Proteases and Cancer

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Secretion of cathepsin B and tumour invasion
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Lysosomal proteases in health and disease
Lysosomal proteases have been implicated in a variety of pathophysiological processes, such as the malignant progression of tumours [1], muscular dystrophy [2], acute inflammation [3] and complications accompanying the ageing process [4]. In nonpathological situations, these proteolytic enzymes are generally found inside cells, i.e. within the endosomal/lysosomal compartment. They have a broad activity spectrum and their major role is, therefore, believed to be in the intracellular degradation of endocytosed proteins [5] and possibly in the processing of class II major histocompatibility complex (MHC)-restricted antigens [6]. Mammalian lysosomes contain many proteases, among them the cathepsins B, C, H, L and S, which are all members of the papain family of cysteine proteases [7]. Cathepsin B has been the cysteine protease most widely studied in health and disease for two reasons. First, its trypsin-like substrate specificity made it possible to assay the enzyme with existing synthetic substrates for serine proteinases at an early stage in the studies [5]. Secondly, cathepsin B is known to be less tightly controlled by specific endogenous inhibitors, the cystatins, than other lysosomal cysteine proteases [8], making it easy to detect in tissue homogenates and complex biological fluids.

Cathepsin B: biosynthesis and processing
Cathepsin B is encoded by a single copy gene that is under the control of a 'housekeeping' type promoter. Multiple mRNA species are produced from this gene by alternative splicing in the 5' and 3' untranslated regions, suggesting that the expression of cathepsin B may be regulated, in part at least, at the level of mRNA processing [9]. Cathepsin B is synthesized as a preproenzyme [10] in the rough endoplasmic reticulum, and then undergoes several post-translational modifications before being packed into lysosomal vesicles [11]. Activation of the proenzyme, procathepsin B, is assumed to take place within lysosomes either by active cathepsin B itself, or by the action of a cathepsin D-like aspartic proteinase [12]. Although cathepsin B does not appear to be stable in vitro at pH values above 6.5, precise kinetic measurements have shown that the enzyme is, in fact, most active at pH = 8.0 [13]. Instability seems, therefore, to be mainly due to rapid autolysis of the enzyme at pH values above 6.5 [14].

Secretion of active cathepsin B by tumour tissues
More than 20 years ago, active lysosomal cathepsin B was reported to be released into the interstitial fluid of growing, solid tumours in the mouse. In addition, at pH 7.1, this enzyme was shown to be able to detach cells grown in vitro in a very similar way to trypsin [15]. This led to the hypothesis that extracellularly released cathepsin B might contribute directly to the invasiveness of tumour cells and to the destruction of adjacent host tissue. Other investigators have reported that secretion of active cathepsin B from human breast carcinoma tissue explants was much higher than from fibroadenomas or normal breast tissue [16]. Subsequent characterization of the enzyme activity secreted from these carcinoma explants revealed that the enzyme was similar to human liver cathepsin B. The tumour enzyme, however, had a higher molecular mass and was more stable at pH values ≥ 7.0 [17]. In another study, a comparison of the preoperative serum level of cathepsin B activity, with that secreted by the iso-
lated human cervical carcinoma cells excised at surgery, showed a clear correlation between the secreted activity levels in vitro and in vivo. Additionally, these cancer cells were shown to secrete 23-fold greater cathepsin B activity than control cervical epithelial cells [18]. Since these pioneering investigations, numerous other laboratories, including our own [19], have shown an increased expression of cathepsin B in cancer specimens when compared with adjacent normal tissues. Increased expression was observed at both the mRNA and the protein level [20]. In some cases, cathepsin B was found to be selectively relocalized either to the cell surface [1] or to nuclei [21], although the molecular basis for these phenomena is not understood. Therefore, special emphasis will be given here to the secretion of cathepsin B by tumour cells. Our laboratory is mainly interested in the pericellular proteolytic events accompanying tumour invasion.

**Accumulation of latent cathepsin B in human cancerous exudates**

The bulk of cathepsin B secreted by tumour cells accumulates extracellularly as inactive, or latent, enzyme. Latent cathepsin B was shown to accumulate in ascitic fluid of a patient with ovarian carcinoma, and to be secreted into growth medium by ascites cells taken from the same patient. Active cathepsin B could be generated in vitro upon treatment of this extracellular fluid with the aspartic proteinase pepsin, and activation was accompanied by a shift in molecular mass, from 40 kDa to 33 kDa. Thus, it was reasonable to assume that the latent form represented a precursor of cathepsin B that had been secreted instead of being targeted to lysosomes [22]. Latent pepsin-activatable cathepsin B was subsequently also found in pleural effusions from breast cancer patients, and reported to have a molecular mass of about 45 kDa. It was shown to be sensitive to the cysteine proteinase-specific inhibitors E-64 and Z-Phe-Ala-CH₂F, only after activation [23]. We decided to purify this enzyme from the ascitic fluid of a patient with advanced ovarian carcinoma. In contrast to the purification of the pepsin-activated enzyme, which closely followed that of lysosomal cathepsin B [24], isolation of latent cathepsin B proved to be a very difficult task. Initial attempts to dialyse the ascitic fluid at pH 7.4 resulted in a complete loss of latent enzyme, and during purification considerable amounts of latent cathepsin B were converted to active enzyme. Therefore, E-64 was added at each step of the purification procedure and, thus, the spontaneous appearance of active enzyme was dramatically decreased. This permitted the isolation of a 45–47 kDa cathepsin B precursor that remained pepsin-activatable [25]. Polyclonal antibodies raised in sheep against reduced and carboxymethylated human liver cathepsin B, strongly cross-reacted with the purified protein on Western blots [25] and gave a reaction of total identity with the purified pepsin-activated enzyme in double immunodiffusion experiments (unpublished work). We concluded that the latent enzyme indeed represented a true proenzyme of cathepsin B.

**Secretion of latent cathepsin B and cystatin C in vitro by colon carcinoma cells**

Further studies in our laboratory showed that human colon carcinoma cells not only secreted latent cathepsin B into culture media [19], but also one of its endogenous inhibitors, cystatin C [26]. Cystatin C is a member of the cystatin superfamily, which comprises a group of proteinase inhibitors that form tight and reversible complexes with cysteine proteinases [8, 27]. Cystatin C belongs to family 2 of secreted inhibitors and has been reported to be synthesized in many tissues [28] and to be present in most biological fluids [29]. However, neither this inhibitor, nor other secreted cystatins, had been studied with respect to tumour invasion and metastasis. When ten human colon carcinoma cell lines were analysed, they were all found to express the 2.3 and 4.3 kb mRNAs of cathepsin B, and the 0.8 kb mRNA of cystatin C. These species were also expressed in other human cell types, such as fibrosarcoma HT1080 cells, promyelomonocytic U937 cells, and spontaneously immortalized HaCaT keratinocytes [26] (D. Keppler, unpublished work). In ten human colon carcinoma cell lines, secretion of latent cathepsin B and cystatin C was found to be correlated (r = 0.755) (D. Keppler and M. Abrahamson, unpublished work). Since cystatin C has high affinity for cathepsin B (Kᵢ = 0.25 nM), and appears to be the most important physiological inhibitor of extracellularly released cathepsin B [29], its contribution to the latency of secreted cathepsin B had to be assessed. Therefore, we analysed whether secretion of latent cathepsin B and cystatin C was co-regulated. When human colon carcinoma SW 480 cells were treated with various cytokines, such as transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), secretion of the two antagonists was not significantly altered, nor was expression of their respective mRNA species (P. Waridel, D. Keppler and M.
Abrahamson, unpublished work). In contrast to these biological response modifiers, two agents, phorbol 12-myristate 13-acetate (PMA) and the acidotropic agent ammonium chloride, significantly increased secretion of latent cathepsin B without affecting secretion of the inhibitor in vitro. The protein synthesis inhibitor, cycloheximide, drastically inhibited secretion of both the enzyme and the inhibitor (D. Keppler and M. Abrahamson, unpublished work). Our results clearly show that latent cathepsin B is a true proenzyme rather than a cathepsin B-cystatin C complex. They do not exclude the possibility that latent cathepsin B may interact with cystatin C; this aspect clearly needs more detailed biochemical analysis using purified proteins. The cathepsin B and cystatin C genes have both recently been reported to be under the control of housekeeping type promoters [9, 28], which may explain the lack of modulation of their expression by cytokines. On the other hand, PMA is a well-known agent that activates numerous transcription factors and alters the genetic programme of the cells [31]. To elicit a response similar to that obtained with PMA, a synergistic action of different endogenous cytokines may be necessary.

**Accumulation of latent cathepsin B and cystatin C in experimental mouse ascites**

Since colon carcinoma cells secrete latent cathepsin B and cystatin C in vitro, we wondered whether they would do so in vivo, after intraperitoneal injection into nude mice [32]. For this, five congenitally athymic female nude mice, maintained in barrier isolators, were used for each cell line. 5 × 10^6 human colon carcinoma cells (Col112 or Col115), or two monoclonal antibody (mAb) secreting mouse hybridoma clones (for comparison), in 0.5 ml Dulbecco's modified Eagle's medium (DMEM) and with a viability >95% were injected intraperitoneally, without any pretreatment of mice. Ascitic fluids (3-5 ml/mouse) were collected 42 days later, and tumour cells pelleted by centrifugation at 300 g for 5 min. The clear, yellowish and cell-free ascitic samples were then pooled and stored at −20°C. A pool of normal nude mouse serum was used as the control. Latent cathepsin B was assayed with Z-Arg-Arg-NHMec, after 1:16 dilution of fluids followed by pepsin activation, as described in [19]. Cystatin C was quantified using a sandwich enzyme immunoassay described previously [33]. A modified version of this assay was elaborated to specifically quantify human cystatin C in mouse samples. For this, the monoclonal antibody was biotinylated and subsequently detected using peroxidase-labelled avidin.

From Figure 1a it is evident that not only the experimentally induced ascitic fluids, but also normal nude mouse serum, contained latent cathepsin B. This was surprising since we could not detect any latent cathepsin B in normal human serum (D. Keppler, unpublished work), and others had

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**Figure 1**

Accumulation of latent cathepsin B and cystatin C in mouse experimental ascitic fluids

Ascitic fluids were induced in nude mice by intraperitoneal injection of human colon carcinoma cells (Col112 and Col115) or mouse monoclonal hybridomas (mAb1 and mAb2). Ascitic fluid from five mice was pooled and assayed for (a), cathepsin B activity, after 1:16 dilution and pepsin treatment of pooled fluids; and (b), total cystatin C, using an enzyme immunoassay specific for the human inhibitor. A pool of normal nude mouse serum was used as control. Values represent the mean ± S.D. of triplicates.
reported the presence of only trace amounts of active enzyme in human serum [3]. In the murine system, when production of ascites was induced by injection of mouse monoclonal hybridomas, latent mouse cathepsin B was also found to accumulate intraperitoneally. Thus, with the tools presently available, the precise cellular origin of cathepsin B could not be established. When cystatin C was analysed in the same biological fluids (Figure 1b), its cellular origin could be defined unambiguously after modification of the enzyme immunoassay to abolish interference by mouse immunoglobulins present in the samples. As a consequence, no cystatin C was measured in mouse serum and ascitic fluids induced by mouse cells (Figure 1b). Colon carcinoma cells secreted human cystatin C in vivo at levels comparable to those in vitro [26].

When 1 ml of Co115 cell-induced mouse ascitic fluid was passed over a size-exclusion chromatography Sephacryl S-200 HR column (2.6 cm x 95 cm), equilibrated in 50 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl and 0.1% (v/v) Triton X-100, a single peak containing latent, pepsin-activatable cathepsin B was recovered (Figure 2a). The latent enzyme from this fluid eluted closely behind ovalbumin (43 kDa) that was used for the calibration of the column. It did not elute at the same position as active bovine cathepsin B (28 kDa). As an additional control, a preformed complex of bovine cathepsin B and recombinant human cystatin C was run under the same conditions (Figure 2b). Active bovine cathepsin B dissociated from this complex during chromatography and was eluted at the same position as free cathepsin B. Recombinant cystatin C was eluted in two peaks: one minor peak corresponding to dimerized cystatin C and one large peak corresponding to monomeric cystatin C. Active bovine cathepsin B, after preincubation with mouse ascitic fluid (induced by Co115 cells) for 30 min at 37°C, eluted in two peaks: one main peak (72%) represented free cathepsin B, the other peak (28%) eluting close to

**Figure 2**

Size-exclusion chromatography on Sephacryl S-200 HR of latent cathepsin B, and a preformed complex of active cathepsin B and cystatin C

(a). 1 ml of pooled mouse ascitic fluids, induced by Co115, was applied to the Sephacryl S-200 HR gel chromatography column and eluted at pH 6.5. Fractions were assayed for active and latent cathepsin B. Since no active cathepsin B could be detected, only latent cathepsin B is represented. (b). A preformed complex of bovine cathepsin B and recombinant human cystatin C (a molar excess of inhibitor over enzyme that had been incubated for 30 min at 20°C) was eluted under identical conditions. For comparison, elution of bovine cathepsin B is also represented in (a). Elution of recombinant cystatin C was assessed by its ability to inhibit bovine cathepsin B added to each collected fraction.
the void volume (at 160 ml) was typical for the α2-macroglobulin-entrapped enzyme (data not shown) [24]. Since latent cathepsin B did not elute in the void volume together with α2-macroglobulin, and did not cleave the fluorescent substrate even after extensive dilution (1:128), it does not appear to have endopeptidase activity.

**Activation of latent cathepsin B by neutrophil elastase**

The role of tumour cell-secreted latent cathepsin B has remained very speculative since the only proteinases known to be capable of activating this enzyme, pepsin and cathepsin D, require very acidic conditions for activity. Recently, however, it has been shown that treatment of sputum (from patients with bronchiectasis) with polymorphonuclear neutrophil (PMN) elastase generated a 5–6-fold increase in cathepsin H activity. This increase was accompanied by a shift of a 40 kDa molecular mass cathepsin B precursor into a 37 kDa component [34]. Cystatin C was also present in the sputum, and it has been shown that PMN elastase catalytically inactivates cystatin C with respect to inhibition of cathepsin B [35]. In a complex biological system like sputum, it is unclear whether PMN elastase acts directly on the precursor, or indirectly via inactivation of cystatin C, to release cathepsin B activity. We therefore decided to separate latent cathepsin B from cystatin C, present in a 100-fold concentrated colon carcinoma cell-conditioned medium, by the use of the same gel-permeation column as used above (Figure 2). Colo205 medium was chosen because it contained high levels of latent cathepsin B but no detectable cathepsin H or L [19]. Activation by human PMN elastase was then studied on the concentrated pool of latent cathepsin B. Elastase activated latent cathepsin B in a dose-dependent manner (D. Keppler and M. Abrahamson, unpublished work). Even at the physiological concentration of 0.1 μM, a significant activation was detected. This concentration is about

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**Figure 3**

Schematic representation of the extracellular potentiation of tumour cell-secreted cathepsin B activity by PMN elastase.
four times lower than the concentration observed when PMNs are induced to degranulate in vitro [36], and about 33 times lower than the PMN elastase concentration measured in purulent sputum of patients with bronchiectasis (600 U/ml ≈ 3.3 μM [37]. In the absence of elastase, and during the incubation time of the assay (30 min at 37°C), latent cathepsin B underwent barely detectable autoactivation [30].

**Cathepsin B and pericellular proteolysis: perspectives**

Several systems have been described for the quantification of tumour invasion and pericellular proteolysis in vitro [38], and in most cases single cell populations were investigated. When colon carcinoma Co15 cells alone were studied for extracellular matrix degradation, we could not detect any cathepsin B-dependent proteolysis, unless cell-conditioned media were pre-treated with pepsin [26]. Taken together, our results indicate that in the future such assays should be performed in vitro in a co-culture system, in which at least neutrophils, or some other elastase-secreting host cells, are present.

Tumour tissues are most often infiltrated by inflammatory cells, such as PMNs [39]. Our finding that human PMN elastase can directly activate tumour-cell-secreted latent cathepsin B offers new clues towards the understanding of the role of cathepsin B in tumour invasion and metastasis. At the tumour-host interface, activation of latent cathepsin B may occur under physiological conditions and, since PMN elastase also has the capacity to inactivate cystatin C, cathepsin B would escape immediate control (Figure 3). In addition, our observations may explain earlier reports showing that tumour explants in organ culture secreted active cathepsin B [15, 16, 18]. Since cathepsin B is able to degrade extracellular matrix components at physiological pH [14, 26] it may, together with other proteinases, have a direct role in tumour invasion and metastasis, by favouring a loss of normal tissue constraints to cell movement [15].

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A novel matrix-degrading protease in hormone-dependent breast cancer
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The process of metastasis in breast cancer accounts for the inability of surgery to cure all but the earliest stages of the disease. As breast epithelial growth becomes dysregulated within the ducts and lobules, the stromal-epithelial tissue compartmentalization can also become disturbed. The destruction of the epithelial basement membrane signals local invasion of the disease, while blood vessel infiltration (angiongenesis) parallels the ability of the tumour to spread, via lymphatic and hematogenous means, to nearby lymph nodes, distant viscera and bone. Plural and ascites growth can also characterize the later behaviour of the metastatic disease [1–3]. Metastasis is proposed to depend upon alterations in cell adhesion, motility, the elaboration of proteases and the ability to survive in non-mammary environments [4]. This article focuses on the identification and characterization of proteases.

Proteases appear to be required for the degradation of the basement membrane and stromal matrix as breast cancer invades locally, enters nearby vasculature (intravasation), exits the vasculature in metastatic sites (extravasation) and expands through the site of metastases. Enzymes from the four general classes of protease have been proposed to be involved in breast cancer metastases: cathepsins B and L represent the cysteine proteases [5, 6]; cathepsin D is a member of the aspartyl protease class [7]; collagenases, specifically the type IV collagenases and stromelysins, represent the metal-dependent proteases [8]; finally, urokinase and plasminogen represent the serine proteases [9]. Each of these enzymes is over-expressed or inappropriately expressed in breast cancer, but none has been fully proven to actually mediate degradation of basement membrane or stromal matrix in breast cancer metastases. The most extensive work has been on aspartyl and metal-dependent proteases; thus they have been primary subjects of our work.

Immunohistochemistry of normal human breast issue has localized the 72 kDa type IV collagenase [matrix metalloproteinase-2 (MMP-2), an enzyme of neutral pH optimum] to myo-epithelial cells; epithelial cells stained negative for MMP-2 [10]. Cathepsin D, an enzyme with a strongly acidic pH optimum, is found in the lysosomes of most normal cells [11]. Ductal epithelial cells stain positive for MMP-2 in carcinoma in situ, as do the majority of invasive breast carcinomas and metastatic lymph node deposits [10, 12]. Immunohistochemical staining has also suggested a prognostic role of MMP-2 for local recurrence of the disease [13, 14], but the rate of distant metastases and patient sur-

Abbreviations used: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; UK, urokinase; PAI-1, plasminogen activator inhibitor-1.