Degradation of Tumor-associated Antigens Shed by Human Melanoma Cells in Culture

Amal M. Docter and Jean-Claude Bystryn

Department of Dermatology, New York University School of Medicine, New York, New York 10016

ABSTRACT

The fate of cell surface tumor-associated antigens shed by viable human melanoma cells was studied in vitro. Labeled surface material shed by radio-iodinated melanoma cells was incubated with a variety of unlabeled human cells for 24 hr. Both melanoma-associated antigens (MAAs), quantitated by specific immunoprecipitation, and unrelated surface macromolecules were degraded or inactivated by normal and malignant cells including the melanoma cells themselves. The MAAs studied were particularly susceptible to degradation. Following incubation with a variety of cells, immunoreactive MAAs decreased 2 to 3 times more rapidly than did unrelated surface macromolecules shed concurrently by melanoma cells. However, melanoma cells had a selective defect in their ability to degrade MAAs. Though catabolically active, these cells degraded non-MAA surface macromolecules shed by themselves or by allogeneic cells much more rapidly than they inactivated MAAs. These observations suggest that the ultimate amount of soluble tumor antigens that accumulate in body fluids will depend on the balance between the rate of their release and that of their degradation and that as a result of a selective defect in the catabolic activity of melanoma cells some tumor antigens may be particularly prone to accumulate in the extracellular fluid bathing these tumors.

INTRODUCTION

Soluble tumor antigens are present in body fluids of animals (2, 32) and humans (13, 14) with progressing cancers. They are believed to have a major impact on host resistance to cancers since they can both stimulate and block tumor-specific immune responses (1, 4, 19). Thus, the factors which influence the accumulation of soluble tumor antigens in body fluids may have an important influence on tumor growth. One of these is the release or shedding of soluble tumor antigens by viable tumor cells (3, 11, 15). It is well documented that tumor cells including melanoma can release a variety of macromolecules including tumor antigens (3, 5, 6, 11, 16, 17, 25, 27). The release of cell surface components is particularly rapid (6). Released tumor antigens can be biologically active. Immunization of mice to partially purified tumor antigens released by B16 melanoma cells can markedly and specifically inactivate MAAs. These observations suggest that the ultimate amount of soluble tumor antigens which accumulate in body fluids will depend on the balance between the rate of their release and that of their degradation and that as a result of a selective defect in the catabolic activity of melanoma cells some tumor antigens may be particularly prone to accumulate in the extracellular fluid bathing these tumors.

MATERIALS AND METHODS

Cells. Human melanoma cells HM29, HM31, and HM34 were obtained from surgically excised metastatic nodules and established in tissue culture as described previously (9, 10, 32). Human melanoma line SK-MEL-23 was obtained from Dr. J. Fogh, Memorial Sloan-Kettering Institute, Rye, N. Y. Murine B16 melanoma cells and neonatal human fibroblasts (HS-1) obtained from circumcisions were established in culture as described previously (5). Lymphocytes were prepared from fresh heparinized human blood by Ficoll-Hypaque separation (24). The separated lymphoid cells were washed 3 times and incubated for 24 hr at 37°C in 100-mm culture dishes, following which lymphocytes were present predominantly as floating cells and macrophages as adhering cells. All cells were grown as monolayers in plastic culture dishes with Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum, 200 units penicillin per ml, 2 mg streptomycin per ml, and 0.25 μg Fungizone per ml.

Labeling of Cells. Surface macromolecules were radiolabeled by the lactoperoxidase technique. Prior experiments have shown that this procedure labels only external macromolecules on viable melanoma cells (10). Cells were labeled in suspension at a concentration of 1 × 10^6 cells per ml with 1 μCi lactoperoxidase, 100 μg glucose, 0.1 μCi Na125I, and 5 μg lactoperoxidase. Following labeling the cells were washed 3 times in PBS and then resuspended in PBS at a concentration of 10^6 cells per ml. The labeled cells were then incubated with a variety of cells for a 24 hr period. Following incubation the cells were washed 3 times in PBS and then resuspended in PBS at a concentration of 10^6 cells per ml. The labeled cells were then incubated with a variety of cells for a 24 hr period. Following incubation the cells were washed 3 times in PBS and then resuspended in PBS at a concentration of 10^6 cells per ml. The labeled cells were then incubated with a variety of cells for a 24 hr period. Following incubation the cells were washed 3 times in PBS and then resuspended in PBS at a concentration of 10^6 cells per ml.
10⁷ cells/ml in PBS, pH 7.4. To 0.3 ml of cell suspension was added sequentially 0.5 mg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) in 0.25 ml of PBS, 0.5 mCi of ¹²⁵I-sodium (New England Nuclear, Boston, Mass.) in 0.05 ml of PBS, and 0.02 ml of 0.3% hydrogen peroxide. The cells were incubated at 25° with gentle agitation, and additional 0.02-ml aliquots of hydrogen peroxide were added at 3-min intervals. After 10 min, the reaction was stopped by the addition of 10 ml ice-cold PBS, and the cells were sedimented and washed 3 times with 10 ml of PBS. Viability of labeled cells was over 90% by trypsin blue exclusion. In some experiments, cells were labeled metabolically by incubating confluent monolayers in 100-mm plastic dishes with 0.5 mCi of (¹¹C)jucine (30 to 50 Ci/mmol; New England Nuclear) in 10 ml of complete medium at 37° for 48 hr. In some cases, labeled cells were immediately lysed in 0.5% Nonidet P-40 (Shell Chemical Corp., New York, N. Y.). This procedure has been shown in prior experiments to solubilize over 90% of the radioactivity associated with labeled macromolecules.

Collection of Shed Macromolecules. Approximately 1 × 10⁷ labeled cells were resuspended in 10 ml of complete medium and incubated in 100-mm plastic dishes at 37° under 5% CO₂. After 3 hr, the medium containing labeled material including MAAs released during this time and henceforth called "labeled medium" was collected. Prior studies have shown that over 60% of the radioactivity associated with labeled macromolecules and over 40% of that associated with MAAs is released during this time with cells remaining over 95% viable. Floating cells and particulate matter were removed by centrifugation at 4000 x g for 10 min. Labeled medium was collected from metabolically labeled cells after 48 hr of incubation.

Degradation of Shed Material. Replicate 3-ml aliquots of labeled medium, diluted 1:2 (v/v) with fresh complete medium, were added to 15- x 60-mm culture dishes containing 1 to 5 × 10⁶ unlabeled cells or to control dishes lacking cells and incubated at 37° in a 5% CO₂ atmosphere. After 24 hr, the medium was collected, the cells were removed by centrifugation, and the amount of radioactivity associated with acid-insoluble macromolecules and with MAAs was assayed as described below. The percentage of degradation was calculated from the following formula:

\[
\text{Degradation} = \left(1 - \frac{\text{Acid-insoluble or MAA cpm in presence of cells}}{\text{Acid-insoluble or MAA cpm in absence of cells}}\right) \times 100.
\]

All experiments were performed on duplicate plates and the average value was used.

Assays. Protein concentration was measured by the method of Lowry et al. (22) using bovine serum albumin as a standard. Radioactivity associated with macromolecules was determined by precipitation with 10% trichloroacetic acid (11). Radioactivity associated with MAAs was measured by specific immunoprecipitation using a double antibody-antigen binding assay. The assay was performed as described previously (9, 10). The anti-melanoma serum used was directed to common antigens expressed by a variety of allogeneic normal, fetal, and unrelated malignant allogeneic cells that differed quantitatively and/or qualitatively from surface antigens of human melanoma tumor cells (30). The anti-melanoma serum used was prepared by the repeated injection of partially purified MAAs into a New Zealand white rabbit. The MAA was partially purified by sequentially fractionating on phenylsepharose, Sepharose 6B, concanavalin A:Sepharose, and Sephadex G-150 the material shed into culture medium by viable human melanoma cells (30). The antiserum was directed to common antigens expressed by a variety of allogeneic normal, fetal, and unrelated malignant allogeneic cells. Briefly, the antiserum reacted strongly to surface antigens on 5 of 12 human melanoma cell lines. It reacted, but much more weakly, to neonatal fibroblast, embryonic lung tissue, one of 2 colon carcinomas, and baby hamster kidney cells. The antiserum did not react at all to 11 other normal or malignant allogeneic cells including neural tumors (astrocytoma, medulloblastoma, and neuroblastoma) and xenografts B16 melanoma cells. Specific binding was calculated by subtracting the cpm bound by an equal volume of normal rabbit serum. All proteins and MAAs were assayed in triplicate and the average values were used. Variation among replicate samples was 10%. Statistical evaluation was performed by Student's t tests.

RESULTS

Quantitation of MAAs. To determine the ability to quantitate soluble MAAs shed by melanoma cells by specific immunoprecipitation, replicate aliquots of anti-melanoma and normal rabbit serum were incubated with serial dilutions of labeled medium. The results of a representative experiment are illustrated in Chart 1. There was an essentially linear relationship between specifically bound radioactivity and the amount of labeled medium added, indicating that the assay provided a quantitative estimate of MAA concentration.

Degradation of Shed Surface Macromolecules and MAAs by Melanoma Cells. In earlier studies, we have shown that surface macromolecules and MAAs are rapidly released by human melanoma cells in culture. To study the fate of the shed material, labeled medium containing radiiodinated surface components shed by melanoma cells (HM29) in 3 hr was incubated for 24 hr with confluent monolayers of unlabeled melanoma cells. The radioactivity associated with labeled macromolecules and MAAs was measured and compared to that in labeled medium incubated without cells. The results of a number of experiments are summarized in Table 1. There was a marked reduction in radioactivity associated with acid-insoluble macromolecules and immunoreactive MAAs in shed material incubated with melanoma cells. On the average, approximately

![Chart 1: Assay of human MAA by double antibody-antigen binding assay. Relationship between increasing concentration of ¹²⁵I-MAA and specifically immunoprecipitated radioactivity. Increasing volumes of labeled medium containing shed radiodiornated MAAs were incubated with a fixed volume (11) of anti-melanoma or normal rabbit serum, and bound MAAs were immunoprecipitated with anti-immunoglobulin serum. Specific binding was calculated as described in "Materials and Methods."](chart1.png)

**Table 1**

<table>
<thead>
<tr>
<th>Degradation of shed MAAs by autologous melanoma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shed protein</td>
</tr>
<tr>
<td>MAAs</td>
</tr>
<tr>
<td>Membrane macromolecules</td>
</tr>
</tbody>
</table>

* Mean percentage of degradation per confluent plate of HM29 melanoma cells ± S.E.

# Statistically significant difference (p < 0.001) by Student's t test.
39% of the total radioactivity associated with labeled macromolecules and 11% of that associated with MAAs was lost in 24 hr. In addition, approximately 7 to 8% of macromolecule- and 5% of MAA-associated radioactivity was lost due to binding to cells and/or to the surface of the culture plate. By contrast, less than 5% of either macromolecule- or MAA-associated radioactivity was lost following 24 hr of incubation of labeled medium without cells. Loss in macromolecule-associated radioactivity was a result of degradation, since it was associated with a concomitant increase in acid-soluble radioactivity and with a downward shift in the molecular weight profile of the shed material (results not shown). On the other hand, loss in MAA-associated radioactivity cannot be ascribed unequivocally to degradation, since denaturation could also decrease immunoreactivity. MAAs were degraded or inactivated much more slowly than were unrelated surface macromolecules released concurrently by melanoma cells and the difference was statistically significant. Similar experiments were conducted with 3 other lines of melanoma cells (HM31, HM34, and SK-MEL-23). The results obtained with HM31 are presented in the following section. In all instances, melanoma cells degraded surface material which they released.

These results indicate that surface macromolecules, and to a lesser extent MAAs, released by melanoma cells can subsequently be degraded or inactivated by these cells.

**Degradation of MAAs by Nonmelanoma Cells.** Since the poor degradation or inactivation of MAAs by melanoma cells has important biological implications, several experiments were conducted to study its causes. Two possibilities were initially considered. The first was that the poor degradation resulted from the resistance of MAAs to catabolism; the second was that there was a defect in the ability of melanoma cells to handle this material. To differentiate between these possibilities, labeled material shed by HM31 melanoma cells in 3 hr was split into replicate aliquots and incubated for 24 hr with HM31 melanoma cells as well as with a variety of allogeneic cells including fresh normal allogeneic lymphocytes and macrophages and cultured fibroblasts and colon carcinoma cells and with control plates lacking cells. The results of several experiments are summarized in Table 2. Because of the varying number of cells used in different experiments, the results are expressed as the proportion of labeled macromolecules or MAAs in a standard amount of labeled medium degraded or inactivated per million cells per 24 hr. Both MAAs and unrelated melanoma surface macromolecules were degraded by all cells. However, the rate of this process varied widely. MAAs were degraded or inactivated most readily by fibroblasts and most poorly by melanoma. There was a 40-fold difference between these 2 types of cells. This was not simply a result of the poor catabolic activity of melanoma cells. Although their catabolic activity was low, as measured by their ability to degrade acid-insoluble macromolecules which they had released, it was still greater than that of lymphocytes and colon carcinoma cells which both degraded or inactivated MAAs more rapidly.

MAAs were particularly susceptible to degradation or inactivation. Following incubation with a variety of nonmelanoma cells, the radioactivity associated with MAAs was lost approximately twice as rapidly as that associated with unrelated acid-insoluble macromolecules released concurrently by melanoma (see Table 2, last column). However, because the material shed by melanoma cells is complex (12), the possibility that minor shed components are as susceptible to degradation as MAAs was not excluded. In contrast, the reverse occurred following incubation of shed material with melanoma cells. The radioactivity associated with MAAs decreased much more slowly than that associated with unrelated macromolecules.

These results indicate that the poor degradation or inactivation of MAAs by melanoma cells was not due to an inherent resistance of MAAs to catabolize but rather to a defect in the ability of melanoma cells to handle this material.

**Degradation of Surface Material Shed by Allogeneic Cells.** Because cell-cell recognition and adhesion is mediated by surface components, it is possible that cells may preferentially bind and consequently degrade their own surface macromolecules. Thus, we considered the possibility that melanoma cells, rather than having a defect in their ability to handle MAAs, had an increased ability to degrade normal macromolecules shed from their surface. To test this hypothesis, we performed cross-over experiments to compare the ability of melanoma (HM31) and normal allogeneic fibroblasts (HS-1) to degrade radioiodinated surface macromolecules released by each cell. The results are summarized in Table 3. As described earlier, fibroblasts were more active catabolically than melanoma cells. They degraded both their own and melanoma-shed macromolecules more rapidly. However, both melanoma and fibroblasts degraded their own and material shed from the other cells at similar rates. Thus, cells do not appear to have increased ability to degrade material shed from their own surface.

**DISCUSSION**

The principal finding of this study is that surface macromolecules, and to a lesser extent MAAs, shed by viable human melanoma cells can be degraded or inactivated by these cells following their release.

Surface components of tumor cells are involved in a variety of physiological events that relate directly to neoplastic transformation and tumor progression (23). The expression and

**Table 2**

<p>| Degradation of cell surface macromolecules and MAAs shed by human melanoma cells |
|---------------------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th><strong>Degradation</strong></th>
<th><strong>Macromolecules</strong></th>
<th><strong>MAAs</strong></th>
<th><strong>Degradation ratio</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>5</td>
<td>17.6 ± 3.3</td>
<td>51.0 ± 15.5</td>
</tr>
<tr>
<td>Macrophages</td>
<td>4</td>
<td>8.1 ± 2.2</td>
<td>19.5 ± 4.8</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>1</td>
<td>1.7</td>
<td>3.8</td>
</tr>
<tr>
<td>Melanoma</td>
<td>4</td>
<td>3.4 ± 0.6</td>
<td>1.2 ± 0.5</td>
</tr>
</tbody>
</table>

* Percentage of degradation of MAAs divided by percentage of degradation of macromolecules.

* Mean percentage of degradation per 10^6 cells per 24 hr ± S.E.

**Table 3**

<p>| Degradation of shed surface macromolecules by melanoma and fibroblasts |
|---------------------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th><strong>Degradation</strong></th>
<th><strong>Shed macromolecules</strong></th>
<th><strong>Degradation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>Melanoma</td>
<td>21.5</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Fibroblasts</td>
<td>25.2</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Fibroblasts</td>
<td>7.6</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Fibroblasts</td>
<td>7.4</td>
</tr>
</tbody>
</table>

* Percentage of degradation per 10^6 cells per 24 hr.

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turnover of these components may consequently play a key role in modulating the growth of cancer cells. While the composition of tumor cell membranes, and more recently the shedding of surface components, has been the object of considerable attention (3, 11, 15), the fate of surface material once released by tumor cells is obscure. However, we have observed previously that surface macromolecules shed by melanoma cells can be degraded by these cells following their release (8). This appears to be a general process since we have now found that all melanoma cells we studied could degrade surface material which they released. The current studies indicate that shed MAAs will also lose immunoreactivity following incubation with cells. It is not possible to directly ascribe the loss in immunoreactivity to degradation, since denaturation without degradation could also decrease immunoreactivity. However, the biological implications of either process are similar, since both lead to loss of immunoreactive antigen.

The melanoma cells we studied appeared to have a selective defect in their ability to degrade the MAAs they released. Though catabolically active, melanoma cells degraded or inactivated MAAs much more slowly than unrelated surface macromolecules which they released. This was not due to an inherent resistance of MAAs to degradation since MAAs were degraded by a variety of nonmelanoma cells 2 to 3 times more rapidly than were unrelated surface macromolecules shed concurrently by melanoma. Nor was this due to a preferential ability of melanoma cells to degrade nonantigenic macromolecules originating from their surface, since they degraded equally well surface macromolecules unrelated to MAAs shed by themselves or by normal allogeneic fibroblasts. As a result, MAAs were inactivated most poorly by the cells which had released them. There was a 40-fold difference in degradation or inactivation of MAAs between melanoma and fibroblasts.

The mechanism of MAA degradation or inactivation was not elucidated. In prior studies, we have shown that the degradation of shed surface macromolecules requires the presence of cells, is not due to enzymes in fetal calf serum, is temperature dependent, and is at least in part an active process requiring cell surface and metabolic activity (8). These observations suggest that MAAs may be degraded by a process similar to that by which most exogenous proteins are degraded by cells, namely endocytosis followed by degradation in lysosomes. Other mechanisms which could be involved include proteolysis by cell surface proteases (18, 20, 21, 26) or active proteases formed as a result of interactions between fetal calf serum components and cells.

These observations have several biological implications. It is evident that the amount of soluble tumor antigens that accumulates in body fluids will depend on the balance between the rate of their release and that of their degradation. The poor catabolic activity of melanoma cells coupled with their selective inactivity to inactivate some MAAs suggest that there may be a selective local accumulation of MAAs adjacent to these tumors. This could favor blocking and escape from immune defense mechanisms, an effect that might be all the more important for being operative immediately adjacent to the tumor. Our observations also broaden our understanding of the turnover of cell surface macromolecules. It is currently believed that the mechanisms involved in this process include shedding of surface material to the outside of cells, proteolysis of macromolecules still attached to cell membranes (28), dissociation from the membrane and reentry into the cytoplasm (28), and internalization of membrane macromolecules forming the wall of endocytic vesicles during pinocytosis (29, 33). Our findings suggest that, in addition, surface components can turn over by being degraded following their release by the cells which have released them. The autocatabolism of shed surface material is not a manifestation of malignant transformation since we observed that it also occurs with normal fibroblasts.

ACKNOWLEDGMENTS

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Degradation of Shed MAAs


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