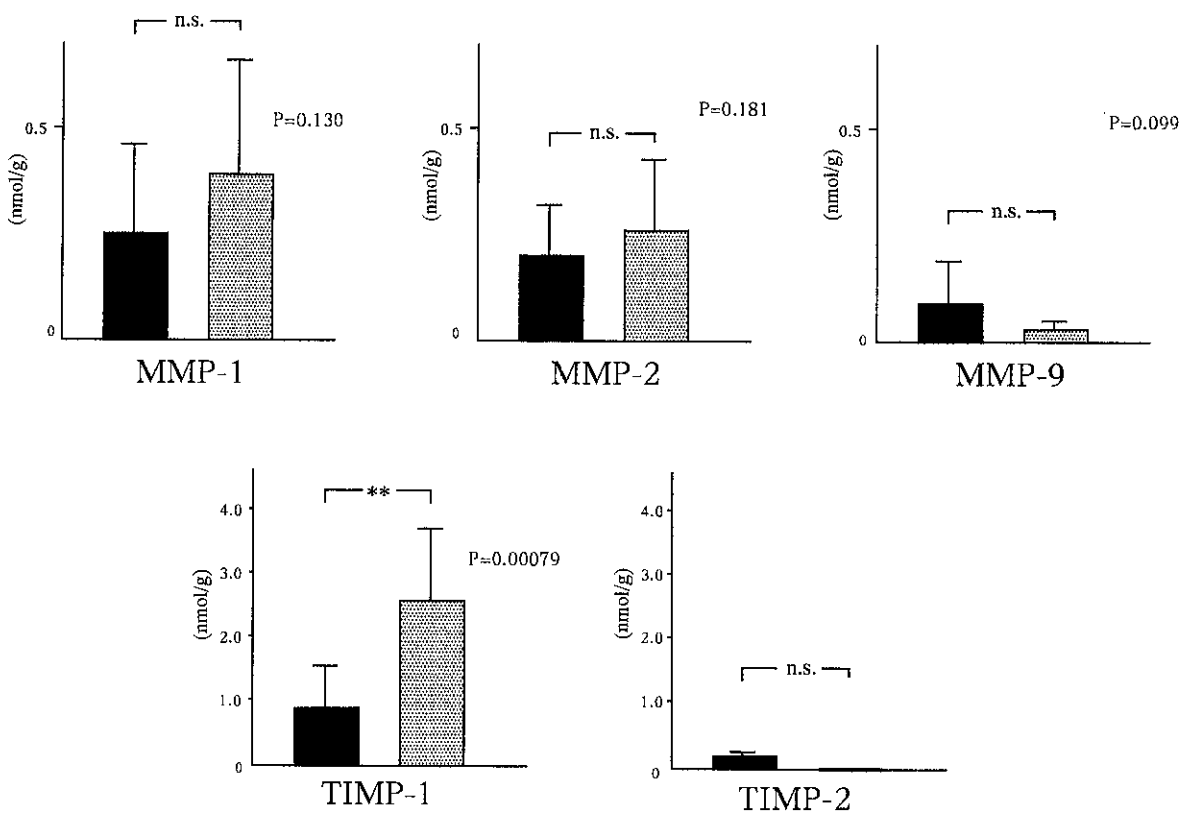

Fig. 4. Amounts of MMPs and TIMPs in the culture media secreted by carcinoma and fibroadenoma tissues. MMP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in the culture media from breast carcinomas (dark bars) and fibroadenomas (light bars) were measured by the corresponding EIA systems. The values (nmol/g weight) were calculated as described in "Materials and Methods." The mean±SD is shown. **, $P<0.01$; n.s., not significant.

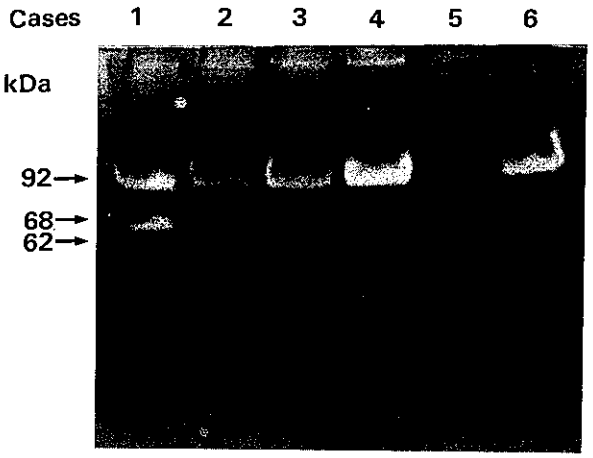

Fig. 5. Detection of MMP-2 and MMP-9 species in the culture media of breast carcinoma and fibroadenoma tissues by gelatin zymography. Cases 1, 2 and 3 are samples from fibroadenoma, and cases 4, 5 and 6 are from breast carcinoma. Gelatinolytic activity of the 92 kDa species corresponding to proMMP-9 and activities of the 68 and 62 kDa species corresponding to proMMP-2 and MMP-2 are indicated.

Inhibitor studies demonstrated that the gelatinolytic activities of the 92, 83, 68 and 62 kDa forms are almost completely inhibited with 10 m*M* EDTA and 1 m*M* 1,10-phenanthroline, but not with 2 m*M* phenylmethylsulfonyl fluoride (data not shown). In addition, incubation of the culture media with 1.0 m*M* 4-aminophenylmercuric acetate prior to electrophoresis caused processing of the proMMP-9 and proMMP-2 proteolytic bands. The data indicate that these gelatinolytic activities belong to a class of metalloproteinases.

Enzyme activities The proteinase activities in the culture media were measured by using ^{14}C-gelatin, ^{14}C-collagen and ^{3}H-Cm-Tf substrates in the presence of inhibitors of serine and cysteine proteinases. However, only negligible or no activities were detected in the samples (data not shown).

DISCUSSION

The present studies demonstrate that MMP-1, MMP-2 and MMP-9 are highly expressed in human breast carcinomas. Previous studies have shown that MMP-2 is expressed in many carcinoma tissues including breast carcinoma.[10,11,37] Although expression of MMP-1, MMP-2, MMP-9 and MMP-10 in human breast cancer cell lines *in vitro*[9] is known, there are few reports about production of MMP-1 and MMP-9 in human breast carcinomas. It is noteworthy that MMP-1 is intensely positive in scirrhous carcinoma compared with papillotubular and solid-tubular carcinomas. Scirrhous carcinoma contains much interstitial connective tissue, which is composed mainly of fibroblasts and ECM components including interstitial collagens, fibronectin, laminin, and various proteoglycans.[23,38] Since the major substrate of MMP-1 is such collagens,[2] MMP-1 may play a role in the stromal invasion by scirrhous carcinoma cells. MMP-9 production is known to be remarkably increased in highly metastatic cell lines as compared with low-metastatic cell lines.[5] Nakajima *et al.*[39] also reported that high levels of plasma proMMP-9 are associated with dissemination of rat adenocarcinoma cells. In addition, it has been reported that MMP-9 production correlates with tumor cell invasion and metastasis of human malignant tumors such as brain tumor,[14] lung carcinoma[16] and bladder carcinoma.[7] In the present studies, MMP-9 localization in the breast carcinoma cells was significantly higher in the cases with lymphnode metastasis than in those without metastasis. Thus, it seems likely that MMP-9 may be implicated in facilitating the lymphnode metastasis of breast carcinomas.

The present studies showed that MMP-2 is immunostained both in and on the carcinoma cells and within the stromal fibroblasts. Previous studies have noted a discrepancy of the expression of MMP-2 protein and its mRNA: MMP-2 was immunolocalized in both carcinoma cells and stromal cells, while the mRNA was detected only in the stromal fibroblasts.[10,37] The disparity could be explained by the possibility of (a) uptake of fibroblast-derived MMP-2 by carcinoma cells, (b) a difference in the rates of mRNA translation and capacity for intracellular storage of the protein or (c) a difference of the threshold of detection by *in situ* hybridization and immunohistochemistry.[36] Polette *et al.*[40] recently proposed that production of MMP-2 by peritumor fibroblasts in breast cancer results in the apparent immunolocalization of MMP-2 in the carcinoma cells according to immunohistochemistry using confocal microscopy and *in situ* hybridization. In addition, Sato *et al.*[4] have reported a new metalloproteinase with a transmembrane domain, i.e., membrane-type MMP, which can active proMMP-2 by capturing it on the cell membranes. Thus, cell membrane localization of MMP-2 in the present study may be due to cell membrane binding and activation of proMMP-2 secreted by stromal fibroblasts and/or carcinoma cells.

Davies *et al.* have reported that activation of proMMP-2 correlates with tumor grade in breast[36] and bladder cancers.[7] Garbisa *et al.*[16] have also reported that MMP-2 levels in the serum are significantly higher in lung cancer patients with distant metastasis than in those without metastasis. In the present studies, the activation ratio of proMMP-2 was significantly enhanced in carcinoma groups with lymphnode metastasis compared with the metastasis-negative group and the fibroadenoma

group. However, neither MMP-2 immunolocalization in the carcinoma cells nor MMP-2 levels in the culture media showed a correlation with clinicopathological factors (data not shown). Actually, the levels of MMP-2 in the media of the carcinoma tissue were not significantly different from those in the fibroadenoma samples. This can be explained by the fact that our EIA system for MMP-2 detects free proMMP-2 and proMMP-2-TIMP-2 complex but not active MMP-2,[25] since culture media of carcinomas contained active MMP-2 more frequently than those of fibroadenomas as detected by zymography. The present studies have shown that MMP-3 staining in breast carcinoma cells is negligible. The data are compatible with the finding that MMP-3 is not expressed in breast carcinoma cell lines.[9] On the other hand, MMP-3 immunostaining was strongly positive in T-lymphocytes that infiltrated the carcinoma stroma. As Woessner[38] have reported that mouse T-lymphocytes secrete metalloproteinase capable of degrading proteoglycan, we speculate that MMP-3 is involved in infiltration of T-lymphocytes in ECM of the breast cancers.

In the present studies, TIMP-1 and TIMP-2 were also immunostained in carcinoma cells and stromal fibroblasts. Visscher *et al.*[41] claimed that breast carcinomas with diffuse TIMP-2 immunostaining show recurrence more frequently than those with weak or no staining. However, we found no such correlation between TIMP-2 staining and carcinoma recurrence (data not shown). Actually, no significant difference was seen between TIMP-2 staining and any clinicopathological factor. When the TIMP-1 levels in the culture media were compared between the samples from carcinomas and fibroadenomas, carcinomas produced significantly less TIMP-1 than fibroadenomas. However, immunolocalization study did not indicate decreased TIMP-1 production in the carcinoma tissues, since both carcinoma cells and stromal fibroblasts were immunostained in about 60% of the cases. This discrepancy is difficult to explain. One possibility is that TIMP-1 secreted from the cases might be trapped by active MMPs and proMMP-9 in the extracellular milieu of the carcinoma tissues, hindering TIMP-1 release into the culture media.

It should be noted that expression and production of MMP-1, MMP-2 and MMP-9 *per se* are not sufficient to account for malignant characteristics of breast tumors, since we found neither specific staining patterns of those MMPs, nor enhanced production in the carcinomas compared with fibroadenomas. No enzymic activities were detected in the assays using radio-labeled substrates in the culture media from carcinoma and fibroadenoma samples, even if they were first treated with APMA to activate proMMPs. This is probably because the amounts of TIMPs are higher than those of MMPs, as shown by EIA assays. Thus, it seems likely that actions of MMPs in the breast cancer tissues may be pericellular. This possibility should be clarified by further studies using new methodology such as *in situ* zymography.[42]

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