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Expression of Cathepsin B and Cystatin C in Human Breast Cancer

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Abstract Cathepsin B, which was originally found to be a lysosomal cysteine protease, is also an important matrix protease. In this study, we investigated the expression of cathepsin B and cystatin C, the strongest inhibitor of cathepsin B, and measured the relative amounts of each in human breast cancer tissues. Cystatin C expression relative to cathepsin B expression was found to be decreased. This finding could be associated with the looseness of cancerous interstitial tissue, which might play a role in cancer invasion and metastasis. This report documents the first simultaneous investigation of cathepsin B and cystatin C in breast cancer tissues.

Key words Cathepsin B · Cystatin C · Breast cancer

Introduction

For cancer cells to invade and metastasize, the proteins that compose basement membranes and connective tissues[1] must be degraded. Cathepsin B, which was originally found to be a lysosomal cysteine protease, is an important matrix protease frequently expressed in highly metastatic cancer cells.[2–14] An elevation of plasma procathepsin B activity in breast cancer patients has also been described. Furthermore, cathepsin B has been reported to be present not only in lysosomes, but also on the surface of cancer cells.[11] Activated cathepsin B is thought to be involved in cancer invasion and the destruction of basement membranes by degrading collagen, laminin, and proteoglycan, and promoting the activities of procollagenase[15] and the urokinase type of plasminogen activator.[16]

In normal tissues, the function of cathepsin B is considered to be strictly controlled by stefin A and cystatin C, which are cysteine protease inhibitors.[10,17] In particular, cystatin C is known to be the strongest inhibitor of cathepsin B with a very low inhibition constant of 0.25.[18]

A number of reports have focused on either cysteine protease[2–10] or cysteine protease inhibitor[19] in cancers. Therefore, based on the hypothesis that a loss of balance between cysteine protease and the cysteine protease inhibitor leads to cancer invasion, we investigated the expressions of cathepsin B and cystatin C in human breast cancer tissues.

Materials and Methods

Materials

The subjects of this study comprised 141 patients with primary invasive carcinoma (t1, t2) of the breast who underwent surgical excision between June 1989 and April 1994, none of whom had received preoperative neoadjuvant therapy. Patients were classified according to the General Rules for Clinical and Pathological Recording of Breast Cancer issued by the Japanese Breast Cancer Society. There were 47 patients with papillotubular carcinoma (pap), 19 with solid-tubular carcinoma (sol), 49 with scirrhous carcinoma (sci), and 16 with other carcinomas, as well as 10 with mastopathy. As normal (benign) control tissues, noncancerous tissues surrounding the cancer lesions and excised specimens from benign diseases of the mammary gland were used.

Specimens were fixed in 10% formalin solution and embedded in paraffin (a), or fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C, for 6 h, followed by penetration with 30% sucrose-

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supplemented PBS at 4°C, for 18 h, stepwise from 10% to 30%, freezing in OCT compound (Tissue Tek; Sakura, Torrance CA, USA), then cryopreservation at −80°C (b), or cryopreserved as fresh specimens without fixation (c).

Staining Methods

H&E Staining

The 10% formalin-fixed, paraffin-embedded specimens and 4% paraformaldehyde-fixed specimens described above were cut into 3–4-μm-thick sections, and stained.

Immunohistochemical Staining

The above preparations were stained by the streptavidin-biotin complex method (sABC method). The formalin-fixed preparations were treated with microwaves in 0.1 M citrate buffer, pH 6.0, to activate antigens at appropriate time points. The antibodies used were anti-cathepsin B antibody (The Binding Site, Birmingham, UK, ×50),[20,21] and anti-cystatin C antibody (BioPur, Bubendorf, Switzerland, ×200), and reacted using sABC kits (Nichirei, Tokyo, Japan). The other primary antibodies used were: anti-ER (Immunotech, Marseille, France, ×2), anti-Von Willebrand factor (Dako, Glostrup, Denmark, ×300), anti-c-erbB2 gene product (Nichirei, ×60), and anti-mutant p53 oncoprotein (Novocastra, Newcastle upon Tyne UK, ×800).

Semiquantification by the Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Method

Total RNA was extracted from 2-mm-square freshly frozen materials, and cDNA was synthesized using first-strand cDNA synthesis kits (Boehringer Mannheim, Mannheim, Germany). RT-PCR was performed using this cDNA as a template.

For human cathepsin B, two oligonucleotides, 5′-TTC GAT GCA CGG GAA CAA TG-3′ (sense), and 5′-GGC CTT TTC TTG TCC AGA AG-3′ (antisense), were prepared and used as primers.[22] For human cystatin C, the oligonucleotides, 5′-GCT CTT TCC AGA TCT ACG CT-3′ (sense) and 5′-AGG CAG CCG ATG CTA CTA TT-3′ (antisense), were prepared and used as primers.[23] As an endogenous control, the primers for β-actin, 5′-TAC ATG GCT GGG GTG TTG AA-3′ (sense) and 5′-AAG AGA GGC ATC CTC ACC CT-3′ (antisense), were used.[24] To determine the PCR conditions, using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA), and a 50–100 ng template DNA, 200 μM dNTP, 2 pM of each primer, and 2 U Taq DNA polymerase (Takara Shuzo, Tokyo, Japan), we performed 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 2 min. The product was electrophoresed on 2% agarose gel, and bands of cathepsin B, cystatin C, and β-actin were confirmed at positions corresponding to approximately 252, 277, and 218 bp, respectively. Using image analysis software NIH Image version 1.56, each band was converted to a numerical value, and the ratios of cathepsin B and cystatin C to the endogeneous control β-actin were calculated for semiquantification.[25,26] The number of PCR cycles selected was 30 because expotential amplification of all PCR products was maintained for 30 cycles in preliminary experiments. As a negative control, a sample to which avian myeloblastosis reverse transcriptase (AMV-RT) was not added during cDNA synthesis was used.

Results

On immunohistochemical examination, cathepsin B was found to be localized in the cytoplasm and on the cell membrane of cancer cells in human breast cancer tissue (Fig. 1). Cystatin C was also localized in the cytoplasm and cell membrane of the cancer cells as well as in the stromal fibroblasts of the cancer tissue (Fig. 2). Therefore, expressions of both cathepsin B and cystatin C were detected in the cancer cells of breast cancer tissue.

Analysis of the expressions of cathepsin B and cystatin C mRNAs by RT-PCR showed that cathepsin B and cystatin C mRNAs were expressed in both cancer and noncancerous tissues (Fig. 3). As the endogenous control, β-actin was analyzed using image analysis software NIH Image version 1.56, and (cathepsin B mRNA expression level) / (β-actin mRNA expression level), (cystatin C mRNA expression level) / (β-actin mRNA expression level), and (cystatin C mRNA expression level) / (cathepsin B mRNA expression level) were found in the tissue. According to the *t*-test, significantly lower expression of cystatin C relative to cathepsin B expression was detected in the cancer tissue (P(2-tail) $<$ 0.05, Table 1). The cathepsin B mRNA expression tended to be higher in the cancer tissue than in the noncancerous tissue, but no significant difference was observed (Table 1). The cystatin C mRNA expression tended to be lower in the cancer tissue than in the noncancerous tissue, but there was no significant difference (Table 1).

There were no correlations between the histological classification of cancer and the cathepsin B mRNA level, cystatin C mRNA level, or cystatin C mRNA level / cathepsin B mRNA level. Nor were there any correlations between cathepsin B and cystatin C expression in individual subjects, or between the ratio of cystatin C to cathepsin B and lymph node metastasis, histologic

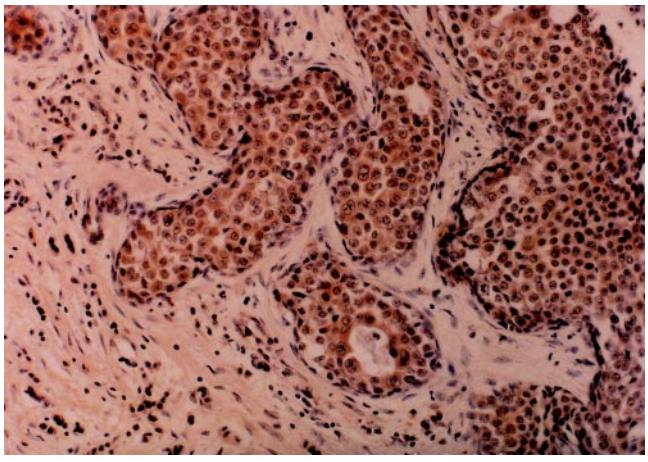

Fig. 1. Immunohistochemical staining. Cathepsin B was localized in the cytoplasm and on the membrane of cancer cells (×100)

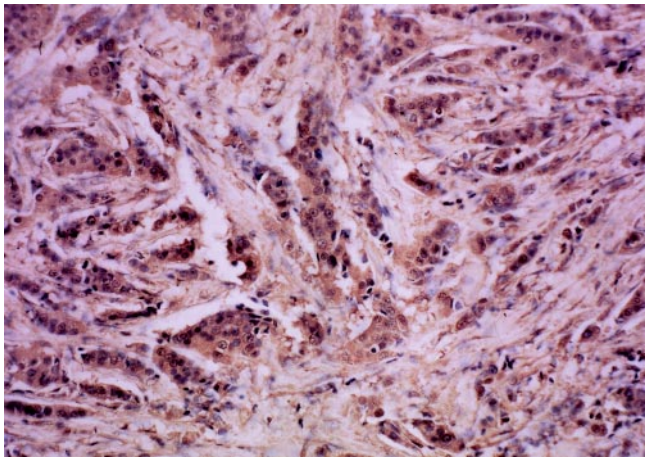

Fig. 2. Immunohistochemical staining. Cystatin C was localized in the cytoplasm and on the membrane of cancer cells, as well as in the stromal fibroblasts (×100)

Table 1. Expression of cathepsin B and cystatin C mRNAs in human breast tissues by semiquantitative reverse transcriptase–polymerase chain reaction (mean)

	CathepsinB/ β-actin	CystatinC/ β-actin	CystatinC/ cathepsin B
Cancer	1.32 (ND)	1.26 (ND)	2.54 ($P < 0.05$)
Noncancer	0.63	4.62	5.69

ND, not determined

grade, hormone receptor, tumor diameter, c-erbB2 overexpression, or mutant p53 expression.

Since venous invasion was observed in only three patients, the significance of the difference could not be determined.

Discussion

The invasion of cancer cells requires an activation of matrix proteases that locally degrade proteins composing the basement membranes and connective tissues.[1] Cathepsin B, primarily a lysosomal cysteine protease, is an important matrix protease frequently expressed in highly metastatic cancer cells.[2–14] An elevation of plasma procathepsin B activity in breast cancer patients has also been reported, while cathepsin B is present not only in the lysosomes, but also on the surface of cancer cells.[11] It is known not only to directly degrade collagen, laminin, and proteoglycan at a pH optimally around neutral, but also to activate procollagenase[15] and the urokinase type of plasminogen activator.[16] These mechanisms by cathepsin B are suspected to be involved in cancer invasion or the destruction of basement membranes. A correlation between the cathepsin B mRNA level and cathepsin B activity,[13,14] and a high cathepsin B activity in poorly differentiated adenocarcinoma of gastric cancer have been reported,[4,5] but the difference among histological classifications in breast cancers remains unclear.

In normal tissues, cathepsin B is considered to be strictly controlled by stefin A and cystatin C, which are cysteine protease inhibitors.[10,17] In fact, cystatin C is known to be the strongest inhibitor of cathepsin B, with a very low inhibition constant of 0.25.[18] Generally, cysteine proteases in normal tissue are regulated by the endogenous inhibitors, stefin A and B, and the extracellular inhibitor, cystatin C.[17,27]

In contrast, stefin A activity has been reported to be low in lung adenocarcinoma[28] and breast cancers.[19] Moreover, there have been no reports of localization in human breast cancer tissues or expression of cystatin C, which is the strongest inhibitor of cathepsin B. Although cystatin C expression has been reported in melanoma[29] and lung tumors,[28] it has not been reported in breast cancer. To our knowledge, this report is the first documentation of a simultaneous investigation of cathepsin B and cystatin C in breast cancer tissues.

On immunohistochemical examination, both cystatin C and cathepsin B were expressed in the cytoplasm and on the cell membrane in cancer cells in breast cancer tissue. Furthermore, cystatin C was localized not only in cancer cells but also in fibroblasts. This finding indicates that the understanding of the pathological state of cancer cells in tissue was insufficient in previous studies focusing on either cystatin C or cathepsin B. A quantitative imbalance between matrix metalloproteinase (MMP) and the tissue inhibitor of matrix metalloproteinase (TIMP) was reported to be a factor inducing metastatic invasion.[30] If the relationship between cathepsin B and cystatin C is similar to that between MMP and TIMP, although both substances are highly

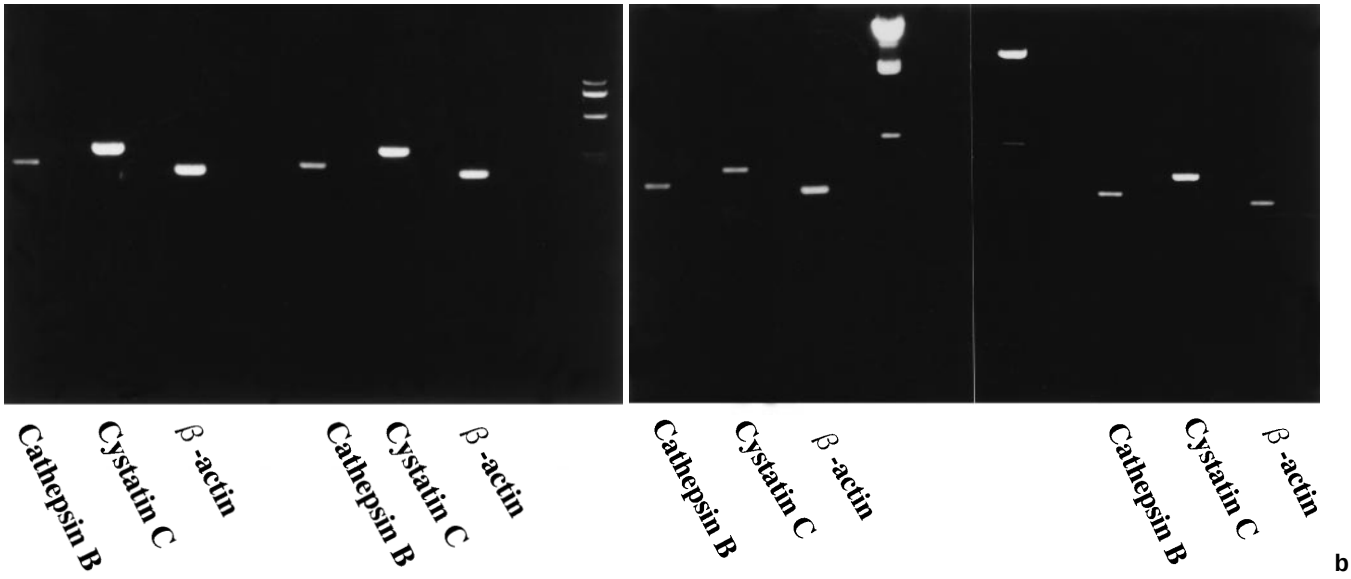

Fig. 3a,b. Reverse transcriptase–polymerase chain reaction analysis of cathepsin B and cystatin C mRNAs. β-Actin was the endogenous control. **a** Noncancerous tissues. **b** Cancerous tissues

expressed, an appropriate balance between cathepsin B and cystatin C may maintain homeostasis. Moreover, the precise mechanism regulating the balance between cystatin C and cathepsin B in cancer tissue in vivo may not be clarified by in vitro experiments using cancer cells only, since the cystatin C expressed by fibroblasts is not taken into consideration.

On investigation of the mRNA expressions of cathepsin B and cystatin C by RT-PCR, it was found that the cathepsin B and cystatin C mRNAs were expressed in both cancerous and noncancerous tissues. Therefore, there were no breast tissues that expressed either cathepsin B or cystatin C.

On analysis of the results obtained by RT-PCR using image analysis software, a significant reduction in relative cystatin C expression to the cathepsin B expression was detected in breast cancer tissue. This finding might be associated with the looseness of cancerous interstitial tissue, caused by a relatively low expression ratio of cystatin C to cathepsin B which therefore plays a role in cancer invasion and metastasis.

There were no correlations between cathepsin B and cystatin C expressions in histological classifications, lymph node metastasis, tumor diameter, or hormone receptors. The imbalance between cathepsin B and cystatin C expression may occur in the early stages of cancer progression and growth.

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