Identification of Cell Surface Cathepsin B-like Activity on Murine Melanomas and Fibrosarcomas: Modulation by Butanol Extraction

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ABSTRACT

Cathepsin B (CB) is a lysosomal cysteine protease that may play a role in the activation of extracellular degradative enzymes involved in the destruction of the subendothelial matrix and extravasation of metastatic tumor cells. In this study we have investigated the cell surface expression of a CB-like enzyme on the surface of tumor cell variants expressing both high and low metastatic potentials. Cell surface CB-like activity was demonstrated by incubation of intact viable cells and isolated plasma membranes with the selective chromogenic substrate N-carboxbenzoxyl-Val-Lys-Lys-Arg-4-methoxy-β-naphthylamide. Cell surface CB activity required thiol activation and was blocked by the CB-selective protease inhibitors leupeptin, antipain, and L-trans-epoxysuccinylleucylamide(4-guanidino)butane, but not by inhibitors inactive against CB. Enzymatic activity was significantly reduced when assayed at pH 7 and greater. Although all tumor lines had detectable CB-like activity, we observed a correlation between the expression of cell surface CB-like activity and metastatic phenotype only with isolated plasma membranes, and not with whole cell preparations. Nocytolytic 2% butanol extraction, a technique known to increase the experimental metastatic propensity, also significantly increased cell surface CB-like activity. Incubation of extracted tumor cells with crude butanol extracts prepared from those cells restored the cell surface CB-like activity to that of the unextracted controls, suggesting that the increased enzyme activity observed following extraction may be due to the release of an endogenous cysteine protease inhibitor. These results demonstrate that a CB-like protease is expressed on the surface of several murine tumor cells and that an endogenous inhibitor may play a role in determining experimental metastatic phenotype.

INTRODUCTION

We have previously shown that nocytolytic extraction of intact B16 melanoma or MCA-F fibrosarcoma cells with 2% 1-butanol results in an increased lung-colonizing potential of these cells in the experimental metastasis assay (1). Reconstitution of the extracted cells with a crude extract protein preparation restores the original metastatic phenotype to that of the unextracted controls (1). One possible explanation for this effect is the butanol-mediated release of endogenous cell surface protease inhibitors.

CB* is a M, 23,000–28,000 lysosomal cysteine protease that requires activation by thiol reagents and is involved in several physiological and biological functions, such as activating proenzymes and prohormones (2) and intracellular protein processing and degradation (3, 4). In addition, cell surface CB has been implicated in the ability of some tumor cells to invade the subendothelial basement membrane matrix and to metastasize to secondary sites (5). Sloane et al. (6) have suggested that CB is located on the cell surface, although their data indicate that the enzyme is found mainly in a purified light mitochondrial fraction of disrupted tumor cells and in vesicles shed from tumor cells (7).

In this study, we have used selective CB substrates and inhibitors to document CB-like activity on the cell surface of several tumor lines. In addition, we have examined the hypothesis that butanol-extracted materials reduce metastatic potential by inhibition of cell surface CB activity. Although no correlation could be found between the CB-like activity of intact viable cells and metastatic potential, the CB-like activity of isolated plasma membranes did demonstrate a correlation, and a significant augmentation in CB-like activity was observed following butanol extraction. Incubation of extracted B16-F10 cells with materials released by B16-F1 cells returned CB-like activity to control levels. Thus, we have positively identified a CB-like activity on the surface of several murine tumors and have obtained circumstantial evidence that an endogenous thiol protease inhibitor is released by the butanol extraction technique.

MATERIALS AND METHODS

Tumor Cell Lines. The B16-F1 and B16-F10 melanoma cell lines, originally isolated by Fidler (8), were obtained from E. G. and G. Mason Research Institute (Washington, DC) and propagated in vitro in Dulbecco’s minimum essential medium supplemented with nonessential amino acids, 1 mM sodium pyruvate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 10% fetal bovine serum. B16-F1 cells produce a low number of experimental pulmonary metastases, but B16-F10 are significantly more malignant (1, 8). UV-2240 and UV-1591 are UV-induced regressor fibrosarcoma cell lines isolated by Kripke (9) from C3H/HeN mice. UV-2240 is not metastatic in adult-thymectomized, 450-R X-irradiated mice. MCA-F is a 3-methylcholanthrene-induced fibrosarcoma (10) syngeneic to C3H/HeN mice. A metastatic variant of MCA-F (clone 9.4) was derived by serial i.v. passage and recovery of isolated lung metastases (1). All cell lines were frequently checked in vivo to test their metastatic potential. Single cell suspensions were prepared from subconfluent monolayers by 2-min incubation at room temperature with 0.05% trypsin in Versene (150 mM NaCl, 5 mM KCl, and 0.6 mM EDTA), followed by addition of fetal bovine serum to a 5% final concentration. Trypsin and serum were removed by at least 3 washings of cells in PBS. In some experiments, cells were harvested using 2 mM EDTA instead of trypsin, without apparent difference in CB-like activity. The resulting cells were counted using a hemacytometer and uniformly displayed greater than 90% viability by trypan blue exclusion. A single cell suspension of thymocytes and splenocytes was prepared from C57BL/6 mice by mincing the tissues in Hanks’ buffer. Erythrocytes were eliminated by brief incubation in 0.15 M NH4Cl buffer.

Butanol Extraction. Tumor cell suspensions were incubated for 5 min in PBS containing 2.0% 1-butanol, as described previously (1, 11, 12). The extracts were subsequently concentrated, dialyzed against PBS, and centrifuged. Cells were immediately washed 3 times in PBS. The viability of extracted cells was always greater than 90%.

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3 To whom requests for reprints should be addressed, at Department of Immunology (Box 178), University of Texas M. D. Anderson Hospital and Tumor Institute, 1515 Holcombe Blvd., Houston, Texas 77030.
4 The abbreviations used are: CB, cathepsin B; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline (140 mM NaCl, 6 mM Na2HPO4, 3 mM KCl, 1.5 mM KH2PO4) without Ca2+ or Mg2+, pH 7.4; E-64, L-trans-epoxysuccinylleucylamide(4-guanidino)butane; N-CBZ-Val-Lys-Lys-Arg-4-methoxy-β-naphthylamide, N-carboxbenzoxyl-valyllysylarginyll-4-methoxy-β-naphthylamide.
Biochemical Assays. CB (EC 3.4.22.1) was assayed as described previously (13) using N-CBZ-Val-Lys-Lys-Arg-4-methoxy-β-naphthylamide (Sigma Chemical Co., St. Louis, MO) as a substrate (14, 15). Purified bovine liver CB (Enzyme Systems Products, Livermore, CA) served as a standard. One unit of CB was defined as the amount of enzyme that will hydrolyze 10 nmol of the substrate per min at 37°C, pH 6.

To assay CB-like activity on the cell surface, 1 or 2 x 10⁶ cells (either untreated or extracted with butanol) or isolated plasma membranes were resuspended in 750 μl of activation buffer (88 mM KH₂PO₄, 12 mM Na₂HPO₄, 1.33 mM EDTA, and 2.7 mM cysteine, pH 6). The volume was adjusted to 1 ml with double distilled water. Thirty nmol of substrate were added and the mixture incubated for 30 min at 37°C. The assay was stopped by the addition of the color reagent and measurement of absorption at 520 nm as described (15). In some experiments, cells were preincubated with butanol extracts (25 μg protein), leupeptin (3 x 10⁻⁷ M), E-64 (5 x 10⁻⁷ M), pepstatin A (10⁻⁷ M), antipain (1.5 x 10⁻⁶ M), or PMSF (2 x 10⁻⁷ M) (all purchased from Sigma) to inhibit the enzymatic activity. Cell viability was checked after incubation in activation buffer and was found to be greater than 90%. Statistically significant differences in enzymatic activity were calculated using Student’s t test.

Preparation of Plasma Membranes. Plasma membranes were prepared as described by Maeda et al. (16). Briefly, 1.5 x 10⁶ cells were suspended in hypotonic buffer (0.01 M Tris-HCl-3 M MgCl₂-0.1 mM PMSF, pH 7.4) for 10 min and then homogenized using 30 strokes of a motorized pestle. The solution was centrifuged (1200 x g for 10 min) and the supernatant was applied on top of a 42% sucrose solution. Separation of membranes was achieved by ultracentrifugation at 22,000 rpm for 1 h. The interface (membranes) was collected, suspended in 0.01 M Tris-HCl + 0.15 M NaCl + 5 mM EDTA (pH 7.4), and washed by ultracentrifugation (100,000 x g, 1 h). Plasma membrane protein concentrations were determined using the Pierce (Rockford, IL) protein assay reagent. The plasma membranes were resuspended in 0.05 M Tris-HCl plus 0.1 M NaCl plus 0.01 mM PMSF and stored at -70°C.

RESULTS

To document the expression of CB-like activity on the surface of murine tumor cells, we examined a panel of murine tumor cells including B16-F1 melanoma and its highly metastatic variant B16-F10, the MCA-F fibrosarcoma and its metastatic variant clone 9.4, and the UV1591 and UV2240 UV-induced regressor fibrosarcomas (Fig. 1). As control cells, we used freshly prepared murine thymocytes and splenocytes. Tissue culture propagated tumor cells were incubated with the CB-selective substrate N-CBZ-Val-Lys-Lys-Arg-4-methoxy-β-naphthylamide in the presence of 2.7 mM cysteine (14, 17). In our laboratory this substrate exhibited an index of specificity for CB 50 to 100 times greater than for trypsin, papain, pepsin, or chymotrypsin. Incubation of tumor cells in CB substrate did not affect the ability of the cells to exclude trypan blue, suggesting that the observed activity was attributable to a cell surface enzyme activity and not to the release of intracellular lysosomal contents.

Significant CB-like activity was observed on the surface of all the tumor cell lines tested, ranging from 0.2 to 0.7 unit/2 x 10⁶ cells (Fig. 1). Using this experimental paradigm, there was no apparent correlation between metastatic phenotype and CB-like activity. However, cell-associated surface CB-like activity was greater on the tumor cell lines tested than on normal murine thymocytes or an unfractionated splenocyte population. In a limited series of experiments using the 10T½ cell line, the cell-associated CB-like activity was equivalent to that observed for the B16 melanomas (data not shown). Fresh splenocytes and thymocytes were used to demonstrate the low level expression of CB by normal cells, as well as to exclude serum as a source of the observed CB-like activity.

In order to confirm the presence of CB on the cell surface, we assayed the CB-like activity associated with isolated plasma membrane fractions prepared from the various tumor cell lines (Fig. 2). Interestingly, the CB-like activity on the plasma membrane fractions did correlate with the metastatic phenotypes of the progressor cells, although there was less than a 3-fold difference between CB-like activity associated with membranes from low and high metastasis lines. Furthermore, when calculated on the basis of CB units/10⁶ cells estimated from the yield of plasma membranes, the enzymatic activity was roughly equivalent to that observed with the whole cells.

Cell surface CB-like activity displayed the same dependence on thiol activation as did isolated bovine liver CB (Fig. 3). Half-maximum activation was observed using 300 μM cysteine at pH 6.0. The membrane-associated CB-like activity was pH dependent, with an acidic pH optimum at about 6 (Table 1). CB-like activity was reduced during incubations above pH 7.0, similar to the lysosomal protease (4, 18).

Extraction of B16-F1 and B16-F10 cells with butanol increased the specific activity of the cell surface CB (Fig. 4), consistent with the previously reported enhancement of metastatic potential by butanol extraction (1). Extraction of the highly malignant variant MCA-F clone 9.4, but not the weakly...
The increased membrane CB-like activity observed following butanol extraction was not attributable to the release of cytoplasmic components, since the cells maintained greater than 90% viability following extraction, as judged by trypan blue exclusion. Despite the relatively high amounts of CB-like activity expressed on the surface of murine tumor cells and the statistically significant increase in CB-like activity following butanol extraction of their metastatic variants, no correlation could be determined between the steady state or the absolute level of cell-associated CB-like activity and experimental metastatic potential.

Addition of the selective thiol protease inhibitors leupeptin, antipain, or E-64 to the chromogenic substrate assay abolished the enzymatic activity observed with the B16-F1 and B16-F10 cell lines (Fig. 5). Addition of other protease inhibitors not active against CB, such as aprotinin, pepstatin A, and PMSF, did not block the surface CB-like activity. Inhibition by leupeptin and E-64 was found to be reversible, since repeated washing of the cells prior to assay returned CB-like activity to control levels (Fig. 6). Incubation of extracted B16-F1 or B16-F10 cells with butanol-extracted materials resulted in an irreversible decrease in cell surface CB-like activity to that of the unextracted controls (Fig. 6) but was not capable of the profound inhibition of cell surface CB activity observed in the presence of leupeptin, antipain, or E-64 (Fig. 5). To demonstrate the modulation of cell surface CB-like activity by removal and re-addition of the putative endogenous inhibitor, we incubated butanol-extracted B16-F1 or B16-F10 melanoma cells with PBS or 25 µg of crude extract protein obtained from the B16-F1 cell line (Table 2). Consistent with our previous findings, butanol extraction significantly increased the CB-like enzymatic activity associated with the cells. The CB-like activity was returned to that of the unextracted controls by brief incubation in butanol-extracted materials (Table 2). Treatment of the unextracted control cells with the butanol-extracted moieties did not affect their CB-like activity (data not shown).

**DISCUSSION**

Cathepsin B has been identified as a lysosomal protease that is present as several isoenzyme forms in a variety of animal tissues (14, 19) and has been demonstrated to be an important

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**Table 1**Dependency of cell surface CB-like activity on extracellular pH

<table>
<thead>
<tr>
<th>Activation buffer pH</th>
<th>Units/2 x 10⁶ cells B16-F1</th>
<th>Units/2 x 10⁶ cells B16-F10</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5</td>
<td>0.41 ± 0.01*</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>pH 6</td>
<td>0.63 ± 0.02</td>
<td>0.53 ± 0.01</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

*Mean ± SE.

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**Fig. 3.** Thiol activation of cell surface and lysosomal CB. Purified bovine liver CB (O) was activated with increasing concentrations of cysteine and assayed using N-CBZ-Val-Lys-Lys-Arg-4-methoxy-/3-naphthylamide as substrate. Similarly, B16-F10 cells (2 x 10⁶/ml) were tested (●) with activation buffer containing varying cysteine concentrations and assayed for CB activity.

**Fig. 4.** Effect of butanol extraction on membrane CB-like activity. Culture-propagated tumor cells were either left untreated (○) or extracted for 5 min with 2% 1-butanol (●). The cells were washed 3 times and assayed for CB activity as described in "Materials and Methods." Values are the means of duplicate determinations ± SE (bars). Statistically significant differences were determined using Student's t test.

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**Fig. 5.** Inhibition of cell surface CB-like activity. B16-F1 and B16-F10 cells were incubated with the indicated protease antagonists for 5 min prior to addition of substrate. CB activity was determined as described in the legend to Fig. 1.

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metastatic MCA-F parental fibrosarcoma, also enhanced the observed CB-like specific activity. Membrane CB-like activity on other tumorigenic but nonmetastatic cell lines, such as UV-2240 and UV-1591, and on fresh murine thymocytes and splenocytes was not influenced by butanol extraction (data not shown).
CELL SURFACE CATHEPSIN B

<table>
<thead>
<tr>
<th>Cells + Treatment</th>
<th>B16-F1</th>
<th>B16-F10</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ CBE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Leupeptin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ E-64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ CBE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Leupeptin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ E-64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Reversible inhibition of membrane CB-like activity. Butanol-extracted B16-F1 and B16-F10 cells (10⁶/ml) were preincubated for 10 min at room temperature in PBS, 10 μg crude butanol extract (CBE) protein, 3 x 10⁻⁶ M leupeptin, or 5 x 10⁻⁶ M E-64. Cells were washed three times and CB-like activity was determined. Results are the means ± SE (bars) of 4 experiments performed in duplicate and were analyzed using Student's t test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells</th>
<th>Butanol extraction</th>
<th>Treatment</th>
<th>CB-like activity (units/10⁶ cells) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B16-F1</td>
<td>−</td>
<td>PBS</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>B16-F1</td>
<td>+</td>
<td>PBS</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>B16-F1</td>
<td>+</td>
<td>B16-F1 extract</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>B16-F10</td>
<td>−</td>
<td>PBS</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>B16-F10</td>
<td>+</td>
<td>PBS</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>B16-F10</td>
<td>+</td>
<td>B16-F1 extract</td>
<td>0.41 ± 0.02</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Our results with the CB-like activity of the isolated plasma membrane fractions and our observation that butanol extraction increased cell surface CB-like activity were both positively correlated with the metastatic phenotypes of the progressor cell lines under investigation. However, the highest plasma membrane-associated CB-like activity was observed using UV-2240, an immunogenic repressor fibrosarcoma that does not cause lung metastases following i.v. injection into immunosuppressed mice. We were unable to detect a difference in cell surface CB-like activity between in vivo- and in vitro-propagated tumor cells (data not shown). The use of cellular homogenates by the other groups (6, 22, 23) may account for the differences reported here. Another difference between the CB-like activity we observed and that reported by other authors was the pH sensitivity of our enzyme (18). In our hands, cell surface CB-like activity was significantly diminished above pH 7, similar to the data reported for mouse liver lysosomal CB (32). Our results may, again, be attributable to the in situ determination of protease activity detailed here.

Butanol extraction significantly elevated the CB-like activity on only three of the six tumors analyzed. The largest increases were observed following extraction of those tumor cell lines that expressed the greatest relative metastatic potentials. Conversely, nonmetastatic tumor cell lines and normal murine lymphoid cells displayed low CB-like activity, with no increase following extraction. The correlation between metastasis and CB-like activity was best demonstrated by plasma membrane fractions of these cells, supporting the hypothesis that CB may be a cell surface marker for some metastatic tumors (5, 22, 29, 30). The augmentation of CB-like activity documented after butanol extraction and its partial inhibition by butanol-extracted moieties suggests that butanol may remove an as yet unidentified endogenous thiol protease inhibitor from the cell surface. Furthermore, these findings together with the observations that neither the CB-like activity of unextracted cells nor purified bovine liver CB are antagonized by the endogenous inhibitor suggests that (a) the thiol protease activity we are modulating by butanol extraction is similar but not identical to the lysosomal enzyme or (b) only a portion of the total cell surface CB is sensitive to antagonism by the endogenous inhibitor. These questions must await further studies.

Although it has been reported that CB is irreversibly inhibited by E-64 (33, 34), we were unable to confirm this conclusion for the membrane CB-like activity under investigation. We observed that three or four PBS washings of the cells treated with the antagonist restored CB activity. Other workers (5, 35) have confirmed that some membrane-associated enzymes can be
reversibly antagonized by specific inhibitors. Conversely, the endogenous surface CB inhibitor was resistant to removal by washing cells in PBS but was released by extraction with a low concentration of organic solvent, perhaps because the inhibitor interacted with both the enzyme and other components of the membrane or was bound to a hydrophobic site on the enzyme. Endogenous thiol protease inhibitors have been described (36, 37) although we believe this is the first report of a cell surface inhibitor released by butanol extraction. It is worth mentioning that the extractable inhibitor is synthesized by the cells, because any inhibition caused by serum components could be easily removed by washing with PBS (data not shown).

The presence of CB or a CB-like enzyme on the cell surface was already suggested by several laboratories. Koppel et al. (22) found an increase in the enzymatic activity of both intra- and extracellular CB-like enzyme using two variants of a rat tumor cell line, AS and ASML. They have suggested that protein synthesis is required to maintain the enzymatic activities. Zucker et al. (38) have investigated the diversity of proteases on human pancreatic cancer cells. Their focus, however, was on the role that tumor cell membrane-bound proteases play in the degradation of normal cells, as measured by erythrocyte lysis. Cavanaugh et al. (7) observed that cysteine proteinase inhibitors blocked the platelet-aggregating activity of 15091A adenocarcinoma cells and their spontaneously shed vesicles. These authors concluded and later verified (6) that a CB-like enzyme is associated with a membrane fraction. A similar enzyme in human ectocervix was characterized by Pietras and Roberts (23) with respect to inhibitor spectrum and pH optimum, although no correlation between plasma membrane CB and metastasis was derived.

The enzyme under investigation here may be considered to be CB or CB-like on the basis of several lines of evidence: (a) the enzyme required thiol activation, with 30 μM cysteine yielding half-maximum activity; (b) the enzyme demonstrated a high selectivity if not specificity for the CB-selective chromogenic substrate N-CBZ-Val-Lys-Lys-Arg-4-methoxy-β-naphthylamide, and it could be inhibited using leupeptin, antipain, and E-64. However, we cannot rigorously exclude the possibility that the observed enzymatic activity, which was enhanced by extraction and antagonized by the readdition of butanol-extracted materials, was not authentic CB but instead a protease with very similar specificities.

The results presented here strongly suggest that CB, or another closely related protease, was expressed on the surface of several neoplastic murine tissues. Butanol extraction of metastatic tumor cell variants, which increases the experimental metastatic potential, also increased the relative expression of CB, or another closely related protease. Consequently, it is possible that the observed enzymatic activity, which was enhanced by extraction and antagonized by the readdition of butanol-extracted materials, was not authentic CB but instead a protease with very similar specificities.

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