Production of Interleukin 1 Activity by Cultured Human Melanoma Cells

Jeanette L. Bennicelli,2 Jack Elias, Jeffrey Kern,3 and DuPont Guerry IV

ABSTRACT

A panel of melanoma cell lines derived from 7 primary and 20 metastatic lesions was tested for the production of interleukin 1 (IL-1) in standard mouse thymocyte costimulation assays. Constitutively produced IL-1 activity was found in the conditioned media of 4 of 7 primary and 5 of 20 metastatic melanoma cell lines tested. Four of 9 cell lines secreting IL-1 were also shown to contain cell-associated activity in their lysates. Melanoma-conditioned media were, however, unable to support the growth of CTLs, an interleukin 2-dependent cell line. The secreted IL-1 activity was significantly inhibited by antibodies to recombinant IL-1α (3 of 3 lines), but not antibody to recombinant IL-1β. When conditioned medium from one cell line was fractionated on a Superose 12 column by fast protein liquid chromatography, a major peak of activity eluted at M, 22,500–27,500. The presence of 2.2-kilobase mRNA hybridizing a probe for IL-1α and 1.6-kilobase mRNA hybridizing a probe for IL-1β was detected by Northern blot in 3 of 4 secreting cell lines but not in a nonsecreting line. Taken together, these results suggest that cultured melanoma cells produce the cytokine IL-1α, although the relationship between melanoma IL-1 and monocyte IL-1 is unclear. The production of IL-1 by melanoma cells is of interest because of its potential roles in the biology of melanoma through direct effects on tumor growth or through indirect effects on adjacent stromal and endothelial cells and infiltrating lymphoid cells.

INTRODUCTION

Melanoma cells in culture have been reported to produce a variety of growth factors and cytokines including platelet-derived growth factor (1), M, 100,000 protein (2), transforming growth factor α and β (3), and melanoma growth-stimulating activity (4). These factors have potential roles in the biology of the disease through direct effects on tumor growth or through effects on adjacent stromal cells and infiltrating lymphoid cells. Reports of the production of IL-1 by gliomas (5), glioblastomas (6), and hepatomas (7) prompted us to investigate the possibility that production of this cytokine may influence tumor progression in melanoma. Therefore, we screened a panel of 27 melanoma cell lines derived from various lesional steps of tumor progression for the production of IL-1 and found activity in cell lines of late stage primary and metastatic disease. Although originally described as a product of monocytes (8), IL-1 activity has been shown to be produced by diverse cell types including normal (8-11) and transformed (12-14) lymphoid cells, Langerhans cells (15), endothelial cells (16), mesangial cells (17), astrocytes (5), fibroblasts (18), thymic epithelial cells (19), keratinocytes (20), and some nonlymphoid malignant cells (5–7). The structural relationship between monocyte IL-1 and the IL-1 activities produced by other cell types is unclear. Monocyte IL-1 activity is itself composed of at least 2 moieties. The predominant monocyctic species share a common molecular weight of 18,000 but vary in their isoelectric points which are approximately 5.0 and 7.0 (21, 22); however, there is evidence for additional charge heterogeneity (22). cDNA cloning experiments have revealed the existence of at least two genes encoding IL-1α (23) and IL-1β (23, 24), the p5.0 and p7.0 species, respectively. Both IL-1α and IL-1β have been shown to bind to a single class of receptors (25) and appear to mediate similar effects on target cells (reviewed in Ref. 26). Several studies (12, 27) have also suggested the existence of IL-1 activities which may be distinct from monocyte-derived IL-1α and IL-1β.

Here we report the production of an IL-1 activity by human melanoma cells in culture which can be neutralized by antibodies to recombinant human IL-1α. By gel filtration chromatography, the activity from one cell line has an apparent molecular weight of 22,500–27,500. Cell lines which produce the activity contain RNA which hybridizes probes for both IL-1α and IL-1β.

MATERIALS AND METHODS

Cells. A panel of 27 melanoma cell lines representing various stages of tumor progression was maintained by weekly passage of monolayer cultures grown in T25 flasks (Corning Glass Works, Corning, NY) in a mixture of 3 parts Eagle’s minimum essential medium (Gibco Laboratories, Grand Island, NY) and 1 part Leibovitz’s L-15 medium (Gibco) supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA) and 2 mM glutamine. Primary melanoma cell lines (WM35, WM39, WM75, WM98-2, WM278, ML853-2, and WM983-A) and metastatic melanoma cell lines (WM9, WM46, WM164, WM266-4, WM373, WM983-B, WM983-C, WM984, and WM1005) were provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA. The metastatic melanoma cell lines CC MEL1154, CC MEL1158, CC MEL1167, CC MEL1168, CC MEL1180-1, CC MEL1193-C, CC MEL1230, and CC MEL7315 were established in our laboratory, as described previously (28). Each of these cell lines was established from surgical specimens obtained from the Pigmented Lesion Clinic, Hospital of the University of Pennsylvania, Philadelphia, PA. The metastatic melanoma cell lines SK MEL21, SK MEL23, and SK MEL37 were provided by K. O. Lloyd, Memorial Sloan Kettering Cancer Center, New York, NY.

Serum-free Conditioned Media and Cell Lysates. For preparation of conditioned media, monolayers of melanoma cells were grown for 4 days after passage until they were 70–90% confluent. The medium was then aspirated, the monolayer was rinsed 3 times with serum-free Eagle’s medium, and cells were scraped free, transferred to test tubes, and sonicated (Heat Systems; Ultrasonics, Plainview, NY) on ice for 2 min at 50% duty cycle on output level 3. The lysates were then prepared and washed as described by the Biomedical Research Support Grant Program, Division of Research Resources, NIH.

Received 7/5/88; revised 11/4/88; accepted 11/10/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was presented in part at the American Association for Cancer Research Meeting, Atlanta, GA, May 1987 (47). This work was supported by Grants CA92500 and B.R.S.G. S07.BR.05415.25 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, NIH.

2 To whom requests for reprints should be addressed, at Cancer Center, 7 Silverstein Pavilion, Hospital of the University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104.

3 Recipient of Physician Scientist Grant 1-K22-HL-01573-01A1 from the National Heart, Lung and Blood Institute.

The abbreviations used are: IL-1, interleukin 1; IL-2, interleukin 2; PHA-M, phytohemagglutinin M; SDS, sodium dodecyl sulfate; Con A, concanavalin A; cDNA, complementary DNA; DMEM, Dulbecco’s minimum essential medium.
filtered through 0.22-µm Millex GS filters (Millipore Corporation, Bedford, MA), aliquoted, and frozen at −70°C.

Bioassay for Interleukin 1. Mouse thymocyte costimulation assays were performed using a modification of techniques described by Mizel (29) with recombinant human IL-1α (Hoffmann LaRoche, Nutley, NJ) as the positive control. Briefly, 5 × 10^4 C3H/HeJ or IL-1β at 2 units/ml or 50 µl of serum-free, melanoma-conditioned medium was filtered through 0.45-µm Nalgene filters (Nalge Company, Rochester, NY) and harvested on glass fiber filter papers with an automatic microharvester (Brandel, Gaithersburg, MD) 16 h thereafter. The filters were dried, placed in vials with 2 ml scintillation fluid, and counted in a scintillation counter (Searle Radiographies, Des Plaines, IL).

For neutralization assays, three antibodies were used: (a) an IgG1 monoclonal antibody to recombinant, human IL-1β (Cistron Biotechnologies, Pine Brook, NJ); (b) an IgG1 monoclonal antibody to yeast-derived, human recombinant IL-1α (Olympus Corporation, Lake Success, NY); and (c) a rabbit polyclonal antibody to recombinant human IL-1α which was the generous gift of Dr. Phillip Simon (SKF Laboratories, King of Prussia, PA). In each assay, 50 µl of recombinant IL-1α or IL-1β at 2 units/ml or 50 µl of serum-free, melanoma-conditioned medium were preincubated for 3 h at 37°C in microtiter wells with 50 µl of various dilutions of each antibody. Afterwards, 5 × 10^4 C3H/HeJ thymocytes were added per well, and the thymocyte costimulation assay was completed as described above. The percentage of inhibition was calculated as

\[
\frac{1 - \frac{X_{\text{no Ab}}}{X_{\text{no Ab}}}}{X_{\text{no Ab}}} \times 100\%\]

where \(X_{\text{no Ab}}\) is (mean cpm of triplicate wells with Con A + test sample preincubated with medium) – (mean cpm of triplicate wells with Con A alone); and \(X_{\text{Ab}}\) is (mean cpm of triplicate wells with Con A + test sample preincubated with antibody) – (mean cpm of triplicate wells with Con A alone). Incubation of thymocytes with Con A alone gave a suboptimal level of proliferation in all experiments.

Gel Filtration Chromatography. A 2-liter batch of serum-free conditioned medium was prepared as described above from a metastatic melanoma cell line (CC MELI154) grown in a multiwell unit (Nunc, Kamstrup, Denmark). The conditioned medium was concentrated 12-fold by positive pressure filtration in a stirred Amicon cell with a YM10 membrane (Amicon, Danvers, MA). The concentrated medium was loaded onto a 1 × 30-cm Superose 12 column (Pharmacia, Piscataway, NJ) in sample buffer (6% formaldehyde-50% formamide-20 mM Tris, pH 7.4) at a flow rate of 0.7 ml/min. The concentrated, conditioned media tested had any activity.

RESULTS

Production of IL-1 Activity by Melanoma Cells

Northern Blot Analysis. Following the culture of melanoma cells as described, total cellular RNA was isolated by a guanidinium isothiocyanate method (31) with cesium chloride centrifugation (32). Equal amounts of RNA, determined by A_{260} nm, were heat denatured at 68°C for 15 min in sample buffer (6% formaldehyde-50% formamide-20 mM morpholinosulfopropylsulfonic acid-5 mM sodium acetate-1 mM EDTA, pH 7). Samples (20 µg/lane) were loaded and size fractionated by electrophoresis in 1% agarose gels containing 6% formaldehyde and 2 µg/dl ethidium bromide. The gels were visualized by UV illumination to determine the positions of the 18S and 28S rRNA bands, to assess the integrity of the RNA, and to verify that equal amounts had been loaded in all lanes. The RNA was transferred to nylon membranes (Gene Screen Plus; New England Nuclear) by capillary blotting for 24 h, and the membranes were dried, baked at 80°C in a vacuum, and prehybridized for 16–25 h at 55°C in prehybridization fluid (10 mM EDTA-0.5% SDS-5 × Denhardt’s solution (0.02% Ficoll-0.02% polyvinylpyrrolidone-0.02% bovine serum albumin-1% SDS-1 mM NaCl-5 mM Tris, pH 7.4]). The membranes were then hybridized for 24 h at 55°C in 1 M NaCl-7 mM EDTA-0.1 M Tris-HCl (pH 8)-5 × Denhardt’s solution-0.1% SDS and 250 µg/ml denatured trNA containing 2 ng/ml of heat denatured, ^32P-labeled synthetic IL-1β oligonucleotide (24). The blots were washed for 10 min at 25°C followed by 1 min at 55°C in 1 mM NaCl-7 mM EDTA-0.1 M Tris HCl (pH 8)-0.5% SDS. This was repeated 4 times, and the membranes were then exposed to Kodak XAR film with Cronex tungstate intensifying screens (DuPont) at −70°C for 2 weeks. The blots were stripped of radiolabeled probe by incubation for 60–90 min at 85°C in 0.2% SDS-10 mM Tris and were then rehybridized with a cDNA probe for IL-1α (33) according to the methods described above.

Bioassay for Interleukin 2. IL-2 activity was assessed by a standard CTLL bioassay (30), using recombinant IL-2 (alanine analogue; Amgen Biologicals, Thousand Oaks, CA) as the positive control. Serial 2-fold dilutions of the control or test samples were prepared in 96-well plates (Corning) using RPMI 1640 (Gibco) as the diluent. Washed CTLL cells (4 × 10^4 per well) were added in 50 µl RPMI containing 10% fetal bovine serum. The cultures were incubated at 37°C in 5% CO2 in air, pulsed with 1 µCi [3H]thymidine (New England Nuclear) per well for 24 h, and harvested 16 h later on glass fiber filter papers using an automatic microharvester (Brandel). The filters were dried, placed in vials with 2 ml scintillation fluid, and counted in a scintillation counter (Searle Radiographies, Des Plaines, IL).

RESULTS

Secretion of IL-1 Activity. Serum-free conditioned media from a panel of 7 primary melanoma cell lines and 20 metastatic melanoma cell lines were tested in the mouse thymocyte costimulation assay for the production of interleukin 1 activity. The results of a representative experiment are shown in Fig. 1. Whereas thymocytes grown in medium alone showed no appreciable proliferation, those grown in medium containing PHA-M proliferated suboptimally, and those grown in medium containing PHA-M and recombinant IL-1α proliferated optimally. The near maximal stimulatory effect of IL-1 was achieved at 1 unit/ml in the presence of 50 µg/ml PHA-M. As shown, IL-1-like activity was found in the conditioned media of 5 melanoma cell lines, including 2 late stage primary melanomas (WM278 and WM983-A) and 3 metastatic melanomas (WM9, CC MELI154, and CC MELI180-1).

On testing in 4 experiments, 2 cell lines from the panel were found to be positive for secretion of IL-1 activity in all assays (WM278 and CC MELI154); whereas WM9 and CC MELI1158 were positive in 3 of 4 assays; WM1065 was positive in 2 of 4 assays; and WM35, WM39, WM983-A, and CC MELI180-1 were positive in 1 of 4 assays (data not shown).

Since IL-2 can cause the proliferation of mouse thymocytes, the melanoma cell-conditioned media were tested by CTLL bioassay (30). As shown in Fig. 2, recombinant IL-2 was able to support the growth of CTLL, an IL-2-dependent cell line, in a dose-dependent fashion; whereas none of the melanoma conditioned media tested had any activity.

Cell-associated IL-1 Activity. Since IL-1 is known to exist in both a secreted M, 18,000 form and a cell-associated, higher molecular weight form (22), the presence of cell-associated activity was assessed in the thymocyte assay using cell lysates prepared by freeze-thaw and sonication. The results of a representative experiment are shown in Fig. 3. Three cell lines were positive for the presence of cell-associated IL-1, including the late stage primary melanoma cell line WM278 and the
PRODUCTION OF IL-1 ACTIVITY BY MELANOMA CELLS

Fig. 1. Thymocyte assay for interleukin 1 in melanoma cell-conditioned media. Melanoma-conditioned media (100 μl) or 100 μl of various concentrations of recombinant IL-1α were incubated with 5 x 10^6 mouse thymocytes in a final volume of 200 μl of DMEM containing 10% fetal bovine serum, 2 mM glutamine, gentamicin, 2.5 x 10^{-4} M 2-mercaptoethanol, and 50 μg/ml PHA-M. The cultures were pulsed with [3H]thymidine on day 2 and harvested on day 3. The results are shown as mean cpm of triplicate wells ± SEM (bars) and include background cpm caused by suboptimal stimulation with PHA-M alone.

metastatic melanoma cell lines CC MEL 1154 and CC MEL 1158. In additional experiments (data not shown), WM9, a metastatic melanoma cell line, was also shown to possess cell-associated IL-1. Although all the cell lines shown to possess cell-associated activity also secreted IL-1 (see Fig. 1), the converse situation was not always observed.

Characterization of Melanoma-derived IL-1 Activity. Mouse thymocyte-costimulating activity in melanoma-conditioned media can be inhibited by rabbit polyclonal antibody to recombinant, human IL-1α. As shown in Fig. 4, a 1:2000 dilution of antibody was able to neutralize the activity of 1 unit/ml recombinant IL-1α by 51%; whereas the activity of 1 unit/ml of recombinant IL-1β was not neutralized. The activities of the three melanoma-conditioned media tested (WM278, CC MEL 1154, and CC MEL 1158) were also inhibited by polyclonal antibody to IL-1α (67, 72, and 87%, respectively).

Monoclonal antibodies to both recombinant IL-1α and IL-1β were also used in neutralization studies. As shown in Table 1, monoclonal antibody to recombinant IL-1α inhibited the activity of 1 unit/ml recombinant IL-1α in a dose-dependent manner, with 47, 40, 25, and 21% neutralization at dilutions of 1:100, 1:200, 1:400, and 1:800, respectively. The activity of recombinant IL-1β was inhibited to a small extent, but this effect did not appear to be dose dependent. The mouse thymocyte-costimulating activity in all three melanoma-conditioned media tested was inhibited by monoclonal antibody to IL-1α in a dose-dependent manner. For example, at a 1:100 dilution of antibody, the activities of WM278, CC MEL 1154, and CC MEL 1158 were inhibited by 96, 57, and 82%, respectively; and at a 1:800 dilution of antibody, they were inhibited by 52, 24, and 46%, respectively.

Results of neutralization studies using monoclonal antibody against recombinant IL-1β are shown in Table 2. A 1:50 dilution of antibody neutralized the activity of 1 unit/ml of recombinant IL-1β by 43% but had no appreciable effect on the activity of 1 unit/ml of IL-1α. As further shown in Table 2, mouse thymocyte-costimulating activity in the melanoma-conditioned media was not significantly neutralized by monoclonal antibody to IL-1β. None of the three antibodies tested significantly inhibited the stimulation of mouse thymocytes caused by IL-2 (data not shown).

To determine the molecular weight of the IL-1 activity secreted by cultured melanoma cells, conditioned medium was prepared from cell line CC MEL 1154, concentrated by Amicon filtration, and fractionated by gel filtration on a Superose F12 column by fast protein liquid chromatography (Fig. 5). When the fractions were tested in the thymocyte assay, IL-1 activity eluted in fractions 16 through 21, with a major peak of activity in fractions 17 and 18 (corresponding to a molecular weight of 22,500 to 27,500) and a minor peak in fraction 21 (corresponding to a molecular weight of 13,000). There was no peak of activity at M, 18,000, the generally accepted molecular weight for secreted monocyte IL-1.

Northern Blot Analysis. When total cellular RNA from cul-
PRODUCTION OF IL-1 ACTIVITY BY MELANOMA CELLS

Fig. 3. Thymocyte assay for interleukin 1 in melanoma cell lysates. Melanoma cell lysate (100 µl) or 100 µl of various concentrations of recombinant IL-1α were incubated with 5 × 10^5 mouse thymocytes in a final volume of 200 µl of DMEM containing 10% fetal bovine serum, 2 mM glutamine, gentamicin, 2.5 × 10^{-5} M 2-mercaptoethanol, and 50 µg/ml PHA-M. The cultures were pulsed with [³H]thymidine on day 2 and harvested on day 3. The results are shown as mean cpm of triplicate wells ± SEM (bars) and include background cpm caused by suboptimal stimulation with PHA-M alone.

Table 1 Neutralization of IL-1 activity by monoclonal antibody to recombinant IL-1α

<table>
<thead>
<tr>
<th>Antibody dilution</th>
<th>IL-1α</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM278</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
<tr>
<td>1:100</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
<tr>
<td>1:200</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
<tr>
<td>1:400</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
<tr>
<td>1:800</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
</tbody>
</table>

Monoclonal antibody specific for IL-1α.

Table 2 Neutralization of IL-1 activity by monoclonal antibody to recombinant IL-1β

<table>
<thead>
<tr>
<th>Antibody dilution</th>
<th>IL-1α</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM278</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
<tr>
<td>1:50</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
<tr>
<td>1:100</td>
<td>531 ± 10.2</td>
<td>30.2 ± 2.3</td>
</tr>
</tbody>
</table>

Monoclonal antibody specific for IL-1β.

Fig. 4. Neutralization of IL-1 activity by polyclonal antibody to recombinant IL-1α. Recombinant IL-1α (50 µl) or recombinant IL-1β (50 µl) at 2 units/ml or 50 µl of melanoma-conditioned medium were combined with 50 µl of medium or antibody at dilutions of 1:25 and 1:50 and were incubated for 3 h at 35°C. Thymocytes (5 × 10^5/well) were added to a final volume of 200 µl containing 10% fetal bovine serum, 2 mM glutamine, gentamicin, 2.5 × 10^{-5} M 2-mercaptoethanol, and 50 µg/ml Con A. The cultures were pulsed with [³H]thymidine on day 2 and harvested on day 3. The results shown are mean cpm of triplicate wells ± SEM (bars) and include background cpm caused by suboptimal stimulation with PHA-M alone.

Fig. 5. Gel filtration chromatography of melanoma-conditioned medium. Conditioned medium of metastatic melanoma cell line CC MELI 154 was fractionated by fast protein liquid chromatography on a Superose 12 column and thirty 1-ml fractions were collected (•••). The column fractions were tested in a mouse thymocyte assay for IL-1 activity, and the results (•••) are shown as the mean cpm of triplicate wells ± SEM (bars) and include background cpm caused by suboptimal stimulation with PHA-M alone.

Fig. 6. mRNA hybridizing probes for both IL-1α and IL-1β was detected in 3 of 4 cell lines which secrete IL-1 activity, but not in a nonsecreting line (Fig. 6). mRNA for IL-1β was identified with an oligonucleotide probe which corresponds to bases 669-689 of IL-1β and which hybridizes to a 1.6-kilobase transcript in lipopolysaccharide-stimulated macrophages (24). In Northern blot analysis of melanoma cell RNA (Fig. 6, right), a 1.6-kilobase band was seen just below the 18S rRNA band in cell
The role of melanoma-derived IL-1 in the biology of the disease is presently speculative and warrants further investigation. Given the function of monocyte-derived IL-1 in T-cell activation (reviewed in Ref. 26), one could hypothesize that melanoma-derived IL-1 is important in the immune response to melanoma. Histologically, lymphocytic infiltrates are seen in precursor lesions and biologically early melanomas, but rarely in metastatic melanomas (37). In an in vitro model, Guerry et al. (38) have demonstrated an autologous lymphocytic response to biologically early melanoma but not to metastatic melanoma. In contrast to these findings, however, we have been able to detect IL-1 activity in both primary (57%) and metastatic (25%) melanoma cell lines, arguing against a role for this factor in selectively recruiting lymphocytes into biologically early lesions.

It is also possible that melanoma-derived IL-1 may play a role in tumor growth, either directly via an autocrine mechanism or indirectly via effects on adjacent stromal or endothelial cells. Melanoma cells in culture have been reported to produce a variety of growth factors including platelet-derived growth factor (1), M, 100,000 protein (2), transforming growth factors α and β (3), and melanoma growth-stimulating activity (4) in addition to the IL-1 activity reported in this study. IL-1 is known to promote the proliferation of a variety of cell types including lymphocytes (39), epithelial cells (40), endothelial cells (41), fibroblasts (42), and astrocytoma cells (43). It is also produced by a number of these same cell types (5-20), suggesting a positive role in autocrine growth regulation as described by Scala et al. (44) for an Epstein-Barr virus-transformed human B cell line. It is tempting to speculate that IL-1 produced by melanoma cells may also function in this manner. Although Lachman et al. (45) and Onozaki et al. (46) have reported that exogenously added IL-1 is cytotoxic for melanoma cells, we have found that the addition of exogenous, recombinant IL-1α or IL-1β at 250 pg/ml is stimulatory for the growth of cultured, human melanoma cells. Therefore, the potential role of IL-1 in autocrine growth regulation of melanoma cells bears further investigation.

ACKNOWLEDGMENTS

We would like to thank Kelvin Gustilo for excellent technical advice and assistance.

REFERENCES


Unpublished data.


Production of Interleukin 1 Activity by Cultured Human Melanoma Cells
