The Osteoclast-associated Protease Cathepsin K Is Expressed in Human Breast Carcinoma

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ABSTRACT

Human cathepsin K is a novel cysteine protease previously reported to be restricted in its expression to osteoclasts. Immunolocalization of cathepsin K in breast tumor bone metastases revealed that the invading breast cancer cells expressed this protease, albeit at a lower intensity than in osteoclasts. In situ hybridization and immunolocalization studies were subsequently conducted to demonstrate cathepsin K mRNA and protein expression in samples of primary breast carcinoma. Expression of cathepsin K mRNA was confirmed by reverse transcription PCR and Southern analysis in a number of human breast cancer cell lines and in primary human breast tumors and their metastases. As this protease is known to degrade extracellular matrix, including bone matrix proteins, it is possible that cathepsin K may contribute to the invasive potential of breast cancer cells, including those that metastasize to bone. Thus, cathepsin K may be a potential target leading to the design of novel drugs for cancer therapy.

INTRODUCTION

Cathepsins are a family of proteases that have been proposed to be involved in the progression of a number of disease processes including rheumatoid arthritis (1) and tumor invasion (2). Recently, a novel cysteine protease, cathepsin K, was cloned from rabbit and human osteoclast-derived libraries (3, 4) and was found to exhibit a variable amino acid homology to other members of the cathepsin family, ranging from 17% with cathepsin D to 48% amino acid homology with cathepsin S.

We have previously demonstrated the osteoclast-specific expression of cathepsin K using immunolocalization and in situ hybridization techniques in various normal and bone disease conditions and also in multinucleated cells in giant cell tumor of bone (5). However, in sections of human bone in which skeletal breast cancer metastases were present, we have also observed a positive immunoreactivity, albeit of lower intensity, in the breast cancer cells that had invaded the bone tissue. This unexpected observation has lead us to investigate further the possible association of the expression of cathepsin K in other human breast cancer tissues and cells. In the current study, we therefore analyzed the expression of cathepsin K using immunocytochemistry, in situ hybridization, and RT-PCR in surgically removed primary breast tumors, bone metastases, and human breast cancer cell lines cultured in vitro.

MATERIALS AND METHODS

Materials. Breast cancer cell lines (purchased from ATCC unless otherwise stated) were classified into subtypes: infiltrating ductal carcinoma (ZR75—1, Hs578T duct, BT474, ZR75—30, BT 549, and T-47D), adenocarcinoma (MDA-MB-231, SK-BR3, MDA-MB-468, and BT20), and breast carcino ma (MVLN and MTLN, transfected MCF-7-derived clones obtained from Dr. J. C. Nicolas (Unit de Recherches sur la Biochemie des Steroides Inserm US8, Montpellier, France), and MCF-7). The MCF-7 cells were obtained from two different sources, ATCC and Dr. Roland Schuele (Tumor Klinik, Freiburg, Germany). These are indicated in the figure legend as clone 1 (ATCC) and clone 2 (Dr. Schuele). Other breast lines consisted of HBL-100 breast epithelial line isolated from the milk of a nursing mother, two different cultures of MCF10A (from two laboratories in Novartis Pharma AG) derived from mammary tissue of a patient with fibrocystic breast disease, the human mammary epithelial line HMEC4144 (Clonetics, San Diego, CA), and MTSV1.7neo, a nonmalignant human breast-derived cell line (6). RNA samples from various primary breast tumors and their metastases were obtained from the Cancer Tissue Bank Research Center (University of Liverpool). All primary carcinoma tissues were graded by the modified Bloom and Richardson system (7).

Immunohistochemistry. Blocks of tissue were fixed with formaldehyde for 24 h prior to paraffin embedding and were further subjected to fixation in methylacarn. Paraffin sections (5 μm) were mounted onto Vectabond (Vector Laboratories, Pethborough, United Kingdom)-treated slides and dried overnight in a 50°C oven before dewaxing with xylene. The immunohistochemistry was performed as described previously (5). A specific chicken anticithepsin K antibody was used for the immunostaining. This antibody has been characterized and validated for selectivity by Western blot analysis, which revealed that the antibody reacted only with cathepsin K and not with any other cathepsins (5, 8).

In Situ Hybridization. An EcoRI/SalI cDNA fragment corresponding to the first 455 bp from the ATG of the human cathepsin K gene was cloned into Bluescript KS+ and checked in GenBank to ensure that there was no homology to other sequences that would result in cross-hybridization of the probes. Sense and antisense RNA probes for in situ hybridization were labeled with the SP6, T7, T3 in vitro transcription kit with DIG-labeled UTP (Boehringer Mannheim) according to the manufacturer’s instructions. The RNA probes were stored at —20°C.

Freshly excised tissue from surgical procedures was submerged in pre-chilled n-hexane, transferred into sterile 50-mL Falcon tubes, and stored at —70°C until use. A cryostat was cooled to —35°C and 10 micron sections were cut and mounted onto 3-aminoPropyltriethoxysilane-coated slides. The in situ hybridization procedure was performed as described by Komminoth (9). For the detection of the DIG-labeled probes, the protocol of the manufacturer’s kit was used (Boehringer Mannheim). The reaction was stopped in 10 mM Tris, 1 mM EDTA, pH 8.1. Slides were dipped briefly in water, counterstained with 1% (w/v) light green in 1% acetic acid, mounted, and photographed.

RT-PCR Analysis. Total RNA was reverse transcribed according to the manufacturer’s instructions (Promega). For the primary breast tumor samples and their metastases, 2.5 μg of total RNA were used for cDNA synthesis in a 50-μL reaction volume. Two primers for cathepsin K were designed and used to amplify an expected product of 617 bp (sense, 5’-TGC CCG CAG TAA TGA CAC C; antisense, 5’-TTT CCC CAG TTT TCC CCC C). In a 100-μL reaction volume, the following components were added: 10 μL of PCR buffer (10× stock, Boehringer Mannheim), 5 μL of DMSO, 2 μL of dNTP mixture (10 μM stock), 0.5 μg of each primer, 0.5 μL of Taq polymerase (Boehringer Mannheim), water to 97 μL, and 3 μL of reverse-transcribed RNA. The PCR was performed so that the amplification was in the log phase. The conditions of denaturation, annealing, and extension used were 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 48°C for 1.5 min, and 72°C for 2.5 min. At the end of the cycling phase, a further extension period of 10 min at 72°C was

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2. The abbreviations used are: RT-PCR, reverse transcription PCR; ATCC, American Type Culture Collection; DSG, digoxigenin.
Fig. 1. Immunoreactivity of cathepsin K in breast cancer cells. A, immunolocalization of cathepsin K in a skeletal metastasis of breast tumor. Positive staining can be seen in osteoclasts (medium arrows), adjacent to bone matrix (small arrow), and in breast cancer cells (large arrow), present in the marrow space. B, invasive carcinoma of no special type (grade 3) showing strong cytoplasmic positivity. The stromal cells are negative. C, intravascular, probably intralymphatic, tumor showing strong cytoplasmic positivity (arrow). The adjacent arteriole shows positivity of the smooth muscle coat. D, nonneoplastic terminal duct lobular units showing microcystic change. There is cytoplasmic positivity that is restricted to the myoepithelial cells. E, a normal terminal duct lobular unit showing no positivity. F, ductal carcinoma in situ of high nuclear grade and comedo growth pattern showing strong cytoplasmic positivity. There is weak staining of the stromal myofibroblasts. G, same tumor as shown in B, exhibiting negativity for cathepsin K in this particular area. There is vascular invasion in this field, and the intravascular tumor is also negative. H, invasive carcinoma of no special type (grade 2) showing strong cytoplasmic positivity. The stroma is negative except for the pericytes of small blood vessels.
CATHEPSIN K IN BREAST CARCINOMA

Fig. 2. Localization of cathepsin K mRNA in an invasive ductal carcinoma (grade 2). All sections were counterstained with 1% (w/v) light green in 1% (v/v) acetic acid. A, in situ hybridization with a DIG-labeled cathepsin K antisense RNA probe. The epithelial cells are expressing cathepsin K as visualized by the purple coloration (arrow). B, serial cathepsin K sense RNA probe hybridized section. This shows no background hybridization of the sense probe.

performed, the PCR reactions were electrophoresed on a 1% (w/v) agarose gel, and the bands were visualized with ethidium bromide.

Southern Analysis. The amplified DNA products were transferred overnight onto a Hybond membrane (Amersham Corp.) in 0.4 M NaOH and 0.6 M NaCl. The membrane was hybridized at 42°C overnight with the full-length cathepsin K cDNA probe labeled with [32P]dATP (Amersham).

RESULTS

Immunolocalization of Cathepsin K in Metastatic and Primary Breast Cancer Tissues. Fig. 1A demonstrates the immunohistochemical localization of cathepsin K in breast cancer cells of a bone metastasis in the bone marrow cavity. Although the intensity of the staining was lower than that observed in the osteoclasts, the protein was clearly present within the tumor cells. This observation led us to extend our study, investigating the expression of cathepsin K in primary breast tumors (Fig. 1, B—H). In sections of all five primary carcinomas and the one metastatic carcinoma studied, moderate to strong positivity was seen in the cytoplasm of between 20 and 90% of tumor cells. Staining was heterogeneous, however, being present in some parts of the tumor (Fig. 1, B and H) but not in others (Fig. 1G). Both positive and negative cathepsin K immunostaining was observed in intravascular tumor (Fig. 1, C and G). Insufficient cases were studied by this method to determine whether there was any relationship between cathepsin K immunopositivity and any other pathological characteristics, such as tumor grade, estrogen receptor status, or metastatic potential. Immunoreactivity was also localized in the cytoplasm of smooth muscle cells forming the walls of stromal blood vessels (Fig. 1C) and in myofibroblasts (Fig. 1F). Four cases of ductal carcinoma in situ accompanying the invasive carcinoma or occurring in the absence of invasive disease showed similar heterogeneous positivity (Fig. 1F). In sections of two normal breast tissue samples, immunostaining was either negative or only weakly positive, localized either in the epithelial or myoepithelial cells (Fig. 1, D and E) of a limited number of lobules or ducts. Sections of a normal male breast were completely negative (data not shown). No staining was observed with the omission of the primary antibody, indicating the specificity of the immunolocalization reaction (data not shown).

In Situ Localization in Primary Breast Carcinomas. In situ hybridization of cathepsin K mRNA showed a histological pattern of expression similar to that observed for the protein as assessed by immunohistochemistry. Hybridization with the antisense RNA probe revealed an intense signal within the tumor cells of an invasive ductal carcinoma (Fig. 2A). No signal was observed in the serial section incubated with the sense RNA control (Fig. 2B).

RT-PCR Analysis of Human Breast Cancer Cell Lines. RT-PCR analysis using specific cathepsin K primers revealed the pres-
The primers were specifically designed to span an intron to eliminate their identity as cathepsin K transcripts. When the RT-PCR different lines were subcloned and sequenced to confirm unequivocally the origin of the tissue and the sensitivity of the detection method used. It thus seems likely that the occasional cathepsin D immunostaining observed in the stromal cells correlated with the increased likelihood of tumor metastasis and decreased survival time of the patient.

Both cathepsin K and cathepsin D are active under acidic conditions and have been shown to be capable of degrading extracellular matrix proteins, providing a possible mechanism by which breast cancer cells can metastasize to secondary sites. Indeed, bone, which is a frequent site for breast tumor metastasis, is rich in osteonectin and collagen, which are both substrates for the degradative action of cathepsin K. The identification of cathepsin K in primary breast cancer cells and in cells that are metastatic to the bone indicates that this protease may play a contributory role in tumor cell invasion.

The mechanisms by which breast cancer metastases induce local bone destruction are unknown. Animal models have been used to demonstrate that tumor cells induce osteolysis by enhancing the process of osteoclast-mediated bone resorption. It is likely that tumor cells release soluble factors, such as cytokines and growth factors, which regulate bone cell metabolism within the vicinity of the metastasis. There is also evidence that tumor cell lines, including MCF-7, and tumor-associated macrophages can directly resorb bone. In the present study, we demonstrated by RT-PCR analysis that MCF-7 cells express cathepsin K, thus providing an indication that breast tumor cells may be able to directly resorb bone via the release of this proteolytic enzyme. Future studies using neutralizing antibodies, antisense oligonucleotides, or inhibitors of cathepsin K should help to clarify this potential association. Thus, demonstrated with cathepsins D, B, and L. In the present study, none of the breast epithelial cell lines derived from noncancerous breast tissue expressed cathepsin K as assessed by RT-PCR followed by subsequent Southern analysis. However, weak immunopositivity was seen in histological sections of normal ducts and lobules, principally in the myoepithelial cells. The reasons for this apparent discrepancy are not yet clear, but myoepithelial differentiation, stromal contact, and the proximity of carcinoma are possible factors based on current observations.

Our cathepsin K in situ hybridization and immunolocalization data parallel other studies that have investigated the expression of cathepsin D in breast cancer tissue. Cathepsin D is a ubiquitously expressed acidic protease that is overproduced by breast cancer cells and has been associated with the proliferative and invasive potential of breast carcinomas. Interestingly, this protease was present in a high proportion of, but not all, breast tumors. Others have demonstrated that the occasional cathepsin D immunostaining observed in the stromal cells correlated with the increased likelihood of tumor metastasis and decreased survival time of the patient.

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![Image](image-url)

**DISCUSSION**

This is the first report demonstrating the expression of cathepsin K in breast cancer tissue and cell lines. Previously, this protein has only been identified in osteoclasts and the cells responsible for bone resorption. Drake et al. (10) failed to detect cathepsin K in a panel of normal human tissues including lung, kidney, heart, spleen, and liver. In addition, they observed little or no expression of cathepsin K in cDNA libraries derived from ovarian, prostatic, and pancreatic cancers. In the present study, cathepsin K immunopositivity was seen in normal breast tissue, but this was weak in intensity and limited to epithelial and myoepithelial cells. It thus seems likely that the detection of cathepsin K in normal tissue may be determined by the origin of the tissue and the sensitivity of the detection method used.

In the current study, we localized cathepsin K in primary and metastatic breast tumors by the techniques of in situ hybridization and immunolocalization. Furthermore, we amplified cathepsin K by RT-PCR in breast cancer cell lines and in primary and metastatic breast tumors, indicating that the gene for this protease is expressed in these tissues. It would be of interest to perform a quantitative study to correlate cathepsin K levels with tumor invasiveness, as others have

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3 Unpublished observations.

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**Table 1** Details of primary breast carcinomas used for RT-PCR

<table>
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<th>Sample no.</th>
<th>Tumor tissue description</th>
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<tr>
<td>2</td>
<td>Infiltrating lobular carcinoma, grade 2</td>
</tr>
<tr>
<td>3</td>
<td>Infiltrating ductal carcinoma, grade 3</td>
</tr>
<tr>
<td>4</td>
<td>Infiltrating ductal carcinoma, grade 3</td>
</tr>
<tr>
<td>5</td>
<td>Infiltrating ductal carcinoma, grade 2</td>
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**Table 2** Details of metastatic breast carcinomas

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<tr>
<td>2</td>
<td>Metastasis of infiltrating ductal carcinoma, grade 3, removed from auxiliary lymph node</td>
</tr>
<tr>
<td>3</td>
<td>Metastasis of infiltrating ductal carcinoma, grade 3, removed from auxiliary lymph node</td>
</tr>
<tr>
<td>4</td>
<td>Metastasis of infiltrating ductal carcinoma, grade 3, removed from auxiliary lymph node</td>
</tr>
</tbody>
</table>

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**Fig. 4.** RT-PCR of breast tumor samples. *Top panel,* ethidium bromide-stained 1% (w/v) agarose gel. A 1-kb DNA ladder (Life Technologies, Inc.) was used as a marker. The 617-bp RT-PCR product was present in the positive control tissue, osteoclastoma, and in five of six primary and four of four metastatic breast tumors. *Bottom panel,* Southern blot of the agarose gel probed with the full-length cathepsin K probe.
cDNA samples.

ACKNOWLEDGMENTS

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REFERENCES


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