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Abstract

The biochemical characteristics of cathepsin B secreted from cultured human liver cancer cells were examined. The enzyme activity of culture medium against a synthetic substrate, N-carbobenzoxy-L-arginyl-L-arginine-4-methyl-coumaryl-7-amide, was dependent on the addition of cysteine, and the optimal pH was found to be 6.0. No activity was observed when the enzyme source was fresh medium not used for culture. These results suggest that the enzyme released from liver cancer cells is the thiol-protease cathepsin B. The molecular weight of the enzyme with 90% of the total activity was 40,000. Two cathepsin B molecules were found in liver tissue from patients with hepatocellular carcinoma (HCC); one was equivalent in size to the secreted enzyme, and a smaller one was the same as normal liver cathepsin B (27,000), which was also obtained from HCC-bearing cirrhotic liver. These results demonstrate that two molecules of cathepsin B are synthesized in liver cancer, and that the larger one is released into the surrounding tissue.

KEYWORDS: cathepsin B, cathepsin B secretion, cultured human liver cancer

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The Secretion of High Molecular Weight Cathepsin B from Cultured Human Liver Cancers

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The biochemical characteristics of cathepsin B secreted from cultured human liver cancer cells were examined. The enzyme activity of culture medium against a synthetic substrate, N-carbobenzoxy-L-arginyl-L-arginine-4-methyl-coumaryl-7-amide, was dependent on the addition of cysteine, and the optimal pH was found to be 6.0. No activity was observed when the enzyme source was fresh medium not used for culture. These results suggest that the enzyme released from liver cancer cells is the thiol-protease cathepsin B. The molecular weight of the enzyme with 90% of the total activity was 40,000. Two cathepsin B molecules were found in liver tissue from patients with hepatocellular carcinoma (HCC); one was equivalent in size to the secreted enzyme, and a smaller one was the same as normal liver cathepsin B (27,000), which was also obtained from HCC-bearing cirrhotic liver. These results demonstrate that two molecules of cathepsin B are synthesized in liver cancer, and that the larger one is released into the surrounding tissue.

Key words : cathepsin B, cathepsin B secretion, cultured human liver cancer

The proliferation, invasion and metastasis by malignant tumors is proceeded by the degradation of surrounding tissue and extracellular matrix. Certain types of proteolytic enzymes, plasminogen activators (1, 2), collagenase (3, 4) and lysosomal enzymes (5) play important roles in these processes. Among the lysosomal proteases, cathepsin B seems to be closely associated with tumor proliferation and metastasis, since its activity in mouse melanoma variants was found to be correlated with metastatic potential (6, 7). We have previously shown that cathepsin B activity is higher in human gastric cancer than in the surrounding tissue, and that activity in poorly differentiated cancers with lymph node or lung metastasis is higher than in well differentiated gastric cancers without metastasis (8).

Cathepsins are contained in the lysosomes of various types of cells and play an important role in intracellular protein degradation. However, Chan *et al.* (9) have reported that preprocathepsin B possesses a presecretory signal sequence, an N-terminal propeptide extension and C-terminal peptide. Furthermore, Gals *et al.* (10, 11) have recently shown that cathepsin L, another major thiol-protease, is a major excretion protein of H-ras oncogene. Altered distribution of this

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enzyme from heavy mitochondrial fractions to plasma membrane-associated lysosomes in malignant melanoma has been reported (12), and the excretion of cathepsin B-like enzyme from established mammary cancer cells into the culture medium has been proposed by Recklies *et al.* (13). Regarding liver cancer cells, we have shown that cathepsin B activity in hepatocellular carcinoma correlates well with the degree of portal tumor thrombosis and lung metastasis (14).

In the present study, we investigated the excretion of cathepsin B from cultured cells of liver cancer, and the biochemical characters of this enzyme.

Materials and Methods

The synthetic substrate for cathepsin B, N-carbobenzoxy-L-arginyl-L-arginine-4-methyl-coumaryl-7-amide (Z-Arg-Arg-NMec), was purchased from Peptide Institute, Inc., Osaka, Japan. Leupeptin and human plasma kallikrein were obtained from Sigma Chemical Co., St. Louis, MO, USA. and Brij-35 from Nakarai Chemical Co., Kyoto, Japan.

The three human liver cancer cell lines used in this study, HuH-6 clone5 (hepatoblastoma)(15), HuH 28 (cholangiocellular carcinoma)(16) and HLE, c-1 (hepatocellular carcinoma)(17), were established in the Pathology Division, Cancer Institute, Okayama University Medical School. All medium specimens, obtained during medium exchange 2 or 3 times a week, were frozen at −80°C until use. HuH-6 clone5 and HuH 28 were cultured in RPMI-1640 supplemented with 20% bovine serum and 0.4 % lactalbumin hydrolysate. HLE, c-1 was cultured in MEM (+ 10% bovine serum).

The assay of cathepsin B activity was performed according to a method modified from that previously described by Higashi (18). The incubation mixture contained 250 μl of buffer (0.2 M phosphate buffer, pH 6.0, containing 1.33 mM disodium EDTA and 2.7 mM cysteine), as well as culture medium as the enzyme source diluted to 250 μl with physiological saline and 250 μl of 0.1% Brij-35. As for the control assay, leupeptin, a thiol-protease inhibitor, was added to the incubation mixture (final concentration, 1 μM). The mixture was preincubated for 15 min at 30°C, followed by the addition of 250 μl of substrate (100 μM). After incubating at 30°C for various periods of time as shown in the Results section, the reaction was terminated by adding 1 ml of a stopping reagent containing 100 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid. A fluorometric spectrophotometer (Model 650-10, Hitachi Co., Ltd., Tokyo) was used to read the fluorescent intensity of free NMec by excitation at 370 nm and emission at 460 nm.

Results

The fluorescent intensity of NMec after 2 h incubation increased in proportion to the culture medium volume added (Fig. 1). The fluorescent intensity generated by 50 μl of fresh medium (+ 10 - 20% bovine serum) not used for culturing of cells was compared with that generated by the same quantity of culture medium to confirm that the enzyme activity was of cultured cell origin. The fluorescent intensity of NMec, when culture medium was used as the enzyme source, increased in proportion to the incubation time (1 - 5 h). The intensity of NMec when fresh medium was used, however, did not significantly increase, indicating that the enzyme activity was released from cultured cells (Fig. 1, inset). Using 50 μl of culture medium as the enzyme source, the temperature stability of this activity was examined. The fluorescent intensity of free NMec increased and resulted in a sigmoid curve when plotted from 4 to 50°C. Furthermore, activity was not evident at 100°C, suggesting that the activity was the result of an enzyme protein (Fig. 2).

Since only kallikrein among various proteases is known to degrade cathepsin B substrate used in this study, we investigated the cysteine dependency, optimal pH and molecular weight of this enzyme in comparison with kallikrein. If the activity in culture medium

is due to cathepsin B, it should be dependent on cysteine and exhibit a different optimal pH and molecular weight from kallikrein. The fluorescent intensity of NMec in culture medium increased 4-fold after adding cysteine, while the degrading activity of kallikrein against this substrate did not significantly change upon the addition of cysteine (Fig. 3). The optimal pH of the enzyme in culture medium was 6, similar to that observed in normal liver tissue, while that of kallikrein was

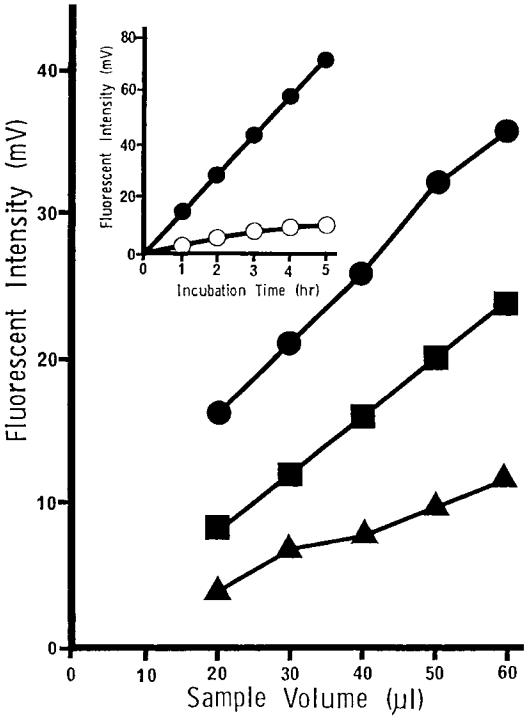

Fig. 1 Effect of sample volume and incubation time (inset) on the enzyme activity in culture medium. The sample volume varied from 20 to 60 μl (each diluted to 250 μl with 0.9% NaCl) with 2 h of incubation to examine the enzyme dose dependency. In the inset, incubation time was varied from 1 to 5 h using a sample volume of 50 μl. Culture medium of liver cancers, HuH-6 clone5 (●——●), HuH 28 (■——■) and HLE, c-1 (▲——▲), and fresh medium (○——○) not used for culture were the enzyme sources. The enzyme activity in liver cancer culture media increased in proportion to both sample volume and incubation time. When fresh medium was used as the enzyme source, however, the observed increases were not statistically significant.

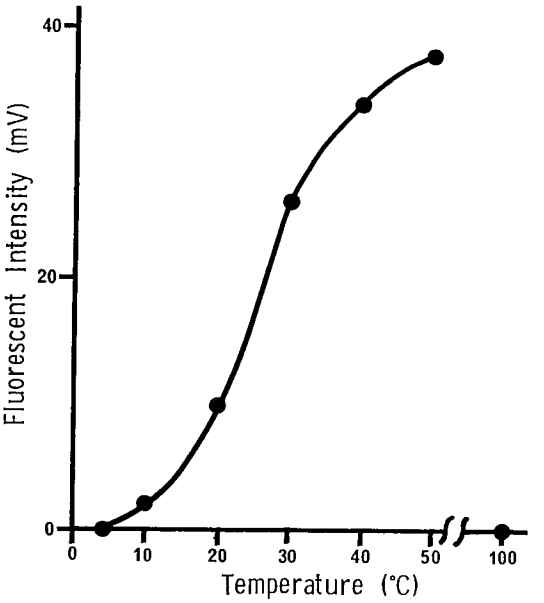

Fig. 2 Effect of assay temperature on cathepsin B activity. Assays were performed at 4, 10, 20, 30, 40, 50 and 100°C. Fifty μl of HuH-6 clone5 culture medium was used as the enzyme source, and a reaction time of 1 h was used. When plotted, the enzyme activity resulted in a sigmoid curve from 4 to 50°C, with no activity being observed at 100°C.

8 (Fig. 4). The molecular weight of human kallikrein used in this study was approximately 99,000, as has been generally reported. However, peaks corresponding to Mr = 40,000 and 7,500 were obtained when culture medium was applied, with over 90% of the activity being concentrated in the former peak (Fig. 5-A). Although we did not clarify what kind of enzyme the smaller peak was, these results mentioned above suggest that most part of the Z-Arg-Arg-NMec degrading activity of the culture medium was the result of cathepsin B, not kallikrein.

Since the molecular weight of the enzyme in culture medium was different from that of the mature form of cathepsin B isolated from rat liver (Mr = 27,000), we investigated molecular weight differences of cathepsin B in human liver cancer and cancer-bearing tissue. Only one peak with Mr = 27,000 was

obtained from non-cancerous (cirrhotic) tissue while two peaks, at Mr = 40,000 and 27,000, were recognized in hepatocellular carcinoma tissue (Fig. 5-B).

Discussion

When fresh medium was used as the enzyme source, a slight increase in Z-Arg-Arg-NMec-degrading activity was detected when the incubation time was long (Fig. 1, inset), and the activity of the fresh medium when incubated for 2 h was not 0 mV on the elution profile of culture medium (Fig. 5-A). We consider, however, that a substrate degrading impurity(ies) different from thiol-protease in the bovine serum had responsible for this phenomenon. Therefore, free NMec fluorescence specific to leupeptin-suppressed enzyme activity in the culture medium demonstrated that cathepsin B was released from human liver cancer cells into the culture medium. The dependency of this activity on time, volume and temperature indicates that this activity is of enzyme origin. Furthermore, the facts that the enzyme activity was dependent on the addition of cysteine and that the optimal pH was 6, suggest that the enzyme present in the culture medium was cathepsin B, not kallikrein.

The molecular weight (40,000) of cathepsin B excreted into the culture medium was larger than that of the enzyme found in nor-

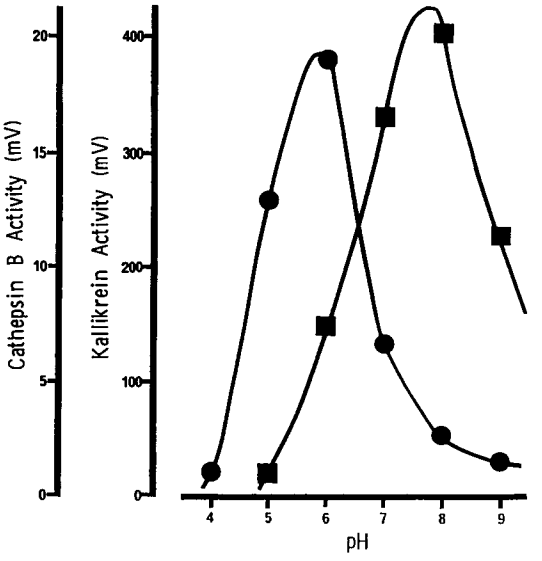

Fig. 4 Optimal pH for Z-Arg-Arg-NMec-degrading activity of cathepsin B and kallikrein. The activity of HuH-6 clone5 culture medium (50 μl diluted to 250 μl with 0.9% NaCl) (●——●), and of human plasma kallikrein (0.005 units diluted to 250 μl with 0.9% NaCl) (■——■) was measured. The enzyme assay was performed after incubation for 2 h using acetate buffer (pHs 4 and 5), phosphate buffer (pHs 6 and 7) and Tris-HCl buffer (pHs 8 and 9). The optimal pH for cathepsin B (in culture medium) was found to be 6, while that for kallikrein was 8.

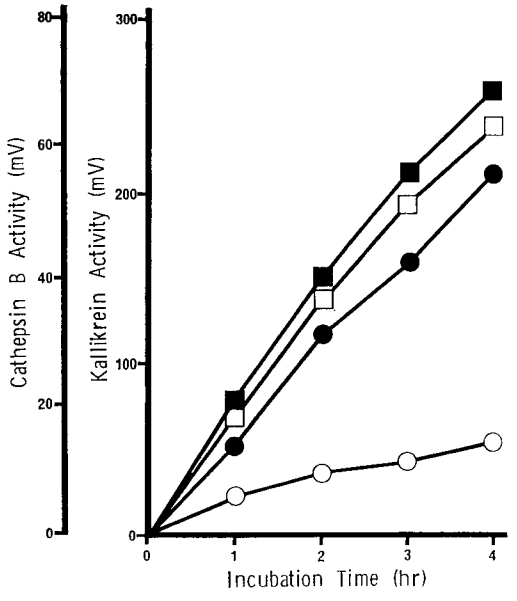

Fig. 3 Effect of the addition of cysteine on the Z-Arg-Arg-NMec-degrading activity of human plasma kallikrein and HuH-6 clone5 cell culture medium. The activity of HuH-6 clone5 culture medium (50 μl diluted to 250 μl with 0.9% NaCl) with (●——●) and without (○——○) cysteine (free base), and of human plasma kallikrein (0.005 units diluted to 250 μl with 0.9% NaCl) with (■——■) and without (□——□) cysteine (free base) was measured. The enzyme assay was performed after incubation for 2 h. Increased enzyme activity upon addition of cysteine was recognized in HuH-6 clone5 culture medium. (Slightly increased activity observed even with no addition of cysteine was due to cysteine contained in bovine serum used for culture.)

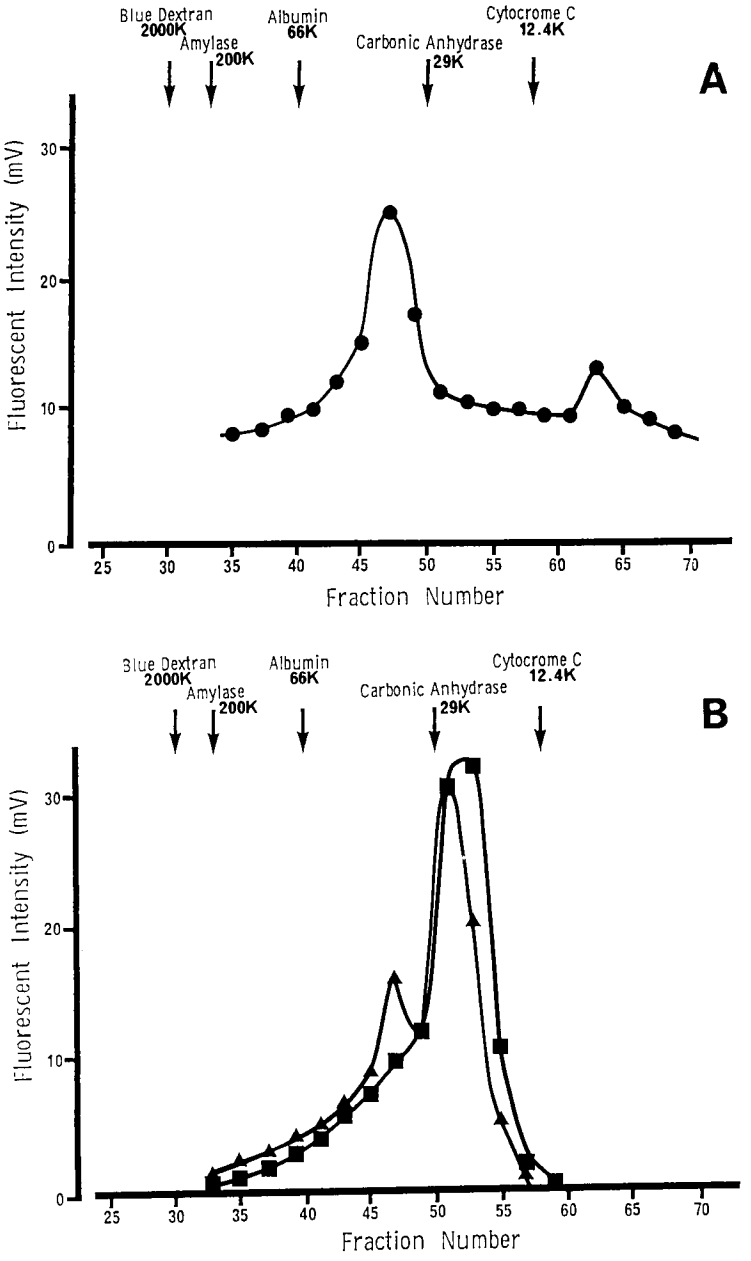

Fig. 5 Sephacryl S-200 elution profile of Z-Arg-Arg-NMec-degrading enzyme from culture medium and liver tissues. A Sephacryl S-200 column (2.6×98 cm) was eluted with 0.9% NaCl at a flow rate of 2.1 ml/min. Samples consisted of HuH-6 clone5 culture medium (A), and liver tissue homogenates (1,000-fold diluted with 0.9% NaCl)(B), *i.e.*, hepatocellular carcinoma tissue (▲——▲) and non-cancerous (cirrhotic) tissue (■——■). Five ml of each sample was applied to the column and 100 tubes of 6 ml fractions were collected. Z-Arg-Arg-NMec-degrading activity was measured using 250 μl of each fraction. Enzyme assay of the sample from culture medium and homogenate took place after incubation for 2 h and 10 min, respectively. Two peaks (Mr = 40,000 and 7,500) were obtained from culture medium (A). Two peaks from hepatocellular carcinoma (Mr = 40,000 and 27,000) were obtained and only one peak (Mr = 27,000) was obtained from non-cancerous tissue (B).

mal liver tissue (27,000). This result is the same as that reported by Mort *et al.* (19) in which the molecular weight of cathepsin B excreted from cultured human and mammary breast cancer cells was found to be 40,000. In human liver cancer tissue obtained during autopsy, two molecular forms of the enzyme were recognized. The larger one was equivalent to excreted cathepsin B(40,000), while the smaller was equivalent to normal liver tissue cathepsin B (27,000). Recently, Freiss *et al.* (20) have shown that a larger cathepsin D molecule, a lysosomal enzyme, is also secreted from breast cancer cells. These observations suggest that two cathepsin molecules are synthesized in malignant cells and that only the larger molecule is secreted into extracellular spaces. Changes in the subcellular distribution of cathepsin B in malignant cells, confirmed by enzyme activity (12) and an immunolocalization study (21), are possibly related to this process. Large molecules of cathepsin B synthesized on ribosomes bound to endoplasmic reticulum and moved into plasma membrane-associated lysosomes seem to be secreted through the membrane.

The mechanisms of the secretion of cathepsin B from malignant cells remain unclear. In a biosynthesis study of cathepsin L in cultured normal hepatocytes, a proenzyme with phosphorylated oligosaccharides and unglycosylated forms was secreted from the cells (22). This result is interesting since tyrosylphosphokinase activity is known to be enriched in transformed cells (23), although it is unknown whether secreted cathepsin B is phosphorylated or not.

Collagenolytic activity of purified cathepsin B has been reported (24). It has not been determined, however, that the secreted high molecular weight cathepsin B possesses the same capabilities. Although some papers have suggested that larger secreted cathepsin molecules may be proenzyme in form (9, 10), we do not regard secreted cathepsin B as a proenzyme since it exhibits Z-Arg-Arg-NMec-degrading activity. Furthermore, it has been revealed that the larger form of cathepsin D secreted from breast cancer cells degrades extracellular matrix (25). It is likely, therefore, that secreted cathepsin B possesses the capability of degrading native tissues and extracellular matrix.

Certain studies have been concerned with the effectiveness of protease inhibitors on tumor proliferation (26, 27) and the malignant transformation of 3T3 cells by H-ras oncogene (28). We also observed that the administration of leupeptin suppressed tumor growth in DMH-induced colon cancer in rats by decreasing cathepsin B activity (Sato *et al.*, unpublished observations).

Our results indicate an alteration in the post-translational processing of cathepsin B in liver cancer, and that the excreted form of this enzyme is released from malignant cells into the surrounding tissue and plays a role in proliferating and metastasizing liver cancer. Further study on the roles of secreted cathepsin B is now underway in our laboratory.

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