Analysis of a Truncated Form of Cathepsin H in Human Prostate Tumor Cells*

Increased expression of proteases has been correlated with the malignant progression of a variety of tumors. We found a significant increase in cathepsin H expression in high-grade prostate intraepithelial neoplasia and carcinoma of the prostate. Two forms of cathepsin H, the full-length form (CTSH) and a truncated form with a 12-amino acid deletion in its signal peptide region (CTSH Δ10–21), were identified by cDNA sequence analysis. This deletion occurred within the genomic level but likely at the RNA processing level. Both forms are expressed in prostate tissues as well as LNCaP, PC-3, and DU-145 prostate cancer cell lines. The deletion within the signal peptide region affected the trafficking of cathepsin H. Fluorescence microscopy, subcellular fractionation, and activity data indicated that the truncated form was perinuclear and secreted and had a reduced lysosomal association as compared with the full-length cathepsin H. Furthermore, the truncated cathepsin H was enzymatically active. Therefore, an increase in overall cathepsin H expression, particularly in the truncated form with a high secretion propensity, may affect cell biological behaviors such as those associated with tumor progression.

Cathepsin H is a lysosomal cysteine protease and, like other members of the family, is involved in lysosomal protein degradation (1). It is synthesized as an inactive proenzyme (41 kDa) and then proteolytically processed to an active single chain, mature form (28 kDa) within the endosomes/lysosomes (2). Cathepsin H is easily distinguished from other endolysosomal cysteine proteases by its unique aminopeptidase activity (3, 4). The crystal structure data of mature cathepsin H (5) have established form with a high secretion propensity, may affect cell biological behaviors such as those associated with tumor progression.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF426247 and AF426248.

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Cell Lines and Tissue—All cell lines used in this study were obtained from the American Type Culture Collection. DU-145, PC-3, and LNCaP prostate tumor cells were maintained in RPMI 1640 medium (Invitrogen) containing 5% fetal bovine serum. Human renal 293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 5% fetal bovine serum. Paraffin-embedded prostate tissue sections were obtained from Harper Hospital, which is affiliated with Wayne State University.

Immunohistochemistry—Paraffin-embedded prostate tissue sections were deparaffinized and stained as described previously (16).

Cloning of Cathepsin H—One µg of total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo(dT). Cathepsin H cDNA was PCR-amplified using 5'-GACGCTCTGGGGCCCACCGCTC-3' and 5'-GGCCGGTGCGACGCTCACACC-3' primers for 26 cycles at 94 °C for 30 s, 62 °C for 30 s, and 68 °C for 90 s. Nested PCR reaction was performed using 5'-ATTAGAATTCATGTGGGGCCACGGCTCGGCT-3' and 5'-GATAGGCTTCGACCCACGAGG-GATGGGAGG-3' primers targeted to the open reading frame of cathepsin H and tagged with the appropriate restriction enzyme sites for 30 cycles with the same PCR profile as described above. The cathepsin H open reading frame was cloned and sequenced.

RT-PCR and Genomic PCR—Briefly, 1 µg of total RNA from DU-145, PC-3, and LNCaP cells was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). One µl of this reaction mixture was PCR-amplified using primers flanking the deleted region (see Fig. 28 for primer sequences).

1 The abbreviations used are: RT-PCR, reverse transcription-PCR; PIN, prostatic intraepithelial neoplasia; YFP, yellow fluorescence protein.

Experimental Procedures

EXPERIMENTAL PROCEDURES

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11533
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Figure 1. Cathepsin H immunohistochemical staining in prostate tissue. Paraffin-embedded tissue sections were incubated with a 1:3000 dilution of anti-cathepsin H antibody and developed using Vectastain Elite ABC (Vector Laboratories, Inc.), followed by hematoxylin counterstain. A, normal prostatic glands showing weak immunostaining. B, compared with the surrounding normal glandular epithelium (arrowheads), foci of high-grade PIN (arrows) demonstrate strong positive immunoreaction for cathepsin H. C–E, low- to high-grade prostatic adenocarcinomas also demonstrating strong immunoreactivity for cathepsin H. C, area of well-differentiated prostatic adenocarcinoma corresponding to Gleason pattern 2. The inset represents a higher magnification of the malignant glands showing enlarged nuclei with prominent nucleoli, characteristic of prostatic adenocarcinomas. D, area of moderately differentiated tumor corresponding to Gleason pattern 3. Notice the presence of a peripheral nerve twig (+) with perineural invasion by tumor glands (arrows). E, poorly differentiated adenocarcinoma with a Gleason pattern of 5. The arrow points to one of the enlarged tumor nuclei. F, immunostaining negative controls, in which the primary antibody was omitted. The left panel shows adenocarcinoma. The right panel shows normal prostate. Magnification bar, 30 μm (A, B, D, and F, left panel) and 15 μm (C, E, and F, right panel). Results are representative of five different cases.
contrast to the weak staining seen in normal prostate glands (Fig. 1). Over 40 glands with focal or diffuse high-grade PIN were examined. All of them exhibited uniform, strong immunostaining. This was particularly evident in glands where high-grade PIN was focal (Fig. 1B). All low-grade (Fig. 1C), moderate (Fig. 1D), and high-grade (Fig. 1E) carcinomas examined showed higher levels of cathepsin H than their matched normal tissue. Cathepsin H protein expression was localized mainly to the columnar epithelial cells; however, some stromal cells were also positive for cathepsin H. Samples from five patients were analyzed, and all showed similar results.

Identification of a Truncated Form of Cathepsin H—Two different forms of cathepsin H cDNA, the full-length form (CTSH) and a truncated form with deletion of 12 amino acids at the signal peptide region (CTSH/H9004-10-21), were identified by PCR amplification and sequence analysis (Fig. 2A; GenBank\textsuperscript{TM} accession numbers AF426247 and AF426248). Sequences of the full-length cDNA were identical to that of cathepsin H reported previously (24). The presence of this truncated form was observed for the first time and further confirmed by RT-PCR and sequencing of the PCR fragments (Fig. 2C). Expression of CTSH appeared to be higher than that of CTSH/H10-21 in tissues as well as DU-145, PC-3, and LNCaP cells (Fig. 2C). Because the deletion was not at a classical splicing donor/acceptor site, we wondered whether this deletion occurred at the genomic level in tumor cells. PCR amplification of genomic DNA showed only one band at the predicted full-length size in all of the three cell lines (Fig. 2, B and C).

**Cathepsin H Gene Structure**—Alignment of the CTSH cDNA sequence with the corresponding genomic sequence in the Human Genome Project data base predicted that the cathepsin H gene has 12 exons spanning 23 kb of genomic sequences (Fig. 2D). All intron-exon junctions conformed to the GT-AG rule. Cathepsin H Expression and Activity in Prostate Tumor Cell Lines—Western blot analysis showed that procathepsin H (41 kDa) was expressed at a similar level in DU-145, PC-3, and LNCaP cells; however, expression of single chain cathepsin H (28 kDa) was higher in LNCaP cells than in DU-145 and PC-3 cells (Fig. 3A). In fact, the 28-kDa protein was detected only after an extended time of exposure in DU-145 and PC-3 cells (Fig. 3A, bottom panel). Therefore, the total amount of cathepsin H protein was significantly higher in LNCaP cells as compared with that in DU-145 and PC-3 cells.

To corroborate the results observed by Western blot analysis, cathepsin H activity was compared in these three cell lines. To demonstrate the specificity of our assay conditions, we used purified human liver cathepsin H and incubated cathepsin H with different substrates: Pyr-Arg-Thr-Lys-Arg-AMC, Z-Phe-Arg-AMC, Z-Arg-Arg-AMC, and L-Arg-AMC (19). Results confirmed the selectivity of L-Arg-AMC toward cathepsin H (Fig. 3B). Using i-Arg-AMC, we demonstrated that cathepsin H activity was inhibited by the diazomethane derivative Ser-
(O-Bzl)-CHN2 (Fig. 3C). This inhibitor was previously shown to be selective for cathepsin H (25). E-64, a general cysteine protease inhibitor, was much less efficient in blocking cathepsin H activity. Nearly 10-fold more of this inhibitor was required to completely block cathepsin H activity (Fig. 3C). The purified enzyme was not sensitive to the cathepsin B-selective inhibitor CA-074 (Fig. 3C).

Validating the above assay conditions with cell lysates prepared from LNCaP cells, we show that the measured enzyme activity in cell lysates was inhibited 98% by Ser(O-Bzl)-CHN2 but not by CA-074 (Fig. 3D). On the basis of these results, we determined the cathepsin H activity in DU-145, PC-3, and LNCaP cells. The cathepsin H activity in DU-145, PC-3, and LNCaP cells was 55, 56, and 80 pmol AMC/min/200,000 cells, respectively (Fig. 3E).

Localization of the Full-length and Truncated Forms of Cathepsin H—PC-3 and 293 cells were transfected with CTSH and CTSHΔ10–21 C-terminally tagged with EYFP or transfected with EYFP tagging vector. CTSH protein distribution showed a discrete compartmentalized pattern in the cytoplasm (Fig. 4A). CTSHΔ10–21 was concentrated in the juxtanuclear region of the cells, yet it was distributed somewhat more throughout the cytoplasm in some cells (Fig. 4A). These results suggested that CTSHΔ10–21 protein had a different localization than CTSH. Cells transfected with the EYFP vector alone exhibited diffuse fluorescence in both the cytoplasm and nucleus (17).

Subcellular Localization of Cathepsin H—To substantiate the immunofluorescence results, transfected 293 cells were fractionated by differential centrifugation. The fractions corresponding to nuclei, mitochondria/lysosomes, microsomes, and cytosol were analyzed for the presence of EYFP-tagged cathepsin proteins (Fig. 4B). Although both forms of cathepsin H were detected in nuclear, mitochondrial/lysosomal, and microsomal fractions, the level of CTSHΔ10–21 appeared higher in the nuclear fraction and lower in the mitochondrial/lysosomal fraction (Fig. 4B). No differences were seen in the microsomal fraction (Fig. 4B). Interestingly, the cytosol appeared to be devoid of cathepsin H, confirming that most of the cathepsin H proteins are compartmentalized. These results supported the immunofluorescence data, i.e. the deletion of 12 amino acids in the signal peptide region affects the intracellular trafficking of cathepsin H.

Protein Expression and Enzymatic Activity of CTSH and CTSHΔ10–21—Western blot analysis detected cathepsin H-YFP fusion proteins (~73 kDa) in both CTSH and CTSHΔ10–21 transiently transfected 293 cells. The protein band in CTSHΔ10–21 was slightly lower than that in CTSH, due to the lack of 12 amino acids (Fig. 4A). Results indicated that cells expressing CTSH-YFP and CTSHΔ10–21-YFP at a similar level.

To determine whether cathepsin H activity is affected due to the deletion in the signal peptide region, we measured the...
cellular and pericellular (cell surface and secreted) activity in transfected 293 cells. Cellular cathepsin H activity was 48/100,000, 57/100,000, and 49/100,000 pmol AMC/min/200,000 cells, and pericellular activity was 10/150,000, 10/150,000, and 16/150,000 pmol AMC/min/150,000 cells in vector-, CTSH-, and CTSHΔ10–21 transfected cells, respectively. Cellular cathepsin H activity was significantly higher in CTSH-transfected cells compared with CTSHΔ10–21-transfected cells. In contrast, the pericellular cathepsin H activity was ~1.6-fold higher in CTSHΔ10–21-transfected cells than in CTSH-transfected cells (Fig. 4C). CTSH expression resulted in an increased cathepsin H activity in cell lysates, whereas CTSHΔ10–21 expression appeared to elevate cathepsin H activity pericellularly (Fig. 4C). These results suggest that the secreted, truncated cathepsin H is enzymatically active.

DISCUSSION

In this study, we have demonstrated the presence of two forms of cathepsin H transcripts in prostate tissues and cancer cell lines, the full-length form (CTSH) and a truncated form with a deletion of 12 amino acids in the signal peptide region (CTSHΔ10–21). To better characterize these two different forms of cathepsin H, CTSH and CTSHΔ10–21 cDNA were transfected into PC-3 and 293 cells. Immunofluorescence microscopy and cell fractionation data indicated that full-length cathepsin H was associated with endosomes and lysosomes, in agreement with previous observations (26). In contrast, the truncated cathepsin H was more concentrated in the perinuclear region, somewhat distributed throughout the cytoplasm in some cells, and reduced in the lysosomal fraction. Although mRNA of CTSHΔ10–21 was expressed at a lower level than that of CTSH in the prostate tissues and tumor cell lines, CTSHΔ10–21 protein tends to be secreted from transfected cells and was active. Therefore, it is conceivable that this secreted CTSHΔ10–21 may play a role in tumor invasion. Additional experiments are warranted to determine the role of this truncated form of cathepsin H in prostate cancer progression.

An alternatively spliced form of cathepsin B that lacks its N-terminal signal peptide and 34 amino acids of the 62-amino acid inhibitory propeptide was reported previously (27). This truncated form of cathepsin B was shown to be associated with nuclei and other membranous organelles (23). Distribution of CTSHΔ10–21 is very similar to that of the truncated cathepsin B. It is unclear, however, whether CTSHΔ10–21 is generated through alternative splicing. Exon-intron junction analysis indicated that the 36-nucleotide deletion was not at a classical splicing donor/acceptor site. In addition, genomic PCR data showed no deletion at the DNA level. Thus, CTSHΔ10–21 may be the result of alternative splicing through an uncommon donor/acceptor site. Several reports, including our serial analysis of gene expres-
sion data, have shown an increased expression of cathepsin H in tumor cells and tissues (9–13). Our immunostaining analysis also demonstrated an increased expression of cathepsin H in intraepithelial and invasive prostate neoplasia. Increased expression of cathepsin H along with cathepsin B and S (28) in high-grade PIN suggests that these proteins may play an important role in the early stage of prostate cancer progression. Furthermore, higher levels of cathepsin H, possibly the secreted form, in carcinoma cells may contribute to tumor invasiveness. We found a similar level of cathepsin H mRNA in LNCaP, DU-145, and PC-3 prostate cancer cells. However, LNCaP cells showed the highest protein expression and activity. DU-145 and PC-3 cells had similar but low levels of protein expression and activity compared with LNCaP cells. Our activity results were different from those described previously (13), in which DU-145 had the highest activity. It is unclear whether the difference is a result of different assay conditions or cell line clonal variation. It is noteworthy that despite the high level of cathepsin H protein in LNCaP (Fig. 3A), cathepsin H activity was only 1.5-fold higher in LNCaP cells than that in DU145 and PC-3 cells (Fig. 3E). This discrepancy could be explained by the presence of endogenous cathepsin H inhibitor(s) in our activity assays. For instance, stefin B has been shown to be a strong cathepsin H inhibitor (25, 29).

Lysosomal cysteine proteases were believed to be involved mainly in intracellular protein degradation. However, recent findings suggest a more expanded role for cysteine proteases, such as a role in apoptosis, major histocompatibility complex class II immune responses, prohormone processing, extracellular matrix remodeling, and tumor invasion (1, 3, 7, 30–32). Expression of alternatively spliced form or truncated variants adds another level of complexity in the function of these diverse cysteine proteases in cell biology.

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