Collagenase-3 Expression in Breast Myofibroblasts as a Molecular Marker of Transition of Ductal Carcinoma in Situ Lesions to Invasive Ductal Carcinomas

Boyé Schnack Nielsen, Fritz Rank, José Manuel López, Milagros Balbin, Francisco Vizoso, Leif Røge Lund, Keld Daniøs, and Carlos López-Otín

ABSTRACT

Collagenase-3 (matrix metalloproteinase 13; MMP-13), a protease originally identified in breast carcinoma, is characterized by a potent degrading activity against a wide spectrum of extracellular matrix proteins. The aim of this study was to locate and identify the MMP-13-expressing cells in invasive human breast carcinoma and to evaluate the role of MMP-13 in transition to invasive lesions by studying ductal carcinoma in situ (DCIS). We found expression of MMP-13 in stromal myofibroblast-like cells in all 21 invasive ductal carcinomas studied and in 4 of 9 invasive lobular carcinomas. In most carcinomas, expression of MMP-13 was limited to small stromal foci in the tumor area. Combined in situ hybridization and immunohistochemistry showed coexpression of α-smooth muscle actin immunoreactivity and MMP-13 mRNA in myofibroblasts. In contrast, cytokeratin-positive cancer cells, α-smooth muscle actin-positive vascular smooth muscle cells, CD68-positive macrophages, and CD31-positive endothelial cells were all MMP-13 mRNA negative. In situ hybridization for MMP-13 in 17 DCIS lesions revealed expression in 10 cases. Immunohistochemical analysis of all DCIS cases identified microinvasion in 8 of the 17 lesions. Seven of the eight lesions with microinvasion were MMP-13 positive. Further analysis showed that MMP-13 expression was often associated with the microinvasive events. This particular expression pattern was unique for MMP-13 among other MMPs analyzed, including MMP-2, -11, and -14. We conclude that MMP-13 is primarily expressed by myofibroblasts in human breast carcinoma and that expression in DCIS lesions often is associated with microinvasive events. On the basis of these data, we propose that MMP-13 may play an essential role during transition of DCIS lesions to invasive ductal carcinomas.

INTRODUCTION

Initial invasion of cancer cells into the host stromal tissue requires the traversing of the basement membrane. This process involves the enzymatic degradation of the dense basement membrane collagen matrix. Continued cancer cell invasion is further facilitated by the degradation of the surrounding extracellular matrix, where the normal host tissue is substituted by tumor tissue. Several extracellular proteases take part in these processes, including MMPs and serine proteases such as plasmin (1–5). The MMPs are zinc-dependent proteases with different but partially overlapping substrate specificities. They are synthesized as inactivezymogens that require proteolytic cleavage to generate the final active form of these proteases (6). At present, a total of 20 distinct human MMPs have been described (7), of which 14 are secreted extracellular proteases and 6 are MT-MMPs (8). Among these enzymes, members of the collagenase subfamily are the principal neutral proteases with the ability to degrade fibrillar collagens. Four human collagens have been identified: fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), the most recently described collagenase-3 (MMP-13; Ref. 9) and MT1-MMP (MMP-14; Ref. 10).

Human MMP-13 was initially identified in breast cancer (9) and later was found overexpressed in other malignant tumors, including chondrosarcoma (11), head and neck carcinoma (12, 13), basal cell carcinoma of the skin (14), malignant melanoma (15), vulvar carcinoma (16), and esophageal carcinoma (17). Furthermore, MMP-13 has been detected in a variety of inflammatory conditions (18–20), in atherosclerosis and aortic aneurysms (21, 22), in skin wound healing (23), and in destructive joint diseases such as osteoarthritis and rheumatoid arthritis (24–26). Biochemical characterization of MMP-13 has revealed that it efficiently degrades the native helix of fibrillar collagens with preferential activity on type II collagen (24, 27). However, MMP-13 is also able to degrade several other extracellular matrix proteins in vitro, including collagens type IV, X, and XIV; fibronectin; tenasin; and fibrillin (28, 29). Biochemical studies have also revealed that pro-MMP-13 is activated at the cell surface by a proteolytic cascade involving MMP-14 and -2 (30), and other studies have indicated that it can also be activated by plasmin (31).

The finding that MMP-13 is overexpressed in several malignant tumors, together with its potent proteolytic activity against numerous extracellular matrix proteins, suggest that it plays a major role in the tissue-remodeling events accompanying tumor progression. However, to date there have been few studies on the nature and functional characteristics of MMP-13-producing cells in human malignancies, and in some of these studies the results are controversial (32). Several studies have indicated that MMP-13 in head and neck squamous cell carcinomas is produced by epithelial tumor cells located at the invading front of the tumors as well as in a subset of the carcinomas in stromal fibroblasts surrounding the tumor cells (12, 13). For invasive breast carcinoma, the picture appears to be more complicated. Some studies have suggested that MMP-13 is produced by fibroblast-like cells located in the stromal compartment of the breast cancer tissue (33), whereas other studies have indicated that MMP-13 is synthesized predominantly by epithelial tumor cells (9, 34). In an attempt to clarify this issue, we performed a detailed study of its expression in a series of invasive ductal and lobular breast carcinomas, and to evaluate the role of MMP-13 in transition to invasive carcinoma, we studied MMP-13 expression during microinvasion, through analysis of DCIS lesions.
noma, and with extensive DCIS. All tumor specimens were obtained at the Departments of Pathology, Rigshospitalet and Bispebjerg Hospital, Copenhagen, Denmark. Tissue samples were dissected from the tumor periphery within 1 h after extirpation as ~1 × 1 cm specimens, fixed overnight at room temperature in Lillie’s neutral-buffered formalin, and embedded in paraffin.

Among the patients with invasive ductal carcinoma, seven were diagnosed with histological malignancy grade I, six with grade II, and eight with grade III (35). The group of invasive lobular carcinomas consisted of classical (three patients), nonclassical (two patients), and mixed classical/nonclassical types (four patients). Malignancy grading of DCIS was according to Holland et al. (36). Five DCIS lesions were grade I, four were grade II, and eight were grade III. All DCIS lesions were selected for being extensive lesions. To identify microinvasion in the DCIS lesions, we immunohistochemically stained three adjacent sections with antibodies against pan cytokeratin (detection of all epithelial cells), CK-14 (detection of myoepithelial cells), and α-sm-actin (detection of myoepithelium), respectively. Microinvasion was then defined as foci of (cytokeratin-positive) carcinoma cells located in the stroma not measuring more than 1 mm in diameter and not being accompanied by (α-sm-actin- and/or CK-14-positive) myoepithelium in accordance with the definition by the National Coordinating Group for Breast Screening Pathology (37). The study was in accordance with permission provided by The Regional Scientific-Ethical Committee for Copenhagen and Frederiksberg, Denmark (journal numbersKF 01-45693).

Antibodies. Antibodies directed against α-sm-actin (clone 1A4), CD68 (clone PGM1), CD31 (clone JC/70A), and cytokeratins (clone MNF116, CK-5, -6, -8, and -17; clone AE1/AE3, CK-2, -4, -5, -6, -8, -10, -14, -15, -16, and -19) as well as biotinylated secondary antibodies and peroxidase-conjugated streptavidin were all purchased from Dako (Glostrup, Denmark). Rabbit polyclonal antibodies against CK-14 were from Babco, Covance (Richmond, CA).

cDNA Subclones and Generation of Radiolabeled RNA Probes. The full-length MMP-13 cDNA cloned in pEMBL (pNt03A; Ref. 9) was used to obtain two nonoverlapping subclones: A 730-bp fragment corresponding to the first half of the open reading frame was isolated upon BamHI digestion and inserted into pBluescript KS (plasmid phMMP13-730). A 790-bp fragment containing the first half of the open reading frame plus 100 bp of the 3′-untranslated region was isolated by digestion with BamHI and HindIII and ligated into BamHI/ HindIII-digested Bluescript KS (plasmid phMMP13-730). A 790-bp fragment containing the first half of the open reading frame was isolated upon BamHI digestion and inserted into BamHI-digested Bluescript KS (plasmid phMMP13-BB). Plasmids containing MMP-2 cDNA (pCol7201; bp 647–1585) and MMP-9 cDNA (pBSII-SK-ZIV; bp 347-2105) have been described (38).

In Vitro Transcription. Antisense and sense riboprobes were labeled with 32P-labeled UTP (NEN, Boston, MA) by in vitro transcription using Sp6, T3, and T7 RNA polymerases (Roche, Basel, Switzerland) as described (41). The DNA template was digested with DNase (Promega, Madison, WI). Unincorporated 32P-labeled UTP and DNA were removed by column chromatography with S-200HR microspin columns (Amersham Pharcia Biotech Inc., Piscataway, NJ). The 32S activity was adjusted for every probe by dilution to 500,000 cpm/μl.

In Situ Hybridization. In situ hybridization was performed essentially as described previously (41) with minor modifications. Three-μm paraffin sections were deparaffinized in xylene, hydrated through graded ethanol solutions, and embedded in paraffin.

To determine the expression of MMP-13 in invasive ductal and lobular breast carcinoma, we performed in situ hybridization with distinct radiolabeled RNA probes. Antisense transcripts obtained by in vitro transcription of two plasmids containing nonoverlapping cDNA fragments of MMP-13 showed an identical hybridization pattern in adjacent paraffin sections from all of five invasive ductal breast carcinomas analyzed (Fig. 1). There was no difference in the signal intensity observed with the two probes. No hybridization signal was seen with two MMP-13 sense probes obtained from the same plasmids (Fig. 1). We therefore concluded that the positive hybridization signal detected in the invasive ductal breast carcinoma depicted in Fig. 1 represents the genuine MMP-13 mRNA. To extend these results, we performed in situ hybridization on a series of 21 invasive ductal carcinomas. These studies revealed that MMP-13 mRNA was detected in all analyzed invasive ductal carcinomas, although with a pronounced variation in the number and size of positive foci (Table 1). There was no obvious relationship between the extent of MMP-13 mRNA expression in the tumor sections and the histological malignancy grade of the individual tumors. The MMP-13 mRNA signal was seen most often in cells with a fibroblast-like morphology, which were located in the tumor stroma. No signal could be ascribed with certainty to the epithelial cancer cells in any of the carcinomas. The expression was most often focal and

<table>
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<th>Tumor category</th>
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Table 1 Semiquantitative evaluation of MMP-13 mRNA signal in human breast carcinomas

The MMP-13 mRNA signal was evaluated in sections from invasive ductal and lobular carcinomas and from DCIS with or without the presence of microinvasive carcinoma. The scores are based on the following criteria: 0, no positive signal; 1, a single positive focus; 2, two or more individual positive foci; 3, many positive cells throughout the entire tumor area. The assessment of microinvasion in the DCIS lesions was based on immunohistochemical staining of three adjacent sections with antibodies against pan cytokeratin, CK-14, and α-sm-actin, respectively.
MMP-13 in Human Breast Carcinoma

Fig. 1. In situ hybridization with two nonoverlapping MMP-13-specific probes in invasive human ductal breast carcinoma. 35S-labeled RNA probes from two nonoverlapping cDNA fragments were hybridized on consecutive sections: antisense (a and b) or sense (d) probes from the plasmid pHMMP13-BB or an antisense probe from the plasmid pHMMP13-730 (c). MMP-13 mRNA-positive cells (arrows) are visualized by brightfield (a) or dark-field (b–d) illumination. An identical hybridization signal is observed with the two antisense probes (arrows in a, b, and c), whereas the sense probe shows no positive signal (d). The MMP-13-positive cells are located close to the foci of carcinoma cells (Ca), which are negative. Note that stromal cells, including inflammatory cells (St), located distally from the carcinoma cells are negative. Magnification, ×90.

was found in the tumor periphery (13 carcinomas), in the paracentral areas (18 carcinomas), and within central sclerotic areas (10 of 19 carcinomas in which central sclerosis was present; Fig. 2, a–f). No signal was detected in the normal, unaffected breast tissue present around the malignant lesion. In contrast to the ductal carcinomas, MMP-13 mRNA was detected in only 4 of 9 invasive lobular breast carcinomas (Table 1). The four positive lobular carcinomas were either nonclassical lesions or mixed classical/nonclassical lesions, whereas three of the five negative lobular carcinomas were classical lesions and the other two mixed classical/nonclassical lesions. The expression pattern in the four positive lobular carcinomas (Fig. 2, g and h) was similar to that described above for the invasive ductal carcinomas, i.e., expression was found in stromal cells located within foci in the malignant area but not in any cancer cells and not in the normal, unaffected breast tissue.

To characterize the MMP-13-positive cells in more detail, six of the invasive ductal carcinomas were analyzed by combined immunohistochemistry and in situ hybridization. Sections were first immunostained with specific cell markers and then hybridized with 35S-labeled MMP-13 antisense RNA probe. We included antibodies against cytokeratins for identification of epithelial cells, α-sm-actin for myofibroblasts, CD68 for macrophages, and CD31 for endothelial cells. With the anticytokeratin antibodies, we could substantiate our preliminary observation, that no cancer cells were MMP-13 mRNA positive in any of the six carcinomas studied (Fig. 3, a and b). However, this double-labeling technique allowed us to detect coexpression of α-sm-actin immunoreactivity and MMP-13 mRNA in all carcinomas studied (Fig. 3, c and d). It was evident that virtually all MMP-13 mRNA-positive cells were α-sm-actin-positive, whereas the majority of the α-sm-actin-positive cells were MMP-13-negative. These α-sm-actin-positive stromal cells could be either myofibroblasts or vascular smooth muscle cells (42). To differentiate myofibroblasts from vascular smooth muscle cells, we considered the association of the vascular smooth muscle cells to vessels, a characteristic morphological parameter (42). Because all MMP-13 mRNA- and α-sm-actin-positive cells were not directly associated with vascular structures, we considered the MMP-13-producing cells as myofibroblasts. In addition, we did not detect the macrophage marker CD68 (Fig. 3, e and f) or the endothelial cell marker CD31 (not shown) in any of the MMP-13-positive cells.

MMP-13 Expression in DCIS of the Breast. DCIS is increasingly diagnosed as a result of mammography. Microinvasive ductal carcinoma, i.e., one or more foci of invasive carcinoma 1 mm or less, is a rare lesion almost exclusively associated with extensive DCIS predominantly of high malignant grade (37). We studied the expression of MMP-13 in 17 DCIS lesions selected for being extensive DCIS lesions, including 5 grade I lesions and 12 grade II/III lesions. Ten of the 17 lesions produced a MMP-13-positive signal, whereas the remaining 7 lesions were negative (Table 1). Although MMP-13 expression was scarce, it was always seen in stromal fibroblasts close to the DCIS foci (Fig. 4, a and b). Because the expression of MMP-13 mRNA in the DCIS lesions was either absent or limited to single foci and because mainly grade II and III lesions were positive, we decided to study the expression pattern with respect to the presence of microinvasion. We analyzed the DCIS lesions for the presence of microinvasive foci by comparing three adjacent sections immunohistochemically stained for cytokeratins (all epithelial cells), CK-14 (myoepithelium), or α-sm-actin (here for myoepithelium), respectively. DCIS lesions were categorized as microinvasive when foci of cytokeratin-positive carcinoma cells (1 mm or less) were identified in the stroma and when, in addition, these carcinoma cells were not associated with myoepithelium, i.e., they were negative for both CK-14 and α-sm-actin. In 8 of the 17 cases, we identified microinvasion (Table 1). Microinvasion was not observed in any of 5 grade I DCIS lesions, but was observed in 8 of the 12 grade II/III DCIS lesions. Comparison of these data with the initial MMP-13 expression study showed that seven of the eight lesions of DCIS with microinvasion had MMP-13-positive cells, whereas the remaining 5 lesions of DCIS without microinvasion had MMP-13-positive cells. The expression of MMP-13 in 17 DCIS lesions selected for being extensive DCIS lesions was compared to the MMP-13 expression in invasive ductal carcinomas using the same immunohistochemical techniques. It was evident that virtually all infiltrative ductal carcinomas were α-sm-actin-positive, whereas the majority of the α-sm-actin-positive cells were MMP-13-negative. These α-sm-actin-positive stromal cells could be either myofibroblasts or vascular smooth muscle cells (42). To differentiate myofibroblasts from vascular smooth muscle cells, we considered the association of the vascular smooth muscle cells to vessels, a characteristic morphological parameter (42). Because all MMP-13 mRNA- and α-sm-actin-positive cells were not directly associated with vascular structures, we considered the MMP-13-producing cells as myofibroblasts. In addition, we did not detect the macrophage marker CD68 (Fig. 3, e and f) or the endothelial cell marker CD31 (not shown) in any of the MMP-13-positive cells.

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ization method alone with those obtained by the double-labeling technique; we found that the in situ hybridization signal intensity was unaffected by the modifications in tissue processing needed for the double-labeling technique. An example of carcinoma cells stained for cytokeratins and subsequently hybridized with an MMP-13-antisense probe is shown in Fig. 4, a–c. From each of the 17 lesions, three adjacent sections were immunohistochemically stained for pan cytokeratin, CK-14, or α-H9251-sm-actin, and subsequently hybridized with an antisense probe for MMP-13. As expected, seven of the eight DCIS lesions with microinvasion were MMP-13-positive, and interestingly, MMP-13-positive stromal cells were found in virtually all of the microinvasive foci. This pattern was observed in both grade II (Fig. 4, d and e) and grade III (Fig. 4, f–i) DCIS. Among the DCIS lesions without microinvasion, a few stromal cells located in the vicinity of a DCIS structure were MMP-13 mRNA-positive in 3 of the 9 cases (Fig. 4, j–l). These studies also confirmed the expression of MMP-13 mRNA in stromal fibroblast-like cells, most of which were α-sm-actin-positive myofibroblasts, and showed the presence of myoepithelial cells positive for both α-sm-actin and CK-14 but negative for MMP-13 mRNA. We therefore conclude that DCIS grade II and III can induce MMP-13 expression in neighboring myofibroblasts, and because microinvasion is usually accompanied by MMP-13 mRNA-positive myofibroblasts, we suggest that MMP-13 enzymatic degradation of extracellular matrix induces microinvasion in these DCIS foci.

Finally, we examined whether the observed association of MMP-13 expression with microinvasion was a characteristic feature of this protease or whether it could be extended to other MMP family members. We analyzed the expression of MMP-2, -11, and -14 mRNAs in six of the DCIS lesions without microinvasion and in four of the DCIS lesions with microinvasion by in situ hybridization. Two of the six DCIS lesions without microinvasion were MMP-13 mRNA-positive, but all six were found to be MMP-2 and -11-positive, and five were MMP-14-positive (see Fig. 5). Three of the four DCIS lesions with microinvasion were MMP-13-positive, but all four were MMP-2, -11, and -14-positive. These four MMPs were all expressed by fibroblast-like stromal cells, but with different expression patterns. MMP-2 showed the most extensive expression pattern, and MMP-13 the most restricted expression pattern. MMP-11 showed increased expression around noninvasive DCIS outgrowths, as has been reported previously by others (43). MMP-2 and -14 are considered the main activators of pro-MMP-13, and by studying their putative colocalization in consecutive adjacent sections, we found that both

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Fig. 2. In situ hybridization for MMP-13 mRNA in invasive ductal and lobular breast carcinomas. Paraffin sections of invasive ductal carcinoma (a–f) and lobular carcinoma (g and h) were hybridized with the antisense (a, b, d–h) or sense probe (c) of phMMP13-730. The framed area in a is shown in higher magnification in d. The MMP-13 mRNA-positive cells (black and white arrows) are identified in the tumor periphery (a–d) and in the central sclerotic tissue (e and f) and are fibroblast-like cells in the stromal compartment (St). Carcinoma cells (Ca; gray arrows in d) are MMP-13 mRNA negative. Magnification, ×45 in a–c and e–h; ×180 in d.
MMP-2 and -14 were expressed at, but not restricted to, microinvasive areas (see Fig. 5).

DISCUSSION

This study was undertaken to clarify the expression and cellular localization of MMP-13 mRNA in invasive human breast carcinoma and DCIS. We based our observations on the use of two specific antisense RNA probes obtained from two nonoverlapping MMP-13 cDNA fragments. Because they showed an identical hybridization pattern in adjacent sections from cases of invasive ductal carcinoma and because application of complementary sense probes as negative controls did not show any hybridization signal, we conclude that the hybridization signal observed represents a genuine MMP-13 mRNA.

The first novel finding in this study was that all of 21 cases of invasive ductal carcinoma investigated contained MMP-13 mRNA-positive cells, in contrast to only 4 of 9 cases of invasive lobular carcinoma. This is interesting because most invasive lobular carcinomas have better prognoses than most invasive ductal carcinomas (44). However, a larger clinical study is required to clarify whether the subgroup of MMP-13-negative invasive lobular carcinomas represents a group of carcinomas of particularly favorable prognosis.

Another significant finding was the characterization of the MMP-13 mRNA-positive cells as myofibroblasts. On the basis of an immunohistochemistry and in situ hybridization double-labeling technique, we concluded that virtually all of the MMP-13 mRNA-positive cells were α-sm-actin-positive fibroblast-like cells not directly associated with vascular structures; therefore, they can be considered as myofibroblasts. Myofibroblasts were originally described in healing wounds and are believed to be involved in wound contraction (45, 46). It is a predominant cell type in the stroma of several cancer types (46), including breast cancer (42, 47), where it has been shown to originate primarily from fibroblasts and vascular smooth muscle cells (48). The possibility that cancer cells also can convert into myofibroblasts on epithelial to mesenchymal transition has recently been discussed (49). Studies of myo- (differentiated) fibroblasts cocultured with breast cancer cells indicated that they can facilitate the migration of cancer cells and thereby promote tumor progression (50). We have previously shown that the myofibroblast is the predominant cell type in human breast cancer expressing uPA mRNA (51). This is noteworthy for at least two reasons: (a) high uPA levels in breast cancer tissue are associated with short survival (52, 53), suggesting that myofibroblasts also have a tumor-promoting role in vivo; and (b) plasmin is one of the few proteases that have been reported to process pro-MMP-13 into active MMP-13 (see below), potentially making the myofibroblasts capable of activating pro-MMP-13 on their own. It does, however, remain to be clarified whether MMP-13 and uPA are expressed by the very same myofibroblasts in human breast cancer.

Our findings are in accordance with a previous study showing that stromal cells adjacent to epithelial tumor cells are the main source of this enzyme (33). In that study, MMP-13 mRNA was detected only in...
30% of invasive ductal carcinomas, whereas we detected MMP-13 expression in all 21 cases studied, although with a pronounced variation in the number of positive cells identified in the individual samples. We believe that this difference is caused by differences either in tissue fixation (54, 55) or by the selection of tissue samples chosen for analysis because MMP-13 expression usually is focal and in some cases limited to one or a few foci within a single sample. A discrepancy with the study by Heppner et al. (34), who found MMP-13 mRNA signal confined to some isolated breast cancer cells, is more difficult to reconcile. However, in that study, no attempts to specifically characterize the positive cells were reported. A preliminary immunohistochemical analysis suggested that MMP-13 immunostaining is associated with breast cancer cells (9). Although such localization may be explained by the uptake of extracellular MMP-13.

Fig. 4. In situ hybridization for MMP-13 mRNA in combination with immunohistochemical staining of epithelial and myoepithelial cytokeratins in DCIS and microinvasive ductal carcinoma. Sections were processed for MMP-13 mRNA in situ hybridization only (a and b), or in situ hybridization combined with pan cytokeratin immunohistochemistry (c–f, i, and j), or CK-14 immunohistochemistry (g, h, k, and l), and are shown with brightfield illumination (a, c, d, f, g, i, j, and l), or darkfield illumination (b, e, h, and k). MMP-13 mRNA signal (arrows in a–c) is seen around foci of DCIS. Note that the carcinoma cells are clearly evident after cytokeratin immunostaining (c) and that a similar MMP-13 mRNA signal intensity is observed (compare a and c) with in situ hybridization and combined immunohistochemistry and in situ hybridization. In one case (d and e; microinvasion and grade II DCIS), carcinoma cells are invading the surrounding stroma (red arrows), and a stromal MMP-13 mRNA signal is observed (black and white arrows). In a second case (f–i; microinvasion and grade III DCIS), carcinoma cells (red arrows in i) are invading the stroma; also here, MMP-13 is expressed in some stromal cells (black and white arrows in g–i). The framed area in f is shown in higher magnification in i. CK-14 immunostaining (g and h) shows the presence of the myoepithelium around the DCIS, but the continuity of the myoepithelium appears interrupted at the site of microinvasion. In a third case (j–l; DCIS grade III), no carcinoma cells have invaded the stroma, but a single MMP-13-positive cell is observed (arrows in k and l). The myoepithelium appears continuous and intact (l). Magnifications, ×90 in a–c and f–h; ×180 in d, e, and j–l; ×360 in i.
protein, produced by stromal cells, by cancer cells, it should be noted that these immunohistochemical data were based on the use of a single preparation of polyclonal antibodies. Our attempts to identify MMP-13 by immunohistochemistry using recently described monoclonal antibodies (56) have failed to provide definitive information on the identity of MMP-13-containing cells, probably because of cross-reactivity with other MMPs, and further studies will be required to clarify the localization of MMP-13 protein in breast cancer.

Probably the most interesting finding of this work came from the studies of DCIS lesions. A consistent diagnostic difficulty in DCIS is to assess whether microinvasion is present. However, the diagnosis is decisive for the treatment. Patients with DCIS without microinvasion generally receive local excision only, whereas patients with microinvasive DCIS are treated as patients with invasive cancer, including axillary dissection and possibly adjuvant drug treatment, depending on the disease stage. Microinvasive events are characterized by carcinoma cells that traverse or already have traversed the continuous layer of basal cells, i.e., the myoepithelium that surrounds DCIS foci (36). In this situation, the myoepithelial cell layer is interrupted by the invasive carcinoma cells, which may appear as single cells or cell clusters in the stroma without any associated myoepithelial cells. The diagnosis of microinvasive carcinoma according to the definition “a tumor in which the dominant lesion is DCIS but in which there are one or more clearly separate foci of infiltration of fibrous or adipose tissue, none measuring more than 1 mm in diameter” (37) is generally based on standard H&E staining, which often is supplemented with

Fig. 5. In situ hybridization for MMP-13, -2, and -14 in DCIS and microinvasive ductal carcinoma. Three adjacent sections from a DCIS lesion without microinvasion (a–d) and DCIS with microinvasion (e–h) were hybridized with antisense probes for MMP-13 (a, b, and f), MMP-14 (c, e, and g), and MMP-2 (d and h). Expression of MMP-13 mRNA was not detected in the pure DCIS lesion (a–d), in contrast to both MMP-2 and -14, which were expressed in stromal cells (St). In the DCIS lesion with microinvasion (e–h), MMP-13 mRNA is expressed together with MMP-2 and -14 mRNA in stromal cells (St) surrounding foci of carcinoma cells (Ca), but MMP-13 shows a more restricted expression pattern than MMP-2 and -14. Magnification, ×45.
identification of myoepithelial cells by immunohistochemical staining for markers such as the basal cell cytokeratins CK-5, -14, and -17 (47, 57–61) as well as muscle-specific proteins such as α-sm-actin, smooth muscle myosin heavy chain, and calponin (47, 62). However, the basal cell cytokeratins are also expressed by a subpopulation of cancer cells in 5–30% of invasive carcinomas (59, 61), and the muscle-specific proteins are also expressed by myofibroblasts and vascular smooth muscle cells (62, 63). Therefore, the use of a single myoepithelial cell marker in the clarification of the presence of intact myoepithelium or microinvasion may not be sufficient, but would require additional supplementary myoepithelial cell markers.

A general method to assess microinvasion in DCIS with certainty has not been established, but the use of different combinations of markers has been reported, including the combination of CK-17 and the tumor cell marker CK-8 (60), pan cytokeratin and α-sm-actin (64), α-sm-actin and calponin (65), and smooth muscle myosin heavy chain and calponin (63). In this study we chose to stain three adjacent sections with one of the following markers: a pan cytokeratin for identification of all epithelial cells, and two supplementary myoepithelial cell markers, one specific for a myoepithelium-specific cytokeratin (CK-14), and one specific for a muscle-specific protein (α-sm-actin). The pan cytokeratin-stained section facilitated the identification of foci suspicious of microinvasion, and the myoepithelial cell markers were used for the final assessment of microinvasion. Thus, microinvasion was defined as foci of (cytokeratin-positive) carcinoma cells traversing the myoepithelial cell layer or foci of carcinoma cells, measuring <1 mm in diameter, that were not accompanied by (CK-14- and/or α-sm-actin-positive) myoepithelium, in accordance with the above definition.

Using the immunohistochemistry and in situ hybridization double-labeling technique to compare the MMP-13 expression with the staining of the three markers, we observed in seven of eight DCIS lesions with microinvasion an association of microinvasive events with MMP-13 expression in surrounding myofibroblasts, whereas little or no MMP-13 signal was observed in nine DCIS lesions without microinvasion. The association of MMP-13 expression with the microinvasive events was very distinct when compared with other MMPs, including MMP-2, -11, and -14, which all showed a broader expression pattern not selectively associated with microinvasion. These findings indicate that MMP-13-catalyzed extracellular matrix degradation may be directly involved in early cancer cell invasion occurring in DCIS lesions.

The induction of MMP-13 expression in the fibroblasts associated with microinvasion may be caused by a specific combination of factors located in the microenvironment of microinvasive foci. In vitro studies have shown that particularly IL-1α, IL-1β, and transforming growth factor-β1 can induce MMP-13 mRNA expression in fibroblasts (33). In breast tumor tissue, it has been reported that IL-1α and IL-1β are located in cancer cells (66), and transforming growth factor-β1 mRNA is expressed in both cancer and stromal cells (67). Taken together, these observations suggest intercellular paracrine mechanisms in association with microinvasion in vivo. Regardless of the mechanism involved in the generation of microinvasive lesions, it seems clear that several extracellular matrix-degrading proteases are involved in the development of the invasive process (5, 68). Because MMP-13 is synthesized as an inactive precursor, it must be activated by other enzymes, such as plasmin, as indicated above. Preliminary data indicate that indeed the plasminogen activator uPA is expressed by stromal fibroblast-like cells associated with DCIS in a pattern partially overlapping that of MMP-13.4 It is therefore noteworthy that recent data (69) suggest that pro-MMP-13 can bind to a type C lectin called endo-180 (70), which is identical with a novel interaction partner for uPA and its receptor, termed uPARAP (71). Whether this interaction can promote pro-MMP-13 activation through plasmin remains to be clarified. MMP-14 and MMP-2 in a membrane-bound complex with MMP-14 are powerful activators of pro-MMP-13 in vitro (30). The finding that both MMP-14 and -2 are expressed by fibroblast-like cells similar to those producing MMP-13 mRNA in DCIS raises the possibility that pro-MMP-13 is secreted from the fibroblasts and subsequently bound and activated at the cell surface by the MMP-14/MMP-2 complex.

It is evident from the present study that MMP-13 is one of several extracellular matrix-degrading proteases expressed by stromal cells in breast cancer. In other types of adenocarcinomas, such as colon adenocarcinoma, similar stromal cells express several different proteases (72). However, this pattern appears to be different in squamous cell carcinoma, where at least some proteases, including MMP-13, MMP-9, and uPA, are expressed by the cancer cells themselves (14, 16, 38, 51, 73). Expression of MMP-13 by stromal cells in association with cancer cell invasion, as reported here in microinvasive ductal carcinoma, suggests that nonepithelial cells can modify particular steps in carcinogenesis (74). Such a hypothesis recently was studied experimentally in MMP-9-deficient mice that develop skin cancer, where MMP-9 supplied by transplanted normal bone marrow cells was found to promote squamous cell skin carcinogenesis (75).

Thus, histochemical studies on the expression of extracellular matrix-degrading proteases in human breast cancer may represent a valuable tool to illuminate steps in breast carcinogenesis. Taken together, our observations show that MMP-13 expression is induced in myofibroblasts during early stromal invasion of breast carcinoma cells in DCIS lesions and that MMP-13 expression is more restricted to microinvasive events than other MMPs, including MMP-2, -11, and -14. According to these data, analysis of MMP-13 expression should be explored to identify DCIS lesions with an increased risk of invasive local recurrence (76), thereby facilitating decisions on the therapeutic strategy to be followed after diagnosis of these early lesions in the multistage process of tumor invasion and metastasis.

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Collagenase-3 Expression in Breast Myofibroblasts as a Molecular Marker of Transition of Ductal Carcinoma in Situ Lesions to Invasive Ductal Carcinomas

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