Release of Surface Macromolecules by Human Melanoma and Normal Cells

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ABSTRACT

Viable human melanoma cells are known to rapidly release cell surface macromolecules and tumor-associated antigens. To study whether this is a phenomenon related to malignant transformation, the release of surface macromolecules by human and murine melanoma cells was compared to that by normal allogeneic cells. Following lactoperoxidase radioiodination of cell surfaces, it was found that the proportion of labeled macromolecules released by human melanoma cells in 3 hr (60.0 ± 9.9% (S.D.)) did not differ significantly from that released in the same time by normal allogeneic keratinocytes (56.0 ± 10.0%) or fibroblasts (42.1 ± 11.1%). Likewise in mice, the release of labeled surface macromolecules by B16 melanoma cells (36% in 3 hr) was no faster than that by normal syngeneic fibroblasts (56% in 3 hr). Control experiments excluded the possibilities that release was the result of cell death or of artifacts of the radioiodination procedure or that it represented release of fetal calf serum proteins adhering to the cells. These results suggest that rapid release of membrane macromolecules is not an expression of malignant transformation but rather a normal process which involves a major portion of macromolecules on the external surface of melanoma and normal cells and which occurs at a similar rate in both instances.

INTRODUCTION

Macromolecules, including tumor-associated antigens, are released by viable murine (2) and human (3, 13, 14, 21, 25, 27) melanoma cells. The release of cell surface macromolecules is particularly rapid. Following lactoperoxidase-catalyzed radiiodination of macromolecules on the surface of human melanoma cells, over 60% of the radioactivity associated with labeled macromolecules and over 40% of that associated with melanoma-associated antigens are released within 3 hr (3). This process contributes to the presence of soluble tumor antigens in body fluids (7), which in turn may stimulate or block host immune defense mechanisms. Thus, the factors that influence the release of material by malignant cells may have an important impact on tumor growth. Unfortunately, little is known about the mechanisms involved. However, it has been reported that in vitro antigen release by malignant cells may correlate with malignant potential (1, 11, 12) and that the ability of some tumors to metastasize may be related to the degree of glycoprotein dissociation from the plasma membrane (19), suggesting that the rapid release of surface macromolecules by melanoma cells may be a phenomenon associated with malignant transformation.

To examine this possibility, we have compared the release of surface macromolecules by human and murine malignant melanoma and normal cells. We did not find any consistent difference in the proportion of surface material which was released by malignant as opposed to normal cells.

MATERIALS AND METHODS

Cells. Human melanoma cells were obtained from surgically excised metastatic nodules, and neonatal human fibroblasts were obtained from circumcisions. The cells were established in tissue culture as described previously (6). All cells were maintained in complete growth medium consisting of Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% (v/v) FCS and penicillin (200 units/ml), streptomycin (200 µg/ml), and Fungizone (0.25 µg/ml) (Grand Island Biological Co., Grand Island, N. Y.). Cultures of confluent human keratinocytes were kindly provided by Dr. Howard Green, Massachusetts Institute of Technology, Boston, Mass. Murine B16 melanoma and syngeneic fibroblasts were established in culture as described previously (4). All experiments were performed on confluent monolayers of cells.

Radiolabeling of Cells. Surface macromolecules were radioiodinated by the lactoperoxidase technique. The cells were labeled in monolayers in 60-mm plastic dishes as described previously (6). Briefly, the cells were washed with HBSS and incubated with 0.5 mCi of sodium [125I]iodide (New England Nuclear, Boston, Mass.) in 0.1 ml of phosphate-buffered saline [0.1163 M NaCl, 0.010 M Na2HPO4, and 0.0032 M KH2PO4 (pH 7.4)], 0.250 mg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) in 0.250 ml of phosphate-buffered saline, and 0.02 ml of 0.3% hydrogen peroxide at 27°C. Additional aliquots of 0.3% hydrogen peroxide were added at 3-min intervals. After 10 min, the reaction was terminated by washing the cells 3 times with 5 ml of HBSS.

In some experiments, melanoma cells were internally labeled with [3H]leucine. Cells (2 × 106) were incubated in culture dishes with 5 ml of complete growth medium containing 0.5 mCi of [3H]leucine (30 to 50 Ci/mmol; New England Nuclear) at 37°C. After 48 hr, the medium was removed, and the cells were washed 3 times with 5 ml of HBSS and used as described below.

Release of Labeled Macromolecules. Labeled monolayers of cells washed 3 times with HBSS were incubated with 1 to 5 ml of complete growth medium in 100-mm plastic plates at 37°C. At intervals thereafter, the medium was removed, floating cells were sedimented by centrifugation, and the volume of the supernatant was adjusted with medium to correct for evaporation. Sedimented cells were transferred to those remaining in the dishes with 1 ml of 0.5% NP-40 (Shell Chemical Corp.,

1 The abbreviations used are: FCS, fetal calf serum; HBSS, Hank’s balanced salt solution; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate.
Measurement of Macromolecule-associated Radioactivity. The radioactivity associated with macromolecules was measured by precipitation with 10% trichloroacetic acid as described previously (4). All measurements were made on triplicate 0.02-ml aliquots, and the average value was used. In experiments involving [3H]leucine-labeled cells, Millipore filters (0.45-μm HA filter) containing trichloroacetic acid precipitates were dissolved by adding to glass scintillation vials containing 8 ml of Cocktail D (Beckman Instruments, Inc., Fullerton, Calif.) and 0.2 ml of methanol and counted in a SL-30 liquid scintillation counter (Intertechnique Instruments, Inc., Dover, N. J.). Acid-soluble radioactivity was taken as the difference between the total amount of acid-insoluble radioactivity in medium or lysate in a sample and that initially present at 0 time.

Assay for FCS Proteins. FCS proteins were measured by double antibody antigen binding assay. Our method of performing this assay has been published (5). Briefly, aliquots of medium to be tested for FCS proteins were incubated with an excess of rabbit anti-FCS serum for 30 min at 37°C. Goat anti-rabbit IgG was added to precipitate immune complexes, and incubation was continued at 37°C for 30 min and at 4°C for 18 hr. Precipitates were washed, and the amount of radioactivity specifically associated with FCS was calculated by subtracting from the cpm bound by anti-FCS serum the cpm bound by an equal volume of normal rabbit serum. All experiments were performed in triplicate, and the average values were used. In preliminary experiments, it was found that there was a linear relationship between the amount of radioactivity bound specifically with FCS and the amount of labeled FCS added, indicating that the results of the assay were quantitative. Over 95% of the radioactivity associated with chloramine T-radioiodinated FCS could be specifically precipitated by an excess of anti-FCS serum.

Gel Electrophoresis of 125I-labeled Macromolecules. The procedure of Cohen et al. (8) was used to make 5 to 15% polyacrylamide gradient slab gels containing SDS. A 2.5% spacer gel (1.5 cm) was formed on top of the gradient gel (24 x 30 cm). Samples were made to 0.1 M Tris-HCl (pH 8.0), 10% glycerol, 1% SDS, 0.01% bromophenol blue, and 1% 2-mercaptoethanol and heated for 3 min at 100°C. Electrophoresis was performed at room temperature for 16 to 18 hr at constant voltage (120 V) until the dye front had migrated 20 cm. Protein components were visualized with 0.1% Coomassie Blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) in a 30% methanol-7% acetic acid solution. Gels were dried under vacuum at 80°C on a Hoefer dryer (Hoefer Scientific Instruments, San Francisco, Calif.). Gels were placed against X-Omat R film (Eastman Kodak Co., Rochester, N. Y.) with a Lighting Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Films were developed in a Kodak automat.

RESULTS

Release of Surface Macromolecules by Melanoma Cells. The initial experiments were conducted to study the kinetics of release of cell surface macromolecules by viable human melanoma cells over 24 hr. Surface macromolecules on replicate plates of melanoma cells were radioiodinated by the lactoperoxidase technique, and the cells were incubated in fresh culture medium. At intervals thereafter, the amount of acid-insoluble radioactivity remaining on the cells and that accumulating in the medium was determined in duplicate dishes. The results of 4 experiments are summarized in Chart 1. Immediately after iodination, all of the acid-insoluble radioactivity was associated with the cells. During the ensuing 3 hr, there was a rapid loss of acid-insoluble radioactivity from cells associated with a concomitant increase in that present in the medium. On the average, 55% of the label was lost from cells in 3 hr. Over 80% of the label lost from cells could be recovered as acid-insoluble radioactivity in the medium, indicating that most of the loss resulted from release of labeled macromolecules into medium rather than from degradation. After 3 hr, acid-insoluble radioactivity on the cells continued to decline but at a slower rate, so that, after 24 hr, less than 30% of that initially present was left on the cells. The kinetics of release appeared to be biphasic with a half-life of approximately 1 hr for the first component and 32 hr for the second.

These results indicate that the bulk of macromolecules on the external surface of human melanoma cells is rapidly released and that most of the material is released within 3 hr.

Is Release of Surface Macromolecules an Artifact? The possibilities that release was an artifact of cell death or of the radiolabeling procedure or that it was due to release of FCS proteins adhering to the cells were considered but excluded by the following observations.

Cell death did not account for the rapid release of labeled surface material since, in separate experiments, it was found that cells released, in 3 hr, only 1.3% of macromolecules metabolically labeled with [3H]leucine which are predominantly of cytoplasmic origin whereas over 95% of the labeled macromolecules were released by lysing the cells with 0.5% NP-40. Furthermore, cell viability remained over 90% by trypan blue exclusion during all experiments.

Chart 1. Release of radioiodinated surface macromolecules by human melanoma cells. Replicate plates of cells were radioiodinated by the lactoperoxidase technique, washed, and incubated in fresh medium. At the indicated times, the medium was collected from duplicate plates, the cells were lysed in NP-40, and acid-insoluble radioactivity was measured in all samples. Results are expressed as the percentage of the initial acid-insoluble radioactivity present on cells at the onset of incubation. Each experiment was performed in duplicate. Time points, means of 4 experiments; bars, S.D.
The possibility that macromolecule release was secondary to their denaturation or to membrane damage during radioiodination was investigated by measuring directly by the method of Lowry et al. the amount of protein released by radioiodinated and untreated melanoma cells. For these studies, labeled and control cells were incubated in FCS-free growth medium so as to permit the measurements of the small quantities of protein released. In 2 experiments (see Table 1), the average amount of protein released per 10^6 radioiodinated melanoma cells in 3 hr (14.6 \mu g) was not significantly different from that released by untreated cells (15.5 \mu g) or by cells which had been mock-iodinated by substituting sodium iodide for \(^{125}\)I (11.7 \mu g) or exposed to \(^{125}\)I alone (14.3 \mu g). These results indicate that macromolecule release is not an artifact due to the procedure or to the reagents used for radioiodination.

Last, the possibility that the released macromolecules were FCS proteins that had adhered to or became incorporated in the cell membrane was examined by measuring the amount of radioactivity associated with FCS proteins in material shed by melanoma cells. In these experiments, release was conducted into FCS-free medium to facilitate the measurement of small amounts of FCS. In several experiments, it was found that 9 to 14\% of the radioactivity associated with shed macromolecules could be specifically immunoprecipitated by an excess of anti-FCS antibodies. These results indicate that FCS proteins can be released by cultured cells but that this process accounts for only a small fraction of the total amount of material released.

Release of Surface Macromolecules by Normal Cells. To determine whether the rapid release of surface macromolecules by melanoma was a phenomenon associated with malignant transformation, the release of surface macromolecules by normal human cells was studied.

In experiments similar to those described above, replicate confluent monolayers of normal human allogeneic fibroblasts or keratinocytes were radioiodinated, washed, and incubated in fresh medium. All experiments were done on duplicate plates in several separate experiments. It was found that the average amount of labeled surface macromolecules released by fibroblasts [42.12 \pm 11.1\% (S.D.)] or keratinocytes [56.0 \pm 10.0\%] did not differ significantly from that by melanoma cells [60.0 \pm 9.9\%] in the same time. In additional experiments, the rate of release of radioiodinated surface macromolecules by confluent monolayers of murine B16 melanoma cells (36\% in 3 hr) was less than that by normal syngeneic fibroblasts (56\% in 3 hr).

These results indicate that a large proportion of externally disposed surface macromolecules is rapidly released by both malignant and normal cells and that the rate of release is similar in both instances.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein released in 3 hr/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18</td>
</tr>
<tr>
<td>Radioiodinated(^a)</td>
<td>17</td>
</tr>
<tr>
<td>Mock-iodinated(^b)</td>
<td>12.4</td>
</tr>
<tr>
<td>(^{131})I-exposed(^c)</td>
<td>17.6</td>
</tr>
</tbody>
</table>

\(^{a}\) By the lactoperoxidase technique, 0.5 mCi \(^{131}\)I used for radioiodination.

\(^{b}\) By substituting cold for sodium \(^{131}\)Ijodide.

\(^{c}\) Exposed to 0.5 mCi of \(^{131}\)I.

Profile of Surface Macromolecules Released by Melanoma and Normal Cells. The profile of labeled macromolecules released by melanoma and normal cells was studied by polyacrylamide gel electrophoresis. Replicate confluent plates of melanoma and human fibroblasts were radioiodinated in monolayers as described previously, and the cells were either lysed immediately in 1.5 ml of NP-40 or incubated for 3 hr in 1.5 ml of FCS-free medium. Aliquots (0.05 ml) of cell lysate and spent medium from each cell line were electrophoresed in 1% SDS on 5 to 15% polyacrylamide slab gels. The results are illustrated in Fig. 1. A variety of macromolecules were labeled on the surface of both melanoma and fibroblasts. Apart from common components (M.W. 70,000 to 80,000) present in the medium of both cells, the material released by melanoma and fibroblasts appeared to be different. Release was selective, since only some of the labeled macromolecules on the surface of melanoma and fibroblasts were released. The macromolecules that were released appeared to be released at different rates since there was no correlation between the amount of radioactivity associated with different bands in the medium and that expressed on the cells. Very little of the labeled material on or shed by either cell line was present in the region corresponding to the molecular weight of fibronectin (M.W. 912).
While the very small amount of fibronectin on fibroblasts was unexpected, there are a number of reports that indicate that the amount of fibronectin expressed by fibroblasts is variable and may be markedly decreased (28, 29), particularly following prolonged culture (28). Thus, although fibronectin may be released by both melanoma and fibroblasts, it accounted for only a very small fraction of the total labeled material released in these experiments. A significant amount of released material had a molecular weight of 70,000 to 80,000, close to that of serum albumin. The nature of this material was not determined. Some of it could be FCS albumin. However, FCS albumin can account for only a small fraction of the total amount of material released. As described above, FCS proteins, of which albumin is the major component, account for only 9 to 14% of the radioactivity associated with shed material. Furthermore, the actual molecular weight of this band (M.W. 70,000 to 80,000) was actually somewhat greater than that of albumin.

These results indicate that a variety of different macromolecules is released by both melanoma and fibroblasts and that these are released at different rates.

DISCUSSION

The most important finding of this study is that melanoma and normal cells both rapidly release cell surface macromolecules and do so at similar rates.

The results confirm earlier observations that macromolecules on the surface of human melanoma cells are released very rapidly. In 3 hr, approximately 55% of the radioactivity associated with radioiodinated surface macromolecules on these cells was released into culture medium. This rate is very similar to the 60% release that we observed in earlier experiments (3). The present studies extend these observations and indicate that most of the surface macromolecules on melanoma cells which can be radioiodinated are eventually released. Approximately 70% of the radioactivity associated with labeled surface macromolecules was released within 24 hr. Release was biphasic with a half-life of approximately 1 hr for the first component and 32 hr for the second. Release was not the result of cell death since only 1.3% of [3H]leucine-labeled macromolecules, which are predominantly of cytoplasmic origin, was released in the same time and cells remained over 90% viable during the course of the experiments. Nor was release an artifact of the iodination process, since there was no difference in the rate at which mock-iodinated and untreated cells released proteins. Last, while it is known that FCS proteins can adhere to cultured cells (16, 17) and were observed in this study to be released, they accounted for only a small fraction (9 to 14%) of the total amount of material released by cells. However, the possibility that the rate of release of surface macromolecules by cultured cells differs from that which occurs in vivo cannot be excluded.

It is already known that normal cells can release a variety of individual membrane macromolecules (9, 10, 15, 18, 20, 24, 26), but the overall proportion of membrane material which is released and the rate at which this occurs is not clearly established. The present study indicates that approximately one-half of the radioactivity associated with labeled membrane macromolecules can be released by normal human fibroblasts or keratinocytes within 3 hr. Thus, a large proportion of externally disposed surface macromolecules can be released rapidly by normal cells.

While the mechanisms of release of surface material by malignant cells are not presently known, it has been suggested that this process occurs much more rapidly in malignant cells. It has been suggested that there is a relationship between the lability of cell surface components as a whole and the capacity of tumors to metastasize. Thus, the rate of release of histocompatibility antigens by murine lymphoma cells (12) and of tumor-specific transplantation antigens by rat fibrosarcoma (11) has been reported to be much higher in metastatic variants of these cells. Furthermore, the glycocalyx of metastatic mammary carcinomas in rats has been reported to dissociate more easily than that on nonmetastatic variants of the same tumor (19). In contrast, we have not observed any gross differences in either the proportion or rate at which surface macromolecules are released by metastatic human melanoma, normal human fibroblasts, or keratinocytes in tissue culture. In 3 hr, the proportions of labeled surface macromolecules released by these cells were 60, 56, and 42%, respectively, rates which do not differ significantly statistically. Nor was the release of surface macromolecules by murine B16 melanoma cells any faster than that by syngeneic fibroblasts. Thus, our experiments suggest that malignant transformation is not associated with increased release of surface material. However, it must be noted that we did not study release by normal, transformed, or “supermalignant” variants of the same cell which would provide a more ideal comparison.

Polyacrylamide gel electrophoresis of the labeled macromolecules present on the surface of the cells and of those shed into the medium indicate that a variety of surface macromolecules was released by both melanoma and normal fibroblasts. Examination of the gels indicates that there are obvious differences in the rates at which different surface macromolecules are released. However, tumor antigens do not appear to be particularly prone to release since, in prior experiments, we have found that human melanoma-associated antigens are released consistently more slowly than are unrelated macromolecules released concurrently by the same cells (3). Relatively little of the shed material was present in regions corresponding to the molecular weight of fibronectin (M.W. 220,000), so that even though this protein may be released by cultured melanoma cells (22) it does not account for the bulk of the labeled macromolecules released by either normal or melanoma cells. The proportion of the shed macromolecules which was structural, peripheral, or in transit components of the cell membrane was not determined.

In conclusion, the result of our present observations indicates that release of surface proteins is a phenomenon which involves a major portion of macromolecules expressed on the external surface of normal and melanoma cells. They do not support the concept that macromolecules on the external surface of metastatic malignant cells are in general more labile than are those of normal cells. Rather, they suggest that release of surface material is one of the normal pathways that accounts for the turnover of cell membrane components on normal and malignant cells and that the overall rate of this process is similar in both instances.

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