Inhibitory Effects of Antisense Cathepsin B cDNA Transfection on Invasion and Motility in a Human Osteosarcoma Cell Line

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ABSTRACT

Increased activity, membrane association, and secretion of cathepsin B have been shown to correlate positively with invasiveness and the metastatic properties of many tumor entities. Cathepsin B is able to directly facilitate invasion by degrading extracellular matrix components or to indirectly facilitate invasion by activating other matrix-degrading proteases like the urokinase-type plasminogen activator. To investigate the role of cathepsin B in bone tumor invasion, the osteosarcoma cell line MNNG/HOS was stably transfected with an expression vector capable of expressing the antisense cDNA transcript of cathepsin B. Five stably transfected antisense cell clones, the control (vector) cell clones, and the parental cells were characterized. At first, the stable incorporation of the constructs was demonstrated by Southern blot analysis. In ELISA assays, all antisense clones showed a significant reduction at the cathepsin B antigen level (about 70%) as compared with the control cell clones and MNNG/HOS. Similar results were obtained for cathepsin B activity in the antisense-transfected cells. In the antisense cell clones, Northern blot analysis and reverse transcription-PCR revealed a considerable decrease of ~50% in the levels of cathepsin B mRNA. Expression of cathepsins L and K (sequence homologies) was not affected. The invasive potential and migration of untransfected and transfected tumor cell clones in vitro were analyzed in Transwell chambers. Antisense-transfected cells showed a markedly lower invasion and motility than did MNNG/HOS and the controls. Adhesion to collagen I and matrigel matrices was not affected. These results demonstrate that cathepsin B is involved in the complex proteolytic processes in invasive osteosarcomas.

INTRODUCTION

Penetration and degradation of extracellular matrix elements during intravasation and extravasation of metastasizing tumor cells are key steps in the metastatic cascade of cancer cells (1). A variety of proteolytic enzymes are involved in these processes. Besides the MMPs (2) and serine proteases (uPA, plasmin; Ref. 3), the cysteine proteases, cathepsin L (4) and cathepsin B (5), are described to be associated with malignancy.

Cathepsin B, which is normally present in the lysosomes of various cell types, participates in the turnover of cellular components as well as in the degradation of molecules assimilated from the extracellular environment. Malignant tumor cells (lung, colon, breast, and ovary) express higher levels of cathepsin B mRNA and protein (for review, see Ref. 6). In addition, cathepsin B changes its localization as the malignancy of human tumors progresses (7, 8). Immunohistochemistry and enzymatical studies have revealed alterations in the intracellular localization of cathepsin B (at the invasive edges) as well as the existence of secreted and membrane-bound cathepsin B in different tumor entities (for review, see Ref. 9) and tumor cell lines (10). It has been shown that under slightly acidic conditions, as is found in the peritumoral environment, the secretion of active cathepsin B is enhanced (11). Furthermore, cathepsin B is able to degrade basement membrane proteins under acidic or neutral pH conditions either directly or indirectly by activating other proteases like the receptor-bound pro-uPA (12). Once activated, uPA can activate plasmin and procathepsin B. Both the morphological findings and the spectrum of cathepsin B substrates suggest that cathepsin B may play an important role in malignant tumor diseases. In in vitro invasion assays, Kobayashi et al. (13) and Kolkhorst et al. (14) tested several cell lines treated with cathepsin B inhibitors or anticalylic antibodies. Decreased cathepsin B activity resulted in a reduced invasion of treated cells in most tumor cell types. However, attempts to down-regulate cathepsin B expression at the mRNA level have not been described thus far.

Therefore, the study presented here shows the specific inhibition of both cathepsin B protein and mRNA expression by antisense RNA in the human osteosarcoma cell line MNNG/HOS. Alterations in invasion, motility, and adhesion representing different steps in the metastatic process were quantified in relevant functional in vitro assays.

MATERIALS AND METHODS

Cloning of Cathepsin B. Total RNA was isolated from cathepsin B-rich BEAS-2B lung carcinoma cells (kindly provided by Dr. F. Bühl, Magdeburg, Germany) using TRizol reagent (Life Technologies, Karlsruhe, Germany) according to the manufacturer’s instructions. One microgram of RNA was reverse-transcribed using oligo(dT)12-18 primer and SuperScript RNase H− reverse transcriptase (Life Technologies). The 1053-bp cathepsin B cDNA fragment was PCR-amplified using the synthetic primers: Cat-B-5′-GATCTAGATGCGCTTCC-3′ (nucleotide 156–173) and Cat-B-3′-CCCACGCGAGATTATGCT-3′ (nucleotide 1190–1208). The reaction mixture was denatured at 94°C for 30 s, annealed at 56°C for 1 min, and extended at 72°C for 1 min. These reactions were repeated in 30 cycles. The Taq-polymerase-amplified PCR product was purified from an agarose gel using the QIAquick Gel Extraction Spin Kit (QIAGEN, Hilden, Germany) and directly ligated into the eucaryotic pcDNA3/V5/His-TOPO expression vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. To distinguish between pTOPO-ssCatB and pTOPO-ascATB plasmids, the orientation of cathepsin B cDNA in plasmid clones was analyzed by sequencing. Sequence analysis showed 100% homology to the published sequence for cathepsin B cDNA (15). As a negative control, the pcDNA3.1/V5/His-TOPO vector without insert was used in subsequent experiments.

Cell Culture. The human osteosarcoma cell line MNNG/HOS was obtained from the American Type Culture Collection (CRL 1547, Rockville, MD). In addition to the cysteine proteases discussed here, this cell line expresses MMPs (MMP-2 and MMP-9), serine proteases (uPA, plasmin), and the aspartic protease cathepsin D. Cells were grown under standard conditions in RPMI 1640 (PAA, Linz, Austria), supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany) and antibiotics/antimycotics (Life Technologies), and incubated at 37°C in humidified atmosphere containing 5% CO2. The adherent cells were detached from the culture flasks using trypsin/EDTA (Life Technologies). Prior to functional assays, 0.02% EDTA (Sigma, Deisenhofen, Germany) was alternatively used to avoid the destruction of cell surface antigens. Cells were counted in the Coulter Counter ZII (Coulter Immunotech, Marseille, France).

Transfection and Selection. MNNG/HOS cells (5 × 106) were transfected with 1 μg of either pTOPO-ssCatB, pTOPO-ascATB, or the host vector using Lipofectin (Life Technologies) according to the manufacturer’s instructions. Selection was initiated 48 h after transfection by adding 500 μg G418 (Life Technologies)/ml of supplemented culture medium. Selection medium was changed every 4 days for 5 weeks until all nontransformed cells died. Resistant cell clones were isolated and expanded for further characterizations.

Received 5/7/99; accepted 10/4/99.

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1 Supported by the Deutsche Forschungsgemeinschaft (SFB387).

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3 The abbreviations used are: MMP, matrix metalloproteinase; RT, reverse transcription.

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Quantification of Cathepsin B by ELISA and Activity Assay. Transfected and parental tumor cells (1 × 10^5) were grown under serum-free culture conditions for 72 h. Supernatants were removed; confluent tumor cells were counted and lysed with 0.5% Triton X-100 and 0.1 m Tris-HCl (pH 8.1) to get 1 × 10^6 extracted cells/ml. The cathepsin B antigen in cytoplasmic fractions was quantified by using the commercially available human Cathepsin B ELISA test (KKRA, d.d. & J. Stefan Institute, Ljubljana, Slovenia) according to the manufacturer’s instructions. A human cathepsin B standard curve was included in each assay.

Enzyme activity of cathepsin B in cell lysates was determined by using the specific substrate Z-Arg-Arg-AMC (Bachem, Heidelberg, Germany). Pellets of cell clones were lysed in assay buffer [50 mM acetate buffer (pH 5.5), 2.5 mM DTT, 2.5 mM EDTA] with 1% Triton X-100. The reaction was terminated, and the resulting fluorescence was measured by using the Fluorolite microplate reader (Dynex, Chantilly, VA). All quantifications were done in triplicate.

Quantification of Cathepsins B, L, and K mRNA by RT-PCR. Total RNA of MNNG/HOS and transfected cells was extracted and reverse-transcribed as described above. Amplification of cathepsin B, L, and K cDNA was done with the following primer pairs: CatBrf-5′-CACACT-TCTAACAGCGTG-3′ and CatBr-3′-GATGATCTCGGCCATGATG-3′; CatLfr-5′-CACACT-GAGAGGTTGGGACGATGGAGGG-3′ and CatLr-3′-CACACT-TCTAAGTCCTTC-3′; and actin-5′-TGACGGGTCACCCACTGTGGCCCTAC-3′ and actin-3′-CTGGAAG-CATTTCGCAGTAGGACCAGGGG-3′ (680 bp). The lengths of the resulting amplification products in bp are given in parentheses. Complete PCR reactions were initially heated to 94°C for 5 min for denaturation, and specific fragments were amplified in 30 cycles (0.5 min at 94°C, 1 min at 56°C, and 1 min at 72°C). The PCR products were analyzed on a 0.8% agarose gel stained with ethidium bromide and scanned. To avoid saturation of the system, only 1% instead of the usual 10% of the first strand reaction was used for amplification. All oligonucleotide primers were custom synthesized by Eurogentec (Seraing, Belgium).

Southern Blot Analysis. High molecular weight DNA was extracted from confluent cultures of transfected and untransfected cells according to standard procedures, digested with EcoRI and BamHI (Eurogentec) at 37°C overnight, and size-fractionated (20 μg/lane) on a 0.7% agarose gel. DNA fragments were blotted onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Freiburg, Germany) and probed with the full-length cathepsin B cDNA (prepared by PCR) labeled with [32P]dCTP by random primer labeling. Hybridization was performed at 65°C for 4 h, and washed blots were exposed to Kodak BioMax MS-1 film (Sigma-Aldrich, Deisenhofen, Germany) at −70°C.

Northern Blot Analysis. Total cellular RNA was extracted from confluent cultures using TRIZol reagent (Life Technologies) according to the manufacturer’s instructions, electrophoresed on 1.2% agarose-formaldehyde gels (10 μg/lane) and blotted to Hybond N+ membranes. The filters were hybridized at 65°C for 4 h with [32P]-labeled cathepsin B (as above). After stripping, the membranes were rehybridized with β-actin cDNA as a semiquantitative control in densitometric analysis. Autoradiography was done as described above.

In Vitro Adhesion. Cellular adhesion of MNNG/HOS cells and their transfecitants was quantified on collagen I matrices. Twenty-four-well culture (in HBSS) for 30 min at 37°C.

Comparison of Cathepsin B Antigen Concentration and Activity of Control and Antisense Clones. To determine the effect of antisense cathepsin B cDNA transfection on the cathepsin B antigen level of clonal cells, we analyzed the cell homogenates in an ELISA assay. As described above, the cathepsin B antigen level in antisense-transfected cell clones was significantly reduced in comparison with the parental cell line MNNG/HOS and the control transfected MNNG/TOPO (Fig. 1). In the antisense cell clones 3, 8, 13, 17, and 24, cathepsin B concentration was reduced by ~70%.

Comparable results were found for cathepsin B activity in untransfected and antisense-transfected cell clones (Fig. 2). The enzyme activity was clearly reduced by 50–60% in the antisense-transfected cell clones 3, 8, 13, 17, and 24 as compared with MNNG/HOS and MNNG/TOPO.

Molecular Characterization of Antisense Clones 3, 8, 13, 17, and 24. Besides the significantly reduced level of cathepsin B antigen in antisense-transfected cells, the cathepsin B mRNA level was with the same pore size. Prior to assays, the matrigel matrix was reconstituted by adding 2 ml of serum-free RPMI 1640 medium for 2 h, and excess media were then removed from the filters. In the lower compartments of the chambers, 5 μg/ml of collagen I was used as a chemoattractant.

Tumor cells were labeled as described above. For invasion assays, 8 × 10^5 cells/well were incubated on the reconstituted basement membrane for 72 h. In motility assays, 1 × 10^5 cells/well were seeded onto the filters for 24 h. Cells passing the filters and attaching to the lower sites of uncoated or matrigel-coated membranes were harvested by using trypsin/EDTA, and cell-bound radioactivity was measured in a liquid scintillation counter. The number of migrating cells was calculated from radioactivity of 8 × 10^5 (invasion) and 1 × 10^5 (motility) labeled cells under identical culture conditions for 72 or 24 h, respectively. All quantifications were performed in triplicate. Statistical comparison was made by the Student’s t test, and the level of significance was established at P < 0.05.

RESULTS

MNNG/HOS cells were transfected with an expression vector (pcDNA3.1/V5/His-TOPO) containing the full cathepsin B coding sequence in antisense and sense orientation downstream of a cytomegalovirus promoter. As negative controls, tumor cells were also transfected with the vector alone. Cell clones that had stably integrated the vector constructs were selected for G418 resistance in 5 days. Nineteen antisense-transfected cell clones and five clones transfected with vector alone and sense-integrated constructs were each isolated and analyzed for cathepsin B expression.

Cellular motility of untransfected and transfected cell clones was evaluated in 24-well Transwell chambers (Costar, Bohemia, Germany). The upper and lower culture compartments were separated by polycarbonate filters with 8-μm pore sizes. Invasion assays were done on 6-well matrigel precoated filters (100 μg/cm²; Becton Dickinson, Heidelberg, Germany) with the same pore size. Prior to assays, the matrigel matrix was reconstituted by adding 2 ml of serum-free RPMI 1640 medium for 2 h, and excess media were then removed from the filters. In the lower compartments of the chambers, 5 μg/ml of collagen I was used as a chemoattractant.

Tumor cells were labeled as described above. For invasion assays, 8 × 10^5 cells/well were incubated on the reconstituted basement membrane for 72 h. In motility assays, 1 × 10^5 cells/well were seeded onto the filters for 24 h. Cells passing the filters and attaching to the lower sites of uncoated or matrigel-coated membranes were harvested by using trypsin/EDTA, and cell-bound radioactivity was measured in a liquid scintillation counter. The number of migrating cells was calculated from radioactivity of 8 × 10^5 (invasion) and 1 × 10^5 (motility) labeled cells under identical culture conditions for 72 or 24 h, respectively. All quantifications were performed in triplicate. Statistical comparison was made by the Student’s t test, and the level of significance was established at P < 0.05.
was observed in the antisense cell clones (results not shown). Expression of cathepsins L and K, which was determined by semiquantitative RT-PCR, was not affected (Fig. 4).

Construct integration was demonstrated for all five antisense cell clones by Southern hybridization. Fig. 5 demonstrates that the cell clones 3, 8, 13, 17, and 24 carry additional cathepsin B-specific DNA incorporated into the cellular genome. In the parental cell line MNNG/
ANTISENSE INHIBITION OF CATHEPSIN B

HOS and the control MNNG/TOPO, only the endogenous cathepsin B sequences were detectable.

Invasion of as Clones and MNNG/HOS. An in vitro matrigel invasion assay was used to assess the invasive potential of parental MNNG/HOS cells and the transfected cell clones MNNG/TOPO, asCB-3, asCB-8, asCB-13, asCB-17, and asCB-24. To distinguish between the cell clones, we chose stringent assay conditions over 72 h with a filter pore size of 8 µm and a thick coating with matrigel (150 µg/well).

Control transfectants (MNNG/TOPO) showed no striking differences in invasiveness as compared with the parental MNNG/HOS cells. In three of the five antisense cell clones, however, the reduced cathepsin B expression caused a significant decrease in cellular invasion (Fig. 6). Whereas 3.5% of the MNNG/HOS cells passed the reconstituted matrigel matrix, only 2.1% of asCB-3, 1.58% of asCB-8, and 1.25% of asCB-17 were detectable on the lower side of the filters.

Motility of as Clones and MNNG/HOS. To test the involvement of cathepsin B in cellular motility, we checked MNNG/HOS cells and transfectants for their ability to migrate over 24 h through uncoated polycarbonate filters with a pore size of 8 µm. As shown in Fig. 7, the antisense-transfected cell clones passed the filter barrier more slowly than did the untransfected MNNG/HOS cells and the control cell clone MNNG/TOPO. Thirty-eight percent of parental MNNG/HOS cells and 41% of MNNG/TOPO cells, initially seeded at a concentration of 1 x 10^5 tumor cells/well, were collected from the lower side of the polycarbonate filter. In contrast to these findings, only 20–29% of the clonal cells from asCB-3, 8, 13, 17, and 24 penetrated the filter surface under identical conditions.

Adhesion on Protein Matrices. Because the antisense-transfected cell clones exhibited reduced migration and invasion, we attempted to clarify if there were differences in the adhesive potential to protein matrices between transfected clones and MNNG/HOS. The effect of antisense cathepsin B transfection was examined by incubating 1 x 10^5 parental or modified tumor cells on precoated surfaces of matrigel or collagen I (both 10 µg/ml). Our results showed that adhesion to both matrices was not influenced by transfection of antisense cathepsin B. Forty-three percent of MNNG/HOS, 40% of MNNG/TOPO, and 37–50% of the antisense cells adhered to the collagen I matrix. Overall, the adhesion of the cell clones to matrigel was lower than collagen I, but the antisense clones showed no tendency to adhere more slowly than did MNNG/HOS (Table 1). These results showed that the reduced invasive potential of antisense-transfected MNNG/HOS cells was not a result of decreased adhesion to matrigel.

DISCUSSION

The correlation between cathepsin B expression levels and cancer aggressiveness (16–18), as well as the results of in vitro (13, 14, 19) and in vivo (20) models, suggest that cathepsin B is an important component of the invasive phenotype of many tumor types. Because the protease is regulated at multiple levels, including transcription, post-transcriptional processing, translation, trafficking, and interaction with endogenous inhibitors (9, 21), we decided to block cathepsin B expression at early stages of mRNA maturation by antisense RNA (for review, see Ref. 22). Using this method, both catalytic and possible noncatalytic functions of cathepsin B are affected. In the study presented here, the osteosarcoma cell line MNNG/HOS was stably transfected with constructs that contained the complete coding region of cathepsin B in antisense orientation under control of the strong eucaryotic cytomegalovirus promoter. Although antisense technique has been widely used within the last few years, defining the best target sequence for antisense RNAs still appears to be a challenge. However, the expression of a selected gene product has successfully been reduced by antisense RNAs complementary to the 5’ translation start and the coding region, as well as 3’ untranslated regions (23, 24).

To exclude possible artifacts resulting from the selection of clonal populations, we isolated nearly 35 antisense clones, 19 of which showed

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<th>Table 1 Adhesion of MNNG/HOS, MNNG/TOPO, and antisense clones to protein matrices of matrigel and collagen I</th>
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<tr>
<td>% Adherent Cells (± SD)</td>
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<tr>
<td>Matrigel (10 µg/ml)</td>
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<tr>
<td>MNNG/HOS</td>
</tr>
<tr>
<td>13.9 (± 1.9)</td>
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<tr>
<td>MNNG/TOPO</td>
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<tr>
<td>16.3 (± 1.2)</td>
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<tr>
<td>asCB-3</td>
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<tr>
<td>13.8 (± 2.0)</td>
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<td>asCB-8</td>
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<td>14.7 (± 1.1)</td>
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<td>asCB-13</td>
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<tr>
<td>13.5 (± 1.2)</td>
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<tr>
<td>asCB-17</td>
</tr>
<tr>
<td>11.6 (± 1.9)</td>
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<tr>
<td>asCB-24</td>
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<tr>
<td>12.7 (± 0.9)</td>
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<tr>
<td>Collagen I (10 µg/ml)</td>
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<tr>
<td>MNNG/HOS</td>
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<tr>
<td>42.8 (± 3.4)</td>
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<tr>
<td>MNNG/TOPO</td>
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<tr>
<td>40.3 (± 1.8)</td>
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<tr>
<td>asCB-3</td>
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<tr>
<td>44.5 (± 1.1)</td>
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<tr>
<td>asCB-8</td>
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<tr>
<td>42.5 (± 2.6)</td>
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<td>asCB-13</td>
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<td>49.2 (± 2.8)</td>
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<td>asCB-17</td>
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<td>39.8 (± 1.8)</td>
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<td>asCB-24</td>
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<td>41.2 (± 3.1)</td>
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similar growth properties (results of proliferation not shown) and sufficient inhibition of cathepsin B protein, mRNA, and enzyme activity (Fig. 2). Furthermore, we compared the results obtained from the antisense clones with those from the cell clones transfected with the original expression vector and the host cell line MNNG/HOS. In both controls, there were no changes either in the cathepsin B protein and mRNA levels or in cathepsin B activity, suggesting that antisense RNA is responsible for the inhibitory effects. Despite extensive sequence homologies and overlapping cellular functions, the mRNA levels of cathepsins L and K were neither down-regulated nor up-regulated.

The findings obtained in the in vitro invasion assay (Fig. 6) are consistent with the results obtained by the cysteine protease inhibitor E-64 and the specific cathepsin B inhibitor CA074 in ovarian cancer cells and bladder tumor cells (13, 19). Kolkhorst et al. (14) tested different tumor cell lines for their protease profile and the potential efficiency of the corresponding antiproteolytic reagents on tumor cell invasion. He found that in cell lines with higher expression of cathepsin B, CA074 efficiently inhibited cellular invasion, whereas in other cell lines, cysteine protease inhibitors were less effective, indicating that proteases such as uPA and MMPs play a more important role in these tumor cells. Evidence suggesting that a network of proteolytic enzymes, together with the corresponding receptors and endogenous inhibitors, play a role in tumor malignancy has been accumulating from several laboratories dealing with different tumor entities (25–28). Therefore, it is possible that in the antisense cell clones showing no significant reduction of the invasive potential, other proteases compensated the reduced cathepsin B activity or that the decreased cathepsin B level induced a reduction of cystatin expression levels. In a previous investigation, our group has shown that urokinase plays an important role in the invasion and metastasis of osteosarcoma cells (29). These results and the findings presented in this study suggest that on the cell surfaces, a cathepsin-uPA-plasmin activation cascade may play a major role in the invasion processes of the MNNG/HOS osteosarcoma cells.

It was also shown by others that cellular adhesion to matrigel and collagen I matrices was not affected by reduced cathepsin B activity (30). Sexton and Cox (30) overexpressed cystatin C, a secreted protease inhibitor and showed a significant reduction of the invasive potential of various cell lines. In our study, we observed no significant differences in the adhesion properties of modified and unmodified cells. The cysteine protease cathepsin B is seemingly not involved in the adhesion to extracellular matrices during metastasization of osteosarcomas.

In all of the antisense-transfected cathepsin B cell clones, cellular motility, a complex process involving the continuous reconstitution of the cytoskeleton and the detachment from and attachment to extracellular matrices, was reduced by ~40%. The mechanisms responsible for the influence of cathepsin B on tumor cell motility have yet to be clarified. Receptor-bound uPA is an important factor of cellular motility in osteosarcoma cells as demonstrated by us and others (25, 31, 32). Therefore, it is possible that cathepsin B influences migration by activating uPA or protein kinases.

Our results suggest that cathepsin B is involved in invasion and metastasization of osteosarcoma cells. Besides the demonstrable direct proteolytic activities of cathepsin B against extracellular matrix components, it is the interaction with other proteases in a complex proteolytic cascade that appears to be important for tumor progression.

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