Melanoma-Derived Interleukin 6 Inhibits In Vivo Melanoma Growth

Cheryl A. Armstrong, Nancy Murray, Michael Kennedy, Sandhya Koppula, David Tara, and John C. Ansel
Department of Dermatology, Oregon Health Sciences University; and Dermatology Service, Veterans Affairs Medical Center, Portland, Oregon, U.S.A.

Malignant melanomas are capable of producing a wide range of cytokines with multiple biologic functions, including interleukin 6 (IL-6). We have observed an inverse relationship between IL-6 production of three B16-derived murine melanoma cell lines (NP133, HFF18, and HFH(M)) and the tumorigenicity of these melanoma cells in syngeneic mice. To further test the effect of IL-6 on melanoma growth, a non-IL-6-producing murine B16-derived melanoma cell line (HFH18) was transfected with a murine IL-6 expression vector, resulting in stable transfectants (HFH18/IL-6(+)) that expressed significant amounts of IL-6 mRNA and secreted high levels of bioactive IL-6. Syngeneic C57BL/6 mice inoculated subcutaneously with HFH18/IL-6(+) cells developed tumors that reached a final mean diameter of less than half the size of tumors that developed in mice inoculated with either HFH18 parental or HFH18 cells transfected with the IL-6 cDNA in the non-coding 3'-5' orientation (HFH18/IL-6(-) cells). In addition, mice bearing IL-6-producing HFH18/IL-6(+) tumors survived twice as long as mice bearing HFH18 parental or HFH18/IL-6(-) tumors. The specificity of melanoma growth inhibition by IL-6 was confirmed by the reversal of the slow-growing phenotype of HFH18/IL-6(+) cells by local peritumoral administration of neutralizing α-murine IL-6 antibody. IL-6-producing melanoma cells exerted a growth-inhibitory effect on distant parental tumors in a dose-dependent manner. The growth of HFH18/IL-6(+) melanomas was also decreased in nude mice, suggesting that melanoma-derived IL-6 may mediate this anti-tumor effect independently of a normal host B- and T-cell immune response. Thus, melanoma-derived IL-6 exerts a significant inhibitory effect on cutaneous melanoma growth and progression. These results indicate that melanoma cytokines may have a profound effect on tumor pathogenesis. Key words: interleukin-6/melanoma/cytokines. J Invest Dermatol 102:278-284, 1994

Manuscript received May 14, 1993; accepted for publication October 14, 1993.
Reprint requests to: Dr. Cheryl A. Armstrong, Dermatology Service, 11C2-P, 3710 SW U.S. Veterans Hospital Road, Veterans Affairs Medical Center, Portland, Oregon, 97207.

Copyright © 1994 by The Society for Investigative Dermatology, Inc.

0022-2023/94/0700 Copyright © 1994 by The Society for Investigative Dermatology, Inc.
Melanoma Cell Proliferation Assays  Melanoma cell lines were plated at a cell concentration of $2 \times 10^5$ cells/well in triplicate wells of 24-well plates in DMEM with 1% calf serum alone, or with 100 μl/ml recombinant murine IL-6 (Biosource International, Camarillo, CA) added every 24 h. In selected experiments, 10 μg/ml neutralizing α-murine IL-6 antibody (R & D Systems) was added to cultured cells every 48 h. Cells were trypsinized and counted with a hemocytometer and Coulter counter at days 1, 3, and 6 to determine the number of cells per well.

Northern Blot Analysis  RNA was extracted from murine melanoma cell lines that were cultured as above [5]. Poly A+ mRNA was prepared by the oligo (dT)-cellulose affinity chromatography method as previously described [25]. Northern blot analysis was performed as described previously [5]. The murine IL-6 cDNA probe used in these studies was generously provided by J. van Snick (Brussels, Belgium). Equivalent loading of RNA in each lane was confirmed by hybridizing the blots with the cDNA probe for the gene cyclophilin (CYP), which was generously provided by J. Douglas (Oregon Health Sciences University, Portland, OR).

Transfection of Melanoma Cells  The pHP185 plasmid containing murine IL-6 cDNA was generously provided by J. van Snick (Brussels, Belgium). The pZIPNeo SV(X) expression vector used in the transfection of the HFF18 murine melanoma cell lines was provided by R. Mulligan (Massachusetts Institute of Technology, Cambridge, MA) [26]. An 1100-base pair (bp) Eco R1 fragment of pHP185 containing the entire murine IL-6 cDNA coding sequence was subcloned into the Bam H1 site of pZIPNeo SV(X). An asymmetric Bgl II restriction site within the Eco R1 insert was used to orient the resulting plasmids. Plasmids were then constructed with 1) the murine IL-6 cDNA in the 5'-3' orientation for expression of IL-6 protein and 2) the IL-6 cDNA in the 3'-5' orientation, which results in no IL-6 protein synthesis. These IL-6 plasmids as well as pZIPNeo SV(X) vector alone were transfected into the non-IL-6 producing HFH18 parental cell line by electroporation. Cells were plated in non-selected media for 48 h, then replated in media containing 1 mg/ml G418. Neomycin-resistant clones were isolated with glass cloning rings after the IL-6 cDNA in the 5'-3' orientation that secreted no IL-6 was designated HFH18/IL-6(-) for use in further experiments.

Animals  Specific-pathogen-free 6-week-old female C57BL/6 mice or athymic C57BL/6 nu/nu (nude) mice were obtained from Jackson Animal Laboratories (Bar Harbor, ME) and housed in the animal facilities at the Portland Veterans Affairs Medical Center.

Measurement of Tumor Size and Host Survival  Cultured murine melanoma cells (NP133, HFF18, HFF(H), or transfected HFH18 cells) were transplanted into the anterior flank of 6-week-old female C57BL/6 mice (or athymic C57BL/6 nu/nu (nude) mice). The secretion of IL-6 by these cell lines was measured in the B9 bioassay (Fig 1). As indicated, the NP133 cell line constitutively secretes significant amounts of IL-6 bioactivity. Parallel differences were noted in mRNA levels (data not shown). The ability of NP133, HFF18, and HFF(H) murine melanoma cell lines to generate tumors in syngeneic mice after subcutaneous inoculation was determined. Mice inoculated with the IL-6-secreting NP133 cell line develop tumors in only one of 10 treated animals following up to 60 d after tumor cell injection (Table I). In contrast, mice injected with the non-IL-6-secreting HFF18 and HFF(H) cell lines developed tumors in 100% of treated animals within 15 d post injection. The single tumor that developed in one animal inoculated with the NP133 cell line was approximately one third the size of the HFH18 or HFF(H) melanomas at 30 d after injection. These results suggest that IL-6 production by murine melanoma cells may be related to decreased tumor growth and progression in vivo. Because other tumor cell phenotypic differences could also account for differences in melanoma tumorigenicity, we generated melanoma cell lines that differed only in their ability to produce IL-6.

RESULTS

Heterogeneity of IL-6 Production and Tumorigenicity in Murine Melanoma Cell Lines  Three B16-derived murine melanoma cell lines (NP133, HFF18, and HFF(H)) were characterized in terms of in vitro IL-6 production and tumorigenicity in syngeneic C57BL/6 mice. The secretion of IL-6 by these cell lines was measured in the B9 bioassay (Fig 1). As indicated, the NP133 cell line constitutively secretes significant amounts of IL-6 bioactivity. Parallel differences were noted in mRNA levels (data not shown). The ability of NP133, HFF18, and HFF(H) murine melanoma cell lines to generate tumors in syngeneic mice after subcutaneous inoculation was determined. Mice inoculated with the IL-6-secreting NP133 cell line develop tumors in only one of 10 treated animals following up to 60 d after tumor cell injection (Table I). In contrast, mice injected with the non-IL-6-secreting HFF18 and HFF(H) cell lines developed tumors in 100% of treated animals within 15 d post injection. The single tumor that developed in one animal inoculated with the NP133 cell line was approximately one third the size of the HFH18 or HFF(H) melanomas at 30 d after injection. These results suggest that IL-6 production by murine melanoma cells may be related to decreased tumor growth and progression in vivo. Because other tumor cell phenotypic differences could also account for differences in melanoma tumorigenicity, we generated melanoma cell lines that differed only in their ability to produce IL-6.

Table 1. Tumorigenicity of Murine Melanoma Cell Lines in Syngeneic Mice

<table>
<thead>
<tr>
<th>Melanoma Cell Line</th>
<th>Number of Mice Developing Tumors</th>
<th>Tumor Mean Diameter$^a$</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP133</td>
<td>1/10</td>
<td>11.5 mm</td>
<td>7/7</td>
</tr>
<tr>
<td>HFF18(M)</td>
<td>10/10</td>
<td>37.6 ± 10.3 mm</td>
<td>28.4 ± 6.5 d</td>
</tr>
<tr>
<td>HFF18</td>
<td>15/15</td>
<td>22.1 ± 4.7 mm</td>
<td>26.9 ± 8.9 d</td>
</tr>
</tbody>
</table>

$^a$ C57BL/6 mice were inoculated sc in the anterior flank with $1 \times 10^5$ murine melanoma cells. Tumor growth and host survival were determined (±SD).

$^b$ Tumor mean diameter was determined at 30 d after tumor cell injection or at the time of death if before d 30.

$^c$ All mice were alive at 60 d after tumor cell inoculation.

Figure 1. IL-6 secretion by melanoma cell lines. Serial dilutions of 48-h supernatants from NP133, HFF18, and HFF18(M) murine melanoma cell lines were assayed for IL-6 bioactivity in the B9 mouse plasmacytoma proliferation bioassay. These data are representative of triplicate experiments measured in three perpendicular axes with calipers at 2-d intervals and tumor volume was calculated as above [27].
Establishment of IL-6-Producing Melanoma Cell Lines
Genetically identical melanoma cell lines that differed only in IL-6 production were established. A murine IL-6 eDNA expression vector was transfected into the non-IL-6-producing HFH18 melanoma cell line. A transfected clone designated HFH18/IL-6(+) was found to constitutively secrete high levels of IL-6 as determined in the B9 IL-6 bioassay and thus was selected for further studies (Fig 2). Parental HFH18 cells transfected with the pZipNeoSV vector alone (HFH18/neo) or with the IL-6 cDNA gene insert in the reverse (3'→5') orientation (HFH18/IL-6(-)) did not secrete IL-6 (Fig 2). The production of IL-6 by the HFH18/IL-6(+) cell line was confirmed by immunohistochemistry (data not shown). The proliferation rate of cultured IL-6-producing melanoma cells does not differ significantly from that of the parental HFH18 cell line or the HFH18/IL-6(-) line. Additionally, the in vitro proliferation of the parental cell line is not affected by the addition of recombinant murine IL-6 nor is the in vitro proliferation of the transfected HFH18/IL-6(+) cell line altered by the addition of neutralizing α murine IL-6 antibody to the cultured cells (data not shown). Similar levels of IL-6 receptors are observed in HFH18 parental, HFH18/IL-6(+), HFH18/IL-6(-), and NP133 cell monolayers by immunohistochemistry (data not shown). These data indicate that tumor cell growth is not directly altered in vitro by the transfection process itself or by IL-6.

Transfection of the IL-6 Gene into Melanoma Cells Results in Decreased Tumor Growth and Improved Host Survival
Mice inoculated with either the HFH18 parental melanoma cells or HFH18/IL-6(+) cells develop tumors that reach a final mean tumor diameter of approximately 25 mm by d 30 (Fig 3). In contrast, animals inoculated with HFH18/IL-6(-) cells develop significantly smaller tumors with a final mean tumor diameter of only 12 mm by d 30 (p < 0.001; HFH18/IL-6(+) versus HFH18/IL-6(-)). Tumor development in mice injected with HFH18/neo cells parallels the growth of the HFH18 line itself (data not shown). The consequence of IL-6 secretion by HFH18 melanoma cells on host survival is summarized in Fig 4. Mice injected with the parental HFH18 cell line or the HFH18/IL-6(-) cell line have a median survival of 25 d. In contrast, mice injected with the HFH18/IL-6(+) cell line have a significant increase in survival with a median survival time of 55 d (p < 0.001; HFH18/IL-6(+) versus HFH18 or HFH18/IL-6(-)). Thus, IL-6 secretion by the HFH18 murine melanoma cell line results in decreased tumor growth and increased survival of the recipient mice.

IL-6 Production of HFH18/IL-6(+) Melanomas
The production of IL-6 was compared by immunohistochemistry in tumors that developed in animals injected with NP133 cells, HFH18 cells, and HFH18 transfected melanoma cells. HFH18/IL-6(+) tumors stain strongly positive for IL-6 (Fig 5A). In contrast, little IL-6 staining is detected in HFH18 (Fig 5B) or HFH18/IL-6(-) tumors (data not shown). As a positive control, NP133 tumors that constitutively produce IL-6 in vitro also stain strongly positive for IL-6 (Fig 5C). HFH18/IL-6(+) tumor explants continue to secrete IL-6 bioactivity in tissue culture whereas HFH18 or HFH18/IL-6(-) tumor explants secrete no IL-6 bioactivity in culture (data not shown). These studies indicate that tumors that develop from injected HFH18/IL-6(+) cells continue to secrete IL-6 in vivo.

Elevated Serum IL-6 in Mice Bearing HFH18/IL-6(+) Tumors
To determine whether local tumor production of IL-6 results in increased serum levels of this cytokine, serum IL-6 levels in mice inoculated with either the HFH18 melanoma cell line or the HFH18 transfected cell lines were measured in the B9 bioassay. As indicated in Fig 6, serum IL-6 levels of mice developing tumors after injection with HFH18 and HFH18/IL-6(-) cells are similar to those of mice injected with saline at 5, 10, and 15 d after tumor cell injection (Fig 6). In contrast, serum IL-6 levels in mice developing tumors after inoculation with HFH18/IL-6(+) cells are markedly elevated above control levels by d 15 and continued to increase up to the time of death.
Figure 5. Immunohistochemical detection of IL-6 in excised melanoma tissue. Tumor tissue was obtained at d 15 from C57BL/6 mice inoculated sc with $1 \times 10^6$ HFH18/IL-6(+) melanoma cells (A), HFH18 parental melanoma cells (B), or NP133 murine melanoma cells as a positive control (C). Immunohistochemistry was performed using a primary monoclonal rat α-murine IL-6 antibody. The controls were tumor tissue treated with rat IgG and tissue treated with secondary antibody only (data not shown). Bar, 10 μm.

Figure 6. Serum IL-6 levels in melanoma-bearing mice. Groups of C57BL/6 mice were inoculated sc with $1 \times 10^6$ HFH18 parental (○), HFH18/IL-6(-) (△), or HFH18/IL-6(+) (■) cells. Control mice were injected sc with saline alone at day 0 (▪). Serum samples were obtained when mice were euthanized at 5, 10, 15, and 30 d after tumor cell injection. Serum IL-6 bioactivity was determined by the B9 murine plasmacytoma proliferation assay. These data are the average of serum samples from four animals per cell line injected at each time point expressed as units/ml, with SD indicated by error bars.

d 30 after tumor cell inoculation (Fig 6). Interestingly, by d 30, the serum IL-6 levels of mice bearing HFH18 or HFH18/IL-6(-) melanomas are also elevated above control animal levels. At this stage the tumor weight of these non-IL-6-producing tumors is approximately 50% of the weight of the mouse. Increased serum IL-6 levels with a large tumor cell burden have been previously reported in various types of neoplasms [29,30]. The tumor burden of mice bearing small HFH18/IL-6(-) tumors is significantly less than that of mice bearing the significantly larger HFH18 parental or HFH18/IL-6(-) melanomas at all time points after tumor cell inoculation. In spite of this small tumor size, however, these neoplasms are associated with high levels of serum IL-6. These results indicate that the mice with HFH18/IL-6(-) melanomas have significantly higher levels of serum IL-6 compared to animals with HFH18 or HFH18/IL-6(-) melanomas ($p < 0.01$).

Histologic Examination of Melanomas Injected HFH18 and HFH18/IL-6(-) tumor cells develop into large, poorly circum-
IL-6 and M~

Figure 8. IL-6 inhibition of melanoma growth is reversed by injection of peritumoral α-IL-6 antibody. Groups of C57BL/6 mice were inoculated sc with 1 X 10^6 HFH18 parental or HFH18/IL-6(-) melanoma cells. Experimental mice in each group received peritumoral injections of 10 μg of neutralizing monoclonal rat α-murine IL-6 antibody every 48 h for 30 d. Control mice received peritumoral injections of 10 μg of rat IgG isotype standard antibody every 48 h beginning at d 0. Data are represented as tumor volume at d 30 with SD indicated by error bars.

dscribed neoplasms by d 10. In contrast, the small tumors formed by the HFH18/IL-6(-) cell line are sharply circumscribed and have no evidence of an infiltrative tumor cell pattern. Both IL-6–producing and non–IL-6–producing melanomas are associated with a moderately dense peritumoral inflammatory cell infiltrate composed primarily of lymphocytic cells as determined by light microscopy of H&E-stained sections.

Systemic Anti-tumor Effect of Melanoma-Derived IL-6 To determine whether HFH18/IL-6(-) melanomas inhibit the growth of HFH18 melanoma cells at a distant site, mice were inoculated sc with both cell types at separate sites and tumor weight was measured at d 30 post injection. Mice co-injected with equal numbers of HFH18 cells (1 X 10^6) in one flank and HFH18/IL-6(-) in the opposite flank demonstrate a 40% decrease in the weight of HFH18 tumors compared to HFH18 tumors in control animals (Fig 7). Mice co-injected with 1 X 10^6 HFH18 cells in one flank and half as many HFH18/IL-6(-) cells (5 X 10^5) in the opposite flank develop HFH18 tumors that are only slightly decreased in size compared to controls. Thus, IL-6 produced by the HFH18/IL-6(-) melanoma cells exerts a systemic growth-inhibitory effect on the growth of distant parental tumors in a dose-dependent manner.

IL-6 Inhibition of Melanoma Growth is Reversed by Injection of Peritumoral α–IL-6 Antibody To confirm the specificity of the apparent IL-6–mediated inhibition of melanoma growth, mice were inoculated sc with either the HFH18 cell line or the IL-6–producing HFH18/IL-6(-) cell line and peritumoral injections of 10 μg α-murine IL-6 antibody were administered every 48 h. The treatment of HFH18 tumors with α-mIL-6 antibody has no effect on tumor volume at d 30 (Fig 8). In contrast, the treatment of IL-6–secreting HFH18/IL-6(-) tumors with α-mIL-6 antibody results in tumors that grow to the same size as the parental HFH18 cell line at d 30. The control isotype-matched rat immunoglobulin (IgG) administered peritumorally every 48 h has no effect on tumor growth. The augmented growth of the HFH18/IL-6(-) tumors by local injection of neutralizing α-IL-6 antibody provides further evidence that IL-6 is responsible for the decreased in vivo growth of these melanomas.

Growth of Murine Melanomas in Nude Mice Our studies indicate that IL-6 does not have an apparent direct in vitro inhibitory effect on melanoma cell growth. The observed decrease in in vivo melanoma growth may be mediated by IL-6 through other mechanisms such as the induction of other inhibitory cytokine(s) or of anti-tumor immune cell subsets. To begin to address this issue, nude mice were inoculated with HFH18 and HFH18/IL-6(-) cells. These animals develop tumors that steadily progress in size to a mean tumor of approximately 4050 mm^3 by d 32 (Fig 9). In contrast, nude mice injected with the HFH18/IL-6(-) cell line develop sc tumors that reach a mean tumor volume of only 2000 mm^3 by d 32 (Figs 9, 10). Thus, the anti-tumor effect mediated by melanoma-derived IL-6 appears not to be dependent on a normal host B- and T-cell immune response.

**DISCUSSION**

Previous studies indicate that there is significant heterogeneity in the cytokine production profile of malignant melanomas [5,9]. Using a well controlled animal model, we tested the hypothesis that a specific tumor-derived cytokine may have a profound effect on the growth and progression of cutaneous melanomas. We found that melanoma-derived IL-6 exerts a significant inhibitory effect on subcutaneous melanoma growth and progression. This is consistent with our observation of an inverse relationship between IL-6 production of three B16-derived murine melanoma cell lines (NP133, HFH18, and HFH(M)) and the tumorigenicity of these melanoma cells in syngeneic mice.

IL-6 is a pleotropic cytokine with a wide variety of inflammatory properties. These include the activation of B and T cells, induction of immunoglobulin production, stimulation of hepatocytes to produce acute phase proteins, and the proliferation of hematopoietic progenitor cells [31–35]. Additionally, IL-6 activates natural killer (NK) cells [36] and human lymphokine-activated killer (LAK) cells [37], and serves as a cytotoxic lymphocyte (CTL) differentiation factor [38].

The role of IL-6 in neoplasia has been the topic of a number of recent reports. IL-6 production has been reported to occur in several different types of malignant human cells including cardiac myxomas, bladder carcinomas, and epidermoid carcinoma cell lines [10,11]. We have shown that human melanoma cell lines produce IL-6 and that there are distinct differences in the IL-6 production between two human metastatic melanoma cell lines tested [5]. One melanoma cell line constitutively expresses IL-6 mRNA and secretes IL-6 activity, whereas the other cell line does not produce a significant amount of IL-6. In another recent study, it was also noted that one of two human melanoma cell lines tested did not constitutively produce IL-6, but did secrete IL-6 after induction with rIL-1α [6]. There is evidence to support both tumor-promoting and tumor-inhibitory properties of IL-6, which may depend...
transfected melanoma cells formed tumors with distinct parental cells
both on the tumor cell type and inherent limitations of experimental
tumor cell type and inherent limitations of experimen-
these photographs are representative of four mice injected
reduced growth and progression of sc malignant melanoma. This
ex vivo proliferation of an IL-6—producing melanoma cell line [6].
believe that IL-6 may mediate this antitumor response by nonspecific proinflammatory mechanisms. This study is
The role of IL-6 in the biologic behavior of malignant melanoma has been evaluated from different experimental approaches. Recent
We thank Dr. Alan Kaynard for his review of the manuscript and Patricia O’Hare, M.D., for technical assistance.
This work was supported by grants from the Dermatology Foundation, the American Cancer Society, Oregon Division, Inc., and the Oregon Medical Research Foundation.

REFERENCES
6. Morinaga Y, Suzuki H, Takatsuki F, Akiyama Y, Taniyama T, Matsuishima K,
Oonoda K: Contribution of IL-6 to the antiproliferative effect of IL-1 and tumor necrosis factor on tumor cell lines. J Immunol 143:3538–3542, 1989


