Endometase/Matrilysin-2 in Human Breast Ductal Carcinoma in Situ and Its Inhibition by Tissue Inhibitors of Metalloproteinases-2 and -4: A Putative Role in the Initiation of Breast Cancer Invasion

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ABSTRACT

Local disruption of the integrity of both the myoepithelial cell layer and the basement membrane is an indispensable prerequisite for the initiation of invasion and the conversion of human breast ductal carcinoma in situ (DCIS) to infiltrating ductal carcinoma (IDC). We previously reported that human endometase/matrilysin-2/matrix metalloproteinase (MMP-26)-mediated pro-gelatinase B (MMP-9) activation promoted invasion of human prostate carcinoma cells by dissolving basement membrane proteins (Y. G. Zhao et al., J. Biol. Chem., 278: 15056–15064, 2003). Here we report that tissue inhibitor of metalloproteinases (TIMP)-2 and TIMP-4 are potent inhibitors of MMP-26, with apparent Kᵢ values of 1.6 and 0.62 nM, respectively. TIMP-2 and TIMP-4 also inhibited the activation of pro-MMP-9 by MMP-26 in vitro. The expression levels of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins in DCIS were significantly higher than those in IDC, atypical intraductal hyperplasia, and normal breast epithelia adjacent to DCIS and IDC by immunohistochemistry and integrated morphometry analysis. Double immunofluorescence labeling and confocal laser scanning microscopy revealed that MMP-26 was colocalized with MMP-9, TIMP-2, and TIMP-4 in DCIS cells. Higher levels of MMP-26 mRNA were also detected in DCIS cells by in situ hybridization.

INTRODUCTION

Matrix metalloproteinases (MMPs) are known to be associated with cancer cell invasion, growth, angiogenesis, inflammation, and metastasis (1, 2). MMP-26 is a novel enzyme that was recently cloned and characterized by our group (3) and others (4–6). It has several structural features characteristic of MMPs, including a signal peptide, a propeptide domain, and a catalytic domain with a conserved zinc-binding motif, but it lacks the hemopexin-like domain (3–6). A unique “cysteine switch” sequence in the prodomain, PHCGVPD as opposed to the conserved PRCGXXD sequence found in many other MMPs, keeps the enzyme latent. MMP-26 mRNA is primarily expressed in cancers of epithelial origin, such as endometrial carcinomas (3, 7), prostate carcinomas (7), lung carcinomas (7), and their corresponding cell lines (3–6), and in a small number of normal adult tissues, such as the uterus (3, 5), placenta (4, 5), and kidney (6). Some parallels exist with MMP-7, which is also expressed epithelially and also lacks the hemopexin-like domain. We have also reported that the levels of MMP-26 gene and protein expression are higher in a malignant choriocarcinoma cell line (JEG-3) than in normal human cytotrophoblast cells (8). Recently, we found that the levels of MMP-26 protein in human prostate carcinomas from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate tissues (9). MMP-26 is capable of activating pro-MMP-9 by cleavage at the Ala133-Met154 site of the pro-enzyme, and this activation facilitates the efficient cleavage of fibronectin (FN), promoting the invasion of highly invasive and metastatic androgen-repressed prostate cancer cells through FN or type IV collagen (9). The activation is prolonged but persistent, which is consistent with the process of tumor cell invasion. These findings indicate that MMP-26-mediated pro-MMP-9 activation may be one biochemical mechanism contributing to human carcinoma cell invasion in vivo.

MMP activities are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). Four mammalian TIMPs have been identified: (a) TIMP-1 (10); (b) TIMP-2 (11); (c) TIMP-3 (12); and (d) TIMP-4 (13). The hydrolytic activity of MMP-26 against synthetic peptides is blocked by TIMP-1, TIMP-2, and TIMP-4 (5, 6, 8), but the inhibitory potential of TIMP-1 is lower than that of TIMP-2 and TIMP-4 (5). TIMP-1 and TIMP-2 also inhibit the cleavage of denatured type I collagen (gelatin) by MMP-26 (6). TIMPs are expressed in human breast cancer cells (14–16). Here, we continue to explore the possible roles of MMP-26 and the coordination of MMP-26 with MMP-9, TIMP-2, and TIMP-4 in human breast carcinoma invasion.

In the present study, we showed that TIMP-2 and TIMP-4 completely inhibited the activation of pro-MMP-9 by MMP-26. The expressions of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins in human breast ductal carcinomas in situ (DCIS) were significantly higher than those in infiltrating ductal carcinoma (IDC), atypical intraductal hyperplasia (AIDH), and normal breast epithelia around the DCIS and IDC. Furthermore, MMP-26 was colocalized with MMP-9, TIMP-2, and TIMP-4 in human breast DCIS.

MATERIALS AND METHODS

Inhibition Assays of MMP-26 by TIMP-2 and TIMP-4. The quenched fluorescence peptide substrates, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂ and Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Arg-Lys(Dnp)-NH₂, were purchased from Calbiochem. The MMP-26 used in this experiment is recombinant and partially active. Briefly, MMP-26 was expressed in the form of inclusion bodies from transformed Echerichia coli cells as described previously (3). The inclusion bodies were isolated and purified using B-PER bacterial protein extraction reagent according to the manufacturer’s instructions. The insoluble protein was dissolved in 8 M urea to ~5 mg/mL. The protein solution was diluted to ~100 μg/mL in 8 M urea and 10 mM DTT for 1 h, dialyzed in 4 M urea, 1 mM DTT, and 50 mM HEPES (pH 7.5) for at least 1 h; and then folded by dialysis in 1× HEPES buffer [50 mM HEPES, 0.2 mM CaCl₂, and 0.01% Brij-35 (pH 7.5)] with 20 μM ZnSO₄ for 16 h. To enhance the activity of MMP-26, the folded enzyme was dialyzed twice for 24 h at 4°C in the folding buffer without Zn²⁺ ions. The total enzyme concentration was measured by UV absorption using ε₂₈₀ = 57,130 M⁻¹ cm⁻¹, which was

Received 6/30/03; revised 10/29/03; accepted 11/7/03.

Grant support: Department of Defense Congressionally Directed Medical Research Programs Grant DAMD17-02-1-0238, NIH Grant CA78646, American Cancer Society Grant, Florida Division Grant F011103U1 (to Y.-G. M.), Florida State University Research Foundation grant (to Q.-X. A. S. and Y.-G. Z.), and Department of Defense Congressionally Directed Medical Research Programs Grants DAMD17-01-0129 and DAMD17-01-0129 (to Y.-G. M.).

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The data were fitted to the Morrison equation to calculate the apparent inhibition constants (apparent $K_i$).

The apparent inhibition constant, $K_i^{app}$, is given by:

$$K_i^{app} = \frac{v_{max} - v}{v}$$

where $v_{max}$ is the maximum velocity, $v$ is the observed velocity, and $K_i$ is the dissociation constant.

**Pro-MMP-9 Activation by MMP-26 and Inhibition of the Activation by TIMP-2 and TIMP-4.** Zymography and silver staining were performed as reported previously (3, 7, 19, 20). MMP-26, pro-MMP-9, and active MMP-9 were purified in our laboratory (3, 21). The molar concentration ratios of MMP-26:pro-MMP-9:FN:TIMP were 10:1:4:10 and 1:4:1:10. Aliquots were removed and treated with a reducing sample buffer and boiled for 5 min. After electrophoresis on 9% SDS-polyacrylamide gels, the protein bands were visualized by silver staining (19).

**FN Cleavage Assay.** MMP-26, pro-MMP-9, MMP-26-activated MMP-9, and TIMPs were prepared as described above. Active MMP-9, purified from human neutrophils (21), was used as a positive control. FN was incubated with MMP-26, pro-MMP-9, active MMP-9, or MMP-26 plus pro-MMP-9 in the presence or absence of TIMP-2 or TIMP-4 in 1× HEPES buffer at 37°C for 18 h. The molar concentration ratio of MMP-26:pro-MMP-9:FN/TIMP was approximately 1:4:10:10. Aliquots were removed and treated with a reducing sample buffer and boiled for 5 min. Samples were then loaded onto 9% polyacrylamide gels in the presence of SDS, electrophoresed, and subjected to silver staining (19).

**In Situ Hybridization.** The DCIS samples were classified according to our previous reports (23, 24). Briefly, the formalin-fixed, paraffin-embedded samples were sectioned on 5-μm thickness and fixed onto slides. The full-length MMP-26 sense cDNA and antisense cDNA were amplified in PCR 3.1 and purified as described in our previous report (9). The sense and antisense plasmids were linearized with XhoI and XbaI, respectively. The sense and antisense digoxigenin-labeled RNA probes were generated by in vitro transcription with T7 polymerase. In situ hybridization was performed as per our previous report (22). Briefly, the paraffin-embedded sections (5 μm) were deparaffinized with xylene and treated with proteinase K solution (50 μg/ml) in 0.2 M Tris-HCl (pH 7.5), 2 mM MgCl$_2$ for 15 min at room temperature. After prehybridization, the sections were hybridized to digoxigenin-labeled MMP-26 antisense cRNA probes for 18 h at 45°C and 100% humidity. The

**Fig. 1. Inhibition kinetics of matrix metalloproteinase 26 by tissue inhibitor of metalloproteinases (TIMP)-2 and TIMP-4.** Zymography and silver staining were performed as reported previously (3, 7, 19, 20). MMP-26, pro-MMP-9, and active MMP-9 were purified in our laboratory (3, 21). The molar concentration ratios of MMP-26:pro-MMP-9:FN:TIMP were 10:1:4:10 and 1:4:1:10. Aliquots were removed and treated with a reducing sample buffer and boiled for 5 min. After electrophoresis on 9% SDS-polyacrylamide gels, the protein bands were visualized by silver staining (19).
MMP-26 sense RNA probe was used under the same hybridization conditions as the control. After hybridization, the slides were washed with saline sodium citrate buffer and blocked [1% blocking reagents in Tris-buffered saline (pH 7.5)] for 30 min. Then the slides were stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science, Mannheim, Germany). The expression signals were photographed under a microscope.

**Immunohistochemistry.** The human breast DCIS, IDC, and hyperplasia tissue samples were classified according to our reports (23–26). Immunohistochemistry was performed on consecutive sections according to our previous report (9). Briefly, the formalin-fixed, paraffin-embedded samples were sectioned to 5-μm thickness and fixed onto slides. After dewaxing and rehydrating, the slides were blocked with 3% BSA/Tris-buffered saline for 1 h at room temperature before incubation with affinity-purified, polyclonal rabbit antihuman MMP-26, MMP-9, TIMP-2, and TIMP-4 antibodies (all 25 μg/ml) at room temperature for 90 min. Sections were then incubated with alkaline phosphatase-conjugated secondary antirabbit antibody (1:500; Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. The signals were detected with Fast-red (Sigma, St. Louis, MO), purified preimmune IgGs from the same animal were used as negative controls.

**Double Immunofluorescence and Confocal Laser Scanning Microscopy.** Double immunofluorescence staining was performed as per our previous description (9). Briefly, the slides were incubated with a rabbit antihuman MMP-26 IgG (25 μg/ml) and a goat antihuman MMP-9 antibody (1:200 dilution; R&D Systems) or a mouse antihuman MMP-26 IgG (25 μg/ml; R&D Systems) and a rabbit antihuman TIMP-4 (25 μg/ml) or a rabbit antihuman TIMP-2 antibody (30 μg/ml) overnight at 4°C. The slides were then incubated with a goat antimouse-IgG IgG for 30 min at room temperature. Secondary Rhodamine Red-X-conjugated donkey antirabbit IgG and FITC-conjugated donkey antigoat IgG (Jackson Immunoresearch) were subsequently applied at a 1:50 dilution for 30 min at room temperature. SlowFade mounting medium was added to the slides, and fluorescence was analyzed using a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss, Heidelberg, Germany) equipped with a multi-photon laser. Images were processed for reproduction using Photoshop software version 6.0 (Adobe Systems, Mountainview, CA). Purified preimmune IgG and normal goat serum were used as negative controls.

**Densitometric and Statistical Analysis.** Four to 16 pictures were taken from the glandular epithelia after immunostaining by each of the four antibodies and the two preimmune IgGs in the DCIS, IDC, AIDH, and normal glands around the DCIS and IDC. Quantification of the immunostaining signals was performed using the Metamorph System (version 4.6r8; Universal Imaging Corp., Inc., West Chester, PA) according to our previous description (9). Briefly, an appropriate color threshold was determined (color model, HSls; hue, 230–255; saturation and intensity, full spectrum). The glandular epithelia...
from each image were isolated into closed regions, and the signal areas and intensities of staining in compliance with the chosen parameters were measured by integrated morphometry analysis. The selected epithelial area was obtained by region measurement. The signal intensities were expressed as integrated absorbance (IOD, the sum of the optical densities of all pixels that make up the object). The ratio of the IOD to the selected epithelial area was determined, and the average ratios from each case were then calculated and used for statistical analysis. Statistical analysis of all samples was performed with the least significant difference correction of ANOVA for multiple comparisons. Data represent the mean ± SE, and $P < 0.05$ was considered significant.

RESULTS

Determination of the Apparent Inhibition Constants of TIMP-2 and TIMP-4 against MMP-26. The apparent $K_i$ values of MMP-26 were measured and calculated to be 1.6 and 0.62 nM for TIMP-2 and TIMP-4, respectively (Fig. 1), using the Morrison equation (18). The apparent $K_i$ values show that TIMP-4 is a slightly more potent inhibitor of MMP-26 than TIMP-2.

Activation of Pro-MMP-9 by MMP-26 and Inhibition of the Activation by TIMP-2 and TIMP-4. To explore the inhibition of MMP-26-mediated MMP-9 activation by TIMP-2 and TIMP-4, purified pro-MMP-9 and MMP-26 were incubated with these TIMPs, and the samples were subsequently analyzed by SDS-PAGE. MMP-26 cleaved pro-MMP-9 (94 kDa) to yield an enhanced active form 86-kDa band on a silver-stained gel under reducing conditions as per our recent report (Ref. 9; Fig. 2A, Lane 4). Zymography revealed that pro-MMP-9 presented as 225-, 125-, and 94-kDa gelatinolytic bands under nonreducing conditions. The 225-kDa band is a homodimer of pro-MMP-9, the 125-kDa band is a heterodimer of pro-MMP-9 and neutrophil gelatinase-associated lipocalin, and the 94-kDa band is a...
monomer of pro-MMP-9 (21, 27, 28). The new active 215-, 172-, 115-, and 86-kDa bands were generated after incubation with MMP-26 (Fig. 2B, Lane 3). The 215-, 115-, and 86-kDa bands are the active forms of the 225-, 125-, and 94-kDa forms, respectively. The 172-kDa band is a dimer of the 86-kDa forms. The activation of pro-MMP-9 by MMP-26 was completely inhibited by recombinant TIMP-2 and TIMP-4 (Fig. 2A, Lanes 5 and 6; Fig. 2B, Lanes 4 and 8). The blocking efficiencies of TIMP-2 and TIMP-4 were comparable with those of two broad-spectrum metal chelators/metalloproteinase inhibitors, 1,10-phenanthroline and EDTA (Fig. 2A, Lanes 7 and 8).

To further confirm the inhibition of MMP-26-mediated pro-MMP-9 activation by TIMP-2 and TIMP-4, in vitro FN cleavage assays were performed. MMP-26 slowly cleaved FN to generate 125- and 58-kDa bands (Fig. 3, Lane 10), whereas pro-MMP-9 did not cleave FN (Fig. 3, Lane 11). However, MMP-26-activated MMP-9 cleaved FN completely, generating at least seven new bands (Fig. 3, Lane 12). Both TIMP-2 and TIMP-4 completely blocked the activation of pro-MMP-9 by MMP-26, which subsequently resulted in inhibition of FN cleavage (Fig. 3, Lanes 13 and 15).

Expression of MMP-26 mRNA in Human Breast Tissues. In situ hybridization showed that MMP-26 mRNA was localized in human breast DCIS (Fig. 4A). On a serial section of the same tissue, MMP-26 protein was also detected in human breast DCIS (Fig. 4C). The MMP-26 sense probe and pre-immune IgG from the same animal as the MMP-26 antibody were used as controls (Fig. 4, B and D).

Expressions of MMP-26, MMP-9, TIMP-2, and TIMP-4 Proteins in Human Breast Tissues. MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins were detected in human breast epithelia (Figs. 5–7). The expressions of the four proteins were extremely high in human breast DCIS (20 cases) cells (Fig. 5E; Fig. 6, E and F; Fig. 7E) but
very low in the normal glandular epithelial cells (25 cases) around DCIS and IDC [Fig. 5A; Fig. 6, A and B; Fig. 7A] and also in AIDH [15 cases (Fig. 5C; Fig. 6, C and D; Fig. 7C)]. Their expressions were substantially decreased in IDC [23 cases (Fig. 5G; Fig. 6, G and H; Fig. 7G)]. Statistical analysis revealed that the signal intensities of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins in DCIS were significantly higher than those in IDC, AIDH, and normal epithelia around the DCIS and IDC (P < 0.05 or P < 0.01; Fig. 8). There was no significant difference for the signals of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins among the normal epithelia around the DCIS and IDC, or in the AIDH and IDC samples (P > 0.05; Fig. 8). Pre-immune IgGs from the same animals as the MMP-26 or TIMP-4 antibodies were used as controls (Fig. 5, B, D, F, and H; Fig. 7, B, D, F, and H). There was no significant difference (P > 0.05) for the pre-immune IgG signals among the normal, AIDH, DCIS, and IDC samples.

Coexpression of MMP-26 and MMP-9 in Human Breast Carcinoma. To confirm the distributions of MMP-26, MMP-9, TIMP-2, and TIMP-4 within carcinoma cells, double immunofluorescence staining assays were performed in human breast DCIS samples. MMP-26 protein was localized mainly in the cytoplasm of the cancerous cells (Fig. 9A, red; Fig. 9B, green), which is consistent with our previous report (9). MMP-9 was localized both in the cytoplasm of the cancerous cells and at the cell surface, mainly on the cell membrane (Fig. 9C, green). The merged picture shows that MMP-26 and MMP-9 were coexpressed in the cytoplasm of the cancerous cells (Fig. 9E, yellow). The high magnification pictures in Fig. 9, A, C, and E, clearly demonstrate MMP-26 and MMP-9 colocalization (indicated by ar-
Matrix Metalloproteinase 26 in Human Breast Cancer

The epithelial component of the normal and noninvasive human breast is physically separated from the stroma by myoepithelial cells and the basement membrane, which is composed of a group of fibrous proteins, including type IV collagen, FN, laminin, and proteoglycans. The disruption of both the myoepithelial cell layer and the basement membrane is an essential prerequisite for the invasion of DCIS. In this present investigation, we found that the levels of MMP-26 and MMP-9 proteins in human breast DCIS were significantly higher than those in human breast IDC, normal mammary glands, and AIDH. MMP-26 and MMP-9 were colocalized in human breast DCIS cells. MMP-9 is a powerful enzyme associated with human breast cancer development and invasion (29, 30). Scorilas et al. (29) and Soini et al. (31) demonstrated that MMP-9 mRNA and protein were highly expressed in human breast carcinoma cells. MMP-9 protein is also expressed in the breast carcinoma cell lines MCF-7 (32, 33), SKBR-3 (34), MDA-MB-231 (35, 36), and MCF10A (36) and in normal breast epithelial cell lines HMT-3522 and T4-2 (32, 37). Our recent study (9) demonstrated that the level of MMP-26 protein in human prostate carcinomas is also significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate tissues. MMP-26 and MMP-9 are not only coexpressed in human prostate carcinomas and in androgen-repressed prostate cancer cells, but they also promoted the invasion of androgen-repressed prostate cancer cells across FN or type IV collagen via MMP-26-mediated pro-MMP-9 activation.

Nguyen et al. (38) showed that active MMP-9 accumulates in the cytosol of human endothelial cells, where it is eventually used by invading pseudopodia, and it is possible that endogenous, self-activated MMP-26 serves as an activator for intracellular pro-MMP-9. The active form of MMP-9 may then be stored inside the cell, ready for rapid release when it is required to initiate invasion of human breast DCIS cells. Although DCIS is not invasive cancer, it may have the potential to develop into IDC, given time. The localization of MMP-26 mRNA and protein in carcinomas was confirmed by in situ hybridization and immunohistochemistry, providing evidence that MMP-26 is an epithelial cell-derived enzyme (3–6).

We demonstrated that the hydrolysis of synthetic peptides by MMP-26 is inhibited by TIMP-2 and TIMP-4, which is consistent with previous reports (5, 6, 8). TIMP-2 and TIMP-4 were also able to completely block MMP-9 activation by MMP-26, as well as the cleavage of FN by MMP-26-activated MMP-9. Therefore, one consequential function of TIMP-2 and TIMP-4 may be their inhibition of MMP-9 activation by MMP-26. TIMP-2 and TIMP-4 possess several distinct cellular functions, but their most widely appreciated biological functions are their roles in the inhibition of cell invasion in vitro (39–42) and their in vivo contributions to tumorigenesis (43, 44) and growth and metastasis (44–47). The underlying molecular mechanism for the tumor-suppressing activities of the TIMPs is thought to be dependent on their anti-MMP activities.

In our experiments, the expressions of TIMP-2 and TIMP-4 were all increased significantly in human breast DCIS but decreased in IDC, mimicking the expression of MMP-26 and MMP-9 in DCIS and IDC, which indicates that these four proteins are highly coordinated during human breast carcinoma development and progression. It may also suggest that remodeling of the extracellular matrix by MMP-26 and MMP-9 stimulates the expression of TIMP-2 and TIMP-4, implying self-regulation through a negative feedback loop. The consistently high expression of TIMP-2 and TIMP-4 proteins in human breast DCIS is in agreement with reports that the expression of TIMP-2 and TIMP-4 in human breast carcinomas is increased (14, 16, 42) and their contributions to tumorigenesis (43, 44).

**DISCUSSION**

Fig. 8. Densitometric quantification analyses of matrix metalloproteinase 26, matrix metalloproteinase 9, tissue inhibitor of metalloproteinases 2, and tissue inhibitor of metalloproteinases 4 protein expression. The quantification analysis was described in “Materials and Methods.” The epithelial regions were isolated, and the staining area and total selected area were obtained by Integrated Morphometry Analysis (IMA) and analyzed by one-way ANOVA with least significant difference (LSD) correction. Each value represents the mean ± SE. *, P < 0.05; **, P < 0.01. Normal, normal breast tissue; AIDH, atypical intraductal hyperplasia; DCIS, ductal carcinoma in situ; IDC, infiltrating ductal carcinoma; n, the number of cases.

Fig. 9. Coexpression of matrix metalloproteinase (MMP)-26 with MMP-9 or tissue inhibitor of metalloproteinases (TIMP)-4 in human breast ductal carcinoma in situ (DCIS). In A, C, and E, the primary antibodies are rabbit antihuman MMP-26 IgG and goat antihuman MMP-9 IgG, the secondary antibodies are Rhodamine Red-X-conjugated donkey antirabbit IgG and FITC-conjugated donkey antigoat IgG. A, red indicates MMP-26 protein staining, which is localized mainly in the cytoplasm of DCIS cells. C, green indicates MMP-9 protein staining, which is localized mainly on the cell surface of the cancerous cells. E, merged images show a color shift to orange-yellow, indicating colocalization between MMP-26 and MMP-9 proteins in DCIS. In B, D, and F, the primary antibodies are mouse antihuman MMP-26 IgG and rabbit antihuman TIMP-4 IgG. After incubation with primary antibodies, the slides were incubated with goat antimouse-IgG1 IgG. The secondary antibodies are the same as those described in A, C, and E. B, red indicates MMP-26 protein staining, which is localized mainly in the cytoplasm of DCIS cells. D, red indicates TIMP-4 protein staining. F, merged images show a color shift to orange-yellow, indicating colocalization between MMP-26 and TIMP-4 proteins in DCIS. Scale bars, 50 μm. ST, stroma; LU, lumen.
The decreased MMP-26 and MMP-9 expression levels in IDC suggest that these two enzymes may play a role in the very early stages of DCIS invasion, but once the basement membrane has been breached, cancer cells become less dependent on the activities of MMP-26 and MMP-9. Nielsen et al. (50) demonstrated that MMP-9 immunostaining or in situ hybridization signals were not detected in human breast ductal carcinoma cells but were seen in tumor-infiltrating stromal cells including macrophages, neutrophils, and vascular pericytes. This indicates that MMP-9 may be transiently expressed in cells during the early stages of human breast carcinoma (DCIS), but not in the later stages of breast carcinoma (IDC). The controversy surrounding the expression of MMP-9 found in the literature (29, 31, 50), inclusive of our current findings, might also suggest that these different expression patterns arise from DCIS and IDC representing genetically distinct disease forms, raising the possibility that DCIS does not simply designate a transitory disease state that invariably leads to IDC.

Down-regulation of TIMPs contributed significantly to the tumorigenic and invasive potentials of cancer cells (43, 44). Decreased production of TIMP-2 resulted in increased MMP activity, leading to increased invasiveness by cancer cells (39). Therefore, maintenance of the balance between MMPs and TIMPs appears critical for the suppression of cancer cell invasion and metastasis. Once the balance between MMP-26/MMP-9 and TIMP-2/TIMP-4 in DCIS is destroyed, the inhibition by TIMP-2 and TIMP-4 may be inadequate to block the degradation of extracellular matrix components by MMP-26 and MMP-9 in DCIS, resulting in degradation of basement membrane components and initiation of the invasive processes of DCIS cells. MMP-26, MMP-9, TIMP-2, and TIMP-4 may be spatially and temporally expressed in the very early stages of DCIS invasion, whereas other enzymes/inhibitors are responsible for the late-stage invasion of IDC cells. Further investigations will provide a more complete understanding of the functions of MMP-26, MMP-9, and TIMPs in human breast cancer progression.

ACKNOWLEDGMENTS

We thank Dr. L. Jack Windsor of Indiana University for kindly providing us with TIMP-2 protein and the polyclonal anti-TIMP-2 antibody, Dr. Weiping Jiang of R&D Systems for the monoclonal anti-MMP-26 antibody, Dr. Jianzhou Wang of University of Oklahoma College of Medicine for critical review of the manuscript, and S. C. Monroe at our laboratory at Florida State University for editorial assistance with manuscript preparation. We are also grateful to Kimberly Riddle and Jon Ekman at the Department of Biological Sciences Imaging Resources at Florida State University for their excellent assistance with confocal microscopy and integrated morphometry analysis.

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_Cancer Res_ 2004;64:590-598.