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Matrix-metalloproteinases 1, 2 and 3 and their tissue inhibitors 1 and 2 in benign and malignant breast lesions: an in situ hybridization study

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Abstract Invasive growth requires degradation of extracellular matrix. Altered expression of matrix degrading enzymes may indicate an increased potential for invasive growth. We determined the expression patterns of matrix-metalloproteinases (MMP)-1, -2, and -3 and of the tissue inhibitors of metalloproteinases (TIMP)-1 and -2 by in situ hybridization with isotopically labeled RNA probes in normal breast tissue (*n*=6), fibrocystic disease (*n*=20), five cases of which contained radial scars, lobular carcinoma in situ (CLIS; *n*=5), ductal carcinoma in situ (DCIS; *n*=9) and invasive carcinomas (*n*=24). Only a few cells displayed MMP-1- and MMP-2-specific labeling in normal breast tissue and fibrocystic disease. Noninvasive ductal carcinomas showed elevated MMP-2 transcript levels in peritumor stromal cells in the absence of significant MMP-1 specific signals. In general, compared with adjacent normal breast tissue, a gradual increase of MMP-2 was found in noninvasive to invasive cancers. Invasive ductal and lobular carcinomas displayed co-expression of MMP-1 and MMP-2 by stromal cells, mainly of the invasion front, with high signal intensity particularly in high-grade invasive carcinomas. Tumor cells and peritumor stroma showed low MMP-3 transcript levels, especially in medullary carcinomas. TIMP-1 and -2 transcript levels were increased in invasive carcinomas correlating with the histological grade. These RNA expression patterns suggest an increased invasive potential in breast carcinomas even prior to histologically overt invasive growth.

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Introduction

Degradation of interstitial and basement membrane extracellular matrix (ECM) represents a key element in the multistage process of tumor invasion and metastasis [23]. To metastasize, a cancer cell must separate from its neighbors, penetrate the basement membrane (BM), force its way through the surrounding stroma and, subsequently, enter into the circulation [39]. During formation of any metastasis these steps must be repeated in reverse order. This process requires extensive degradation and modification of BM-ECM components, such as collagen type IV and laminin, and also of interstitial ECM, such as fibrillar collagen types I and III, fibronectin, tenascin, and proteoglycans. In early stages of neoplastic epithelial breast lesions an intact basement membrane is still present separating proliferating epithelial cells from the surrounding mesenchymal stroma. The transformation from noninvasive to invasive carcinomas is accompanied by focal disruption and discontinuity of the BM. Overt tissue invasion then requires the expression of proteinases specific for interstitial ECM. Altered levels and deranged expression patterns of such proteinases and their specific inhibitors may thus represent morphologic correlates for an increased invasive potential.

Among ECM-degrading enzymes, matrix-metalloproteinases (MMPs) have a central role. MMPs are a family of zinc-dependent neutral proteinases with overlapping, but distinct substrate spectra [24]. In addition to their role in tumor invasion and metastasis, they are involved in various physiological connective tissue remodeling processes, such as embryonic development, postpartum involution of the uterus, ovulation, wound healing and joint destruction in rheumatoid diseases. Most MMPs are secreted as inactive zymogens and require proteolytic activation. Four subfamilies are known, collagenases, gelatinases, stromelysins and membrane MMPs. Except for

bone, cartilage tissues and granulocytes, MMP-1 (synonyms: interstitial or fibroblast collagenase) is the major collagenase able to degrade native fibrillar collagens [5]. The gelatinase MMP-2 cleaves collagen type IV, the major BM constituent, as well as degraded collagen, i.e. gelatin, and some native noncollagenous ECM glycoproteins. MMP expression is controlled at the levels of transcription, secretion, activation, and inhibition of activated enzyme. Proteolytic activation of MMP-1 and -3 is primarily mediated by plasmin, the conversion of which from plasminogen is, in turn, stringently regulated by locally produced activators and inhibitors. MMP-2 is activated by MMP-14, a membrane MMP. MMP-3 has a similar spectrum of ECM substrates to MMP-2 and does not degrade native collagen type I. As a co-activator of MMP-1, however, MMP-3 is a pivotal element in the degradation of native fibrillar collagen. Proenzymes and active forms of MMP are controlled by stoichiometric binding of specific, locally produced tissue inhibitors of metalloproteinases, TIMPs. In addition to some other MMP species, TIMP-1 inactivates MMP-1 and MMP-3. TIMP-2 forms a complex with MMP-2, designated large inhibitor of metalloproteinases (LIMP), which serves as an additional inhibitor for MMP-1 [20, 35, 39]. Evaluation of the invasive potential of individuals or groups of cancer cells in situ thus requires the assessment of the expression patterns of relevant MMPs, their activators, and their inhibitors. The balance between these molecules ultimately determines the extent of ECM degradation and tissue remodeling in the course of invasive growth [12].

Previous immunohistochemical studies have led to reports of elevated levels of MMP-2 expression in breast carcinoma cells [9, 26]. However, MMP-2-specific RNA was localized in the peritumor fibroblasts of breast carcinomas rather than in tumor cells [30]. MMP-1 and TIMP-1 RNA has been detected in a small number of breast carcinoma cases. It was the objective of this study to establish the expression patterns of a series of functionally related MMP and TIMP genes by semi-quantitative in situ hybridization (ISH) both in fibrotic nonmalignant breast lesions and in pre-invasive and invasive stages of breast cancer.

Materials and methods

Tissues

Formalin-fixed breast tissue was embedded in paraffin wax by routine procedures. Paraffin blocks from normal breast tissues (n=6), fibrocystic disease with ductal hyperplasia but without epithelial atypia (n=20), 5 also containing radial scars, CLIS (n=5), DCIS (n=9), invasive ductal carcinomas (n=19), and invasive lobular carcinomas (n=5) were drawn from the archives of the Division of Gynecopathology of the University Hospital Eppendorf. Sections 6 µm thick were cut and transferred onto baked slides previously coated with aminopropyltriethoxysilane (APES; Sigma, Gillingham UK), air-dried, fixed in freshly prepared 4% paraformaldehyde for 20 min, and stored at –70°C until required. Clinical data were not collected.

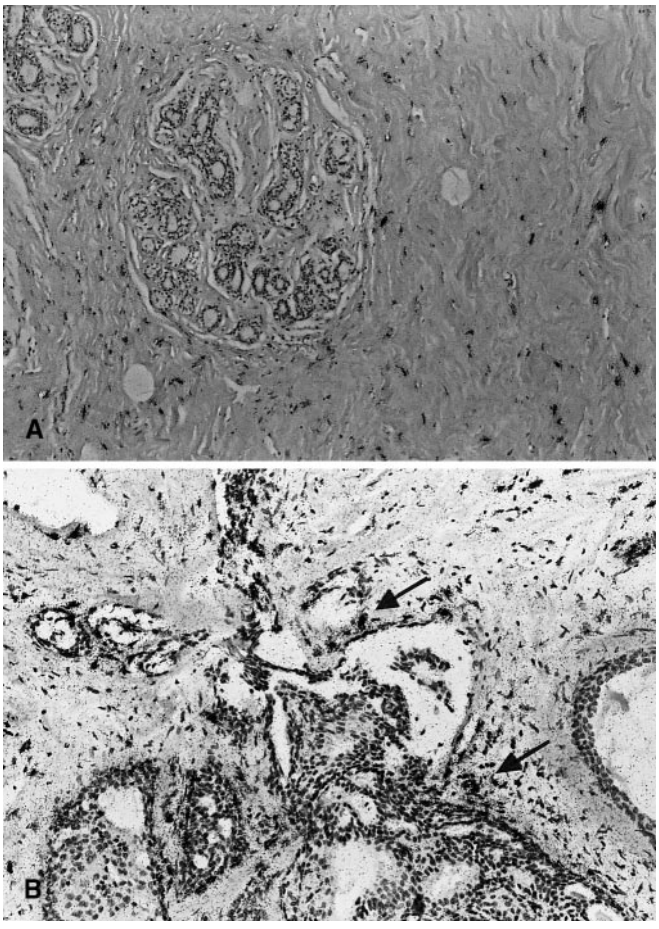

Fig. 1A, B MMP expression in non-neoplastic breast lesions. **A** MMP-2 expression in stromal fibroblasts of slightly atrophic, but otherwise normal breast tissue. **B** MMP-1 expression in fibrocystic disease with ductal epithelial hyperplasia with accentuation in fibroblasts bordering on a radial scar (*arrows*). Paraffin sections, **A** autoradiographic exposure 4, **B** 5 weeks, original magnification ×105

Ribonucleotide probes

pGEM1 (Promega Biotech, Madison, Wis.)-based run-off transcription expression vectors were constructed by subcloning of the 735-bp *Sst*I-*Eco*RI fragment of pCllaseI [37], obtained from the American Type Culture Collection, Rockville, Md (#57685), to generate a probe for human MMP-1. Similarly, the 1300 bp *Eco*RI-*Bgl*II fragment of pK121, harboring a human MMP-2 cDNA [16] and kindly provided by Dr. K. Tryggvason, and the 600 bp *Eco*RI-*Hin*dIII fragment of pTR1 [25], a generous gift from Dr. L.M. Matrisian, were subcloned into pGEM1 to generate probes for MMP-2 and MMP-3, respectively. This rat MMP-3 probe displays identity with the rat MMP-10 (stromelysin-2) gene over 310 bp of its sequence and is highly homologous to human MMP-3 and -10 [7]. The generation of the human TIMP-1 and TIMP-2 gene probes has been described previously [14]. All constructs were subjected to nucleic acid sequence analysis and conformed to the published sequences. After linearization of the plasmids with either *Hin*dIII or *Eco*RI restriction endonuclease, T7 or SP6 RNA-polymerase (Gibco-BRL, Eggenstein, Germany), respectively, was employed to obtain run-off transcripts of either the anti-sense (complementary to mRNA), or the sense (anti-complementary, negative control) strands. Transcription and labeling of RNA probes were performed as described previously [14] using

Table 1 Distribution of MMP and TIMP RNA transcripts in normal, benign and malignant breast lesions [*CLIS* lobular carcinoma in situ, *DCIS* ductal carcinoma in situ, – no labeling above background as defined by sense hybridizations, (+) faint labeling, up to 10 grains per cell, + weak labeling, up to 25 grains per cell, ++ moderate labeling, up to 60 grains per cell, +++ strong labeling, more than 60 grains per cell]

	n=	MMP-1	MMP-2	MMP-3	TIMP-1	TIMP-2
Normal breast tissue	6	–	+	–	+	+
Fibrocystic disease	15	–	+	–	+	+
Scar areas including radial scars	5	+	(+)	–	+	+
CLIS	5	–	+	–	+	+
DCIS	9	–	++	–	+	+
Invasive carcinoma	24	+++	+++	+	++	+++

[^{35}S]uridine-5'-(α-thio)-triphosphate (1250 Ci/mmol, New England Nuclear, Dreieich, Germany). The specific activity routinely obtained was 1.2–1.4×10^9 cpm/µg.

In situ hybridization

Deparaffinized sections were pretreated with 0.2 M HCl for 20 min, digested with pronase (Boehringer, Mannheim, Germany) at 600 µg/ml for 10 min at room temperature, postfixed with freshly prepared paraformaldehyde (4%) in PBS for 20 min on ice and acetylated. After dehydration in graded ethanols and drying, hybridizations were performed at 50°C for 16–18 h with 4×10^5 cpm antisense or sense probe in 25 µl of hybridization solution containing 50% formamide, 10% dextrane, 10 mM Tris/HCl (pH 7.5), 10 mM sodium phosphate (pH 6.8), 300 mM NaCl, 5 mM EDTA, 10 mM DTT, 1 mg/ml yeast tRNA (Boehringer) and 1× Denhardt's solution (Sigma, Deisenhofen, Germany). Slides were subsequently washed in modified hybridization buffer for 4 h at 50°C, subjected for 20 min to RNase (20 µg/ml) at 37°C followed by further washing for 30 min at 37°C in 100 mM Tris/HCl (pH 7.4), 500 mM NaCl, 1 mM EDTA and further washes in 2×SSC and 0.2×SSC. After dehydration in graded ethanols/600 mM ammonium acetate and drying slides were coated with Kodak NTB 2 emulsion diluted at equal volumes with 600 mM ammonium acetate. Autoradiography was for 14–42 days at 4°C. Slides were counterstained with H&E.

Pre-hybridization and hybridization washing procedures including removal of nonspecifically bound probe by RNase A digestion were performed for both anti-sense and sense strand [^{35}S]-labeled RNA probes as described in detail. Sections were always processed simultaneously using the same batches of probes and reagents. Hybridization of tissues pretreated with *Micrococcus* nuclease verified that cellular RNA was the target of hybridization [38].

Microscopic semi-quantitative evaluation

Evaluation of conventional histology and autoradiographic signals was performed on serial sections by counting the grains over 100 cells of the epithelial and the mesenchymal compartments of the lesions and calculating the mean value. The same procedure was carried out with the corresponding control sections (sense probes) to evaluate the intensity of background signals. After subtraction of background signals, cases were grouped as absent (-, number of grains equivalent to background), faint [(+) up to 10 grains per cell], weak ("+" up to 25 grains per cell), moderate "++" (up to 60 grains per cell) and strong "+++" (more than 60 grains per cell).

Results

Normal tissue

Very few periductal and perilobular stromal cells displayed weak autoradiographic signals specific for MMP-2 (Fig. 1A), TIMP-1 and TIMP-2 in a similar distribution pattern. Epithelial cells were not labeled. MMP-1 and MMP-3 were not expressed at levels detectable even after extended autoradiographic exposure of up to 3 months (Table 1).

Fibrocystic disease and radial scars

Compared with normal breast tissue, slightly higher numbers of cells expressing MMP-2, TIMP-2 and TIMP-1 were found in cases of fibrocystic disease without epithelial atypia. The relative transcript levels were similar to those observed in normal tissue. Scarred areas including radial scars displayed a distinct pattern with prominent MMP-1 expression in stromal cells surrounding the (myo)epithelial compartment in the absence of similarly elevated MMP-2 transcript levels. This distinct pattern was observed in four out of five cases of fibrocytic diseases also containing radial scars (Fig. 1B). MMP-3 transcripts were below the threshold of detection.

CLIS and DCIS

MMP-2, TIMP-1 and TIMP-2 transcript levels were slightly elevated in the neighborhood of CLIS lesions compared with adjacent normal breast tissue. MMP-1 and -3 transcripts were not detectable. All cases of DCIS showed strong MMP-2-specific labeling in stromal cells surrounding the neoplastic ductal epithelial formations and in myoepithelial cells adjacent to the tumor islands (Fig. 2A). Neoplastic epithelial cells, however, did not display MMP-2 transcripts. With respect to intralesional differences, we observed that some of the DCIS areas, in particular high-grade lesions surrounded by desmoplastic stroma, displayed the expression levels described above, whereas neighboring areas displayed no or only weak

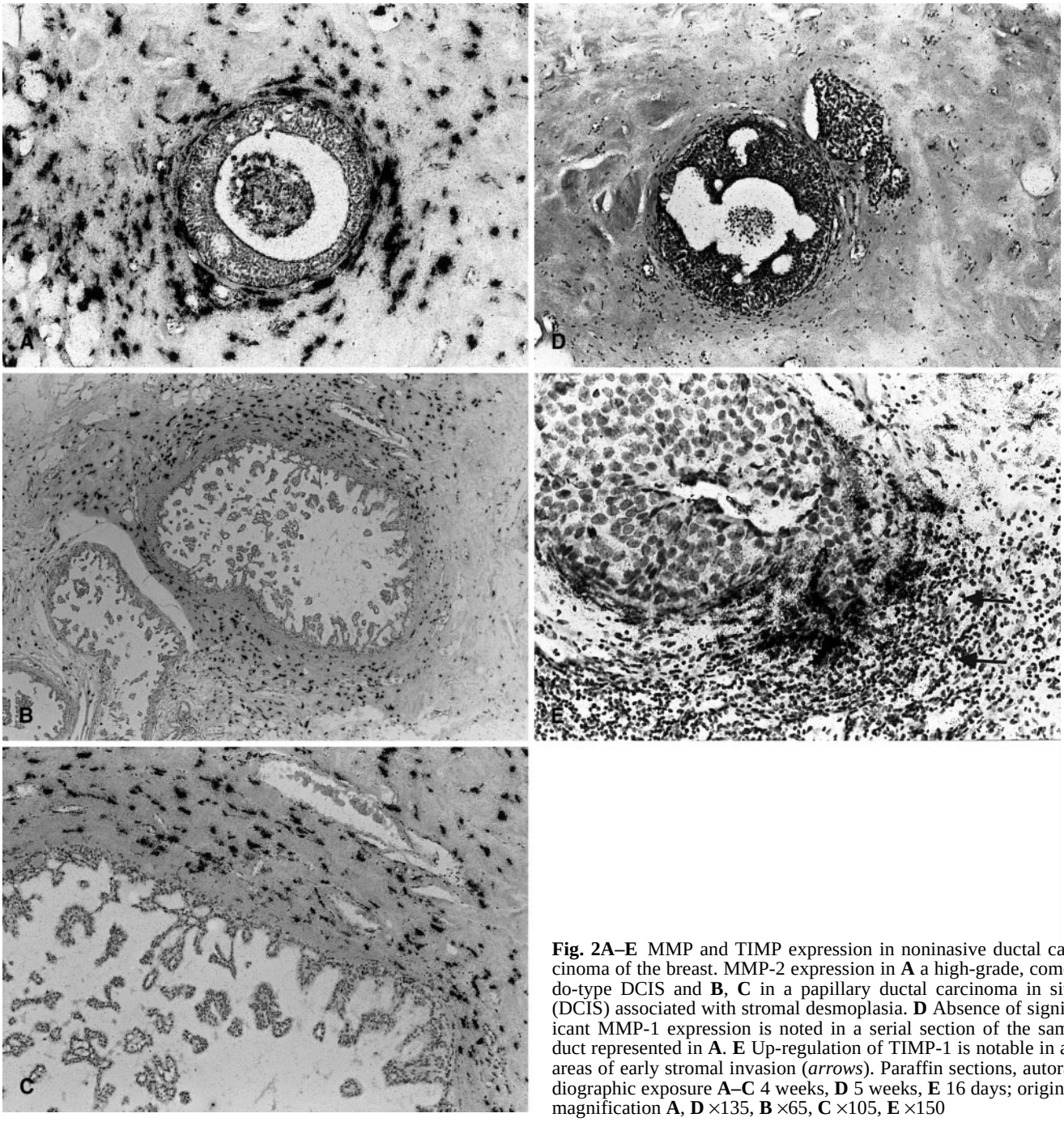

Fig. 2A–E MMP and TIMP expression in noninasive ductal carcinoma of the breast. MMP-2 expression in **A** a high-grade, comedo-type DCIS and **B**, **C** in a papillary ductal carcinoma in situ (DCIS) associated with stromal desmoplasia. **D** Absence of significant MMP-1 expression is noted in a serial section of the same duct represented in **A**. **E** Up-regulation of TIMP-1 is notable in an areas of early stromal invasion (*arrows*). Paraffin sections, autoradiographic exposure **A–C** 4 weeks, **D** 5 weeks, **E** 16 days; original magnification **A**, **D** ×135, **B** ×65, **C** ×105, **E** ×150

expression (Fig. 2B, C). MMP-1 and MMP-3 transcripts were not detectable (Fig. 2D). TIMP-1 and TIMP-2 transcripts were present at moderately elevated levels directly adjacent to the epithelial compartment. Two cases with early stromal invasion showed intensive labeling with the TIMP-1 probe within the area of focal invasive growth (Fig. 2E).

Invasive carcinomas

All invasive carcinomas showed high MMP-2 transcript levels, not only in the peritumor stroma, but also in more peripherally localized stromal cells (Fig. 3A). There were also moderate signals in capillary endothelial cells. In 20 of 24 invasive carcinomas a co-expression of MMP-2 and MMP-1was found. A high level of MMP-1 transcripts was found in carcinoma cells and fibroblasts at the invasion front (Fig. 3B, C). Reduced MMP-1 transcript levels were observed within the central tumor areas. The signal density and distribution of labeled cells correlated with the histological grade of tumor differentiation with high-grade carcinomas displaying the highest levels of MMP-1 and MMP-2 transcripts (Table 2). MMP-3 displayed low transcript levels in tumor cells

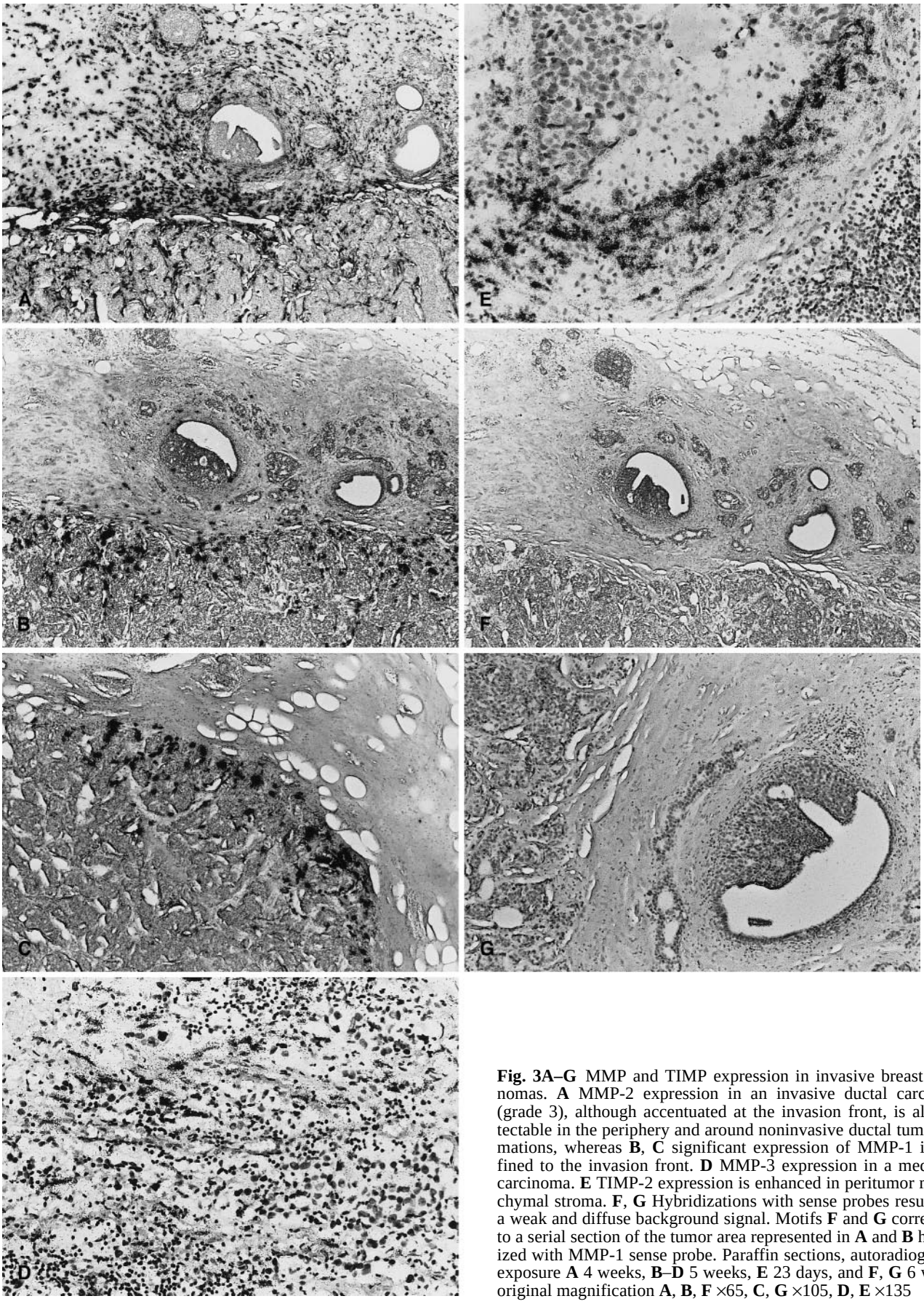

Fig. 3A–G MMP and TIMP expression in invasive breast carcinomas. **A** MMP-2 expression in an invasive ductal carcinoma (grade 3), although accentuated at the invasion front, is also detectable in the periphery and around noninvasive ductal tumor formations, whereas **B**, **C** significant expression of MMP-1 is confined to the invasion front. **D** MMP-3 expression in a medullary carcinoma. **E** TIMP-2 expression is enhanced in peritumor mesenchymal stroma. **F**, **G** Hybridizations with sense probes resulted in a weak and diffuse background signal. Motifs **F** and **G** correspond to a serial section of the tumor area represented in **A** and **B** hybridized with MMP-1 sense probe. Paraffin sections, autoradiographic exposure **A** 4 weeks, **B**–**D** 5 weeks, **E** 23 days, and **F**, **G** 6 weeks; original magnification **A**, **B**, **F** ×65, **C**, **G** ×105, **D**, **E** ×135

Table 2 Distribution of MMP and TIMP RNA transcripts in carcinomas of different levels of differentiation

	n=	MMP-1	MMP-2	MMP-3	TIMP-1	TIMP-2
Ductal carcinoma G1	2	++	++	–	++	++
Lobular carcinoma G2	5	++	++	–	++	+
Ductal carcinoma G2	9	++	+++	+	+++	+++
Ductal carcinoma G3	5	+++	+++	+	+++	+++
Medullarycarcinoma G3	3	+++	+++	++	+++	+++

and peritumor stroma, especially in medullary carcinomas (Fig. 3D). TIMP-1 and TIMP-2 were expressed in the peritumor stroma (Fig. 3E), and TIMP-2 was also found in the peripheral stroma. Tumor cells displayed TIMP-1 RNA at low transcript levels.

Control hybridizations with all of the probes transcribed in "sense" orientation displayed few and scattered autoradiographic grains (Fig. 3F, G).

Discussion

Matrix metalloproteinases represent a group of enzymes responsible for normal connective tissue remodeling and also involved in the increased turnover of ECM proteins associated with tumor progression. MMP expression is tightly regulated because of the destructive potential of these enzymes. It has previously been established that the levels of urokinase-type plasminogen activator (uPA) and its type-1 inhibitor (PAI-1) are elevated in tissue extracts of invasive breast carcinomas, and that this up-regulation correlates with poor prognosis [17, 18]. Furthermore it has been shown that urokinase gene expression indicates early invasive growth potential in squamous cell lesions of the uterine cervix [31]. uPA and PAI-1 are involved in the conversion from plasminogen to plasmin which, in turn, is a co-activator of MMPs [28, 36]. Therefore, it is reasonable to look more closely at downstream components of the proteolytic cascade required for interstitial ECM degradation. It was the aim of this study to examine the patterns of expression and to semiquantify relative transcript levels of metalloproteinases and their inhibitors in reactive lesions and during different stages of invasive growth. It was also important to look more closely at the cellular sources of these enzymes, for tumor cells must traverse the barrier of epithelial and endothelial BMs [22].

Normal breast tissue as well as fibrocystic disease showed weak levels of MMP-2, TIMP-1, and TIMP-2 suggesting a limited degree of normal connective tissue remodeling. Interestingly, a distinct expression pattern with increased MMP-1-specific signals combined with weak MMP-2 transcript levels became evident in scarred areas, including radial scars in tissue specimens with fibrocystic disease in four out of five such cases. Radial scar is a benign lesion characterized by a pseudoneoplastic growth of the (myo)epithelial cells. The expression of MMP-1 by fibroblasts in this lesion indicates that MMP-1 is likely to be relevant for ECM remodeling not only in malignant breast tissue, and that MMP-1 expression in other benign conditions may be present, but below the threshold of detection. The near absence of MMP-2-specific signals is in good agreement with the finding of an intact BM in radial scars.

Moderate labeling for MMP-2 in CLIS and strong labeling for MMP-2 in desmoplastic stroma surrounding DCIS and in myoepithelial cells may indicate a degradation potential at the BM. Of particular interest is the expression of TIMP-1 RNA in two cases of DCIS with early stromal invasion. TIMP-1 was localized in the fibroblasts directly surrounding the area of stromal invasion. These findings lend support to the idea that TIMP-1 expression is a tumor-induced host response to ECM perturbation caused by invasion [13].

Invasive carcinomas displayed co-expression of transcripts for MMP-1, MMP-2 and both inhibitors with accentuation at the invasion front. The relative levels of transcripts correlated with the histological grade of the invasive ductal carcinomas. In medullary carcinomas, we were also able to detect MMP-3 transcripts clearly, whereas in other lesions the levels were below the limits of detection. As MMP-3 is a co-activator for MMP-1, this indicates the presence of an extended activation potential for MMP-1. The increased TIMP-1 and -2 signals furthermore illustrate that the matrix-degrading capacity of breast carcinomas not only depends on the presence of MMPs, but is also influenced by proteinase inhibitors [34]. Whether these increased relative levels of MMP correspond to increased proteolytic activity has to be investigated in future studies employing methods such as zymography and immunoblot analysis.

The various elements of the MMP cascade and their respective inhibitors represent a tightly balanced system in which a reduction of inhibitory capacity can lead to enhanced collagenolytic activity. It has been shown that the invasiveness of primitive embryonic mesenchymal cell lines can be increased by inactivation of the TIMP-1 gene [1]. Alexander and Werb also proposed that the ability of cancer cells to invade the surrounding stroma may depend on the balance of the synthesis of proteases and their inhibitors. Khokha et al. [19] demonstrated that transfection of melanoma cell lines with a TIMP-1 overexpression vector induced decreased invasiveness of the tumor cells.

ISH demonstrated that – even in invasive carcinomas – MMP-2 RNA is scarcely expressed by the tumor cells, but rather by fibroblasts, myoepithelial cells and macrophage-like cells in the surrounding stroma. These results

correspond with observations recorded by Davies et al. [8]. The expression of MMP-2 by fibroblasts has previously been documented in vitro and in vivo [3, 11, 32]. In human carcinomas, immunolocalizations of MMP, including their zymogen forms, showed increased activity in tumor cells of colorectal and mammary carcinomas [4, 15, 21, 29]. Wooly et al. [40] showed immunohistochemical staining for collagenase in both tumor and stromal cells. Monteagudo [26] detected MMP-2 protein in benign and malignant breast lesions. It was localized in epithelial and myoepithelial cells, in normal breast tissue and in benign lesions. Tumor cells of in situ and invasive carcinomas displayed weak staining.

Our finding of a predominance of MMP-2 RNA in peritumor fibroblasts rather than in tumor cells, however, may seem inconsistent. The results of our study correspond to the observations of Poulsom et al. [30], who found MMP-2 mainly in desmoplastic fibroblasts around tumor islands, and Soini et al. [34], who detected that MMP-2 RNA was more abundant in stromal fibroblasts and endothelial cells than in carcinoma cells. Our findings are also in line with results of Autio-Harmainen et al. [2] and Soini et al. [33], which showed that stromal cells are also the main site of MMP-2 synthesis in lung and ovarian cancer. These results indicate that the production of these proteases is not confined to malignant cells, and that fibroblasts play a more decisive role in degradation of BMs in breast carcinomas than previously thought. The observations that MMP-2 RNA is localized in peritumor stroma cells and that MMP-2 protein occurs in carcinoma cells strongly suggest that there is an interaction between tumor and stroma with respect to the production of MMP-2. This statement has been supported by Biswas [6] and Nabeshima et al. [27], who identified a tumor cell-derived factor that stimulates collagenase production in fibroblasts. Also Emonard et al. [10] presented a cell surface receptor for MMP-2 in breast carcinoma cells.

In summary, the up-regulation of MMP-2 RNA in pre-invasive lesions, followed by MMP-1 and TIMP-1 in invasive cancers, suggests a gradually increasing invasive potential. MMP-2 expression, when focally observed in the pre-invasive stage of breast cancer (DCIS, CLIS), may indicate tumor areas with an increased risk for invasive growth.

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