Cathepsin B-like Activity in Viable Tumor Cells Isolated from Rodent Tumors

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ABSTRACT

The cathepsin B-like cysteine proteinase activity which has been implicated in tumor malignancy has been attributed to several cellular sources, including viable tumor cells, necrotic tumor cells, and host-inflammatory cells. We have isolated subpopulations of cells from eight rodent tumors of five histological types, using centrifugal elutriation, and verified the cellular composition of the subpopulations cytologically. Ninety-two % or greater of the cathepsin B-like activity was associated with the isolated fractions containing ≥95% tumor cells of 86 ± 2% (SE) viability (β fractions). The isolated fractions consisting of necrotic tumor cells and inflammatory cells (α fraction) apparently contain a cysteine proteinase inhibitor, since both cathepsin B-like and cathepsin H activities in the β fraction of B16 amelanotic melanomas could be inhibited by addition of the α fraction.

INTRODUCTION

Activity of a CB3 has been linked to tumor malignancy and/or the metastatic capability of tumors (for review, see Refs. 1 and 2). In our laboratories we have demonstrated that CB activity in the murine B16 melanoma variants B16-F1 and B16-F10 correlates with their lung colonization potential (3, 4), and that CB activity in metastatic variants of murine B16a and 3LL tumors correlates with their potential to spontaneously metastasize to the lung (5). Takenaga (6) and Koppel et al. (7) have demonstrated a correlation between CB activity and metastatic capability in variants of murine 3LL and a rat spontaneous anaplastic sarcoma, respectively. In contrast, Lowe and Isaacs (8) were not able to demonstrate any correlation between CB activity and metastasis in variants of a rat prostatic adenocarcinoma, nor were McLaughlin et al. (9), in variants of a murine methylcholanthrene-induced sarcoma.

Studies by Recklies et al. (10, 11) and by Pietras et al. (12–14) have provided evidence that tumor CB activity is associated with viable tumor cells in murine and human mammary and human cervical carcinomas, respectively. In contrast, in papers by Graf et al. (15) and Baici et al. (16) on the rabbit V2 carcinoma, it is suggested that in this tumor line, CB is associated primarily with host cells. Studies in our own laboratory indicate that tumor CB activity is associated with viable tumor cells in B16-F1 and B16-F10 variants, since we were able to establish that CB activity correlates with B16-F1 and B16-F10 lung colonization potential, whether measured in homogenates of solid s.c. tumors, of viable tumor cells isolated from the s.c. tumors by centrifugal elutriation, or of tumor cells in primary culture (3, 4).

For further characterization of the role of CB in tumor invasion and metastasis, we wanted to determine whether a primary cellular source of CB could be identified in homogenates of rodent tumors. Viable tumor cells were isolated by centrifugal elutriation from 8 rodent tumors of 5 histological types. Ninety-two % or greater of CB activity was associated with these viable tumor cell fractions.

MATERIALS AND METHODS

Tumor Lines. Murine B16a, murine 3LL, and rat W256 were obtained from the Division of Cancer Treatment (National Cancer Institute) human and animal tumor bank. Variants of the murine B16 melanoma (B16-013, B16-0L6, and B16-B15b), the M5076 reticulum cell sarcoma, and the 15091A mammary adenocarcinoma were provided through the courtesy of Drs. Garth L. Nicholson (M. D. Anderson Hospital and Tumor Institute, Houston, TX), Lance Liotta (National Cancer Institute), and Gabriel Gasic (Pennsylvania Hospital, Philadelphia, PA), respectively. The tumor lines were frozen down in liquid N2 immediately upon receipt. Line 15091A was passaged as an ascites tumor in male CAF1/J mice (The Jackson Laboratory, Bar Harbor, ME). All other tumor lines were propagated in vivo by s.c. injection of cells from frozen stocks or cellular brei from s.c. tumors into the right axillary region of male (B16a, 3LL, B16-BL6, B16-B15b) or female (B16-013, M5076) syngeneic C57BL/6J mice (Jackson Laboratory, Hazlett, MI). In order to maintain their metastatic phenotype, all tumor lines were routinely restituted from liquid N2 frozen stocks after 6 iso transplant generations in vivo.

Preparation of Monodispersed Cells from Tumors. Cell suspensions were prepared from s.c. tumors by sequential collagenase digestion as previously described (3). Ascites fluid was drawn from the peritoneal cavity of anesthetized 15091A tumor-bearing mice using a syringe equipped with a 26-gauge needle. Harvested cells were washed twice with sterile Eagle’s minimal essential medium and resuspended in minimal essential medium.

Isolation of Viable Tumor Cells. Our published procedure for isolating tumor cells from solid murine tumors by centrifugal elutriation (3) was modified to enable us to collect purified tumor cell fractions from 8 rodent tumor lines. Solid tumors are heterogeneous with respect to average tumor cell size and the proportion of either host cells, i.e., RBC, macrophages, etc., and of necrotic tumor cells. Thus, for this study we used 3 separate protocols for isolation of tumor cells (see below). Elutriation was performed with a Beckman J6E elutriator rotor fitted with a Sanderson separation chamber, and operated at a constant speed of 1480 ± 10 (SD) rpm in a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, CA). The separation medium consisted of Eagle’s minimal essential medium with Hanks’ salts, containing 1% (w/v) bovine serum albumin. The medium was pumped through the system with a Cole-Parmer Master Flex pump with Head 7014 (Cole-Parmer, Chicago, IL). The pump control was modified with a 10-turn potentiometer. The rotor chamber, the cell mixing chamber, and the collection tubes were kept at 4°C.

Single cell suspensions (2 to 8 x 10⁶ viable cells) were injected into the mixing chamber and then introduced into the rotor through an inline T-fitting placed just before the rotor inlet. In the first protocol for the
B16a, 3LL, and W256 tumor lines, 4 fractions were pooled as the α fraction; 15 ml were collected at 3.2 ml/min, 50 ml at 7.5 and 10.0 ml/min, and 100 ml at 13.0 ml/min. Eight 100-ml fractions were collected at 15, 20, 26, 31, 36, 41, 46, and >70 ml/min. Not all tumor cells yielded fractions at the >70-ml/min flow rates. Cells isolated at each flow rate were collected by centrifugation, were counted using a Model ZBi Coulter Counter, and were resuspended in Eagle’s minimal essential medium. Aliquots of these cells were removed for determination of cell viability and for differential cell counts. Cell viabilities were determined by Trypan blue dye exclusion.

Differential Cell Counts. Cell aliquots (see above) were spun on a Shandon-Elliot Cytospin (Sewickley, PA). Carbocyan-fixed specimens were stained by the Papanicolaou technique and were air dried; unfixed specimens were stained with Leishman’s hematological stain. Monocytes were identified by staining for nonspecific esterase in the non-fixed, air-dried specimens (17). Two cytotechnicians independently counted ≥100 cells each in the cell aliquots collected at the flow rates given above. Therefore, for any given tumor line, 1000 to 1600 cells were identified and enumerated in the fractions designated as β fractions.

Morphological Criteria for Cellular Identification. The tumor cells, polymorphonuclear leukocytes, lymphocytes, and monocyte-macrophages were readily differentiated by the 2 cytological stains using conventional morphological criteria. The nonspecific esterase staining of the monocyte-macrophage cells confirmed their identification on the basis of morphology. The Papanicolaou stain was valuable for identifying the tumor cells, since the nuclear characteristics of malignancy, including irregular distribution of nuclear chromatin, enlarged and/or multiple nuclei, and altered nuclear:cytoplasmic ratios are emphasized by this stain. The Leishman’s hematological stain facilitated identification of inflammatory cells and confirmed the separation of tumor cells from inflammatory cells by centrifugal elutriation (see “Results”).

Electron Microscopy. Pooled suspensions of elutriated α and β fractions were pelleted, fixed with Trump’s fixative (18), postfixed in 1% (w/v) osmium tetroxide, dehydrated with a graded series of ethanols and propylene oxide, and then embedded in Embed 812 plastic resin (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with aqueous uranyl acetate and lead citrate, and were examined in a Model 10 CA Zeiss electron microscope.

Homogenization Procedure. Single-cell suspensions and the elutriated fractions (~10^6 cells/ml) were homogenized at 4°C in 250 mM sucrose and 1 mM EDTA (pH 7.2), using two 5-sec bursts of a Tekmar homogenizer (Cincinnati, OH) at maximum speed. The 2 bursts were separated by an intermediate cooling period in an ice slurry. Triton X-100 (0.2% v/v) was added, and the samples were stored frozen at −20°C until assay.

Biochemical Assays. Lysosomal enzymes (cathepsin B, EC 3.4.22.1; cathepsin H, EC 3.4.22.16; cathepsin D, EC 3.4.23.5; and β-NAG, EC 3.2.1.30), protein, and DNA were assayed as previously described (4, 19). Concentrations of reaction products were determined by linear regression analysis, using standard curves run with each assay.

RESULTS

Isolation of Viable Tumor Cells by Centrifugal Elutriation. Eight rodent tumors representing 5 histological types (melanoma (B16a, B16-BL6, B16-B15b, and B16-013), adenocarcinoma (15091A), carcinoma (3LL), carcinosarcoma (W256), and a reticulum cell sarcoma of macrophage origin (M5076)) were studied. The 15091A adenocarcinoma was grown as an ascites tumor, and the other 7 lines were grown as s.c. tumors. Viable tumor cells were isolated from nonviable or necrotic tumor cells and host cells (lymphocytes, monocytes, and polymorphonuclear leukocytes) by centrifugal elutriation (see “Materials and Methods”). Elutriated fractions were individually analyzed for cellular content. Data from fractions containing viable tumor cells were pooled (designated β fractions); data from the remaining fractions were also pooled (designated α fractions). The cellular composition of the β fractions of the 8 tumors is tabulated in Table 1. Greater than 95% of the cells in the β fractions were tumor cells. Cell viability of the β fractions was 86 ± 2%, whereas cell viability of the initial dispersed tumors was 55 ± 5%.

Ultrastructural examination (data not shown) of pooled α and β fractions of the 8 tumors verified the differential cell counts determined at the light microscopic level. The β fractions consisted of homogeneous populations of tumor cells with only rare inflammatory cells (compare with Table 1). Composition of the α fraction was primarily cellular fragments and inflammatory cells.

Lysosomal Enzyme Activities in α and β Fractions. Activities of 4 lysosomal enzymes (the cysteine proteinases CB and cathepsin H, the aspartic proteinase cathepsin D, and the glycosidase β-NAG) were determined in homogenates of the elutriated fractions (Table 2). The percentage of enzyme activities associated with the viable tumor cells isolated from the 8 rodent tumor lines was calculated by adding the enzyme activities in the viable tumor cell (β) fractions and dividing by the total enzyme activity in all the elutriated fractions (α plus β). Greater than 92% of the CB activity was associated with the viable tumor cell (β) fractions in all 8 rodent tumor lines (Table 2). Association of the activities of the 3 other lysosomal enzymes with viable tumor cells varied with tumor type. In general, 85% or greater of the activities of cathepsins H and D and β-NAG was associated with the viable tumor cell (β) fractions of the 8 rodent tumors studied.

The activities per μg DNA of CB, cathepsins H and D, and β-NAG for the B16 melanoma lines are depicted in Charts 1 to 4, and for the remaining tumor lines are listed in Table 3. One hundred % or greater (see "Cysteine Proteinase Inhibitor") of the CB activity in the monodispersed cell homogenates of every
Lysosomal enzyme activities associated with the viable tumor cell (total β) 
fractious isolated from rodent tumors by centrifugal elutriation

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Cathepsin B-like</th>
<th>Cathepsin H</th>
<th>Cathepsin D</th>
<th>β-NAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>W256</td>
<td>94.7 ± 1.5</td>
<td>97.3 ± 0.7</td>
<td>93.8 ± 1.9</td>
<td>87.6 ± 4.5</td>
</tr>
<tr>
<td>B16a</td>
<td>98.1 ± 1.3</td>
<td>96.6 ± 2.4</td>
<td>99.6</td>
<td>88.5 ± 4.7</td>
</tr>
<tr>
<td>B16-BL6</td>
<td>93.6</td>
<td>89.0</td>
<td>97.4</td>
<td>95.6</td>
</tr>
<tr>
<td>B16-B15b</td>
<td>92.8</td>
<td>93.2</td>
<td>99.7</td>
<td>92.2</td>
</tr>
<tr>
<td>B16-B103</td>
<td>95.0</td>
<td>90.8</td>
<td>72.6</td>
<td>85.9</td>
</tr>
<tr>
<td>3LL</td>
<td>96.1</td>
<td>98.0</td>
<td>97.5</td>
<td>54.1</td>
</tr>
<tr>
<td>15091A</td>
<td>92.4</td>
<td>75.0</td>
<td>84.7</td>
<td>90.8</td>
</tr>
<tr>
<td>M5076</td>
<td>92.7</td>
<td>96.8</td>
<td>75.3</td>
<td>90.6</td>
</tr>
</tbody>
</table>

*Expressed as a percentage of the total activities

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Cysteine Proteinase Inhibitor. Mean β-fraction activities of CB and cathepsin H in the B16a tumor line were 10- and 6-fold greater, respectively, than the monodispersed cell activities (Charts 1 and 2). For the other tumor lines studied, CB activities in the mean β fractions were from 1.1- to 1.6-fold greater than in the monodispersed cells, and cathepsin H activities in the mean β fractions were from 0.6- to 1.5-fold greater than those in the monodispersed cells. The enhancement of cysteine proteinase activity in the B16a tumor β fraction suggested that we might be eliminating a source of endogenous cysteine proteinase inhibitor by separating host cells from the tumor cells.

We tested this hypothesis by conducting a series of add-back experiments. CB and cathepsin H activities were measured in B16a pooled β fractions and α fractions separately, and then in mixed β and α fractions. CB activity in the mixed fractions was 63.2 ± 4.2% less than would be expected by adding the activities in the 2 fractions. Cathepsin H activity was similarly inhibited by 58.2 ± 7.8%. Since the α fractions had a higher concentration of protein than did the β fractions, we also tested the ability of bovine serum albumin to decrease cysteine proteinase activity, using up to 10 times more albumin than the level of protein in the α fraction. Neither CB or cathepsin H activities were reduced in the presence of albumin. These data suggest that the levels of an endogenous cysteine proteinase inhibitor, which has been localized to the cytoplasm of normal cells (20) and human melanoma cells (21), may be low in B16a tumor cells.

The presence of a cysteine proteinase inhibitor in the cells of the α fraction was not unique to the B16a tumor line. In an experiment in which CB activity was assayed in the B16a β fraction and the M5076 α fraction separately and then in combination, CB activity in the combined α and β fractions was 63% lower than if the 2 activities were additive. Thus, endogenous cysteine proteinase inhibitors in host inflammatory cells may prevent an accurate assessment of CB (or cathepsin H) activity in homogenates of solid tumors (see Ref. 22 for an extended discussion of this problem). Interestingly, the M5076 β (viable tumor cell) fraction also inhibited CB activity in the B16a β fraction by 12 to 44%, depending on the experiment. The presence of a
Cysteine proteinase inhibitor in M5076 tumor cells could account in part for the low CB activity in the M5076 tumor (Table 3).

Correlation of CB Activity with Lung Colonization Potential.
The B16 melanoma lines syngeneic to the C57BL/6 mouse (B16a, B16-BL6, B16-B15b, and B16-O13) were obtained from Dr. G. L. Nicolson as frozen stocks and grown in vivo as s.c. tumors. The lung colonization potential, invasive capabilities, and heparanase activities of these B16 variants follows the order: B16-BL6 > B16-B15b > B16-O13 (23). CB activity in the viable tumor cell (β) fractions of these variants also correlated with their potential to form lung colonies (Chart 1). This was true whether the activities were expressed as mean β-fraction activities or total β-fraction activities. In contrast, CB activity in the nonelu-triated, monodispersed cells did not correlate with lung colonization potential (Chart 1). This may reflect the presence of an endogenous cysteine proteinase inhibitor in the host inflammatory cells separated from the viable tumor cells by centrifugal elutriation (see above). In contrast to CB activity, activities of 3 other lysosomal enzymes (cathepsin H, cathepsin D, and β-NAG) did not correlate with lung colonization potential of these B16 variants (Charts 2 to 4).

DISCUSSION

The ability of tumor cells to invade into the host stroma and blood and lymph vessels is thought to be due to hydrolytic enzymes associated with tumor cells or released from host cells in response to tumor cells. One group of hydrolytic enzymes that...
has been implicated in tumor malignancy is lysosomal enzymes (24). Weiss (25) has demonstrated that lysosomal enzymes released from necrotic tumor cells can facilitate tumor cell detachment from solid tumors. Dobrossy et al. (26) found that activities of lysosomal glycosidases in solid 3LL tumors are elevated coincident with the onset of metastasis. Activity of a lysosomal proteinase, CB, in elutriated tumor cells or tumor cells in primary culture has previously been correlated with the lung colonization potential of B16-F1 and B16-F10 variants (3, 4). CB activity has been correlated with the lung colonization potential of cultured, cloned 3LL cells (6), and the metastatic potential of cultured BDX sarcoma variants (7). Studies of CB activity in homogenates of solid tumors have resulted in conflicting evidence for correlations between CB activity and metastasis, either experimental or spontaneous (3–5, 8, 9). Several studies have demonstrated that CB activity may be elevated in extracellular fluid of tumor-bearing animals (27) and cancer patients (28–30), and in culture medium of tumor explants (10, 11) and tumor cells (4, 14). Mort and Leduc (31) have recently demonstrated that “CB activity” in human serum may be the result of 2 peptidases acting sequentially rather than of CB. CB has, however, been characterized biochemically and immunologically in culture medium of tumor explants (32, 33). In ascites fluid of women with ovarian carcinomas (28), and in urine of women with gynecological cancers (34).

The cellular source(s) of lysosomal enzyme activities associated with tumors has been a matter of controversy. Tumor CB activity has been linked to the presence of necrotic tumor cells (25), viable tumor cells (3), infiltrating inflammatory cells (15), and/or fibroblasts (15). If CB is associated with necrotic tumor cells one would expect its release to be a passive process. However, Recklies et al. (10, 11) established that release of CB from tumor explants is an active process requiring protein synthesis. In addition, we have demonstrated that CB release from tumor cells can be stimulated by hydroperoxy fatty acids and inhibited by lipoxygenase inhibitors (35), confirming that CB release is an active process. In the present study we have established in 8 rodent tumor lines of 5 histological types that the major cellular source of CB (≥92%) is viable tumor cells. This confirms our earlier findings with the B16-F1 and B16-F10 tumor lines (3). The remainder of the CB activity in tumor homogenates may be derived from macrophages (36). In this study, 100% of the CB activity in the monodispersed cells was recovered with the cells isolated by centrifugal elutriation, suggesting that extracellular CB does not constitute a significant proportion of the CB activity measured in a solid tumor homogenate.

Our results are in conflict with the studies by Graf et al. (15) and Baici et al. (16), which indicate that CB is not associated with viable tumor cells in the rabbit V2 carcinoma. By immunohistochemistry and histochemistry, Graf et al. (15) localized CB in fibroblasts and in the extracellular matrix adjacent to invading tumor cells. Baici et al. (16) found that CB activity is released from host cells into the extracellular matrix in response to a diffusible factor produced by the V2 carcinoma cells. However, the V2 carcinoma may be an exception; the antibody to cathepsin B purified from liver as recently described by Graf et al., which does not stain tumor cells in the rabbit V2 carcinoma, was found to stain tumor cells in sections of human colonic tumors.4

Host cells could have both a negative and a positive effect on tumor-associated CB activity in vivo. As we demonstrate in this study, host cells may be a source of cysteine proteinase inhibitors. However, host inflammatory cells, i.e., macrophages and neutrophils, that are capable of producing large quantities of lipoxygenase products (37, 38) may also stimulate release of CB from tumor cells (35). Urban (39) demonstrated that macrophages can increase lysosomal enzyme activities in tumor cells, but he did not assay CB activity.

Numerous investigators have reported positive correlations between hydrolytic enzyme activities and metastatic potential, and have proposed models for predicting metastatic behavior based on these activities. Although CB activity has been correlated with metastatic behavior in several tumor lines (3–7), activity measurements of CB in tumor homogenates may be subject to misinterpretation for several reasons, including (a) the presence of endogenous cellular or humoral inhibitors of cysteine proteinases; (b) the presence of alternative substrates for CB; (c) compounds which can directly interfere with measurement of reaction products; and/or (d) nonspecificity of substrates. Giraldi et al. (22) have previously discussed several of the problems in interpreting proteinase activity measured in homogenates of s.c. 3LL tumors. The discrepancies between the cysteine proteinase activity measured in whole tumors and in isolated tumor cells in the present study make it apparent that one must use caution in attributing a biochemical property to a particular tumor when using tumor homogenates. Our demonstration that approximately 90% of the CB activity in rodent tumors could be attributed to tumor cells does indicate that CB isolated from s.c. tumors will be primarily of tumor cell origin. Thus, although activity measurements of CB in tumor homogenates may be problematic, for the biochemical and immunological characterization of tumor CB, as well as for localization of tumor CB by subcellular fractionation, nonelutriated solid tumors can be used. Such studies to further examine the role of CB in tumor invasion and metastasis are in progress in our laboratory.

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