Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 556-561

MEMBRANE-ASSOCIATED CATHEPSIN L: A ROLE IN METASTASIS OF MELANOMAS

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Received September 5, 1989

Subcellular distribution of cathepsin L, the major protein released by transformed or *ras* transfected fibroblasts, was examined in murine liver, murine B16 amelanotic melanoma and human A2058 melanoma after sequential differential and Percoll density gradient centrifugation. In both murine and human melanomas, cathepsin L activity was found to be enriched in plasma membrane fractions; cathepsin L in these fractions was in both native and acid activatable forms. Plasma membrane fractions from B16 melanoma subpopulations of "low" and "high" metastatic potential were assayed for activity of cathepsin L and of heat stable endogenous inhibitors. The relative specific activity of cathepsin L was 7-fold greater in the subpopulation of "high" metastatic potential, whereas cysteine proteinase inhibitory activity was 5-fold less. Since cathepsin L can degrade intact basement membrane, this membrane-associated cathepsin L may well contribute to metastatic spread of melanomas .

Cathepsin L is the major protein synthesized by *ras* transfected murine fibroblasts (1). An acid activatable precursor form of cathepsin L is the major protein released from transformed murine fibroblasts (2). Gottesman and colleagues have hypothesized that cathepsin L plays a role in malignant transformation; however, upregulation of the expression of cathepsin L in murine NIH 3T3 fibroblasts was not sufficient to induce a transformed phenotype as measured by anchorage or serum independent growth or tumorigenicity in nude mice (3). Denhardt *et al.* (4) have suggested that cathepsin L plays a role in the proteolytic steps of the metastatic cascade since elevated mRNA for cathepsin L in *ras* transfected fibroblasts correlates with the metastatic capability of the transfected cells. A role for cathepsin L in metastasis would appear a reasonable hypothesis as this cysteine proteinase readily degrades intact basement membranes (5,6), one of the major barriers crossed by metastasizing tumor cells.

We have shown that enhanced activities of another cysteine proteinase, cathepsin B, in metastatic tumor cells (human and murine) correspond with metastatic potential as well as with increased association of this proteinase with the plasma membrane fractions of these cells (for review see 7). Since cathepsin B like cathepsin L is released from tumor cells, we have

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Abbreviations: B16a, B16 amelanotic melanoma; E-64, L-t-epoxysuccinylleucylamido(4-guanidino)butane; RSA, relative specific activity; Z-Phe-Arg-NHMec, carbobenzyloxy-phenylalanyl-arginyl-7-amino-4-methylcoumarin; Z-Phe-Phe-CHN_2, carbobenzyloxy-phenylalanyl-phenylalanyl-diazomethylketone.

0006-291X/89 $1.50

examined the subcellular distribution of cathepsin L in metastatic melanomas of human and murine origin. We report here that cathepsin L (native and acid activatable) was associated with a plasma membrane fraction in murine and human melanomas suggesting that this enzyme could participate in focal, pericellular proteolysis of the basement membrane during the metastatic cascade.

MATERIALS AND METHODS

Reagents and tissue. Z-Phe-Arg-NHMec and Z-Phe-Phe-CHN_2 were purchased from Enzyme Systems Products (Livermore, CA); papain, Percoll, Brij 35 and E-64 from Sigma (St. Louis, MO); and tissue culture media from Gibco (Grand Island, NY). All other chemicals were of reagent grade and were obtained from commercial sources.

Murine B16a tumors were propagated in syngeneic male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) according to our published procedure (8). Normal livers were obtained from non-tumor-bearing mice of the same strain. B16a tumors were dissociated and tumor cell subpopulations separated by centrifugal elutriation as previously described (9). Human A2058 melanoma cells (a kind gift of Dr. Elliott Schiffmann, NCI) were grown in Dulbecco's Modified Eagle Medium supplemented with L-glutamate and sodium pyruvate.

Subcellular fractionation. B16a tumor or liver homogenates were fractionated by sequential differential centrifugation and Percoll density gradient centrifugation of the light mitochondrial fraction as we have described previously (8). Elutriated B16a or cultured A2058 cells (1-4 x 10^7 cells) were homogenized and fractionated by the same procedure using volumes ten-fold smaller.

Experimental metastasis assay. The metastatic potential of B16a subpopulations was verified by methods described previously (8). Briefly, 3.7 x 10^4 B16a cells were injected into the tail vein of syngeneic male mice (12 mice per group). At 21 days post tumor cell injection, the mice were sacrificed by cervical dislocation, the lungs removed and fixed in Bouin's solution and macroscopic pulmonary colonies counted using a dissecting microscope.

Enzyme, inhibitor and protein assays. Cathepsin L activity was assayed by a modification of the procedure of Mason *et al.* (10). For this assay we utilized Z-Phe-Arg-NHMec (final concentration, 8 or 8-32 μM) as the substrate as well as the inhibitor Z-Phe-Phe-CHN_2 (6 nM) in order to differentiate activity of cathepsin L against this substrate from that of cathepsin B against the same substrate. Acid activatable cathepsin L was assayed after incubation at pH 3.6 for 3 h (10). In order to improve the quantitation of cathepsin L activities in subpopulations and subcellular fractions of B16a tumors, activities were determined as V_{max} to minimize interactions with endogenous reversible inhibitors.

To determine the levels of endogenous cysteine proteinase inhibitors, we dissociated inhibitor-cysteine proteinase complexes by taking advantage of differences in stability of cysteine proteinases and their inhibitors (11). We tested two methods: 1) exposure to pH 11.5 for 2 h at 37° C and 2) heating at 100° C for 5 min. Since the results were qualitatively the same, we used the simpler procedure of heating; denatured proteins were removed by centrifugation (13,000 *g* x 10 min). Thus the inhibitor activities reported in this paper represent only those of heat stable cysteine proteinase inhibitors and were determined against the plant cysteine proteinase papain; prior to assay of inhibitory activity, the concentration of active papain was determined using the active site titrant E-64 (12). The assay procedure used was a slight modification of the method employed by Nicklin and Barrett (13). Papain was incubated for 10 min at 37° C in 0.13 M acetate buffer, pH 5.5, containing 1.3 mM EDTA, 2.6 mM dithiothreitol, inhibitor (5-40 μl) and 0.05% Brij 35 in a total volume of 300 μl. Substrate (20 μM Z-Phe-Arg-NHMec; 100 μl) was added and the reaction terminated after 20 min with 400 μl of 100 mM sodium monochloroacetate-100 mM sodium acetate, pH 4.3. Activity of the inhibitors is expressed as inhibitory units with one unit representing the amount of inhibitory protein which will completely inhibit the release by papain of one μmole of product/min.

Protein was measured by the Bradford procedure (14) using bovine serum albumin as a standard.

RESULTS

We examined the subcellular distribution of cathepsin L in a normal murine tissue (liver) and in murine B16a tumors (Fig. 1). The degree of purification in each fraction is represented by RSA, i.e., the ratio of the specific activity in the fraction to the specific activity in the homogenate. The specific activities and RSAs of cathepsin L in both liver and B16a tumors were highest in the lysosomal fractions. In liver and in B16a tumors, the RSAs for cathepsin L in the lysosomal fraction were 17 and 19, respectively. However, cathepsin L in B16a tumors was also enriched 4-fold in the plasma membrane fraction. This is similar to the degree of enrichment of cathepsin B in the plasma membrane fraction of B16a tumors (15). Since cathepsin L is released from transformed or transfected fibroblasts in the form of an acid activatable precursor (2), we also assayed the various subcellular fractions for acid activatable cathepsin L. Interestingly, acid activatable cathepsin L activities were enriched in both the plasma membrane and the nuclear and heavy mitochondrial fractions, but not in either the lysosomal or supernatant fraction (data not shown). We presume the acid activatable cathepsin L activity in the nuclear and heavy mitochondrial fraction is due to the cosedimentation of endoplasmic reticulum with this fraction (8) and the presence of a latent cathepsin L precursor in microsomes as has been demonstrated by Nishimura *et al.* (16).

In murine melanomas the specific activity of cathepsin B in the plasma membrane fraction seems to correlate with the metastatic capability of those melanomas (15). Therefore we determined whether the specific activity of cathepsin L in the plasma membrane fraction might exhibit a similar correlation with metastatic capability. For this study we utilized B16a tumor cells separated by centrifugal elutriation into discrete cell subpopulations that differ in their metastatic capability (10). The two subpopulations used in the present study exhibited a 11-fold difference in their ability to form lung colonies, forming a median (range) of 6 (2-14) and 68 (36-82) lung colonies per mouse for the "low" and "high" subpopulations,

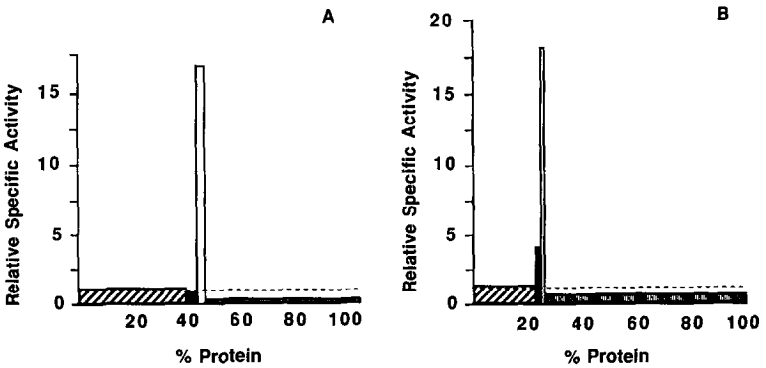

Fig. 1. Distribution of cathepsin L activity in subcellular fractions obtained by sequential differential and Percoll density gradient centrifugation of normal murine livers, A, and B16a tumors, B. The histogram bars from left to right depict nuclear and heavy mitochondrial (diagonally striped bars), plasma membrane (solid bars), lysosomal (open bars) and supernatant (vertically striped bars) fractions. RSA of each fraction is plotted against the relative protein content of each fraction. An RSA > 1 indicates enrichment of cathepsin L in the fraction. Note that there is no cathepsin L in the plasma membrane fraction of normal liver.

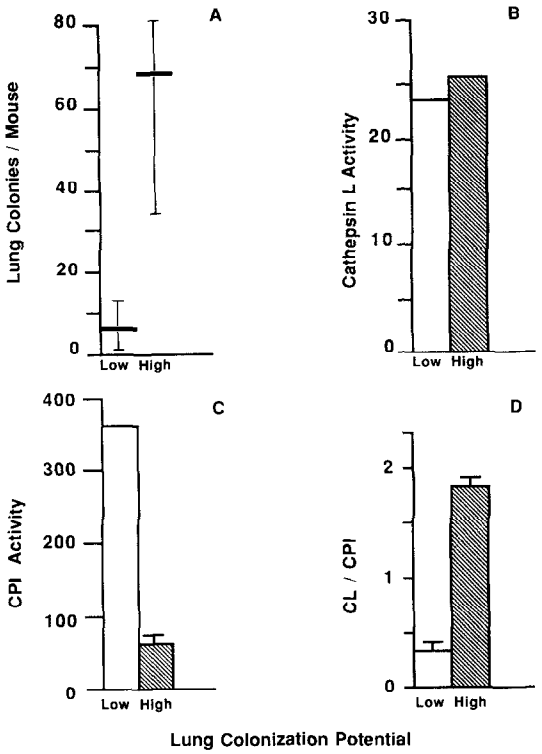

Fig. 2. Correlation of lung colonization potential with activities of cathepsin L, endogenous heat stable cysteine proteinase inhibitors and the ratio of cathepsin L and inhibitory activities in plasma membrane fractions of two B16a tumor cell subpopulations isolated by centrifugal elutriation. Panel A, lung colony formation; median and range. Panel B, cathepsin L activity;nmoles/min·mg protein. Panel C, cysteine proteinase inhibitor (CPI) activity; munits/mg protein. Panel D, the potential "working" activity of cathepsin L (CL) or CL/CPI.

respectively (Fig. 2A). The specific activity of cathepsin L in plasma membrane fractions isolated from the "low" and "high" subpopulations was similar (Fig. 2B). However, the RSA of cathepsin L in the "high" subpopulation was 7-fold higher than in the "low" subpopulation, suggesting that the distribution of cathepsin L in the more metastatic tumor cells was altered.

Endogenous cysteine proteinase inhibitors bind very tightly to cathepsin L, thus obscuring the activity of this cysteine proteinase in samples that also contain inhibitors (17). Since we have previously shown that plasma membrane fractions of tumors possess cysteine proteinase inhibitory activity (18), we measured the activity of heat stable cysteine proteinase inhibitors in plasma membrane fractions of the two B16a subpopulations. In the "high" subpopulation there was 5-fold less cysteine proteinase inhibitory activity than in the "low" subpopulation (Fig. 2C). Thus cysteine proteinase inhibitory activity exhibited an inverse correlation with the ability of the B16 subpopulations to form lung colonies.

The strikingly reduced inhibitory activity in the plasma membrane fraction of the "high" subpopulation could mean that the activity of the cathepsin L associated with the plasma membrane fraction would be greater than one would predict based on simply measuring cathepsin L. Since the binding of cysteine proteinases to the endogenous low Mr cysteine proteinase inhibitors (cystatins) is 1:1 (13) and their K_i's against cathepsin L orders of

Table 1
Activity of Native and Latent Cathepsin L in Human A2058 Melanoma Cells

ACTIVITY	SUBCELLULAR FRACTION			
	Nuclear+Mitochondrial	Membrane	Lysosomal	Supernatant
Specific[a]				
Native	0.32 ± 0.02	7.03 ± 0.36	17.0 ± 1.0	0.02 ± 0.00
Latent[b]	1.06 ± 0.00	1.97 ± 0.05	0.0 ± 0.0	0.00 ± 0.00
RSA[c]				
Native	0.6	13.7	32.4	0.0
Latent[a]	2.4	4.6	0.0	0.0

[a]Mean ± SD of triplicate samples.

[b]Latent in this case refers to increase in activity after exposure to acid pH, i.e., acid activatable activity.

[c]An RSA > 1 indicates enrichment of cathepsin L in the fraction.

magnitude less than against cathepsin B (11), one can arbitrarily obtain an estimate of the "working activity" of cathepsin L by determining the ratio of the activities of cathepsin L and the endogenous inhibitors. In the "high" B16a subpopulation the "working activity" of cathepsin L could be as much as 5-fold greater than in the "low" subpopulation (Fig. 2D).

To determine whether our observations in murine melanomas have any relevance to human melanomas, we determined the subcellular distribution of cathepsin L in human A2058 melanoma cells (Table 1). Because the specific activity in the homogenate was low, RSA for the A2058 cells is expressed as the ratio of specific activity in the fraction to the sum of the specific activities in all fractions. The specific activity and RSA of cathepsin L were greatest in the A2058 lysosomal fraction as seen above for both murine liver and B16a melanoma. In addition, cathepsin L activity was enriched 14-fold in the plasma membrane fraction of the A2058 melanoma (Table 1), i.e., an enrichment considerably greater than the 4-fold observed in the B16a melanoma (Fig. 1). In the A2058 melanoma as in the B16a melanoma, acid activatable or latent cathepsin L activity was enriched in both the plasma membrane and nuclear and heavy mitochondrial fractions, but not in the lysosomal or supernatant fractions (Table 1).

DISCUSSION

In the present study we have shown that plasma membrane fractions of both murine and human melanoma cells contain activity (native as well as acid activatable) of cathepsin L, a lysosomal cysteine proteinase. This proteinase has been shown by several groups to be the major protein synthesized and released from murine transformed or *ras* transfected fibroblasts (1-4). We had previously established that synthesis and activity of a related lysosomal cysteine proteinases, cathepsin B, is enhanced in metastatic murine tumors (19,20) and that the metastatic potential of murine melanomas correlates with increased activity of cathepsin B and of a lysosomal glycosidase, ß-hexosaminidase, in their plasma membrane fractions (15). This apparent alteration in subcellular localization of these three lysosomal enzymes in metastatic tumors (8 and present study) does not extend to all lysosomal enzymes (8).

Associations of lysosomal enzymes with plasma membrane fractions or plasma membrane-associated vesicles have been reported in a number of disease states such as I-cell disease and cancer and in cells like macrophages, a cell type involved in local proteolysis during

pathological states. Recently, Reilly *et al.* (21) have suggested that cathepsin L in macrophages may be bound to the membrane, but they provide no experimental evidence for this assertion. In addition, Maciewicz *et al.* (6) have demonstrated by immunofluorescence at the light microscopic level that the distribution of cathepsin L in cultured human colon carcinoma cells appears to be at the cell surface. The process of tumor cell metastasis requires the movement of tumor cells through basement membranes containing collagens, glycoproteins and proteoglycans. We speculate that the presence of cathepsins L and B and ß-hexosaminidase in plasma membrane-associated vesicles may lead to the establishment of a local microenvironment in which focal degradation of the basement membrane is enhanced.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants (CA 36481 and CA 48210) and a grant from Harper/Grace Hospitals. BFS is the recipient of Research Career Development Award (CA 00921) from the National Institutes of Health. We thank Dr. Peter Bohley for the impetus to undertake these studies and Dr. J.M. Onoda and Mr. K.K. Nelson for providing the elutriated B16a subpopulations and performing the experimental metastasis assays.

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