Cathepsin B Activity in B16 Melanoma Cells: A Possible Marker for Metastatic Potential

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ABSTRACT

In solid s.c. tumors of a variant of the murine B16 melanoma with high metastatic potential (B16F10), there was a 2- to 7-fold elevation of lysosomal cathepsin B activity when compared to the B16F1 variant with low metastatic potential. The highest activities (based on either protein or DNA) of cathepsin B were found in tumors of less than 1 g. When B16F1 and B16F10 melanoma variants were grown in tissue culture, the metastatic differential in cathepsin B activity was lost as the cells were subcultured. However, this differential in cathepsin B activity could be restored by reestablishing the cultured cells as s.c. tumors.

The activities of four other lysosomal enzymes (cathepsin D, β-N-acetylglucosaminidase, β-glucuronidase, and acid phosphatase) showed little evidence of a positive correlation with the metastatic potential of the B16 melanoma variants. Eighty to 90% of cathepsin B activity has been localized to a fraction containing viable tumor cells which was isolated by centrifugal elutriation. In contrast, only 50% of cathepsin D activity was in the viable tumor cell fraction, and from 30 to 70% of β-N-acetylglucosaminidase, β-glucuronidase, and acid phosphatase. Elevated levels of cathepsin B in the high metastatic B16F10 variant are consistent with the idea that cathepsin B may play a direct or a regulatory role in tumor metastasis.

INTRODUCTION

Lysosomes may play important roles at several steps in the metastatic cascade due to their complement of hydrolytic enzymes which can degrade collagen and proteoglycans (for review, see Ref. 26). Release of lysosomal hydrolases into the extracellular matrix may facilitate invasion at primary and secondary sites as well as cause detachment of cells from the primary tumor. The lysosomal glycosidases, β-NAG and β-GLU, will degrade hyaluronic acid and chondroitin sulfate. An increase in the activity of these lysosomal glycosidases has been correlated temporally with onset of pulmonary metastasis from Lewis lung carcinoma (9), and inhibitors of β-NAG and β-GLU have been shown to reduce growth of s.c. tumors (8). The lysosomal cysteine proteinase, cathepsin B, will degrade pericellular protein at pH 7.1 (37) and collagen at pH 7 (7). In addition, cathepsin B could play a regulatory role in collagen degradation, since it can convert inactive procollagenase to its active form (11). Poole et al. (21, 27, 29) have found that cathepsin B is released from explants of malignant human breast tumors and that a gradient of cathepsin B activity within the tumor explant is suggestive of release at the growing, and thus invading, tumor periphery (29). We have shown that there is a positive correlation between cathepsin B activity and metastatic potential of the s.c. B16 melanoma and that cathepsin B is located in the tumor cells themselves and not in stromal cells or invading macrophages (33). Although in vivo studies suggest that lysosomal enzyme activities correlate with tumor malignancy (4, 16, 36, 39, 40), studies of tumor cells in vitro have reported both a correlation between lysosomal enzyme activities and metastatic potential (3) and lack of such a correlation (22).

Therefore, in the present study, we determined the activities of 5 lysosomal enzymes in low (B16F1) and high (B16F10) metastatic variants of the B16 melanoma at various stages of growth both in vivo and in vitro.

MATERIALS AND METHODS

Tumor Lines. Low (B16F1) and high (B16F10) metastatic variants of the murine B16 melanoma developed by Fidler (12-14) were obtained from the Division of Cancer Treatment (National Cancer Institute) tumor bank and propagated in vivo by s.c. injection into male syngeneic C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine).

Cell Separation. Cell suspensions were prepared from s.c. tumors as described previously (17, 33). After dispersal, the cells were either separated into an α and β fraction by centrifugal elutriation (33) or propagated in vitro (17). The β fraction contained viable tumor cells, and the α fraction contained macrophages, stromal cells, and nonviable tumor cells (33). Culture Techniques. Freshly dispersed tumor cells were adapted for growth in tissue culture medium (Eagle's MEM with Hanks' salts (Grand Island Biological Co., Grand Island, N. Y.), supplemented with sodium pyruvate, MEM nonessential amino acids (Grand Island Biological Co.), 150 units penicillin G per ml (Sigma Chemical Co., St. Louis, Mo.), 100 g neomycin sulfate per ml (Sigma), 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma), and 10% FCS (M. A. Bioproducts, Walkersville, Md.). Growth medium in stock cultures was changed twice per week, and the cells were subcultured once per week. For subculture, the cells were detached by a 1-min trypsinization (0.25% trypsin (Worthington Biochemical Corp., Freehold, N. J.); 0.02% EDTA in MEM), pelleted at 100 x g, and resuspended in MEM supplemented with FCS. B16F1 and B16F10 cells were allowed to grow to confluency before harvesting or subculturing. Forty-eight hr before the cells were harvested, the medium was changed to medium containing 10% FCS which had been heat treated and then acid treated to inactivate α2-macroglobulin (15), an inhibitor of cathepsin B (35).

Homogenization Procedure. Whole tumors, dispersed cells, α and β fractions, and cultured cells were homogenized in 250 mM sucrose
with 5 mM EDTA, pH 7.3, at 4° using two 5-sec bursts of a Tekmar homogenizer at maximum speed. Homogenates were fractionated by differential centrifugation, with lysosomes sedimenting in the light mitochondrial fraction at 23,000 x g for 15 min (33).

**Enzyme Assays.** Five lysosomal enzymes were assayed fluorometrically in homogenates, light mitochondrial fractions, and culture media. Cathepsin B (EC 3.4.22.1) was assayed at pH 6.2 using carbenzoxyl-oxalylalanylarginyl-4-methoxy-β-naphthylamine (Enzyme Systems Products, Livermore, Calif.) as substrate (33). β-NAG (EC 3.2.1.30), β-GLU (EC 3.2.1.31), ACPase (EC 3.1.3.2), cathepsin D (EC 3.4.23.5), protein, and DNA were determined as described previously (32). Enzyme assays were standardized to ensure linearity. Triton X-100 [0.2% (v/v)] was added to the homogenates to disrupt the lysosomal membranes.

**Statistical Analysis.** All data were analyzed by a 2-tailed Student's t test; p values are listed in chart and table legends. Concentrations of reaction product, protein, and DNA were derived from standard curves by linear regression analysis.

**RESULTS**

**In Vivo.** Activity of cathepsin B, a lysosomal cysteine protease, was significantly greater (2- to 3-fold) in homogenates of the high metastatic B16F10 melanoma line than in the low metastatic B16F1 line for tumors in all weight ranges (Chart 1). Cathepsin B activity based on DNA was greatest for both B16F1 and B16F10 tumors less than 1 g in wet weight.

β-NAG, a marker enzyme for lysosomes, did not differ in homogenates of B16F1 and B16F10 tumors of greater than 1 g in weight (Chart 2). However, the activity was significantly greater for the B16F10 melanomas less than 1 g in weight. The same pattern of activity was observed for β-GLU (Chart 2). No significant differences between B16F1 and B16F10 lines in any weight range were found for cathepsin D, another major lysosomal proteinase, or for ACPase (Table 1).

Since tumors consist of many cell types, we used centrifugal elutriation to separate viable tumor cells (β fraction) from stromal cells, macrophages, and nonviable tumor cells (α fraction). Only the β fraction produced tumors upon s.c. injection. Seventy to 90% of the lysosomal enzyme activity for cathepsin B (33), β-NAG, β-GLU, and ACPase (Table 2) was present in the tumor cells (β fraction) of the B16F10 melanoma. In contrast, only 30 to 55% of the activity of β-NAG, β-GLU, or ACPase was found in B16F1 tumor cells. Fifty percent of cathepsin D occurred in the tumor cells themselves (Table 2). The differential between the specific activity of cathepsin B in the B16F1 and B16F10 lines could be increased up to 400 or 700% by isolating lysosomes from the dispersed tumor cells or from β fractions, respectively.

**In Vitro.** Cathepsin B activity was 7-fold greater in the lysosomes of the high metastatic B16F10 tumor line than in the B16F1 tumor line grown in vivo. However, some authors have failed to demonstrate a difference between B16F1 and B16F10 for the activity of any lysosomal enzyme when the tumor lines are in tissue culture. Therefore, we investigated whether tumor cell lysosomal enzyme activities might be altered under tissue culture conditions. All assays were run on confluent cells, since Kaplan (18) has demonstrated that lysosomal enzyme activities decrease after cells are subcultured but recover after a lag period.

In primary culture, the activity based on DNA of cathepsin B was 4-fold higher in the B16F10 tumor (Chart 3). The differential between B16F1 and B16F10 was no longer present when the tumor cells had been subcultured 3 times. Cathepsin B activity in the cultured tumor cells was always less than the activity present in tumor cells isolated from in vivo tumors. Cathepsin B activity could not be detected in the media of B16F1 and B16F10 cells in primary culture. B16F10 cells which had been subcultured 3 times released 2-fold more cathepsin B into the media than did B16F1 cells.

β-NAG activity was significantly greater in cultured B16F10 cells than in B16F1 cells at all stages measured (Chart 4). B16F1 and B16F10 cells in culture released β-NAG into the culture media. β-GLU and ACPase activities were lower in primary culture than in vivo (Chart 4), and with time in culture, the activities approached their in vivo values (compare Chart 2 and Table 1). Both tumor lines released β-GLU and ACPase into the culture media. Cathepsin D activity was reduced in tissue culture and remained 50% lower than in vivo after 6 passages in vitro (Chart 4). Cathepsin D activity could not be measured in the culture media by our methodology, due to the presence of primary amines (2) in MEM.

**Reestablishment of in Vivo Tumors.** The greater activity of cathepsin B in the high metastatic B16F10 tumor line was lost as the cells were passaged in vitro. We attempted to restore the differential between the B16F1 and B16F10 tumor lines by reintroducing sixth passage cultured cells s.c. In the initial tumors, the activity of cathepsin B in the B16F10 tumor was 2-fold higher than in the B16F1 tumor (Table 3). A second passage in vivo by s.c. reinjection of first passage tumor cells did not result in further elevation of cathepsin B activity. The absolute value of cathepsin B activity in both B16F1 and B16F10 was less after one and 2 passages in vivo than was found in our initial in vivo
Chart 2. Effect of tumor wet weight on activity of β-NAG or β-GLU in homogenates of B16F, and B16F,0 metastatic variants. Enzyme activity is expressed as concentration of reaction product formed per mg protein or μg DNA per mm. Bars, S.E. Asterisks indicate that the value for B16F,0 is significantly different than for B16F, (0.025 > p > 0.005; n = 3 to 7). 4-MeUmb, 4-methylumbelliferone.

Table 1
Cathepsin D and ACPase activity as function of tumor weight

<table>
<thead>
<tr>
<th>Tumor wt (g)</th>
<th>Tumor line</th>
<th>μM tyrosine/mg protein x min</th>
<th>nm tyrosine/μg DNA x min</th>
<th>n</th>
<th>μM 4-MeUmb/a</th>
<th>μM 4-MeUmb/μg DNA x min</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>B16F,</td>
<td>6.6 ± 1.4 b</td>
<td>391 ± 89</td>
<td>7</td>
<td>62.1 ± 2.4</td>
<td>3.69 ± 0.34</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>8.0 ± 1.5</td>
<td>681 ± 131</td>
<td>6</td>
<td>58.0 ± 2.3</td>
<td>5.14 ± 0.73</td>
<td>6</td>
</tr>
<tr>
<td>1-4</td>
<td>B16F,</td>
<td>6.6 ± 0.4</td>
<td>327 ± 69</td>
<td>6</td>
<td>53.0 ± 2.1</td>
<td>2.62 ± 0.55</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>7.0 ± 0.7</td>
<td>373 ± 52</td>
<td>7</td>
<td>50.3 ± 3.3</td>
<td>2.78 ± 0.44</td>
<td>7</td>
</tr>
<tr>
<td>&gt;4</td>
<td>B16F,</td>
<td>9.6 ± 0.4</td>
<td>414 ± 95</td>
<td>3</td>
<td>50.5 ± 5.4</td>
<td>2.17 ± 0.52</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>9.4 ± 0.6</td>
<td>417 ± 59</td>
<td>4</td>
<td>40.3 ± 4.3</td>
<td>1.86 ± 0.46</td>
<td>4</td>
</tr>
</tbody>
</table>

* 4-MeUmb, 4-methylumbelliferone.
* Mean ± S.E. (p < 0.010).

Table 2
Lysosomal enzyme activity in tumor cells of metastatic variants of B16 melanoma

Enzyme activity was measured in homogenates of dispersed tumor cells and of elutriated fractions (β, viable tumor cells; α, macrophages, stromal cells, and nonviable tumor cells). n = 3 in all cases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tumor line</th>
<th>Dispersed tumor cells</th>
<th>α fraction</th>
<th>β fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μM 4-MeUmb/μg DNA x min</td>
<td></td>
<td>μM 4-MeUmb/μg DNA x min</td>
</tr>
<tr>
<td>β-NAG</td>
<td>B16F,</td>
<td>3.07 ± 0.41 b</td>
<td>1.42 ± 0.57</td>
<td>0.64 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>3.64 ± 0.30</td>
<td>0.61 ± 0.14</td>
<td>1.44 ± 0.10</td>
</tr>
<tr>
<td>β-GLU</td>
<td>B16F,</td>
<td>1.07 ± 0.17</td>
<td>0.44 ± 0.20</td>
<td>0.19 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>1.08 ± 0.07</td>
<td>0.17 ± 0.04</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>ACPase</td>
<td>B16F,</td>
<td>2.16 ± 0.36</td>
<td>0.46 ± 0.17</td>
<td>0.56 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>2.41 ± 0.22</td>
<td>0.25 ± 0.03</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>B16F,</td>
<td>282 ± 61</td>
<td>87 ± 26</td>
<td>95 ± 45</td>
</tr>
<tr>
<td></td>
<td>B16F,0</td>
<td>449 ± 57</td>
<td>142 ± 44</td>
<td>143 ± 12</td>
</tr>
</tbody>
</table>

* 4-MeUmb, 4-methylumbelliferone.
* Mean ± S.E.
* p = 0.05 as determined by a 2-tailed Student's t test.
studies (see Chart 1). After passaging the tumors in vivo for 5 times, the specific activity of cathepsin B was greater than found in our initial in vivo studies (compare Table 3 and Chart 1), and cathepsin B activity based on DNA had returned to or above its initial values in both the B16F1 and B16F10 tumor lines (compare Table 3 and Chart 1 for tumors of comparable wet weight).

In contrast to cathepsin B, the specific activity of β-NAG was elevated in B16F1 and B16F10 tumors from the initial and second passages in vivo (Table 3). After 5 passages, the specific activity in the B16F1 tumor was reduced to a value found in our initial in vivo studies. Specific activity of β-NAG in the B16F10 tumor was significantly less than measured previously. β-NAG activity based on DNA was not restored to previous values after one, 2, or 5 passages in vivo (see Chart 2). The activities of cathepsin D, β-GLU, and ACPase were measured in the initially reestablished tumors (Table 4) and followed the same pattern as found for β-NAG. The specific activities were elevated above the original in vivo values (compare Chart 2 and Table 1), but the activities based on DNA were less than found originally.

**DISCUSSION**

We had demonstrated previously that there is a positive correlation between cathepsin B activity and the metastatic potential of B16 melanoma variants grown in vivo as solid tumors and that 90% of this cathepsin B activity was derived from viable tumor cells (33). In the present study, we have shown that the high metastatic B16F10 tumor line has 2- to 7-fold more cathepsin B activity than the low metastatic B16F1 tumor line for solid tumors in any weight range. The absolute levels of cathepsin B were found to be highest in tumors weighing less than 1 g. Although many reports in the literature have suggested that elevated lysosomal enzyme activity in tumors is due to the presence of necrotic tumor cells or of invading macrophages (9, 41), our results (Ref. 33; present study) confirm the reports by Sylven and Malmgren (38) as early as 1957 and by Shamberger and Rudolph (31) that the youngest and most rapidly growing tumors have the highest
lysosomal proteinase activities. Furthermore, we were unable to detect cathepsin B activity in peritoneal macrophages obtained from the same strain of mice (male C57BL/6J; The Jackson Laboratory). Morland and Pedersen (19) have also reported that murine resident peritoneal macrophages have negligible cathepsin B activity.

When the B16F1 and B16F10 metastatic variants were grown in tissue culture, the differential in cathepsin B activity was lost as the cells were subcultured. The restoration of a metastatic differential in cathepsin B activity when the tumor lines were reestablished in vivo suggests that cathepsin B activity may be regulated by a reaction of the host to the growing tumor or by cofactors missing in our culture medium. The change in cathepsin B activity could be the proteinase released from the L1210 cells.

In contrast to cathepsin B, the activity of the other lysosomal enzymes measured showed little evidence of a positive correlation with metastatic potential. Both β-NAG and β-GLU were significantly higher in tumors of less than 1 g from the Bi 6F10 tumor line and, at another stage, higher in the Bi 6F1 tumor line. Release of the lysosomal enzymes into the culture media did not necessarily correlate with their respective levels in the cultured cells of the 2 tumor lines. Earlier studies reporting both a difference (3) and no difference (22) in lysosomal glycosidase and proteinase activities between the B16F1 and B16F10 variants in culture may have both been correct, with the variance in results due to how many times the tumor cells had been subcultured or to their relative confluency, as suggested by Bosmann et al. (3). In our hands, the confluency of the B16F1 and B16F10 tumor cells did not affect the lysosomal enzyme activities.

Poole et al. (27, 29) have shown that up to 11 times more cathepsin B is released from malignant human breast tumors than from normal breast tissue or nonmalignant tumors. In contrast, release of cathepsin D from the 3 tissues was not significantly different. A positive correlation between cathepsin B activity in pancreatic fluid and pancreatic cancer has been reported (30). In addition, Pietras et al. (24) have found an apparent elevation of cathepsin B levels in the serum of women with vaginal clear-cell adenocarcinoma and invasive neoplastic diseases in diverse tissues (23, 25). Burger (6) also reported that mouse leukemia L1210 cells also release proteinases but did not characterize the proteinases. However, Bowers et al. (5) found that cathepsin D activity in mouse leukemia L1210 cells is primary nonsedimentable, suggesting that cathepsin D could be the proteinase released from the L1210 cells.

Degradation of the extracellular connective tissue matrix is a necessary prelude to the invasion of surrounding tissues by tumor cells. Cathepsins B and D can degrade 2 of the major components of the matrix, collagen and proteoglycans (7, 20). In addition, cathepsin B can degrade pericellular protein (37).
Release of either cathepsin B or D from neoplastic cells could account for modifications in the cell surface of such cells (28) and result in escape from contact inhibition of growth. Normal cells in vitro will lose their contact inhibition if exposed to proteolytic enzymes (6). Although cathepsin B has significant activity at pH 7.0 (7), cathepsin B purified from tumors seems to be modified to increase its stability at alkaline pH (21). Cathepsin D has a very acid pH optimum ranging from 2.0 to 5.0 (1), which would suggest that cathepsin D may not have enough activity to play a role in tumor invasion in vivo. Furthermore, we have shown that, while 90% of cathepsin B activity is located in the B16F10 tumor cells, only 50% of cathepsin D activity is in the tumor cells, suggesting that cathepsin B may play the more important role in vivo in tumor cell invasion.

Although Poole et al. (27) did not find an increase in the total activity per µg tissue DNA of either cathepsin B or D in homogenates of breast carcinoma in comparison to normal breast tissue or nonmalignant breast tumors, several investigators have demonstrated an increase in lysosomal enzyme activities in other types of cancer. Shamberger and Rudolph (31) found an increase in the total activity per mg tissue protein of cathepsin D, β-GLU, and ACPase in actively growing skin carcinomas. Drewa et al. (10) have also found that the total activities per mg tissue protein of cathepsin D, β-GLU, arylsulfatases A and B, and ACPase were significantly greater in a highly invasive melanotic melanoma than in a less invasive amelanotic melanoma. Cathepsin B was not assayed in these studies.

The metastatic variants isolated from the B16 melanoma by Fidler (12-14) form differing numbers of lung colonies after i.v. injection (tail vein). A tumor cell which is injected directly into circulation must retain the ability to invade normal tissue in order to establish a successful lung colony. Presumably, the increased level of cathepsin B in the high metastatic line, B16F10, could facilitate extravasation into the lung matrix tissue by the B16F10 tumor cells. It is not clear otherwise how increased cathepsin B activity could aid in lung colony formation from tail vein-injected tumor cells. The B16F6, and B16F10 metastatic variants are not an ideal model in which to study the relationship of cathepsin B to tumor metastasis, since their development by tail vein injection eliminated the need for the initial steps in the metastatic process (see also Ref. 34 for a discussion of this model). However, the positive correlation between elevated cathepsin B levels and metastatic potential suggests this may be an area for further study. We are currently assaying cathepsin B in 2 additional murine tumors, the B16 amelanotic melanoma and the Lewis lung carcinoma, both of which spontaneously metastasize from a s.c. tumor as well as in high metastatic cell lines developed from spontaneous pulmonary metastases of these 2 tumor lines.8

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