Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2483–2487, April 1986
Cell Biology

Cathepsin B: Association with plasma membrane in metastatic tumors

(cysteine proteinase/lysosome/melanoma/glycosidase/Percoll)

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Communicated by George B. Koelle, December 12, 1985

ABSTRACT **The subcellular localization of cathepsin B activity (EC 3.4.22.1) in three murine melanomas of increasing metastatic potential (Cloudman < B16-F1 < B16 amelanotic) was determined. Cathepsin B activity was localized in the heavy mitochondrial fraction of normal murine liver but in the light mitochondrial fraction of the metastatic melanomas; the localization of three other lysosomal hydrolases did not shift. Further purification of the light mitochondrial fraction into L-1 (density = 1.045 g/ml) and L-2 (density = 1.07 g/ml) fractions was achieved on a 30% iso-osmotic Percoll gradient. The L-1 fraction of liver and melanomas contained Na^+,K^+-ATPase activity; the L-2 fraction of liver contained four lysosomal hydrolase (cathepsins B and H, *N*-acetyl-β-glucosaminidase, and β-glucuronidase) and glucose-6-phosphatase activities. Ultrastructural examination revealed that the L-1 fraction consisted of membrane vesicles and the L-2 fraction of secondary lysosomes. In the B16 melanomas cathepsin B and *N*-acetyl-β-glucosaminidase activities were found in both L-1 and L-2 fractions. Specific activities of the two enzymes in the plasma membrane (L-1) fractions increased in correspondence with metastatic potential. Cathepsin H and β-glucuronidase activities were not localized in the plasma membrane fractions of the B16 melanomas. Localization of hydrolytic enzymes in the plasma membrane of metastatic tumor cells could result in focal dissolution of the extracellular matrix and thereby invasion and metastasis.**

The ability of tumor cells to invade into the extracellular matrix has been linked to enzymes that are released by tumor cells or associated with the plasma membrane of tumor cells. The role of a tumor cell heparanase in this process(es) has been extensively evaluated by Nicolson *et al.* (1). Proteinases of three classes (metallo-, serine, and cysteine) have been implicated in tumor cell invasion and metastasis by numerous laboratories (see refs. 2–4 for review). Our laboratory has been exploring the role of a cathepsin B-like cysteine proteinase in metastasis. We have shown that cathepsin B activity (EC 3.4.22.1) in tumor cells correlates with the lung colonization potential of B16 melanoma variants and with the metastatic potential of variants derived from an amelanotic B16 melanoma and the Lewis lung carcinoma (4–6).

Although cathepsin B is a lysosomal cysteine proteinase (7), cathepsin B activity (mainly latent) has been measured in the media of tumor cells and explants in culture (5, 8) and in ascites fluid of women with ovarian carcinoma (9). Macrophages release mature lysosomal enzymes in response to stimuli (10); other cell types seem to release only a small proportion of their lysosomal enzymes (latent and mature), perhaps due to a defect in intracellular processing (11). The presence of tumor cathepsin B extracellularly might indicate that tumor cells are defective in the intracellular processing of this enzyme, resulting in its delivery to plasma membrane rather than to the lysosome. The fact that the cathepsin B released from breast carcinoma explants and present in ascites fluid is latent and has a higher molecular weight (8, 9) lends support to this hypothesis. Cathepsin B activity has been found in a plasma membrane fraction of squamous carcinoma cells from human ectocervix but not of non-neoplastic cervical cells (12). Bohmer *et al.* (13) reported acid cysteine proteinase activity in plasma membrane fractions of bovine lymphosarcoma cells and normal lymphoid cells; the acid cysteine proteinase was not identified. Koppel *et al.* (14) have found that the spontaneous BDX rat anaplastic sarcoma (BSp 73) manifests membrane-associated cathepsin B activity. In our laboratory we had established that membrane vesicles spontaneously shed from murine tumor cells (15091A mammary adenocarcinoma) in culture possess cathepsin B activity (15). In the present study we performed a systematic analysis of lysosomal hydrolase activity in subcellular fractions of murine melanomas of various metastatic potentials. We found that the subcellular localization of cathepsin B and *N*-acetyl-β-glucosaminidase activities shifted to a plasma membrane fraction in correspondence with increasing metastatic potential.

MATERIALS AND METHODS

Reagents. Percoll was purchased from Sigma. Substrates for assay of cysteine proteinases were obtained from Enzyme Systems Products (Livermore, CA), and substrates for assay of glycosidases were from Research Products International (Elk Grove Village, IL). Embedding medium (Embed 812) and chemicals for electron microscopy were purchased from Electron Microscopy Sciences (Fort Washington, PA). All other chemicals were of reagent grade and were obtained from commercial sources. Mice were purchased from The Jackson Laboratory.

Tissues. An amelanotic variant of the murine B16 melanoma (B16a), the B16-F1 metastatic variant, and the Cloudman S91 melanoma were obtained from the Division of Cancer Treatment (National Cancer Institute) human and animal tumor bank and were frozen in liquid N_2 immediately upon receipt. The tumor lines were propagated *in vivo* by subcutaneous injection of cells from frozen stocks or cellular brei from subcutaneous tumors into the left axillary region of syngeneic male mice. B16 tumors were propagated in C57BL/6J mice and Cloudman, in DBA/2 mice. To maintain their metastatic phenotypes, the tumor lines were routinely restarted from liquid N_2 frozen stocks after six isotransplant generations *in vivo*. The livers were from C57BL/6J male mice without tumors.

Subcellular Fractionation. A 5-g pool of normal livers or subcutaneous tumors (weight range 0.8–2.5 g) was minced in

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ice-cold homogenization buffer (250 mM sucrose/25 mM Hepes/1 mM EDTA, pH 7.3). All further manipulations were at 4°C. The minced tissue was homogenized by two 5-sec bursts of a Tekmar (Cincinnati, OH) homogenizer at maximum speed separated by an intermediate cooling period in an ice slurry. The homogenate was filtered through four layers of cheesecloth and the volume was adjusted to 10% (wt/vol) with the homogenization buffer.

The homogenate was centrifuged at 530 × *g* for 11 min (Beckman Ti 45 rotor) to yield a nuclear pellet; the pellet was washed in 10 ml of buffer and recentrifuged. The two nuclear supernatants were pooled and centrifuged at 6800 × *g* for 7 min to yield a heavy mitochondrial pellet; this pellet was washed and recentrifuged. The pooled supernatants were then centrifuged at 15,100 × *g* for 19 min to yield a light mitochondrial pellet. The light mitochondrial pellet was also washed and recentrifuged.

The final light mitochondrial pellet was further purified by density gradient centrifugation on 30% (vol/vol) iso-osmotic Percoll prepared in homogenization buffer. The self-forming gradient was generated by centrifugation for 16 min at 60,000 × *g* (Beckman Ti 50 rotor). Fractions were collected in one of two ways: (*i*) two visible bands (L-1 and L-2) were aspirated with a Pasteur pipette or (*ii*) 0.3-ml fractions were collected from the bottom of the tube by using a peristaltic pump. Density marker beads were centrifuged in parallel gradients to determine density. The L-1 and L-2 bands collected by aspiration were separated from the Percoll medium after dilution in the homogenization buffer and recentrifugation at 120,000 × *g* for 55 min. Fractions were resuspended in or diluted with homogenization buffer and quick frozen at −70°C. Triton X-100, 0.1% (vol/vol) final concentration, was added to all aliquots except those for assay of Na^+,K^+-ATPase. Samples were stored at −70°C until assay.

Electron Microscopy. Aliquots of the L-1 and L-2 bands were fixed with 2% (vol/vol) glutaraldehyde in 75 mM sodium cacodylate buffer (pH 7.4) for 2 hr at 4°C. The subcellular fractions were postfixed for 1 hr in 1% (wt/vol) OsO_4, dehydrated in a graded series of ethanols and propylene oxide, and embedded in Embed 812. Thin sections were cut on a Sorvall MT-5000 ultramicrotome, stained with aqueous uranyl acetate and lead citrate, and examined in a Zeiss 10CA microscope.

Enzyme Assays. Cathepsin B was assayed at pH 6.2 using carbobenzoxyarginylarginyl-4-methoxy-β-naphthylamide as substrate (7); the enzyme was activated for 30 min at 37°C in the presence of 15 mM dithiothreitol and 7 mM EDTA at pH 5.2. V_{max} was determined from Lineweaver and Burk plots of initial velocities from the rate of enzyme reactions. Corrections were made for quenching by the sample and for nonenzymatic hydrolysis of substrate. Cathepsin H (EC 3.4.22.16) was assayed at pH 6.8 by using L-arginyl-4-methoxy-β-naphthylamide as substrate in the presence of 0.1 mM puromycin to inhibit arylamidases (7). Otherwise, the procedure was identical to that for the cathepsin B assay. Two lysosomal glycosidases [*N*-acetyl-β-glucosaminidase (EC 3.2.1.30) and β-glucuronidase (EC 3.2.1.31)] were determined by using 4-methylumbelliferyl substrates as described previously (16). Activity of ouabain-sensitive (Na^+/K^+-activated) ATPase (EC 3.6.1.3) was determined according to Jorgensen (17). Glucose-6-phosphatase (EC 3.1.3.9) was assayed as described by Aronson and Touster (18). Protein was determined by the Bradford procedure (19), using bovine serum albumin as standard. Concentrations of reaction products were derived from standard curves by linear regression analysis.

Metastasis Studies. The metastasis studies were performed as previously described (20). Briefly, viable tumor cells were isolated from subcutaneous tumors by sequential collegenase digestion and subsequent centrifugal elutriation (21). Tumor cells were counted on a model ZBI Coulter Counter and viability was determined by trypan blue dye exclusion. Cell aliquots were injected subcutaneously into the left axillary region of syngeneic male mice. At 28 days after tumor cell injection the mice were sacrificed by cervical dislocation, the lungs were removed and fixed in Bouin's solution, and macroscopic pulmonary metastases were counted by using a dissecting microscope. A minimum of 12 mice were used per tumor line per metastasis study. Over a period of 4 years these experiments have been repeated 5–100 times for the three melanoma lines.

RESULTS

Pulmonary Metastasis. The three melanomas used in this study exhibited a spectrum of capability for spontaneous pulmonary metastasis from subcutaneous tumors. The Cloudman melanoma rarely metastasized even when 5×10^5 viable tumor cells were injected subcutaneously. The B16a line formed 40 ± 8 pulmonary metastases from an inoculum of 10^5 viable tumor cells. The metastatic capability of the B16-F1 variant was intermediate; 0–5 pulmonary metastases resulted from an inoculum of 10^5 viable tumor cells.

Differential Centrifugation. Homogenates of liver and melanomas were separated into four fractions (nuclear, heavy mitochondrial, light mitochondrial, and supernatant) of decreasing sedimentation coefficient s_{min}. The recovery of protein for the four tissues was 87–98%; recovery of enzyme activities was 81–118%. The distribution of lysosomal hydrolases in liver and Cloudman, B16-F1, and B16a melanomas is depicted in Fig. 1. The degree of purification in each fraction is represented by the relative specific activity—i.e., the ratio of the specific activity in the fraction to the specific activity in the starting material.

Cathepsin B activity in the liver was distributed primarily in the heavy mitochondrial fraction. In the metastatic tumors the distribution of cathepsin B activity was shifted to the light mitochondrial fraction in correspondence with metastatic potential (Fig. 1). The ratio of cathepsin B activity in the light mitochondrial fraction to that in the heavy mitochondrial fraction was 0.5 for liver, 1.0 for Cloudman, 1.5 for B16-F1, and 1.9 for B16a. We also analyzed the subcellular distribution of one additional lysosomal cysteine proteinase, cathepsin H, and two lysosomal glycosidases, *N*-acetyl-β-glucosaminidase (Fig. 1) and β-glucuronidase. Their distribution in the liver was primarily in the heavy mitochondrial fraction. A shift in distribution to the light mitochondrial fraction in correspondence with metastatic potential was not observed.

Percoll Density Gradient Centrifugation. Light mitochondrial fractions obtained from the four tissues were further purified by density gradient centrifugation on a self-generated gradient of iso-osmotic 30% Percoll. The two visible bands were aspirated and assayed. The upper band was designated L-1 and had a density of 1.045 g/ml; the lower band was designated L-2 and had a density of 1.07 g/ml. Na^+,K^+-ATPase was used as a marker for the plasma membrane and glucose-6-phosphatase as a marker for the endoplasmic reticulum. The highest relative specific activities and specific activities of Na^+,K^+-ATPase were found in L-1 (Table 1), whereas the highest relative specific activities and specific activities of glucose-6-phosphatase were found in L-2 (data not shown). The highest specific activities and relative specific activities of the four acid hydrolases were found in the L-2 fractions of the four tissues [cathepsin B and *N*-acetyl-β-glucosaminidase (Table 1) and cathepsin H and β-glucuronidase (data not shown)]. The relative specific activities in L-2 from normal murine liver ranged from a high of 16.4 for cathepsin H to a low of 4.4 for *N*-acetyl-β-

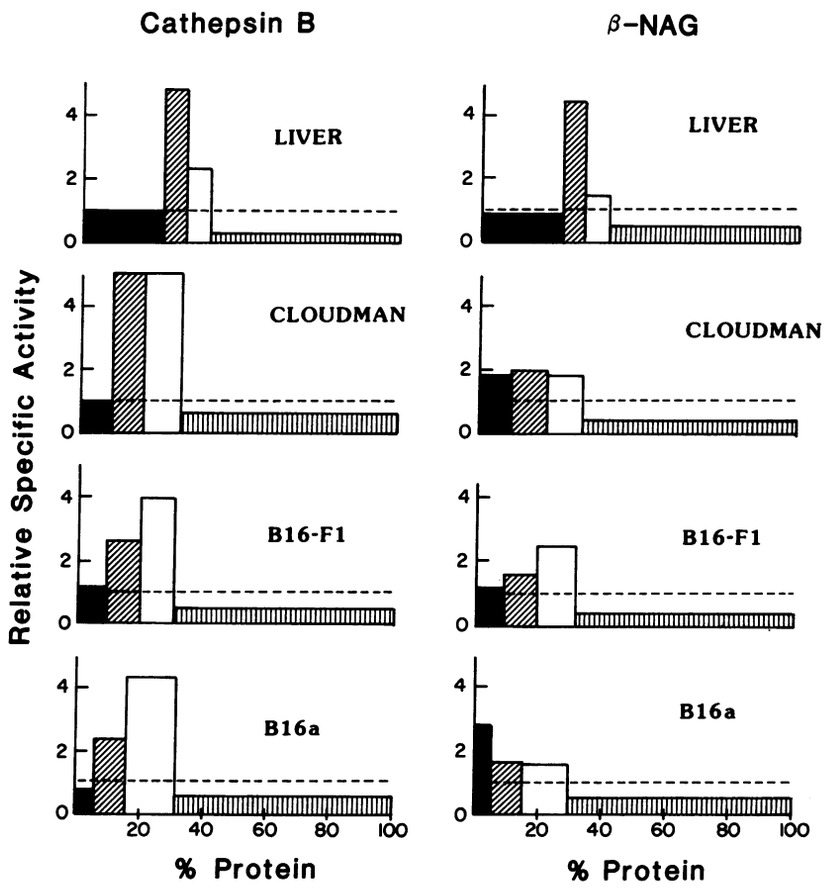

FIG. 1. Distribution of lysosomal hydrolases after differential centrifugation of homogenates of murine liver, Cloudman melanoma, B16-F1 melanoma, and B16 amelanotic (B16a) melanoma. The histogram bars from left to right depict the nuclear, heavy mitochondrial, light mitochondrial, and supernatant fractions. The relative specific activity of the fractions is plotted against their relative protein content. β-NAG, *N*-acetyl-β-glucosaminidase. Results shown are from a representative experiment out of a total of 7 for liver, 2 for Cloudman, 3 for B16-F1, and 10 for B16a.

glucosaminidase. Activities of three lysosomal hydrolases [cathepsin B and *N*-acetyl-β-glucosaminidase (Table 1) and cathepsin H (data not shown)] were minimal in the L-1 fraction of normal murine liver; the relative specific activity for all three hydrolases in L-1 of the murine liver was 0.3. In contrast, β-glucuronidase, which has been shown previously to have both a lysosomal and nonlysosomal localization (22), had a relative specific activity of 1.3 in L-1 of liver (data not shown).

On the basis of the marker enzyme assays of murine melanomas, the L-1 fraction contained plasma membrane but not endoplasmic reticulum and the L-2 fraction contained lysosomes and endoplasmic reticulum. The absence of lysosomes in L-1 was supported by the assays of cathepsin H and β-glucuronidase activities in the three melanomas—i.e., the relative specific activities of cathepsin H and β-glucuronidase in the L-1 fractions of the three melanomas were similar to their relative specific activities in L-1 of liver (0.5 ± 0.2 for cathepsin H and 1.1 ± 0.2 for β-glucuronidase). In contrast, both cathepsin B and *N*-acetyl-β-glucosaminidase were purified in the L-1 fractions of the melanomas (Table 1). Cathepsin B exhibited an approximately 4-fold purification and *N*-acetyl-β-glucosaminidase an approximately 2-fold purification in the L-1 fractions of the metastatic melanomas. The specific activities of cathepsin B and *N*-acetyl-β-glucosaminidase in the L-1 fractions increased in correspon-

Table 1. Distribution of marker enzymes in subcellular fractions isolated by Percoll gradient centrifugation at pH 7.3

		Liver		Cloudman		B16-F1		B16a	
Enzyme	Fraction	SA	RSA	SA	RSA	SA	RSA	SA	RSA
Na^+,K^+-ATPase	L-1	59.7 ± 3.6	3.4	86.7 ± 8.3	4.9	55.3 ± 4.4	4.8	61.2 ± 2.8	3.8
	L-2	42.7 ± 1.6	2.4	12.2 ± 11.5*	0.7	0.0	0.0	13.9 ± 4.9*	0.7
Cathepsin B	L-1	1.9	0.3	3.3	0.8	11.4	3.9	41.2	3.8
	L-2	52.2	9.0	25.0	6.2	22.9	8.0	69.0	6.4
N-Acetyl-	L-1	0.46 ± 0.00	0.3	3.51 ± 0.08*	0.9	6.35 ± 0.16*	1.7	8.33 ± 0.1*	1.5
β-glucosaminidase	L-2	6.91 ± 0.45	4.4	10.2 ± 0.40	2.7	21.0 ± 0.10*	4.4	17.50 ± 1.11*	3.1

Specific activity (SA) values are expressed as nmol of reaction product formed per min per mg of protein. RSA, relative specific activity (relative to starting material). Cathepsin B specific activity = V_{max}; Na^+,K^+-ATPase and *N*-acetyl-β-glucosaminidase specific activities = mean ± SEM of triplicate incubations. Na^+,K^+-ATPase activity was determined in the absence and the lysosomal hydrolases in the presence of 0.1% Triton X-100. Results shown are from a representative experiment out of a total of 7 for liver, 2 for Cloudman, 3 for B16-F1, and 10 for B16a.
*$P < 0.05$ versus liver, evaluated by Student's *t* test (two-tailed).

dence with metastatic potential (Table 1). Cathepsin B in the L-1 fraction did not appear to be latent, as exposure to pepsin did not enhance cathepsin B activity but instead resulted in a reduction in activity.

The association of cathepsin B and *N*-acetyl-β-glucosaminidase activities with the L-1 fractions of the metastatic tumors could not be disrupted by treating the L-1 fractions with isotonic potassium acetate. This suggests that the activities of these two enzymes in the L-1 fraction were not due to solubilized enzyme that had been trapped or adsorbed during the centrifugation procedures.

The substrate used for assay of cathepsin B, carbobenzoxyarginyl-arginyl-4-methoxy-β-naphthylamide, is selective rather than specific—e.g., kallikrein (23) and trypsin (unpublished observation) can also degrade this substrate. Since tumor cells have been reported to have trypsin-like neutral proteinases in their plasma membranes (24), we confirmed that the proteolytic activity in the L-1 fractions of the metastatic tumors was due to cathepsin B by utilizing a specific cysteine proteinase inhibitor, carbobenzoxyphenylalanylalanyl-diazomethylketone (25). At a 5 μM concentration this inhibitor totally blocked the cathepsin B activity in the L-1 and L-2 fractions. We measured the V_{max} of both cathepsin B and cathepsin H activities to eliminate the effects of endogenous competitive inhibitors (26, 27).

Subcellular fractions isolated by differential and density gradient centrifugation exhibit cross-contamination of organelles and membranes. The minimal cross-contamination of the L-1 fraction of murine liver with lysosomes is reflected by the 2–6% activity therein of cathepsins B and H and *N*-acetyl-β-glucosaminidase. To correct for cross-contamination we normalized the data to the Na^+,K^+-ATPase activities in the L-1 fractions. Only cathepsin B and *N*-acetyl-β-glucosaminidase exhibited an increase in this ratio in correspondence with metastatic potential; the mean value of the increase for cathepsin B was 17.5 and for *N*-acetyl-β-glucosaminidase was 7.1. We have recently extended these observations to a Hepa 1 clone of a transplantable hepatoma induced originally in C57BL/6 mice by methylcholanthrene. In this hepatoma clone cathepsin B activity also exhibited a shift to the L-1 fraction; the mean value of this shift was 10.1. In contrast, *N*-acetyl-β-glucosaminidase activity did not exhibit a shift to the L-1 fraction in the hepatoma.

Chakravarthy *et al.* (28) have reported that optimal separation of plasma membrane from neuroblastoma cells on a Percoll gradient is achieved when the separation is performed at pH 8.8. The majority of studies employing Percoll for isolation of lysosomes have been performed at pH 7.4 (29–31). The studies depicted in Fig. 1 and Table 1 were all performed at pH 7.3. However, since the cysteine proteinases cathepsins B and H are inactivated by exposure to pH values above 7.0, we performed additional studies utilizing a homogenization and isolation buffer at pH 6.5 (250 mM sucrose/25 mM 2-(*N*-morpholino)ethanesulfonic acid/1 mM EDTA). A striking redistribution of the activities of cathepsin B and *N*-acetyl-β-glucosaminidase from the bottom of the gradient (equivalent to L-2) to the top of the gradient (equivalent to L-1) was observed in the metastatic B16a melanoma (Fig. 2). Similar redistributions of β-glucuronidase activity, Na^+,K^+-ATPase activity, or protein were not observed (Fig. 2 or data not shown).

Ultrastructural Studies. The purities of the L-1 and L-2 fractions were confirmed by electron microscopy (data not shown). The L-2 fraction contained a population of vesicles with electron-dense contents which had the appearance of secondary lysosomes and a few swollen mitochondria. The L-1 fraction consisted of membrane fragments and translucent vesicles. Secondary lysosomes, multivesicular bodies and mitochondria were not observed in the L-1 fraction. The lysosomes purified in the L-2 fraction were morphologically

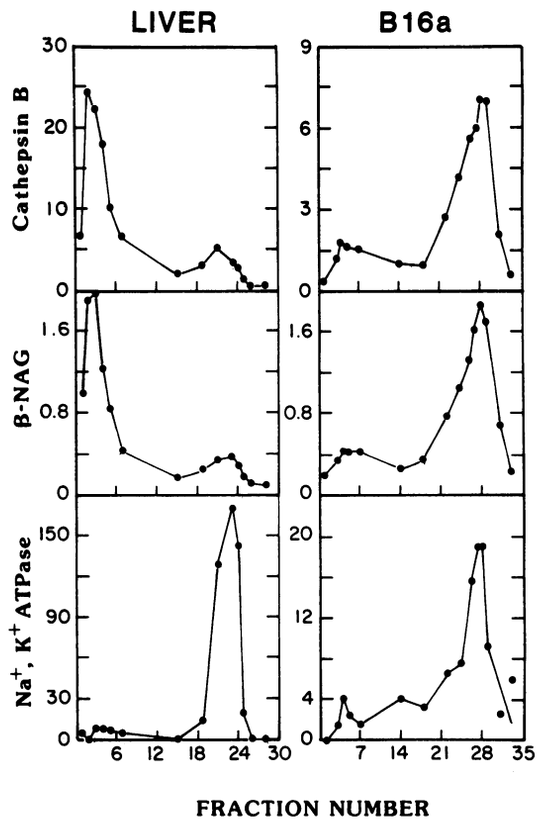

FIG. 2. Elution profiles of enzyme activities after Percoll density gradient centrifugation at pH 6.5 of the light mitochondrial fraction of murine liver and B16 amelanotic (B16a) melanoma. β-NAG, *N*-acetyl-β-glucosaminidase. Fractions (0.3 ml) were collected from the bottom and analyzed for protein and marker enzymes; analyzed fractions contained both Percoll and 0.1% Triton X-100. Results shown are from a representative experiment out of a total of two.

identical to those visible by electron microscopy in the intact tissue prior to subcellular fractionation.

DISCUSSION

Lysosomes have been reported to be separable into two populations by Percoll density gradient centrifugation (29–31). The population of greater density was shown to consist of secondary lysosomes containing mature lysosomal enzymes (29–31). The population of lesser density was shown to contain elements of the Golgi apparatus and endoplasmic reticulum by Rome *et al.* (31) and autophagic vacuoles by Surmacz *et al.* (32). Several studies (11, 29–33) suggest that a bimodal distribution of lysosomal enzymes may reflect the intracellular synthesis and processing of lysosomal enzymes—i.e., precursor or latent forms of the enzymes are found in the lighter fraction and mature enzymes in the heavier. Precursor forms of lysosomal glycosidases have catalytic activity (33). Precursor forms of cathepsin B, however, do not seem to have catalytic activity (9, 34), so the cathepsin B activity measured in both the L-1 and L-2 fractions of the metastatic melanomas in this study is presumably due to mature cathepsin B. Our inability to increase cathepsin B activity by pepsin activation also indicates that the cathepsin B was not latent.

The cathepsin B activity in the L-1 fraction of the metastatic B16 melanomas does not appear to be due to a second population of lysosomes (primary or GERL) since plasma membrane fractions isolated from tumor cells of four species by five separate methods have now been shown to possess cathepsin B-like cysteine proteinase activities (12–15, 35).

Pietras and Roberts (12) purified plasma membrane from human cervical carcinoma cells by using a discontinuous sucrose density gradient. Zucker *et al.* (35) also employed a sucrose gradient. Bohmer *et al.* (13) used a gradient of dextran and Visotrast (metracinamide). Koppel *et al.* (14) attached a rat anaplastic sarcoma to Affi-Gel 731 beads, disrupted the cells by sonication, and assayed the cell membranes, which remained attached to the beads. Differential centrifugation at high speed (100,000 × *g*) was used to isolate membrane vesicles spontaneously shed into the culture medium by 15091A murine mammary adenocarcinoma cells (15). In the present study Percoll density gradient centrifugation was used to purify a plasma membrane fraction. The present study was the only one of the six to measure cathepsin B-like activity in plasma membrane fractions purified from solid tumors.

Further work will be needed to clarify why cathepsin B and *N*-acetyl-β-glucosaminidase activities become associated with the plasma membrane fraction of metastatic B16 melanomas. The process of tumor cell metastasis requires the movement of tumor cells through extracellular matrices containing collagens, glycoproteins and proteoglycans. The presence of cathepsin B and *N*-acetyl-β-glucosaminidase at the cell surface of tumor cells could facilitate focal dissolution of the extracellular matrix and thereby movement to metastatic sites.

The authors thank Dr. James Hatfield for performing the electron microscopy, Ms. Gerri Pawlik for expert technical assistance, and Ms. Janice Clarke for typing the manuscript. This work was supported in part by Grant CA36481 awarded by the National Cancer Institute (to B.F.S.) and a grant from Harper–Grace Hospitals (to K.V.H. and B.F.S.). B.F.S. is the recipient of Research Career Development Award CA00921 from the National Cancer Institute.

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