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In vitro activation of pro-cathepsin B by three serine proteinases: leucocyte elastase, cathepsin G, and the urokinase-type plasminogen activator

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In vitro activation of pro-cathepsin B purified from ascitic fluid of ovarian carcinomas by serine proteinases was studied. Both elastase and cathepsin G from human leucocytes were found to be activators, on the basis of generation of cathepsin B activity and processing of the precursor. These results represent a new cooperative pathway between cancer cells and host cells. The urokinase-type plasminogen activator activated pro-cathepsin B faster than leucocyte proteinases. A new relationship is emerging between the cysteine proteinases and the plasmin-activation system. Both pathways suggest an important role of cathepsin B in the proteolytic cascade associated with tumour invasion.

Pro-cathepsin B; Activation; Processing; Elastase; Cathepsin G; Urokinase

1. INTRODUCTION

Increased level of proteinases is generally associated with malignancy. Tumour cell invasion and metastasis require migration of malignant cells in the body following proteolytic digestion of basement membranes and extracellular matrices. Consequently, proteinases, such as collagenases, cathepsins, plasmin or plasminogen activators, could be associated with tumor spreading [1]. The cysteine proteinase, cathepsin B, is a lysosomal endopeptidase that has been implicated in tumour invasion [1,2]. Cathepsin B is secreted from human and murine tumours either as an active enzyme or as an inactive pro-enzyme. Such a distribution may result from altered post-translational processing and/or intracellular trafficking [2–4].

In this laboratory, we have purified a pro-cathepsin B from ascitic fluid of ovarian carcinomas. This pro-enzyme exhibited two components (45 and 36 kDa) which could correspond to glycosylated and non-glycosylated proforms, respectively [5,6]. Both were activatable in vitro by pepsin or cathepsin D at pH 3.0, leading to a 30–32 kDa cathepsin B-like proteinase, similar to mature single-chain lysosomal cathepsin B from normal tissues [7]. In this report we describe the activation of this same pro-enzyme by both elastase and cathepsin G from human leucocytes at pH 8.6 and 7.5, respectively. This pathway is more favourable to extracellular activation than the aspartic proteinase pathway. Activation of pro-uPA by cathepsin B is known [8]. Similarly, we found activation of pro-cathepsin B by uPA. Both types of proteinases, secreted by or associated with the tumour cell surface, could be linked to abnormal proteolysis. The biological significance of pro-cathepsin B secretion may be a consequence of these reactions: generation of active cathepsin B at near neutral pH could be a step in the proteolytic cascade leading to tissue destruction as a prelude to tumour invasion.

This is the first description of such a pathway, indicating a cooperativity between the cancer cells and the host cells in the generation and regulation of extracellular proteolysis.

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Abbreviations: cathepsin B, EC 3.4.22.1; elastase, EC 3.4.21.11; cathepsin G, EC 3.4.21.20; urokinase, uPA, EC 3.4.21.31; Z, benzyloxycarbonyl; NH-Mec, 4 methyl-7-coumarylamide; EDTA, ethylene diamine tetraacetate, disodium salt; DTE, dithioerythritol; PBS, phosphate buffer saline.

2. MATERIALS AND METHODS

2.1. *Materials*

Trypsin, chymotrypsin, and plasmin were purchased from Boehringer-Mannheim (Meylan, France). Elastase from human leukocytes and the urokinase-type plasminogen activator (uPA) from human urine were provided by Serva (St. Germain en laye, France). Cathepsin G from human leukocytes and the fluorogenic substrate, Z-Arg-Arg-NHMEC, were obtained from Nova-Biochem (Laufelfingen, Switzerland). Nitrocellulose sheets were from Schleicher and Schüll (Dassel, Germany).

Fluorescence measurements were carried out on a Kontron SFM 25 spectrofluorometer at λ_{ex} 347 nm and λ_{em} 440 nm using NH_2Mec for calibration. Electrophoresis was carried out using a vertical tank (Gibco-BRL, Cergy-Pontoise, France). Immunoblotting was carried out using a Novablot transfer-kit from LKB (Les Ulis, France).

2.2. *Activation of pro-cathepsin B by serine proteinases*

Pro-cathepsin B was purified from malignant ascitic fluid as described before [5]. In each experiment, approximately 20 μg of purified precursors were incubated at different times (0–150 min) with 5 μg of one of the following serine proteinases: trypsin, chymotrypsin, plasmin, leukocyte elastase, leukocyte cathepsin G, or uPA. Alternatively, 1 or 2 μg of each serine proteinases was checked. The following buffers were used: 100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl for trypsin; 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl for chymotrypsin; 50 mM Tris-HCl, pH 8.6, containing 100 mM NaCl for elastase; and phosphate-buffered saline (PBS) 100 mM, pH 7.4, containing 150 mM NaCl, for cathepsin G, plasmin, and uPA. The activation step was performed in 100 μl final volume. Cathepsin B activity was thereafter measured by adding 400 μl of phosphate buffer 100 mM, pH 6.8, containing 1 mM DTE, 2 mM EDTA, together with 10 μl Z-Arg-Arg-NHMec (10 mM, stock solution). The reaction was stopped by the addition of 100 μl monochloroacetate, pH 4.3, after 30 min of incubation. Fluorescence of the reaction product was measured as described above. For each proteinase, a control experiment was carried out with pro-cathepsin B alone, which was subtracted from the final value.

2.3. *Immunoblotting*

Slab gel-electrophoresis (15%) in the presence of SDS with reduction (10 mM DTE) was performed according to the method of Laemmli [9]. Proteins were transferred electrophoretically onto nitrocellulose sheets. Following protein transfer, blots were treated with anti-cathepsin B antibodies as described previously [4,5].

3. RESULTS AND DISCUSSION

As shown in Figs. 1A and 2A, elastase cathepsin G and urokinase were able to activate pro-cathepsin B leading to active cathepsin B-like proteinase. The extent of autoactivation was the following: 10% after a 120 min incubation at pH 8.6; 5–10% for the different incubation times at pH 7.4. Consequently, in this pH range, the autoactivation seems to be irrelevant. Chymotrypsin gave little cathepsin B activity and neither plasmin nor trypsin activated the pro-enzyme (data not shown). Nevertheless, in both cases, the time dependency of this activation was different: leucocyte elastase and cathepsin G gave a maximal cathepsin B activity after a 120 min incubation. On the contrary, uPA activated pro-cathepsin B more quickly (10–30 min), followed by a fast decrease of enzymatic activity (Fig. 2A). This activation was associated with the processing of the pro-enzymes (45–36 kDa) into two chain-forms of cathepsin B, as demonstrated by immunoblotting using antibodies directed against human liver cathepsin B. The processing with elastase, cathepsin G and urokinase showed the same temporal pattern as the appearance of cathepsin B activity, i.e. the pro-enzymes were fully processed after 120 min with elastase or cathepsin G, and after only 10 min with urokinase (Figs. 1B and 2B). The molar ratios of pro-cathepsin B vs. uPA and of pro-cathepsin B vs. elastase or cathepsin G were about 5 and 3, respectively. When we used 1 or 2 μg of activating proteinases, no significant activation was found. On the

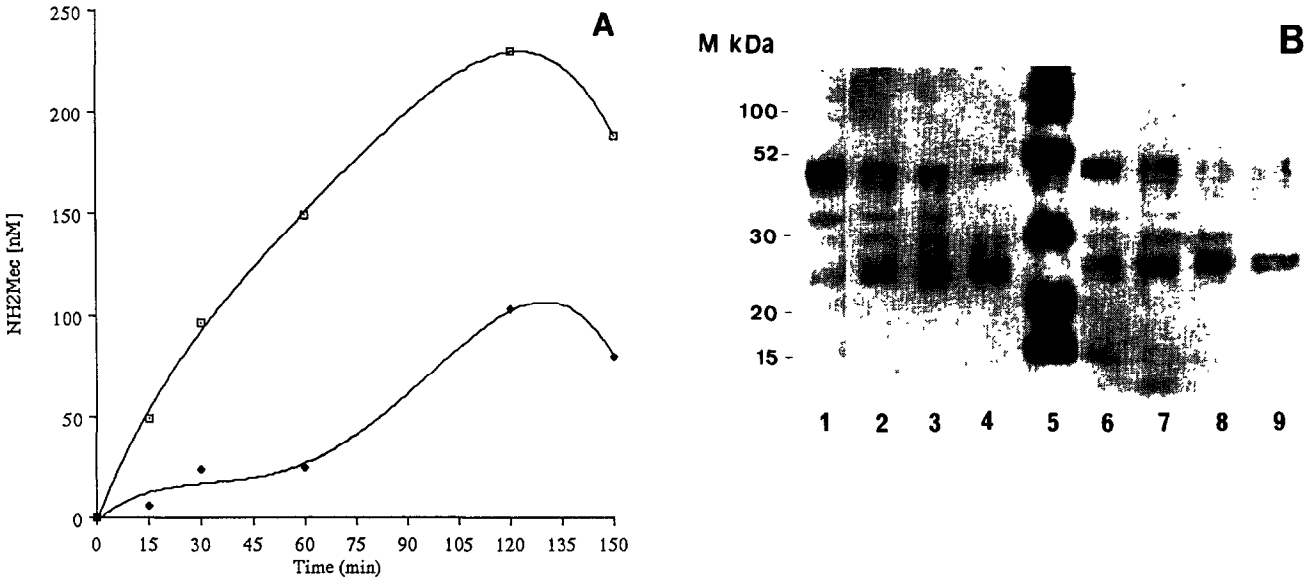

Fig. 1. (A) Generation of cathepsin B from purified pro-cathepsin B as a function of time. Approximatively 20 μg (20 μl) of purified precursor was incubated with 5 μg (5 μl) of leucocyte elastase or cathepsin G in 100 μl (final volume) of 50 mM Tris-HCl buffer, pH 8.6, containing 150 mM NaCl for leucocyte elastase and cathepsin G, respectively. The following incubation times were used: 0, 15, 30, 60, 120, and 150 min. The cathepsin B activity was measured by adding 390 μl of 100 mM phosphate buffer, pH 6.8, containing 1 mM DTE, 2 mM EDTA, 10 μl Z-Arg-Arg-NHMec. After 30 min incubation time, the fluorescence of free NH_2Mec was measured. Activation by elastase (□) and cathepsin G (♦), respectively. (B) Processing of pro-cathepsin B as a function of time studied by the immunoblotting procedure. After incubation as in A the samples were boiled for 5 min at 95°C with 30 μl of 10% SDS/30% glycerol, followed by 15% SDS- PAGE, and transferred to a nitrocellulose sheet (2 h, 300 mA). The sheet was blocked with 2% BSA in PBS overnight. Immunological staining was performed with anti-cathepsin B polyclonal antibodies (40 μg/ml), anti-sheep biotinylated immunoglobulins (2 μg/ml), extravidin (2 μg/ml) and biotin horseradish peroxidase (2 μg/ml). The bands were visualized using 4-chloro-1-naphtol. Lanes 1–4, processing with leucocyte elastase, incubation times 0, 30, 60, 120 min; lane 5, prestained BRL standard proteins; lanes 6–9, processing with cathepsin G, incubation times 0, 30, 60, 120 min.

other hand, chymotrypsin processed the pro-enzymes partially, trypsin digested the pro-enzymes, and plasmin had no effect on the processing of pro-enzymes (data not shown). Pro-cathepsin B is secreted by several transformed cells, including cultured colonic and breast cells [4,10]. Consequently, high concentrations of pro-cathepsin B have been found in ascitic fluids from ovarian carcinomas [5,6].

Recently, a similar pro-enzyme has been purified using ascitic fluids from colon cancer [11]. All studies reported the presence of latent cathepsin B-like species. The in vivo significance of such pro-enzymes could depend on the presence of different activation pathways. Intracellularly, the aspartyl proteinase pathway could be the most important one [12]. Nevertheless, this pathway would be associated with digestion of basement membrane in large acidic vesicles of breast cancer cells [13]. Using a yeast expression system for recombinant rat pro-cathepsin B, activation by cysteine proteinases, such as cathepsins B and L, was found. Processing sites of leucocyte elastase, cathepsin D, pepsin and cathepsins B and L have been identified by means of active site mutants of pro-cathepsin B. However, generation of active cathepsin B from the mutant form was impossible [14]. Results on the activation of pro-cathepsin B by leucocyte elastase from human sputum have been previously reported [15]. In the present report, both leucocyte serine proteinases, located in the azurophil granules, activated pro-cathepsin B following a time-dependent lag phase for the generation of the enzymatic activity and the processing. Elastase was a better activator than cathepsin G on the basis of the activation curves. Other serine proteinases, such as trypsin or chymotrypsin, active in the same pH range, did not activate pro-cathepsin B significantly. From these observations, a leucocyte activation pathway can be postulated. Such a pathway could generate cathepsin B locally around tumour islets, following inflammation and invasion by leucocytes associated with many cancers [1]. These cells are able to liberate elastase and cathepsin G which are active at neutral pH. Both proteinases generate cathepsin B active at near neutral pH, and this could contribute to the invasion through digestion of basement membranes and extracellular matrices [16].

On the other hand, we have studied the action of uPA and plasmin on pro-cathepsin B: only uPA was able to generate enzymatic activity and to process pro-cathepsin B. Plasmin had no effect at either level. The activation and the processing were faster with uPA than with leucocyte proteinases. Another pathway, the activation of soluble and bound Pro-uPA by cathepsin B, has also been described [8]. These pathways could function synergystically, leading to an increased level of both proteinases. The activations occurred at the same time, i.e. the uPA activity generated from pro-uPA with cathepsin B reached a maximum after 30 min [8], and the

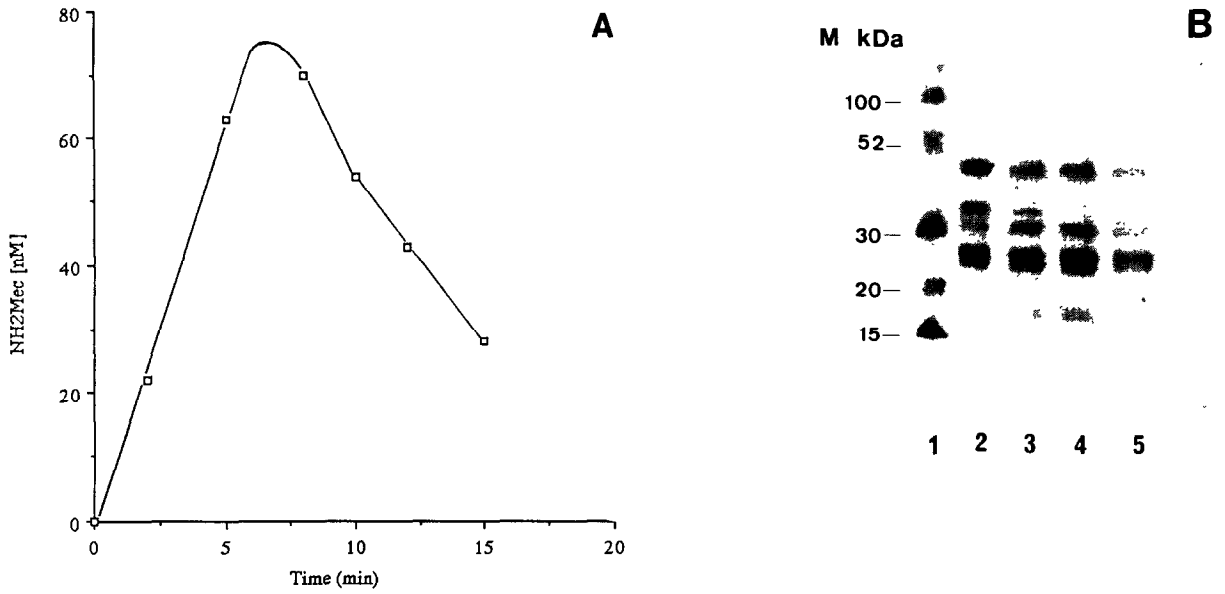

Fig. 2. (A) Generation of cathepsin B from purified pro-cathepsin B as a function of time. Approximatively 20 μg (20 μl) of purified precursor was incubated with 5 μg (5 μl) of uPA in 100 μl (final volume) of 100 mM PBS, pH 7.4, containing 150 mM NaCl. The following incubation times were used: 0, 2, 5, 8, 10, 12, 15 min. The cathepsin B activity was thereafter measured by adding 390 μl of 100 mM phosphate buffer, pH 6.8, containing 1 mM DTE, 2 mM EDTA, 10 μl Z-Arg-Arg-NHMec. After 30 min incubation, the fluorescence of free NH_2Mec was measured. (B) Processing of pro-cathepsin B as a function of time studied by the immunoblotting procedure. After incubation as in A the samples were boiled for 5 min at 95°C with 30 μl of 10% SDS/30% glycerol, followed by 15% SDS-PAGE and transfered to a nitrocellulose sheet (2 h, 300 mA). The sheet was blocked by 2% BSA in PBS overnight. Immunological staining was performed with anti-cathepsin B polyclonal antibodies (40 μg/ml), anti-sheep biotinyled immunoglobulins (2 μg/ml), extravidin (2 μg/ml) and biotin horseradish peroxidase (2 μg/ml). The bands were vizualized using 4-chloro-1-naphtol. Lane 1, prestained BRL standards proteins; lanes 2–5, processing with uPA, incubation times 0, 5, 10, 15 min.

cathepsin B activity generated from pro-cathepsin B with uPA was maximum after about 10 min (Fig. 2A). Expression of both uPA and cathepsin B on the cell surface of HOC1 ovarian cancer cells has been reported [17]. Both proteinases were implicated in the invasive capacity of these cells. This could be, at the cellular level, an illustration of the activation pathways described. Activation of pro-uPA by the cysteine proteinase, cathepsin L, has been shown [18]. A strong relationship between the cysteine proteinases and the plasmin pathway is emerging, which could be important in malignancy, both on the basis of increased levels of these components and reciprocal activation mechanisms. A significant direct degradation of basement membrane type IV collagen by plasmin has been found [19]. Such a result stresses the involvement of a proteolytic cascade dependent on cysteine proteinases in tumour invasion.

Rowan et al. have found that the processing of procathepsin B is a consequence of a peptide bond cleavage in a very short region of the pro-peptide (amino acids −9 to −4): elastase, cathepsin D, pepsin, cathepsins B and L, and papain cleaved and activated pro-cathepsin B in this region [14]. We have noticed the presence of the Arg–Val bond (−9 to −8) which corresponds to the specificity of uPA, suggesting activation of the procathepsin B by uPA at this site. Similarly, cathepsin G, which is a chymotrypsin-like proteinase, could activate pro-cathepsin B by hydrolysing the Phe–Ser bond (−6 to −5). Thus, the different activation pathways of procathepsin B are dependent on the short sequence of this pro-peptide which could be a hinge region located between the pro-peptide and the active proteinase.

In conclusion, as a consequence of the gene overexpression [20], pro-cathepsin B is synthetized in excess and secreted by a lot of human cancer cells. Generation of extracellularly active cathepsin B is dependent on serine proteinases, such as leucocyte elastase, cathepsin G and uPA. These new activation pathways give a more important biological significance to pro-cathepsin B in the proteolytic process associated with tumour invasion.

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