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Gelatinase A expression and localization in human breast cancers. An in situ hybridization study and immunohistochemical detection using confocal microscopy

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Abstract The gelatinase A (72 kDa type IV collagenase) is a matrix metallo-proteinase which degrades basement membrane collagens. Various studies emphasize its role in stromal invasion of cancers, but there is some controversy about its origin. Gelatinase A was localized by immunohistochemistry using confocal microscopy in 15 human mammary carcinomas. In addition, the cells responsible for the synthesis of this enzyme were detected by in situ hybridization. Most invasive and non-invasive tumour cells were labelled by immunohistochemistry. Of particular interest was the pattern observed in some pre-invasive areas. Gelatinase A was found in fibroblasts in close contact with pre-invasive tumour clusters. Confocal observation allowed a more precise localization of gelatinase A to the periphery of tumour clusters along the basement membranes and in peritumour fibroblasts. The malignant epithelial cells were negative by immunohistochemistry in these areas. By in situ hybridization, mRNAs encoding gelatinase A were detected only in fibroblasts in close contact with pre-invasive and well differentiated tumour clusters. These findings support the hypothesis that peritumour fibroblasts produce gelatinase A and that breast cancer cells may bind this enzyme to their cell surface and/or internalize it.

Key words Gelatinase A · Breast carcinoma · Basement membrane · Invasion

Introduction

The ability to degrade the extracellular matrix is an important determinant of the invasive and metastatic phenotype of a tumour [13, 14]. Different proteolytic enzymes such as cathepsins, serine proteinases and matrix metallo-proteinases have been implicated in this process.

The 72 kDa type IV collagenase (now called gelatinase A) is a matrix metallo-proteinase which degrades basement membrane collagens, especially type IV collagen. Various studies emphasize the role of this enzyme in the stromal invasion in cancers [24]. Elevated levels of gelatinase A activity in transformed cells correlate with their ability to cross basement membranes in vitro and produce metastases in animal models [16, 25].

The role of gelatinase A in the progression of breast cancers is supported by different studies in vitro and in vivo. Cultured breast tumour cell lines have been shown to secrete gelatinase A. The more metastatic clones also display a higher type IV collagenase activity [16]. Brown et al. [6] have described the expression of activated gelatinase A in human invasive breast carcinomas by the technique of gelatin zymography. Immunohistochemical labelling for the gelatinase A has been shown to be an effective prognostic indicator for local recurrence of disease. However, no statistically significant difference in the rate of distant metastasis formation or overall patient survival was noted [10].

The cellular source of gelatinase A in vivo remains ambiguous. Gelatinase A has been detected by immunohistochemistry (IH) of human breast carcinomas [2, 15]. In a first study, Barsky et al. [2] found type IV collagenase only in invasive tumour cells. Non-invasive lesions were negative. More, recently, Monteagudo et al. [15] using affinity-purified antipeptide antibodies, showed cytoplasmic immunoreactivity for gelatinase A in myoepithelial cells in normal tissues and in invasive and non-invasive tumour cells in human breast carcinomas. Similar observations have been reported with the localization of gelatinase A in tumour cells of human prostatic adenocarcinoma [4], ovary and thyroid cancer [8, 9]. However, recent studies on human skin and colon cancers, using in situ hybridization (ISH), have detected mRNAs encoding gelatinase A in stromal cells while tumour cells were negative [11, 19, 20, 22, 23]. Therefore the possibility exists that gelatinase A could be secreted

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by peritumour fibroblasts and bind to a specific gelatinase A surface receptor in mammary carcinomas.

The aim of the present work is to study the expression of gelatinase A in human breast cancers employing various complementary morphological techniques applied to the same tumours. ISH is the best methodology to detect in vivo mRNAs encoding the enzyme. IH displays the presence and localization of gelatine A. The tissue sections treated with Ab 45 antibodies against gelatinase A have been examined with confocal laser scanning microscopy providing more precise information on spatial distribution of the labelling. The localization of a specific antigen in a precise cell compartment (cytoplasm or membrane) is sometimes difficult to deduce from IH on 8 µm thick sections. Confocal microscopy which captures images from 0.1 µm thick sections considerably improves the precise localization of a cellular antigen. Therefore, we localized the gelatinase A by means of confocal microscopy. The data obtained with the two morphological approaches were compared in non-invasive lesions (intraductal and intralobular proliferations) and in infiltrating carcinomas. The various approaches indicate a cooperation between stromal and cancer cells in the breakdown of the basement membrane.

Materials and methods

We examined 15 breast carcinomas. Fresh surgical specimens were cut, frozen in liquid nitrogen and then sectioned with a cryostat at −20° C. Tissue sections (5–10 µm) were fixed in acetone, 10 min, for IH and 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.2 for 30 min at 4° C followed by three 2 min washes in PBS. Slides were partially dehydrated in 50% and 70% ethanol.

Gelatinase A antibody used in this study is a rabbit polyclonal antibody (Ab 45) directed against a synthetic peptide containing the sequence NPDVANYNFFPRKPKWDKNQ from human gelatinase A (gift from Stetler-Stevenson, Bethesda, USA). This peptide (beginning with amino acid 75, ending at amino acid 94) overlaps the cleavage site between the propetide and the amino terminus of the activated gelatinase A.

Non-unspecific binding was blocked with 3% bovine serum albumin (BSA)-PBS for 30 min. The sections were treated by Ab 45 antibody (10 mg/ml) for 1 h, washed 3 times for 5 min each in PBS, incubated with a biotinylated secondary antibody (1 h, 1:300, goat anti-rabbit, Dako, Denmark) and followed by incubation by streptavidin fluoresceine complex (1 h, 1:20, Amersham, UK), respectively, at room temperature. Slides were counterstained for 2 sec in haematoxylin and mounted in Citifluor AF1 (Citifluor, UK). Sections were examined under a confocal laser scanning (Biorad MRC 600) microscope.

Stromal and endothelial cells were identified by conventional indirect immunohistochemical staining with an anti-vimentin and an anti-factor VIII-related antigen antibodies (Dakopatts, Denmark; data not shown).

For in-situ hybridization a gelatinase A (700 bp; kindly provided by K. Tryggvason, Oulu, Finland) cDNA insert was subcloned into pGEM5 and used to prepare ^{35}S labelled RNA probes.

Frozen sections were treated with 0.2 N hydrochloric acid (HCl) for 20 min at room temperature, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and hybridized overnight at 50° C with ^{35}S labelled antisense RNA transcripts. Hybridation mixture contained the radioactive RNA probe, 10 mM Dithiotreitol, 10 mM TRIS-HCl, 10 mM sodium dihydrogen phosphate, (NaH_2PO_4) yeast tRNA (0.2 mg/ml), deionized formamide 40% of the volume, dextran sulfate 10% of the volume, 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrrolidone, BSA (0.2 mg/ml). A 30 µl sample of hybridation mixture (2.10^6 cpm/30 µl hybridation buffer) was placed on each section. Hybridizations were followed by RNase treatment (20 mg/ml, 1 h, 37° C) to remove unhybridized probe and four stringent washes (2×SSC and 1×SSC, 10 min at room temperature) before autoradiography using D 19 emulsion (Kodak). Samples were exposed for 15 days prior to development. The controls were performed under the same conditions using ^{35}S labelled sense RNA probes.

Results

The 15 breast carcinomas were classified according to the World Heath Organisation scheme and the Scarf and Bloom grading as follows: 3 ductal carcinomas grade I, 10 ductal carcinomas grade II, 2 ductal carcinomas grade III. No correlation could be established between gelatinase A expression, the level of hormonal receptors, the grade and the stage of the lesions.

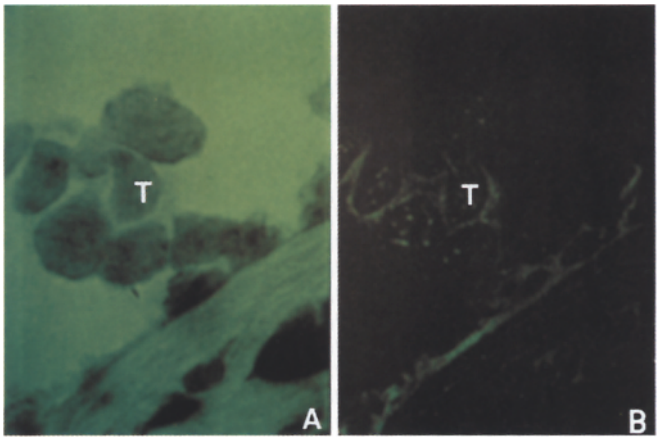


Fig. 1 Confocal laser scanning shows cytoplasmic immunolabelling for gelatinase A of pre-invasive cancer cells (*T*). **A** Counterstaining. **B** Immunofluorescence. (×4000)

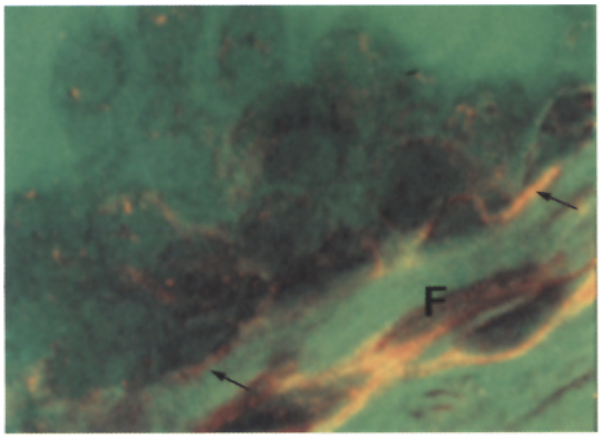


Fig. 2 Confocal laser scanning shows a cytoplasmic immunolabelling of fibroblasts (*F*) and a irregular labelling at the periphery of tumour clusters along the basement membrane (*arrows*). Combination of immunofluorescence and counterstaining. (×4000)

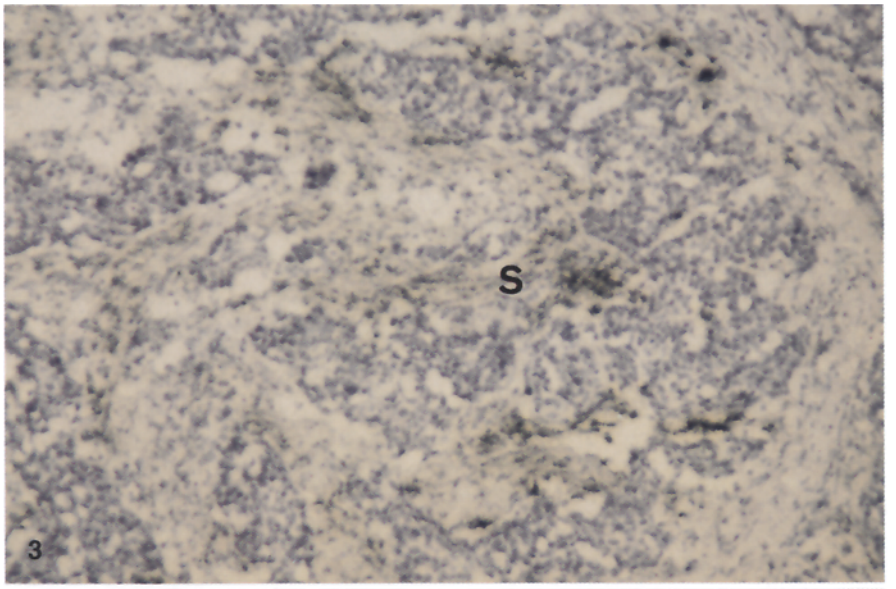
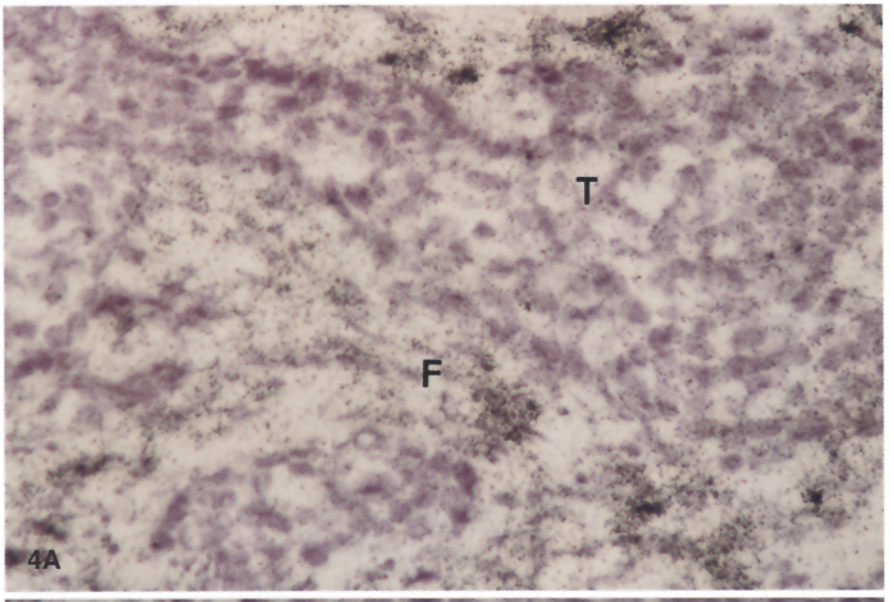


Fig. 3 Stromal spindle cells (*S*) hybridized for mRNAs encoding for gelatinase A near pre-invasive tumour clusters. (×100)



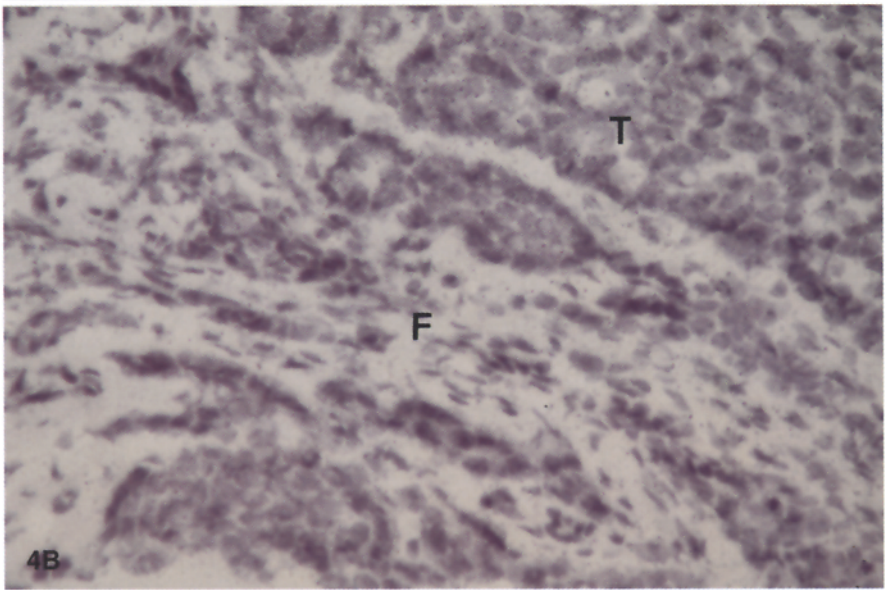


Fig. 4 **A** In situ hybridization signals for mRNAs encoding for gelatinase A were found in the fibroblasts (*F*) in close contact to well differentiated cancer cells (*T*). **B** Same area treated with sense RNA probe as control reaction. (×250)

Most pre-invasive and invasive tumour cells were labelled immunohistochemically in the 15 carcinomas examined. The labelling was cytoplasmic as shown by confocal microscopy (Fig. 1). Some elongated stromal cells considered to be fibroblasts or myofibroblasts were stained.

In some pre-invasive areas of five tumours, intraductal cancer cells were negative whereas stromal (vimentin positive) spindle cells were decorated. In these territories, confocal microscopy showed that gelatinase A was distributed in the fibroblastic compartment and along the basement membrane of the unstained pre-invasive tumour clusters (Fig. 2).

Gelatinase A mRNAs expression was only detected in stromal cells, especially near pre-invasive or well differentiated tumour clusters (Figs. 3, 4A). Specificity of hybridization was confirmed using a sense ^{35}S labelled probe (Fig. 4B). Gelatinase A mRNA expression was not significant in cancer cells and was identical to the low and uniform grain distribution obtained with the control sense probe.

Discussion

Our study on 15 breast carcinomas revealed, a strong and extensive labelling of most cancer cells with a gelatinase A antibody by IH examination using confocal microscopy. These observations are similar to the previous data of Monteagudo et al. [15], except that we found some positivity in occasional stromal cells.

Of particular interest is the pattern observed in some pre-invasive areas. Stromal cells (fibroblasts and/or myofibroblasts), separated from cancer nests by bundles of connective tissue fibres, were labelled by IH. By contrast, in these regions, intraductal tumour cells were negative with IH. We analysed the gelatinase A distribution using the confocal laser scanning microscopy which enhances the resolving power of fluorescence microscopy [1]. A series of optical sections collected in XZ or XY sections confirmed the cytoplasmic labelling of fibroblasts and demonstrated, in these pre-invasive areas, only a faint linear labelling at the periphery of the unstained tumour cells close to the adjacent basement membranes.

Moreover, as demonstrates by the ISH study, we showed that gelatinase A mRNAs were present exclusively in elongated stromal cells associated with bundles of connective tissue fibres, identified as fibroblasts or myofibroblasts, especially near non-invasive and well differentiated tumour clusters. These ISH results are similar to those of Pyke et al. [22, 23] and Poulsom et al. [20] concerning human skin and colon cancers. A significant expression of mRNAs encoding gelatinase A was noted within the stromal cells of these carcinomas. Similarly, Basset et al. [3] detected mRNAs for the metalloproteinase stromelysin 3 exclusively in stromal cells of human mammary cancers.

Our results support the hypothesis that stromal cells can secrete gelatinase A and deliver this enzyme to tumour cells. These cancer cells may either bind gelatinase A at their surface and/or internalize it. Emonard et al. [12] recently described a tumour cell surface associated binding site for the gelatinase A in human breast adenocarcinoma cells. Recent in vitro studies have also revealed a cell membrane associated activation that is selective for the gelatinase A proenzyme and is inhibited by the tissue inhibitor of metallo-proteinases TIMP 2 [5, 7, 26]. Thus, the pattern of gelatinase A distribution, in pre-invasive areas at the periphery of the negative tumour clusters, may be a snapshot of the beginning of a dynamic process leading to the degradation of the basement membranes.

In conclusion, our findings, obtained with two complementary methodologies, plead for an active cooperation between stromal and tumour cells for the expression of gelatinase A. The mechanisms responsible for the production of gelatinase A by the peritumoral host fibroblasts remain to be elucidated. Cancer cells might secrete cytokines to stimulate the production of proteolytic enzymes by stromal cells which participate to the extracellular matrix modifications [17, 18].

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