

Enhanced levels of cathepsin B mRNA in murine tumors

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Relative amounts of mRNA for cathepsin B were measured in normal murine liver and three murine tumors, an invasive liver tumor (hepatoma, Hepa cl 9) and two melanoma variants (B16-F1 and B16 amelanotic melanoma, B16a). Using a human cDNA to the cathepsin B coding region as a hybridization probe, we detected two species of cathepsin B specific RNA transcripts (2.2 and 4.1 kb) in total RNA preparations of all four tissues. The concentrations of the 2.2 and 4.1 kb species were 3.6 and 2.7-fold greater in the highly metastatic B16a melanoma than in normal liver. The concentration of the 2.2 kb species in the invasive hepatoma was 1.7-fold greater than in normal liver. The increased levels of the 2.2 kb message were reflected in increases in activity of cathepsin B in both Hepa cl 9 and B16a.

Cysteine proteinase; Acid hydrolase; Tumor metastasis; Gene expression

1. INTRODUCTION

The cysteine proteinase cathepsin B has been hypothesized to play a role in the focal dissolution of the extracellular matrix by tumor cells during the metastatic cascade. This hypothesis is based in part on a correlation between cathepsin B activity (in subcutaneous tumors, isolated tumor cells and tumor plasma membrane fractions) and the metastatic potential of tumor variants (reviews [1,2]). Enhanced cathepsin B activity in metastatic tumors may reflect changes in the concentrations, forms or subcellular localization of cathepsin B. Here, we have determined whether the enhanced cathepsin B activity reflects increased gene expression. The levels of cathepsin B-specific RNA transcripts in total RNA from liver and three tumors were measured in Northern blots using a cDNA clone for human cathepsin B [3] as a hybridization probe.

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2. MATERIALS AND METHODS

2.1. Tissues

B16-F1, B16a and Hepa cl 9 were propagated in their syngeneic male hosts, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) according to our published procedures [4]. The metastatic (lung colonization) capability of Hepa cl 9 was determined and that of the B16 variants verified as in [4]. Livers were obtained from male C57BL/6J mice without tumors.

2.2. Isolation of the cDNA clone

The human cathepsin B cDNA clone, pCB-1 was screened from a human cDNA library and inserted into the bacterium *Escherichia coli* as described [3]. A subclone of this plasmid, pB-1, containing only the cathepsin B coding region (*Pst*I/*Eco*RI fragment of 593 nucleotides) was selected [3]. *E. coli* harboring the plasmid pB-1 was grown in culture according to Maniatis et al. [5]. The plasmid was amplified in the presence of chloramphenicol (Sigma, St. Louis, MO) and purified as described in [5]. Plasmid pUC19 containing cDNA to human 18 S rRNA [6] was a gift from Dr O.J. Miller (Department of Molecular Biology, Wayne State University).

2.3. RNA isolation

A 5 g pool of fresh tissue was minced in homogenization buffer (4 M guanidine thiocyanate, 0.5% sarkosyl, 25 mM sodium acetate, pH 7.0) and homogenized with a Potter-Elvehjem

homogenizer (Wheaton Scientific, Millville, NJ) as in [4]. Total cellular RNA was isolated according to Chomczynski and Sacchi [7], electrophoresed on a 1% agarose-formaldehyde denaturing gel (30 μ g/lane) according to the procedure of Maniatis et al. [5] and transferred to a GeneScreen Plus nylon membrane (New England Nuclear, Boston, MA) by electroblotting (Hoeffer Scientific, San Francisco, CA) or stained with ethidium bromide (final concentration 0.5 μ g/ml).

2.4. 32 P labeling and RNA blot hybridization of the probes

1 μ g of plasmid pB-1 or plasmid pUC λ 9 was 32 P labeled by nick translation (Bethesda Research Labs, Gaithersburg, MD) to a specific activity of 4.0×10^7 cpm/ μ g. Alternatively, 60 ng of the purified cathepsin B cDNA insert was 32 P labeled by random priming (Amersham, Arlington Heights, IL) to a specific activity of 5.0×10^8 cpm/ μ g. Nylon membranes were prehybridized and then hybridized at 42°C to the radioactive probe as described by Maniatis et al. [5]. Washing was done twice in $2 \times$ SSC ($1 \times$ SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7) at room temperature for 5 min, twice in $2 \times$ SSC-1% SDS at 60°C for 30 min, and twice in $0.1 \times$ SSC at room temperature for 30 min. Autoradiography was performed at -70°C for 30 min to 24 h depending on the intensity of the signal. The relative amounts of cathepsin B-specific RNAs were quantitated (peak area) by scanning laser densitometry (LKB, Bromma, Sweden) of the autoradiograms.

2.5. Cathepsin B and protein assays

Cathepsin B activity (V_{\max}) in tissue homogenates was determined using our published procedure [4]. Subcellular fractionations were performed as in [8]. Protein was measured by the Bradford procedure [9].

3. RESULTS

Based on previous observations of increased cathepsin B activity in plasma membrane fractions of metastatic tumors [1,4,8], we chose to examine three parameters regarding cathepsin B (activity, subcellular localization and mRNA levels) in three representative murine tumors and in normal murine liver. The three tumors were a hepatoma, i.e. a malignant counterpart of the liver, and two variants of the B16 melanoma that differ in their metastatic capacity (B16-F1 and B16 amelanotic melanoma, B16a). The Hepa cl 9 was a locally invasive tumor with limited metastatic capability, forming a median of 2 lung colonies (range, 0-6) at 21 days post-tail vein injection of 3.75×10^4 viable tumor cells into 10 mice. Under the same conditions the B16-F1 and B16a form a median of 25 and 54 lung colonies, respectively [4]. In addition, the B16a is reproducibly metastatic after subcutaneous injection [4]. Thus the three tumors

examined cover a spectrum of metastatic abilities.

In all three tumors examined cathepsin B activity was greater than that in liver. Specific activities (determined as V_{\max}) in liver, Hepa cl 9, B16-F1 and B16a homogenates were 5.5, 10.2, 7.7 and 10.9 nmol/min per mg protein, respectively. The proportion of cathepsin B in the plasma membrane fraction (L-1) as compared to the lysosomal fraction (L-2) was greater in the three tumors and this elevation was proportional to the metastatic capability of all three tumors (fig.1), a finding consistent with previous studies of both rodent and human tumors [1,4,8].

Using a human cathepsin B cDNA probe, we detected two hybridizable RNA species of 2.2 and 4.1 kb in the four murine tissues examined (figs 2,3A). The 4.1 kb transcript can be observed more readily in fig.2. The concentration of the 4.1 kb transcript (fig.3A) in the two less metastatic tumors, Hepa cl 9 and B16-F1 (33 and 33 pg/mg of total RNA, respectively), was similar to that in liver (26 pg/mg), whereas that in the spontaneously metastatic B16a tumor (70 pg/mg) was increased. In contrast, the concentration of the 2.2 kb mRNA (fig.3A) was greater in both Hepa cl 9 and B16a (64 and 150 pg/mg, respectively) than in liver (40 pg/mg), yet not in B16-F1 (47 pg/mg). This apparent increase in expression of the cathepsin B gene in Hepa cl 9 and B16a was reflected in part in increased cathepsin B activity in the respective tumors (see above). The amount of RNA per lane on the Northern blots was judged to be equal by ethidium bromide staining of the ribosomal

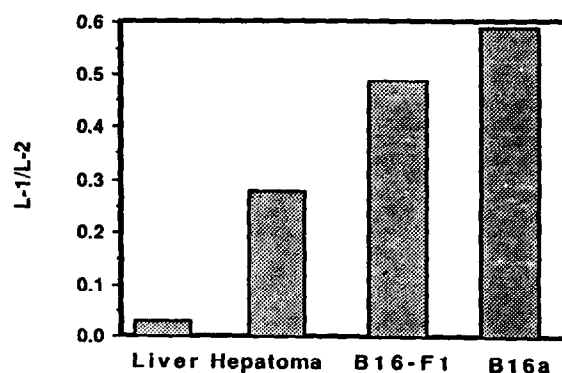


Fig.1. Ratios of cathepsin B activities in plasma membrane (L-1) and lysosomal (L-2) fractions isolated by Percoll density gradient centrifugation as described in [8].

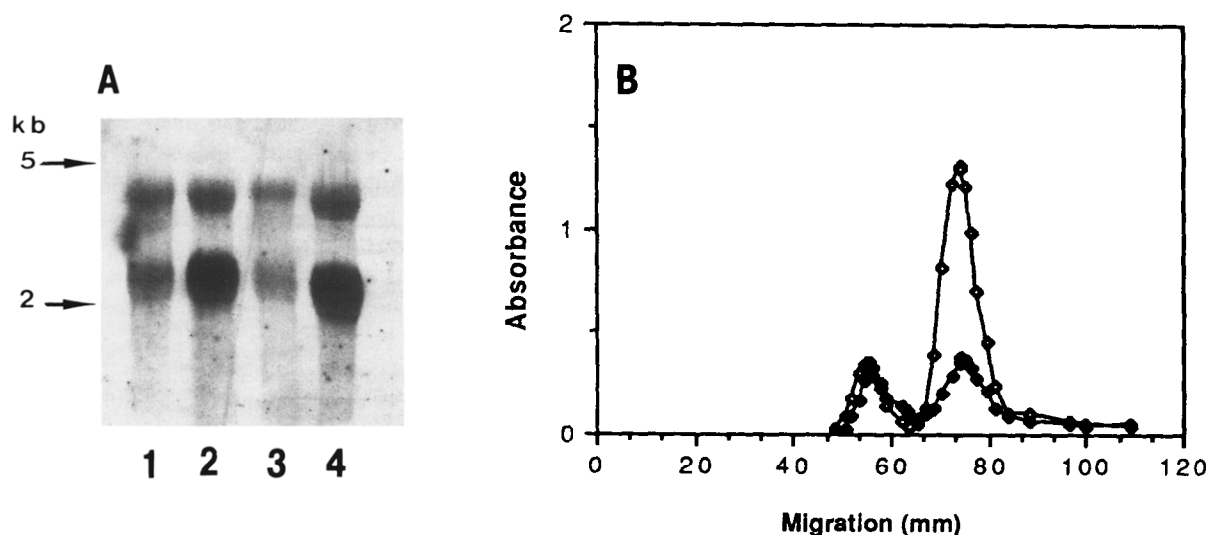


Fig.2. (A) Blot hybridization of total RNAs to human cathepsin B cDNA. Lanes: (1,3) liver RNA; (2,4) B16a RNA. Ribosomal RNA sizes are indicated in kilobases (kb). (B) Laser densitogram of hybridized mRNAs from A illustrating the absorbance peaks corresponding to the 4.1 and 2.2 kb mRNAs in both tissues. (◊) B16a, (●) liver.

bands (not shown) and by hybridization to human 18 S rRNA-specific cDNA (fig.3B). In addition, the amount of hybridizable β -actin RNA in the tissues showed no correspondence to that of cathepsin B-specific RNA transcripts (not shown). These data indicate that the increased levels of cathepsin B-specific RNA transcripts in Hepa cl 9 and B16a were specific and not due to differences in the amount of total RNA per lane.

4. DISCUSSION

Indirect evidence had indicated that cathepsin B synthesis might be increased in tumors. Koppel et al. [10] had reported that cycloheximide reduces the cathepsin B content of a metastatic rat anaplastic sarcoma and Recklies et al. [11] had reported that cycloheximide inhibits cathepsin B release from malignant human breast tumors.

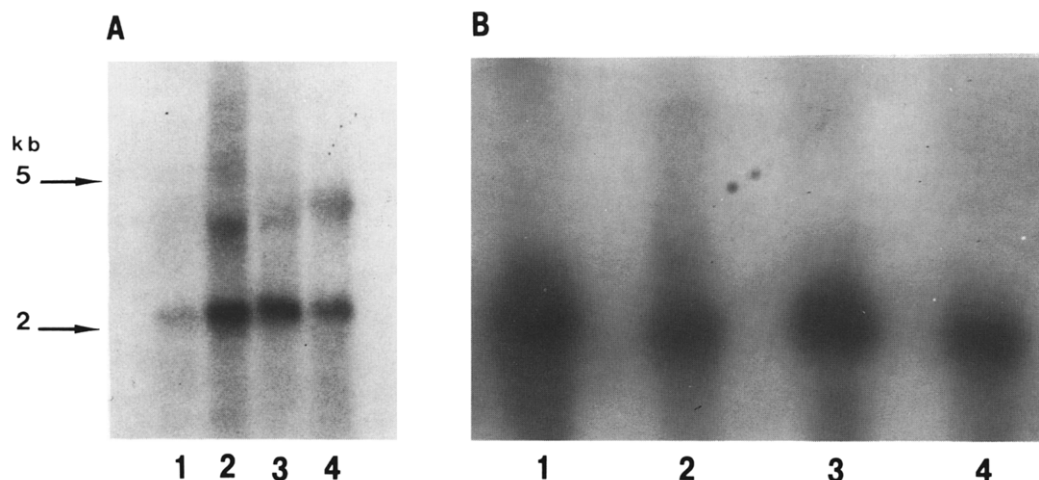


Fig.3. Blot hybridization of total RNAs to human cathepsin B cDNA (A) and human 18 S rRNA cDNA (B). Lanes 1-4 contain RNA from normal mouse liver, B16a, Hepa cl 9 and B16-F1, respectively. Ribosomal RNA sizes are indicated in kb.

Here, we have now provided direct evidence that cathepsin B synthesis may be enhanced in two tumors. The first of these was a hepatoma that arose spontaneously in the liver of C57BL/6J mice [12]. Although of low metastatic potential, in our hands the Hepa cl 9 was highly invasive, invading through the peritoneal wall after subcutaneous implantation and spreading throughout the peritoneal cavity. The second tumor, a melanoma (B16a) also syngeneic to C57BL/6J mice, was one we have shown to be highly metastatic, both upon tail-vein injection and subcutaneous implantation [4]. In the third tumor we examined, the B16-F1, a tumor in which we have previously shown a low metastatic potential and low cathepsin B activity [4,13], the mRNA levels for cathepsin B were not greater than in liver. However, the subcellular distribution of cathepsin B in this tumor was altered so that a significant proportion of cathepsin B was membrane-associated (see fig.1).

The cathepsin B activity measured in the present study in the B16a tumor was similar to that we had reported previously [4], i.e. approx. 2-fold greater than that in normal liver. The apparent discrepancy between the levels of the 2.2 kb mRNA transcripts (1.7- and 3.6-fold greater in Hepa cl 9 and B16a than in liver) and cathepsin B activities in Hepa cl 9 and B16a may reflect an enhanced synthesis of inactive, high- M_r precursor forms of cathepsin B in the B16a tumor and the presence of endogenous inhibitors that may affect activity measurements.

Earlier work had identified only one cathepsin B-specific transcript of 2.3 kb in nine normal rat tissues and one rat tumor [14]. There has now been a report in abstract form of two cathepsin B-specific transcripts of 4.0 and 2.2 kb in murine melanoma but not in murine liver [15]. Thus, the present study is the first to report two cathepsin B-specific transcripts in a normal tissue.

We have speculated that cathepsin B could be one component of a proteolytic cascade that leads to the extravasation of metastatic tumor cells [2]. Several lines of evidence provide support for this hypothesis: (i) the increased activity of cathepsin B in metastatic tumors [1]; (ii) an association of cathepsin B with plasma membrane fractions of metastatic tumors [1] (see also fig.1); and (iii) the ability of cathepsin B to degrade components of the basement membrane under physiologic condi-

tions [16]. In the present study we have demonstrated that the enhanced cathepsin B activity in two tumors reflects an increase in expression of the cathepsin B gene. The present study therefore suggests that alterations in the expression and/or subcellular distribution of cathepsin B in tumors may be linked to their invasive and metastatic capabilities.

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