Interleukin 6: A fibroblast-derived growth inhibitor of human melanoma cells from early but not advanced stages of tumor progression (metastasis/multicytokine resistance)

Chao Lu*, Mark F. Vickers*, and Robert S. Kerbel**††

*Division of Cancer Research, Reichmann Research Building, Sunnybrook Health Science Centre, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada; and †Departments of Medical Biophysics and Molecular and Medical Genetics, and Institute for Medical Science, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

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ABSTRACT Recently we reported that human dermal fibroblasts, orconditioned media obtained from such cells, affect the growth of human melanoma cells as a direct function of tumor progression: melanoma cells obtained from early-stage (metastatically incompetent) primary lesions were growth inhibited, whereas cells obtained from more advanced (metastatically competent) primary lesions, or metastases, were growth stimulated. Ion-exchange and gel-filtration chromatography of fibroblast conditioned medium revealed the inhibitor to be a protein of molecular mass between 20 and 30 kDa and distinct from the stimulator. This is the approximate molecular mass of interleukin 6 (IL-6), a ubiquitous multifunctional cytokine known to affect in particular many kinds of hemopoietic and lymphoid cells. Since this cytokine is known to be made by fibroblasts, we attempted to determine if the human fibroblast-derived growth inhibitor (hFDGI) was identical to IL-6. Neutralizing antibodies specific for IL-6 completely eliminated the inhibitory activity of hFDGI. Moreover, exposure to human recombinant IL-6 was found to inhibit the growth of early-stage melanoma cells obtained from radial growth phase (RGP) or early vertical growth phase (VGP) primary lesions in three of four cases. In contrast, melanoma cells from a number of more advanced VGP primary lesions, or from distant metastases, were completely resistant to this IL-6-mediated growth inhibition. Acquisition of an "IL-6-resistant" phenotype by metastatically competent melanoma cell variants may provide such cells with a proliferative advantage within the dermal mesenchyme (a hallmark of melanoma cells that are malignant), helping them eventually to dominate advanced primary lesions and to establish secondary growths elsewhere.

The growth and spread of cancers can be strongly influenced by surrounding normal tissues in a variety of ways. Tumor angiogenesis—i.e., the process satisfying the absolute requirement of new blood vessel capillaries for solid tumors to grow beyond 1–2 mm in diameter—is perhaps the most striking illustration of this interaction (1). Similarly, a variety of different hormones or locally produced growth factors and cytokines secreted by various normal cells can stimulate tumor growth (2, 3). In addition to stimulating the growth of tumors, surrounding normal cells can, in other circumstances, significantly suppress such growth (4); this is especially evident in the case of an interspersed minority subpopulation of tumor cells surrounded by an excess of normal cells, such as fibroblasts (5).

With few exceptions the effects of a given normal cell population on the growth and behavior of a particular type of tumor have not been studied in the context of different stages of tumor progression. It is possible, for example, that the effect of normal adjacent cells on the growth of tumor cells from primary lesions early in tumor progression is quite different from the effects of more advanced (metastatically competent) primary lesions, or metastases. We recently uncovered an interesting example of this in the context of human malignant melanoma (6). This type of cancer is one of the few where lesional steps in tumor progression are well defined and readily detectable (7–10). Thus, most primary melanomas arise in the epidermis as horizontal plaque-like lesions—the so-called "radial growth phase" (RGP) primary melanoma (7). Despite invasion into the dermis, these melanomas are always curable by surgery and are thought to be composed of poorly proliferating tumor cells incompetent for metastasis (7). The next stage is the vertical growth phase (VGP), in which the cells can grow as an expandable nodule in the dermal mesenchyme (7, 8). This step initially encompasses lesions with little competence for metastasis (usually those with a thickness <0.76 mm, a low mitotic count, and a brisk lymphocytic infiltration) (11) and, subsequently, those likely to acquire metastatic competence (thick lesions with a high mitotic count and an absence of lymphocytic infiltration (11). The latter lesions generally have phenotypic markers associated with metastatic melanoma cells—e.g., up-regulation of intracellular adhesion molecule-1 and the B3 integrin subunit (12–14). The final stage of melanoma progression is distant metastasis formation. We found that coculture of human melanoma cell lines with irradiated human dermal fibroblasts affected the growth of the melanoma cells as a direct function of tumor progression: cell lines derived from RGP or early-stage VGP primary tumors were growth inhibited, whereas a majority of cell lines from more advanced VGP primary or metastatic tumors were growth stimulated under the same conditions (6). These results could be reproduced with fibroblast cell culture conditioned media, thus implicated the involvement of one or more secreted paracrine growth factors (6).

From these results we speculated that a single growth factor may be involved that behaves as an inhibitor for early-stage melanoma cells but as a mitogenic agent for more advanced-stage melanoma cells. Alternatively, two different factors may be involved—one being an inhibitor, active only on early-stage melanoma cells, and the other being a stimulator, active only on the more advanced-stage melanoma

Abbreviations: RGP, radial growth phase; VGP, vertical growth phase; TGF-α, transforming growth factor α; IL, interleukin; hFDGI, human fibroblast-derived growth inhibitor; CM, conditioned medium; TNFα, tumor necrosis factor α.

**To whom reprint requests should be addressed: Division of Cancer Research, Reichmann Research Building, Sunnybrook Health Science Centre, 2075 Bayview Avenue, Toronto, Ontario M4N 3M5, Canada.

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cells. Either possibility would provide metastatically competent melanoma cells with a relative growth advantage. In preliminary experiments we were unable to suppress the inhibitory or stimulatory influences of dermal fibroblasts on melanoma cell proliferation by addition of neutralizing antibodies to a number of known growth factors thought to be involved in the growth of solid tumors. These included transforming growth factor β (TGF-β), interferon α/β and interferon γ, basic fibroblast growth factor (bFGF), platelet-derived growth factor, insulin-like growth factor-1, epidermal growth factor, and nerve growth factor (I. Cornil and R.S.K., unpublished observations). We therefore decided to identify the growth factor(s) involved by means of biochemical purification.

The purpose of the present report is to summarize our findings; they strongly implicate interleukin (IL)-6, a "hematopoietic" multifunctional cytokine, as the fibroblast-derived growth inhibitor for early-stage melanoma cells. This raises the intriguing possibility that IL-6 could be a potential growth regulator of other types of nonhematopoietic human solid tumor, depending upon stage of disease progression.

MATERIALS AND METHODS

Partial Purification of Human Fibroblast-Derived Growth Inhibitor (hFDGI) for Early-Stage Melanoma Cells. A three-step purification procedure was employed involving Q-Sepharose anion-exchange chromatography, SP cation-exchange chromatography, and gel-filtration chromatography, using a Waters 650E HPLC Protein Purification System.

Human neonatal foreskin fibroblasts (5–20 passages) were cultured in Dulbecco’s modified Eagle’s medium (GIBCO) containing 5% fetal bovine serum (GIBCO) until completely confluent. After being washed twice with phosphate-buffered saline, fibroblasts were cultured in serum-free medium W489 (MCDB 153: L-15 = 4:1, Irvine Scientific) at 37°C for 48 hr. Conditioned medium (CM) was then collected and concentrated by using Amicon YM1 membranes. Concentrated CM was dialyzed against 20 mM Tris-HCl buffer, pH 8.5, and loaded onto a Q-Sepharose (Pharmacia) column (2.5 × 8.5 cm; Bio-Rad) at a flow rate of 45 ml/hr. Bound fractions were eluted by a linear gradient (0.0–0.6 M) of KCl for 300 ml and then a step gradient to 2 M KCl. Pooled fractions with high hFDGI activity, as assayed by inhibition of growth of WM 35 melanoma cells) from Q-Sepharose chromatography were dialyzed against 25 mM glycine-HCl buffer, pH 2.5, and then loaded onto a Protein-Pak SP 8HR column (1.0 × 10 cm; Waters) at a flow rate of 60 ml/hr in the same buffer. After being washed with 70 ml of buffer, bound fractions were eluted with KCl (0.0–0.3 M) and then with NaCl (0.0–1.0 M) linear gradients. Fractions containing high hFDGI activities from the SP column were concentrated to about 0.7 ml by using the Centriprep-10 (Amicon) and then injected into a Superose 12 FPLC column (16 × 515 mm; Pharmacia) in 25 mM Tris-HCl, pH 7.2/0.1 M NaCl at a flow rate of 30 ml/hr. One milliliter was collected for each fraction. Protein standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (21.5 kDa), and myoglobin (17 kDa).

Growth Assays. These were done by using [3H]thymidine incorporation, essentially as described previously (6). To monitor hFDGI activity during purification, appropriate amounts (50–200 μl) of concentrated fibroblast-derived CM, or of fractions from the chromatographic studies, were added to dialysis tubing with 1 mg of bovine serum albumin and dialyzed against 20 mM Tris-HCl buffer, pH 7.4, and subsequently against serum-free W489 medium. They were then sterilized through a 0.22-μm-pore filter and hFDGI bioactivity was assayed, using an early-stage (RGP) human melanoma cell line, WM35, as a growth-inhibition-sensitive "target.

Human melanoma cells (5000 cells per well) were plated in 96-well plates and incubated with either hFDGI or cytokines for 36–48 hr. Cells were pulse labeled with [3H]thymidine for 4–6 hr before being harvested.

To assess the effects of cytokines on melanoma cell growth, human cytokines prepared by recombinant DNA methods (Upstate Biotechnical, Lake Placid, NY) at various concentrations were added to cell cultures in ExCell 300 medium (J. R. Scientific; Woodland, CA) and 1% fetal bovine serum. Cytokines and their concentrations used were IL-1α (0.5–50.000 pg/ml, 1 unit = 1 pg/ml), IL-1β (0.05–50.0 ng/ml, 1 unit = 0.1 ng/ml), IL-6 (0.025–250 ng/ml, 1 unit = 0.26 ng/ml), and tumor necrosis factor α (TNFα, 0.025–250 ng/ml, 2 × 10⁶ units/mg). Highly specific polyclonal neutralizing antibodies to IL-6 (R & D Systems, Minneapolis) were used as described in Results. All experiments were repeated at least twice and conducted by triplicate determinations. Controls without added factors were considered as 100%.

Cell Lines. The origins of the melanoma cell lines used in these studies have been described in detail elsewhere by us (6) and by Herlyn and colleagues (9, 10, 15). Some details are also summarized in Table 2 of Results. The WM series of cell lines is kindly provided by Meenhard Herlyn (Wistar Institute for Anatomy and Cell Biology, Philadelphia). Melanoma cell lines derived from RGP or early VGP primary melanoma cell lines of patients who had no recurrence of disease (even 8–10 years later) are classified as "early-stage" lesions. Those derived from distant metastases, or from VGP primaries in which the patient had evidence of distant metastases, are designated as "advanced-stage" lesions (9).

RESULTS

hFDGI for Early-Stage Melanoma Cells Is a Protein of Molecular Mass Between 20 and 30 kDa. Initially we attempted the isolation and purification of both the putative growth stimulator for advanced melanoma cells and the inhibitor for early-stage melanoma cells. Preliminary experiments showed that the stimulator (as assessed using the advanced-stage WM 9 melanoma cell line as the ‘target’) was not stable and could be separated from the inhibitor. Therefore, we decided to try to identify the growth inhibitor (i.e., hFDGI) of early-stage human melanomas, using the early-stage WM 35 cell line to monitor the biological (i.e., growth-inhibitory) activity during the various biochemical purification steps. As summarized in Fig. 1 and Table 1, the three-step purification gave 0.5% total protein recovery and showed that hFDGI has a molecular mass of 20–30 kDa. The relative broad hFDGI activity peak in FPLC gel filtration (Fig. 1C) was reproducible by concentrated CM, indicating that the broad peak was not the result of acidic treatment of hFDGI in SP chromatography (data not shown). The semipurified hFDGI after FPLC was then tested on other human melanoma cell lines (Fig. 2). It will be noted that this semipurified material retained inhibitory activity against three of four early-stage melanoma cell lines (the exception being WM 793), whereas no effect was observed on the growth of a large number of more advanced stage (metastatically competent) melanoma cell lines, whether these were derived from VGP primary tumors or metastases. It also suppressed the growth of a melanoma cell line called WM 75 (data not shown), similar to dermal fibroblasts (6). WM 75 behaves as a relatively early-stage VGP primary melanoma (D. Guerry, personal communication). In a previous paper we had mistakenly identified this cell line, which was called WM 75c, as having been derived from an advanced-stage metastasis (6). The lack of any stimulatory activity on the advanced-stage cell lines implied the existence of a growth stimulator distinct from the inhibitor, which was lost during
Table 1. Summary of purification of hFDGI

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein*, mg</th>
<th>Protein recovery, %</th>
<th>Specific activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>80.0</td>
<td>100</td>
<td>57</td>
</tr>
<tr>
<td>Anion-exchange</td>
<td>14.2</td>
<td>17.7</td>
<td>128</td>
</tr>
<tr>
<td>Cation-exchange</td>
<td>2.0</td>
<td>2.5</td>
<td>1000</td>
</tr>
<tr>
<td>Superose 12 FPLC</td>
<td>0.4</td>
<td>0.5</td>
<td>2632</td>
</tr>
</tbody>
</table>

*Protein measurements were conducted by the enhanced protocol at 60°C as described by the reagent supplier (Pierce). Bovine serum albumin was used as a protein standard and was obtained from the supplier.

†Specific activity is expressed as units per mg of total protein. One unit is defined as the amount of immobilized protein in 150 μl of culture medium that inhibited the proliferation of WM 35 cells 50%.

DISCUSSION

IL-6 is generally viewed as a member of the hemopoietic growth factor/cytokine family (16, 17). In terms of its capacity as a potential regulator of tumor cell growth, most studies have emphasized its possible role as a paracrine or autocrine stimulatory growth factor for various types of hematologic malignancies (19) such as multiple myeloma (20). It might therefore be expected that IL-6 should have...
support this view, as 27 different cell lines obtained from 12 different types of human cancer (mostly carcinomas) were found to be insensitive to exogenously added IL-6. Our results, however, suggest otherwise, with the proviso that stage of disease be taken into consideration when assessing the possible influence of IL-6 on tumor cell growth. Thus the majority of melanoma cell lines from early (metastatically incompetent) stages of tumor progression were found to be highly sensitive to IL-6-mediated growth inhibition. This sensitivity was completely lost, however, in all the advanced-stage (metastatically competent) melanomas that were examined.

The results with the metastatic cell lines are consistent with the results of Serve et al. (21) in that these authors used advanced-stage cancers. However, clearly, it cannot be concluded necessarily that IL-6 is an "inert" growth factor in the context of melanoma, and perhaps other types of solid tumor as well. This underscores the necessity of evaluating the response of tumor cells obtained from different stages of disease progression to a given growth factor when trying to determine the overall effect that factor has on tumor cell growth and behavior. Unfortunately, this has largely been ignored in most growth factor studies in human cancer, in part because it is frequently not possible to obtain early-stage lesions in most types of cancer, as it is in melanoma or colorectal carcinoma. The few studies that have been undertaken with this consideration in mind tend to support the view that increasing resistance to negative growth regulators such as TGF-β occurs with tumor progression (e.g., see refs. 22-24). Since the tumor cells in many solid cancers have been found to express IL-6 activity in situ (25), this suggests that in some cases IL-6 may not only cease being a paracrine growth inhibitor but may also actually behave as an "ectopic" stimulatory autocrine growth factor (26). In this regard experiments are needed to evaluate the possible relationship of IL-6 production and IL-6 responsiveness in the melanoma cell lines studied in this report. Preliminary evidence indicates that cells from a number of advanced-stage melanoma cell lines do indeed make IL-6 and that it functions as an autocrine growth stimulator in these cells (C.L. and R.S.K., unpublished observations). We have also recently undertaken Scatchard analyses of IL-6 receptor numbers and affinities in the melanoma cell lines studied here. No consistent differences were detected between early- and advanced-stage-derived cell lines that could explain their disparate responses to IL-6 (C.L. and R.S.K., unpublished observations).

How might acquisition of an IL-6-resistant phenotype affect the course of melanoma cell growth, progression, and

![Figure 3](https://example.com/fig3.png)

**Figure 3.** (A) Neutralization of hFDGI activity by anti-hIL-6 antibody. Five thousand human melanoma cells were incubated with human IL-6 (12.5 ng/ml), antibody to human IL-6 (ab; 10 μg/ml), hFDGI (0.5 μg per well), or the combinations as indicated for 48 hr. Cells were then pulse labeled with [3H]thymidine; [3H]thymidine incorporation was assessed 4-6 hr later. (B) IL-6 dose–response curves on four independent human melanoma cell lines, MeWo and WM 9 (obtained from advanced-stage metastatic disease) and WM 35 and WM 902B (obtained from early-stage metastatically incompetent primary lesions).

Table 2. Effect of cytokines on [3H]thymidine incorporation by human melanoma cells as a function of tumor progression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>IL-1α Effect</th>
<th>IL-1β Effect</th>
<th>IL-6 Effect</th>
<th>TNFα Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM 35</td>
<td>Early-stage RGP (primary)</td>
<td>↓ (0.11)</td>
<td>↓ (0.5)</td>
<td>↓ (1.5)</td>
<td>↓ (6.5)</td>
</tr>
<tr>
<td>WM 902B</td>
<td>Early-stage VGP (primary)</td>
<td>NR</td>
<td>NR</td>
<td>↓ (1.0)</td>
<td>NR</td>
</tr>
<tr>
<td>WM 1341B</td>
<td>Early-stage VGP (primary)</td>
<td>NR</td>
<td>NR</td>
<td>↓ (14)</td>
<td>NR</td>
</tr>
<tr>
<td>WM 793</td>
<td>Early-stage VGP (primary)</td>
<td>↓ (0.5)</td>
<td>↓ (30)</td>
<td>NR</td>
<td>↓ (15)</td>
</tr>
<tr>
<td>WM 1361A</td>
<td>Advanced-stage VGP (primary)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>↓ (0.9)</td>
</tr>
<tr>
<td>WM 983A</td>
<td>Advanced-stage VGP (primary)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>↓ (0.2)</td>
</tr>
<tr>
<td>WM 120S</td>
<td>Metastasis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>↑</td>
</tr>
<tr>
<td>WM 9</td>
<td>Metastasis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>MeWo</td>
<td>Metastasis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>WM 451</td>
<td>Metastasis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>SKMEL 28</td>
<td>Metastasis</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

*↓ and ↓ show an inhibition in [3H]thymidine incorporation of melanoma cells of more than or less than 50% compared with controls (100%), respectively. Numbers in parentheses are doses of cytokines (ng/ml) that caused 50% inhibition of [3H]thymidine incorporation into melanoma cells (ID50). NR means no response (i.e., no inhibition or stimulation). ↑ indicates a slight stimulation (~150%) of [3H]thymidine incorporation compared with controls (100%).
metastasis? Loss of sensitivity to an inhibitor such as IL-6 represents a form of “progressive emancipation” from external negative growth control (5), and this may endow rare metastatically competent melanoma cell variants with a growth advantage, thereby helping them eventually to overgrow their nonmetastatic counterparts within the primary tumor site (27, 28). We have termed this process “clonal dominance” of primary tumors by metastatically competent tumor cell variants (27, 28), a process that may enhance the probability of formation of distant metastases (29). Human melanoma appears to conform to this type of evolutionary growth pattern (7, 10, 12, 14, 28). Given the cellular ubiquity of IL-6—i.e., it is produced by keratinocytes, endothelial cells, fibroblasts, macrophages, and monocytes (16, 17)—it has considerable potential to facilitate the metastatic cell subpopulation clonal dominance process at the primary site, by impeding the growth of metastatically incompetent tumor cells. This may be particularly true in the dermal mesenchyme, where early-stage primary melanoma cells appear to have limited ability to proliferate, unlike their metastatically competent counterparts (7). This process may be facilitated by the fact that human melanoma cells can release IL-1 (30, 31), which is a major inducer of IL-6 expression (16, 17). Thus there is the possibility of a closed paracrine IL-6/IL-1 loop involving host cells, such as endothelial cells or fibroblasts, and melanoma cells, which could limit the proliferative capacity of metastatically incompetent (i.e., IL-6-responsive) melanoma cells, in both the epidermis and the dermis.

Finally, we point out that acquisition of resistance to the inhibitory effects of IL-6 by metastatically competent cancer cells may be accompanied by resistance to a number of other potential growth inhibitors, such as IL-1α, IL-1β, and TGF-β. This “multicytokine-resistant” phenotype (32) may be crucial to allowing metastatically competent melanoma cells to proliferate readily in the foreign environment of the dermal mesenchyme (where blood vessel capillaries are located and to which they can gain access) and perhaps distant organ mesenchymes as well (32). These considerations also raise the intriguing notion that acquisition of resistance to one particular growth factor—e.g., TGF-β—may lead to expression of resistance to other structurally unrelated growth factors, such as IL-6—conceptually similar to the acquisition of multidrug resistance in cancer (32, 33).

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