Matrix Metalloproteinase 26 Proteolysis of the NH$_2$-Terminal Domain of the Estrogen Receptor $\beta$ Correlates with the Survival of Breast Cancer Patients

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Abstract

Estrogens have many cellular functions, including their interactions with estrogen receptors $\alpha$ and $\beta$ (ER$\alpha$ and ER$\beta$). Earlier, we determined that the estrogen-ER complex stimulates the transcriptional activity of the matrix metalloproteinase 26 (MMP-26) gene promoter. We then determined that ER$\beta$ is susceptible to MMP-26 proteolysis whereas ER$\alpha$ is resistant to the protease. MMP-26 targets the NH$_2$-terminal region of ER$\beta$ coding for the divergent NH$_2$-terminal A/B domain that is responsible for the ligand-independent transactivation function. As a result, MMP-26 proteolysis generates the COOH-terminal fragments of ER$\beta$. Immunohistochemical analysis of tissue microarrays derived from 121 cancer patients corroborated these data and revealed an inverse correlation between the ER$\alpha$-dependent expression of MMP-26 and the levels of the intact ER$\beta$ in breast carcinomas. MMP-26 is not expressed in normal mammary epithelium. The levels of MMP-26 are strongly up-regulated in ductal carcinoma in situ (DCIS). In the course of further disease progression through stages I to III, the expression of MMP-26 decreases. In contrast to many tumor-promoting MMPs, the expression of MMP-26 in DCIS correlated with a longer patient survival. Our data suggest the existence of an MMP-26–mediated intracellular pathway that targets ER$\beta$ and that MMP-26, a novel and valuable cancer marker, contributes favorably to the survival of the ER$\alpha$/ER$\beta$–positive cohort of breast cancer patients. (Cancer Res 2006; 66(5): 2716-24)

Introduction

Human matrix metalloproteinases (MMP) are a family of 24 zinc enzymes that degrade the extracellular matrix and cell-surface molecules (1). The promod of all MMPs exhibits the sequence motif PRICG called the “cysteine-switch” (2). An unpaired Cys sulphydryl group of the PRICG cysteine-switch binds the active site zinc. The Cys-Zn interactions are essential for maintaining the latency of MMP zymogens. There is, however, an exception from this general rule. An unconventional PH$_4$CGVPD cysteine-switch distinguishes human MMP-26 from other members of the MMP superfamily (3–5). The presence of the His-81 in the immediate proximity of the Cys-82 residue, in addition to other atypical structural features, leads to the unorthodox, autolytic mechanisms of the MMP-26 zymogen activation and contributes to the unusual physiologic role of MMP-26 in cells and tissues (3, 6–13). In contrast with other MMPs, which are either secretory, soluble, or membrane-anchored enzymes, MMP-26 primarily accumulates in the intracellular milieu (4, 8, 10, 14).

The promoter of the MMP-26 gene includes the estrogen response element that binds estrogen receptors (ER; ref. 6). Signaling of estrogens, primarily 17$\beta$-estradiol (E2), is transmitted by ERs. ERs are members of a nuclear receptor superfamily and are encoded by two distinct genes, ER$\alpha$ and ER$\beta$ (15). Five ER$\beta$ isoforms, which diverge at a common position within the predicted helix 10 of the ligand-binding domain, have been were identified and cloned (16). Our experimental work was done with the ER$\beta$1 isoform, which was termed ER$\beta$$^1$ for the clarity of presentation in the text below.

ERs consist of five individual domains: the NH$_2$-terminal A/B domain with a 16% sequence identity between the two ERs, the highly conserved central DNA-binding domain (96% sequence identity), the flexible hinge D domain (30% sequence identity), the ligand-binding domain (59% sequence identity), and the short COOH-terminal F domain (18% sequence identity). The A/B domain is responsible for the ligand-independent transactivation function (AF-1). The flexible hinge D domain contains a nuclear localization signal. The multifunctional ligand-binding domain, in addition to its role in ligand binding, is involved in dimerization and the ligand-dependent transactivation function (AF-2; ref. 17).

The E2-ER complex stimulates, via the binding of the estrogen response element motif, the transcriptional activity of the MMP-26 gene promoter in hormone-regulated neoplasms, including breast, ovarian, and endometrial carcinomas as well as the normal reproductive processes and menstrual cycle (6, 18–21). Using immunohistochemical analysis, we determined, however, that an inverse correlation, rather than a direct correlation, frequently occurs between the levels of MMP-26 and ER in biopsy samples from breast cancer patients. These findings prompted us to hypothesize that there is a regulatory loop in hormone-regulated malignancies and that this loop links E2-induced MMP-26 to the proteolysis of the ERs.

Our experiments, which are described in the current article, confirmed this hypothesis. We showed that the NH$_2$-terminal portion of the A/B domain of ER$\beta$ was sensitive to MMP-26 proteolysis in vitro and in cell-based assays. In the breast cancer patient cohort, the expression of MMP-26 correlated inversely with the residual levels of the intact ER$\beta$ in the adenocarcinoma...
Elevated MMP-26 expression ductal carcinoma in situ (DCIS) was strongly associated with a longer overall survival in this patient cohort. Our results suggest that high levels of MMP-26 in the mammary epithelium at the early stages of its malignant transformation are a marker of a favorable prognosis. Conversely, the use of broad-range MMP inhibitors such as Marimastat, which is potent against MMP-26, is not favorable for breast cancer patients, a phenomenon observed in clinical trials (22).

Materials and Methods

Chemicals and cells. Reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Human α1-antitrypsin (AAT) was obtained from Calbiochem (San Diego, CA). GM6001, a hydroxamate inhibitor, and rabbit polyclonal antibody AB1410 against the 1-12 amino acid NH2-terminal sequence region of ERβ was obtained from Chemicon (Temecula, CA). The purified ERα and ERβ were obtained from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibody Ab-24 against the COOH-terminal part of ERβ was obtained from LabVision (Fremont, CA). Mouse monoclonal antibody 14C8 directed against the 1-153 NH2-terminal sequence region of ERβ was from GeneTex (San Antonio, TX). The rabbit polyclonal antibody against the COOH-terminal part of ERα and murine monoclonal antibody 1D5 against ERα were purchased from Santa Cruz (Santa Cruz, CA) and DakoCytomation ( Carpinteria, CA), respectively. MMP-26 and the recombinant catalytic domain of membrane type 1 MMP (MT1-MMP) were expressed in E. coli and then purified from the inclusion bodies and refolded to restore their conformation and their catalytic activity (6, 23, 24). Rabbit polyclonal antibody, raised against the catalytic domain of membrane type 1 MMP (MT1-MMP) were refolded to restore their conformation and their catalytic activity (6), the murine monoclonal antibody 1D5 to 1-antitrypsin (AAT) was obtained from LabVision (Fremont, CA). Mouse monoclonal antibody 14C8 directed against the 1-153 NH2-terminal sequence region of ERβ was from GeneTex (San Antonio, TX). The rabbit polyclonal antibody against the COOH-terminal part of ERα and murine monoclonal antibody 1D5 against ERα were purchased from Santa Cruz (Santa Cruz, CA) and DakoCytomation (Carpinteria, CA), respectively. MMP-26 and the recombinant catalytic domain of membrane type 1 MMP (MT1-MMP) were expressed in E. coli and then purified from the inclusion bodies and refolded to restore their conformation and their catalytic activity (6, 23, 24). Rabbit polyclonal antibody, raised against the catalytic domain of MMP-26, was prepared and affinity purified as previously described (6). The total concentrations of MMP-26 and the catalytic domain of MT1-MMP were measured by absorption at 280 nm and calculated using a molar extinction coefficient of 39,000 and 57,000 L mol⁻¹ cm⁻¹, respectively. MT1-MMP and MMP-26 were each titrated with GM6001 to determine the precise concentration of catalytically active enzymes. Breast carcinoma MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA). Endometrial carcinoma Ishikawa cells were a kind gift of Dr. Minoru Fukuda (The Burnham Institute, La Jolla, CA). Cells were routinely maintained in DMEM supplemented with 10% fetal bovine serum (FBS).

Cleavage assays. AAT, ERα, and ERβ (300 ng each) were coincubated for 2 hours at 37°C with the indicated amounts of the proteases in 20 μL of 50 mMol/L HEPES buffer (pH 6.8), containing 200 mMol/L NaCl, 10 mMol/L CaCl₂, 20 μMOL/L ZnCl₂, and 0.01% Brij-35. The reactions were stopped by adding 2% SDS and analyzed by SDS-PAGE. The digests fragments were identified by Coomassie staining or Western blotting.

Lentiviral expression of MMP-26. The full-length MMP-26 cDNA (10) was inserted into the SpeI-XhoI restriction sites of the pLent6/V5-D-TOPO lentiviral vector under the control of the cytomegalovirus promoter. The lentiviral vector was amplified using a complete ViraPower Lentiviral package (26). For double-labeling experiments, tissue microarrays were stained with the polyclonal antibody against the recombinant catalytic domain of MMP-26 (6), the murine monoclonal antibody 1D5 to ERα (DakoCytomation), and the rabbit polyclonal antibodies AB1410 and Ab-24 against the ERβ. Staining with the primary antibody was followed by a diaminobenzidine (DAB)-based detection method employing the Envision Plus HRP system (DakoCytomation) and an automated Dako immunostainer (26). For double-labeling experiments, tissue microarrays were stained first with the Envision Plus HRP system and a DAB substrate (brown color) and then with the second primary antibody followed by either alkaline phosphatase staining with the Vector 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium development or the avidin-biotin–indolyl phosphate/nitroblue tetrazolium development or the avidin-biotin complex method-HPR system and SG chromagen (gray-black color). The slides were counterstained with Nuclear Red, dehydrated, and mounted with permanent mounting media. For all tissues examined, the immunostaining procedure was done in parallel using either premunize serum or antisera depleted by incubation with recombinant protein immunogen to verify specificity of the results. The immunostaining results were scored according to intensity as 0, negative; 1, weak; 2, moderate; and 3, strong. The scoring of immunostaining was calculated by multiplying the percentage of immunopositive cells (0-100) by the staining intensity score (0, 1, 2, and 3), yielding scores ranging from 0 to 300.

Immunocytochemistry. Cells were subcultured in LabTek chamber slides. The attached cells were fixed twice for 3 minutes with 2% glutaraldehyde (10% zinc-buffered formalin, pH 5.5; Anatech, Battle Creek, MI) and then blocked for 30 minutes with 2% bovine serum albumin and 1% normal goat serum. The slides were then incubated overnight at ambient temperature with the primary antibody diluted 1:2,000 to 1:6,000 in the DakoCytomation antibody diluent (DakoCytomation) supplemented with 1% goat normal serum. The colorimetric reaction was developed by incubating the slides with the HRP-conjugated goat anti-rabbit antibody and a 3,3'-diaminobenzidine substrate (0.25 mg/mL in PBS supplemented with 0.05% H₂O₂). Methyl green was used for counterstaining.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated for 4 hours with the primary antibody diluted with PBS supplemented with 1% FBS and 0.1% sodium azide. The slides were then incubated for 2 hours with the secondary species–specific IgG conjugated with phycoerythrin. 4',6-Diamidino-2-phenylindole was used for nuclear staining. The slides were mounted in VectorShield anti-fading embedding medium (Vector, Burlingame, CA) and fluorescence-labeled cells were examined under a fluorescence microscope.

Patient specimens. Archival paraffin-embedded tissue specimens containing normal mammary epithelium (n = 16), in situ breast carcinomas (n = 23), and invasive breast tumors, represented by the ductal (n = 103), lobular (n = 15), and mucinous (n = 3) histologic subtypes, were obtained from St. Vincent's Hospital (Dublin, Ireland). These specimens represented the residual pathologic materials remaining after the diagnostic and hormone receptor determinations and were derived from women who presented in 2001 with the symptomatic stage I to III breast cancers. These samples were used for the preparation of tissue microarrays. Human breast surgical specimens were obtained under the Institutional Review Board approval of the Department of Surgery and Pathology, University College (Dublin, Ireland). In addition, 16 normal mammary epithelium specimens, excised from surgical margins, and 4 independent normal mammary gland tissue samples were included in the tissue microarrays. The breast cancer specimens have been fixed in 8% formalin and paraffin embedded according to routine procedures.

Tissue microarrays. To construct high-density breast cancer tissue microarrays, each containing 140 to 190 specimens, two to five 1-mm (diameter) cylindrical cores were taken from the representative areas of normal tissue (one core per patient) and of malignant tissues (two to three cores per patient) from archival paraffin blocks and arrayed into a new recipient paraffin block using a custom-built precision microarrayer (Beecher Instruments, Silver Spring, MD). Serial sections (4 μm) of the recipient block were applied to the Superfrost-Plus glass slides (Fisher, Pittsburgh, PA) coated with 3-aminopropyltriethoxysilanes (25).

Immunohistochemistry. Following routine dewaxing, the tissue microarrays were stained with the polyclonal antibody against the recombinant catalytic domain of MMP-26 (6), the murine monoclonal antibody 1D5 to ERα (DakoCytomation), and the rabbit polyclonal antibodies AB1410 and Ab-24 against the ERβ. Staining with the primary antibody was followed by a diaminobenzidine (DAB)-based detection method employing the Envision Plus HRP system (DakoCytomation) and an automated Dako immunostainer (26). For double-labeling experiments, tissue microarrays were stained first with the Envision Plus HRP system and a DAB substrate (brown color) and then with the second primary antibody followed by either alkaline phosphatase staining with the Vector 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium development or the avidin-biotin complex method-HPR system and SG chromagen (gray-black color). The slides were counterstained with Nuclear Red, dehydrated, and mounted with permanent mounting media. For all tissues examined, the immunostaining procedure was done in parallel using either premunize serum or antisera depleted by incubation with recombinant protein immunogen to verify specificity of the results. The immunostaining results were scored according to intensity as 0, negative; 1, weak; 2, moderate; and 3, strong. The scoring of immunostaining was calculated by multiplying the percentage of immunopositive cells (0-100) by the staining intensity score (0, 1, 2, and 3), yielding scores ranging from 0 to 300.
of matrix metalloproteinase 1 (TIMP1), and TIMP2 fully inhibited the proteolysis of ERβ by MMP-26 (not shown).

The cleavage by MMP-26 transformed the 59-kDa ERβ into several digest fragments. The apparent molecular mass of the main digest fragments was in the range of 51 to 54 kDa but the shorter fragments were also observed in the digest samples. To identify the relative position of the cleavage fragments within the ERβ polypeptide chain, we used the antibodies AB1410 and 14C8, and Ab-24, which recognized the NH2-terminal and COOH-terminal epitopes of ERβ, respectively. The ERβ samples were cleaved by increased amounts of MMP-26 and the digest samples were analyzed by Western blotting employing the AB1410, 14C8, and Ab-24 antibodies. As shown in Fig. 1B, the Ab-24 antibody against the COOH-terminal epitope recognized the intact ERβ and the digest fragments whereas the Ab1410 and 14C8 antibodies against the NH2-terminal epitope reacted only with the intact ERβ. These results indicate that MMP-26 proteolysis generated the stable cleavage fragments that represented the COOH-terminal portion of the ERβ molecule. Because there were several closely overlapping digest fragments of ERβ, we were unable to identify their NH2-terminal sequence by NH2-terminal microsequencing (not shown). The size difference in the apparent molecular weight between the intact ERβ and the major ERβ fragments suggested, however, that these stable, NH2-terminally truncated species are missing the first 40 to 60 NH2-terminal residues of the ERβ A/B domain and, accordingly, we believe they are missing the functionality of the A/B domain which normally exhibits

Results

MMP-26 cleaves ERβ in vitro. According to our earlier observations, the AAT serpin is a clinically relevant protein target of proteolysis by MMP-26 (6). Consistent with these data, the catalytic amounts of MMP-26 fully proteolyzed 61-kDa AAT (the enzyme/substrate molar ratio at a range of 1:15-1:150) in 2 hours in our studies and generated a 55-kDa NH2-terminal fragment and an ∼6 kDa COOH-terminal fragment of AAT (Fig. 1A, top right; the 6-kDa fragment, which migrated to the bottom of the gels, was not shown). The potency of MT1-MMP in cleaving AAT was lower, albeit comparable, with that of MMP-26 (Fig. 1, top left). In turn, ERβ was resistant to MT1-MMP but it was sensitive to proteolysis by the catalytic amounts of MMP-26 (the enzyme/substrate molar ratio at a range of 1:30-1:60). In contrast to ERβ, ERα was not susceptible to MMP-26 (Fig. 1A, bottom). GM6001, tissue inhibitor of matrix metalloproteinase 1 (TIMP1), and TIMP2 fully inhibited the proteolysis of ERβ by MMP-26 (not shown).

The cleavage by MMP-26 transformed the 59-kDa ERβ into several digest fragments. The apparent molecular mass of the main digest fragments was in the range of 51 to 54 kDa but the shorter fragments were also observed in the digest samples. To identify the relative position of the cleavage fragments within the ERβ polypeptide chain, we used the antibodies AB1410 and 14C8, and Ab-24, which recognized the NH2-terminal and COOH-terminal epitopes of ERβ, respectively. The ERβ samples were cleaved by increased amounts of MMP-26 and the digest samples were analyzed by Western blotting employing the AB1410, 14C8, and Ab-24 antibodies. As shown in Fig. 1B, the Ab-24 antibody against the COOH-terminal epitope recognized the intact ERβ and the digest fragments whereas the Ab1410 and 14C8 antibodies against the NH2-terminal epitope reacted only with the intact ERβ. These results indicate that MMP-26 proteolysis generated the stable cleavage fragments that represented the COOH-terminal portion of the ERβ molecule. Because there were several closely overlapping digest fragments of ERβ, we were unable to identify their NH2-terminal sequence by NH2-terminal microsequencing (not shown). The size difference in the apparent molecular weight between the intact ERβ and the major ERβ fragments suggested, however, that these stable, NH2-terminally truncated species are missing the first 40 to 60 NH2-terminal residues of the ERβ A/B domain and, accordingly, we believe they are missing the functionality of the A/B domain which normally exhibits

Figure 1. MMP-26 cleaves the NH2-terminal A/B domain of ERβ. A, top. AAT is comparably sensitive to proteolysis by MT1-MMP and MMP-26. AAT was incubated with increasing amounts of MMP-26 and MT1-MMP to generate the 55-kDa cleavage fragment. Bottom. MMP-26 cleaves ERβ (59 kDa), but not ERα (64 kDa), whereas ERα is resistant to the in vitro cleavage by MT1-MMP. The digest samples were separated by SDS-PAGE. The gels were stained with Coomassie to visualize the cleavage fragments. B, the MMP-26 cleavage fragment represents the COOH-terminal part of ERβ. Following coinubcation with MMP-26, the digest samples were analyzed by Western blotting with the antibodies 14C8 and AB1410 to the NH2-terminal part of ERβ (right and middle, respectively) and the antibody Ab-24 to the COOH-terminal part of ERβ(left). C, MMP-26 and the cleavage of ERβ. MMP-26 is an estrogen-inducible gene (6) and the expression of cellular MMP-26 requires the presence of ERα/ERβ in the cells. Estrogen, through either ERα or ERβ or both, induces the expression of cellular MMP-26. MMP-26 cleaves the NH2-terminal sequence of ERβ (59 kDa) and this cleavage generates the COOH-terminal fragments (51-54 kDa) of the receptor. The 1-148 NH2-terminal sequence represents the A/B transactivation domain of ERβ. Shaded boxes, relative positions of the epitopes of the AB1410 (raised against the 1-12 NH2-terminal sequence of ERβ), 14C8 (raised against the 1-150 NH2-terminal sequence of ERβ), and Ab-24 (raised against the COOH-terminal part ERβ) in the ERβ sequence.
the ligand-independent AF-1 transactivation function of ERβ.

Figure 1C shows in a schematic manner the relative positions of the antibody epitopes, the A/B domain, and the MMP-26 cleavage site in the ERβ polypeptide sequence.

**MMP-26 cleaves cellular ERβ.** Although the available antibodies were generated to the specific sequence regions of the ERs or to the recombinant purified receptor proteins, because of the high degree of sequence homology between the ERα and ERβ, we first showed the antibody specificity to the receptor subtypes. Using Western blotting of the purified ERα and ERβ, we confirmed that the antibodies Ab-24, AB1410, and 14C8 to ERβ did not cross-react with ERα. We also showed that the 1D5 antibody to ERα did not recognize ERβ (not shown).

To confirm our *in vitro* cleavage data, we evaluated MMP-26 and ERβ by immunoblotting in endometrial carcinoma Ishikawa cells and breast carcinoma MCF-7 cells. We specifically selected Ishikawa and MCF-7 cells for our studies because, according to our earlier reverse transcription-PCR (RT-PCR) results, these cells express substantial levels of the mRNA of MMP-26 (6, 10, 21). A purified MMP-26 control was included along with the Ishikawa extract in Western blot analysis. In agreement with the results of RT-PCR, total cellular extracts of Ishikawa cells (Fig. 2A, left) and MCF-7 cells (Fig. 2B, left) showed the presence of MMP-26. Consistent with the presence of MMP-26, the degradation products of ERβ (51-54 kDa) along with the intact 59-kDa receptor were detected by immunoblotting with the ERβ antibody Ab-24 in these cells. The molecular weight of the ERβ degradation products observed in Ishikawa and MCF-7 cells was similar to that in the control MMP-26-cleaved samples of the purified recombinant ERβ [Fig. 2A (middle) and B (right)]. The relative quantities of the ERβ degradation products were significantly higher in Ishikawa cells when compared with MCF-7 cells.

We next wanted to confirm the cleavage of the cellular ERβ by MMP-26. For this purpose, we increased, by using cell transfection, the expression of MMP-26 in MCF-7 cells. Transfection of MCF-7 cells with a recombinant lentivirus bearing the full-length MMP-26 cDNA gene caused a noticeable increase in the MMP-26 levels (Fig. 2B, left). This increase correlated with an enhanced degradation of ERβ in the transfected cells when compared with mock-transfected control (Fig. 2B, right). The NH2-terminal 6-kDa cleavage fragment of ERβ was never detected in cell extracts, thus suggesting that this low molecular fragment was sensitive to proteolysis and that it was rapidly degraded by cellular proteinases.

In agreement with our earlier results as well as with the results of others (27–29), immunostaining confirmed the presence of both

**Figure 2.** The presence of MMP-26 correlates with the proteolysis of ERβ in endometrial carcinoma Ishikawa cells and breast carcinoma MCF-7 cells. A, left, Western blotting of MMP-26 naturally expressed by Ishikawa cells and the purified MMP-26 control. Right, Western blotting of ERβ expressed by Ishikawa and MCF-7 cells, intact ERβ and ERβ coincubated with MMP-26. B, left, Western blotting of MMP-26 from MCF-7 cells transfected with the control lentiviral vector (mock), the lentivirus bearing MMP-26 (MMP-26), and the purified MMP-26 control. Right, Western blotting of ERβ from mock- and MMP-26-transfected MCF-7 cells, intact ERβ, and ERβ coincubated with MMP-26.

**Figure 3.** Immunostaining of Ishikawa and MCF-7 cells. Immunoreactivity was evident in Ishikawa cells stained with the MMP-26 antibody and the ERβ antibody AB1410. There was no ERα immunoreactivity in Ishikawa cells. Immunofluorescence staining confirms the expression of MMP-26 and ERβ (AB1410 antibody) in MCF-7 cells. The staining with the antibody control was negative.
MMP-26 and ERβ in Ishikawa cells (Fig. 3). ERα was not detected in Ishikawa cells. Consistent with the presence of the mRNA, as detected by RT-PCR, and the protein, as determined by Western blotting, immunofluorescence staining confirmed the expression of MMP-26 and ERβ in MCF-7 cells (Fig. 3).

Inverse correlations of MMP-26 with ERβ in breast cancer cells. The redundancy of the MMPs as well as the low levels of MMP-26 expression in cells complicates the identification of ERβ cleavage by MMP-26 in vivo. The available broad-spectrum hydroxamate inhibitors cannot specifically target and discriminate MMP-26 from the other structurally similar redundant MMPs. To overcome these experimental difficulties and to support our hypothesis that MMP-26 cleaves ERβ in vivo, we employed an immunohistochemical approach to analyze the expression of MMP-26, ERβ, and ERα in breast tissue specimens derived from stage I-III breast cancer patients and arranged in the tissue microarrays. Our manual immunoscopy method provided highly reliable data when compared with the digital scoring systems (30, 31).

Immunostaining determined that MMP-26 immunoreactivity was high both in in situ and invasive carcinomas when compared with the normal mammary epithelium (mean immunoscores of 71 ± 11.6, 43 ± 11.6, and 5 ± 2.8, respectively; P = 0.000003, ANOVA), with MMP-26 levels in in situ tumors considerably exceeding those in the other histologic categories (Fig. 4A). In invasive carcinomas, high MMP-26 immunoreactivity was associated with early clinical I-II stages compared with the late stage III (P = 0.01; Fig. 4B). These unbiased observations indicated that the up-regulation of MMP-26 is an early event in the pathogenesis of breast cancer.

To correlate MMP-26 expression with the clinical outcome, the immunostaining data were dichotomized into the high versus the low protein levels using the median immunoscore as a cutoff. In the investigated cohort, patients with the enhanced expression of MMP-26 in in situ tumors enjoyed significantly longer disease-free and overall survival when compared with patients with the low levels of MMP-26 in in situ lesions (P = 0.03; Fig. 4C).

To determine possible associations between the expression of MMP-26 and the estrogen status of the tumors, the tissue...
microarrays also were stained for ERα (the antibody 1D5) and ERβ (the antibody AB1410 to the NH2-terminal portion of the receptor). In agreement with our biochemical data which suggested that ERβ is a cleavage target of MMP-26, the immunohistochemical analysis of the breast cancer tissue microarrays revealed an inverse correlation between the MMP-26 expression and the levels of immunoreactivity of the residual intact receptor: the high immunoreactivity of MMP-26 was accompanied by a concomitant loss of ERβ in invasive adenocarcinomas ($r = -0.22, P = 0.01$; Fig. 4D). In agreement with other reports (32–34), our results showed that the presence of high levels of the intact ERβ in the ERα-positive tumors favorably correlated with a patient’s survival (Kaplan-Meier analyses; Fig. 4E). Consistent with volumes of other works, high levels of the ERα immunoreactivity correlated with a longer survival of the patients in the patient cohort available to our study ($P = 0.01$ for the overall survival and $P = 0.04$ for the disease-free survival; data not shown).

Figures 5 and 6 show the representative tissue microarrays immunostained for MMP-26, ERα, and ERβ in invasive ductal carcinomas and DCIS, respectively. In agreement with the regulation of MMP-26 by E2, the presence of ERα is required for the induction of the MMP-26 expression in breast carcinoma cells. In the ERα/MMP-26–positive samples, the AB1410 immunoreactivity of ERβ was low whereas the Ab-24 immunoreactivity of ERβ was high, thus suggesting the predominant presence of the proteolyzed ERβ (Figs. 5A–C and 6A–C). In turn, in the ERα-negative tumor specimens, the immunoreactivity of MMP-26 was minor and the Ab-24 immunoreactivity of ERβ was similar to that of the AB1410 antibody, thus suggesting the predominant presence of the intact ERβ (Figs. 5D–F and 6D–F).

Discussion

E2 and its α and β receptors play a crucial role in the progression of hormone-dependent neoplasms, including breast

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**Figure 5.** Representative immunostaining of ERα, ERβ, and MMP-26 in invasive ductal carcinomas. Tissue microarrays were stained (DAB, brown) with the antibody 1D5 to ERα (B and E) and with the antibody Ab-24 to the COOH-terminal portion of ERβ (C and F). In double-labeling staining, tissue microarrays were stained with the antibody AB1410 to the NH2-terminal portions of ERβ (DAB, brown; A and D) and with the antibody to MMP-26 (SR chromagen, gray-black). Tissue microarrays were counterstained with Nuclear Red (pink). A to F, the bottom portions ($\times 60$ magnification) show the respective enlarged images ($\times 250$ magnification).
cancer (15, 33, 34). The ERs have been targets for breast cancer treatment for years. ERα and ERβ each play complex and distinct roles, which are not understood in detail, in regulating the cell response to E2. A recent comprehensive study of 305 breast cancer patients suggests that low levels of ERβ predict resistance to Tamoxifen therapy in breast cancer (35). These data stimulated our interest in the intracellular proteolytic processes, which might regulate the concentrations and the functionality of ERβ in breast carcinomas, and we focused our attention on MMP-26, a unique MMP, the expression of which is associated with carcinomas and is regulated by E2 (5, 8, 10, 21).

Consistent with the earlier structure-functional features and cellular localization of MMP-26, our current results suggest that MMP-26, naturally expressed by the cells, was predominantly associated with the intracellular milieu (6, 8, 10). According to our additional results as well the observations of other authors (3, 5, 36), the presence of the unorthodox PH₆₋CGVPD cysteine-switch motif in the sequence of MMP-26 stimulates the autolytic mechanism of the protease activation. The promoter of the MMP-26 gene represents the 5'-GGTCACCTCTGCCCC-3' estrogen response element motif (nucleotides 129–117), having a characteristic 13-bp palindromic element consisting of two 5-bp arms separated by a 3-bp spacer (6). In agreement with the presence of the estrogen response element in the MMP-26 gene promoter, E2, via its interactions with the ERs, regulated the MMP-26 gene expression in Ishikawa cells. These results explain the association of the MMP-26 expression with hormone-regulated malignancies and MMP-26 cycling in the course of a menstrual period (6, 9, 19, 20, 37, 38).

Based on these observations, we asked ourselves if MMP-26 proteolysis targets the cellular ERs. In the current study, we determined that MMP-26 proteolysis generates the NH₂-terminal truncated receptor species of ERβ which lack the 40- to 60-amino-acid-long NH₂-terminal fragment. In turn, ERα is resistant to MMP-26. Our data indicate that MMP-26 attacks the NH₂-terminal region of ERβ. This sequence region of ERβ represents a divergent

![Figure 6. Representative immunostaining of ERα, ERβ, and MMP-26 in DCIS. Tissue microarrays were stained (DAB, brown) with the antibody 1D5 to ERα (B and E) and with the antibody Ab-24 to the COOH-terminal portion of ERβ (C and F). In double-labeling staining, tissue microarrays were stained with the antibody AB1410 to the NH₂-terminal portions of ERβ (DAB, brown; A and D) and with the antibody to MMP-26 (SR chromagen, gray-black). Tissue microarrays were counterstained with Nuclear Red (pink). A to F, the bottom portions (×60 magnification) show the respective enlarged images (×250 magnification).](cancerres.aacrjournals.org)
NH2-terminal A/B domain that is responsible for the ligand-independent transactivation AF-1 function of the receptor (39). Consistent with our biochemical in vitro data, endometrial carcinoma Ishikawa cells, which coexpress MMP-26 with ERβ, naturally exhibit the proteolyzed form of ERβ. Following the transfection with the MMP-26 construct, the proteolyzed ERβ species was generated in breast carcinoma MCF-7 cells, which naturally express ERβ.

Having shown the proteolysis of ERβ by MMP-26 in a cellular setting, we did an unbiased immunohistochemical analysis of the tissue microarrays derived from 121 breast cancer patients. Consistent with the estrogen-dependent induction of the MMP-26 expression, we detected the presence of the protease only in the ERα-positive specimens. The proteolytic mechanism of the ERβ regulation by MMP-26 is consistent with our immunohistochemical data. These data indicated that the high levels of MMP-26 expression correlated with the presence of the NH2-terminally truncated species of ERβ, which was undetectable with the antibody to the NH2 end of the receptor but which were readily detectable with the antibody to intact COOH-end portion of the receptor. In contrast, ERα-negative and, consequently, MMP-26-negative biopsy samples exhibited the intact ERβ forms, which were identified with equal efficiency by the NH2-end and the COOH-end targeting antibodies.

Overall, our analyses confirmed that there was an inverse correlation between the levels of MMP-26 and the levels of the intact ERβ in breast cancer biopsies. According to our observations, the expression of MMP-26 was insignificant in normal mammary epithelium. The expression of the protease was high in stage I to II invasive carcinomas, especially in DCIS, whereas in stage III carcinomas the MMP-26 levels decreased. Our data were consistent with the earlier results by Zhao et al. (40) who showed that the expression levels of both MMP-26 mRNA and protein were highest in human breast DCIS compared with other breast tissue samples. Our data are also consistent with the recent report (41) that stated that MMP-26 is expressed by laminin-5-positive keratinocytes in the migrating area during wound repair, in benign skin disorders characterized by inflammation and microdisruptions of basement membrane, and also in grade 1 and 2 squamous cell cancers. MMP-26, however, was not present in dedifferentiated grade 3 tumors. Based on these independent observations, we suspect that MMP-26 is up-regulated during the early stages of cancer and then, as the cancer progresses, the levels of the enzyme decrease. It seems that MMP-26 is a part of an inflammatory response and that its presence contributes to a favorable prognosis of the disease progression. In agreement with this suggestion, we identified an unexpected, but significant, direct correlation between the expression of MMP-26 in ductal carcinomas in situ and patient survival. To the best of our knowledge, MMP-26, in addition to MMP-8 (42), is the only species of MMP that showed antitumor properties. From these perspectives, MMP-26 (matrilysin-2) is very different from MMP-7 (matrilysin-1), a structurally similar enzyme that is directly involved in tumor progression (43, 44). We believe the lack of MMP-26 in DCIS could be a novel and independent marker of aggressive growth of ERα/β-positive breast carcinomas.

Taken together, our data suggest the presence of an MMP-26-mediated intracellular regulatory pathway that targets ERβ in hormone-regulated malignancies. We suggest that this pathway plays an important role in E2 signaling by regulating the levels and the functionality of cellular ERβ. Our results warrant further studies because they add another level of complexity to understanding and precisely deciphering the potential effects and clinical outcomes of using MMP inhibitors and ER antagonists in breast cancer therapy.

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