In Vivo and in Vitro Characteristics of Interleukin 6-transfected B16 Melanoma Cells

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Abstract

Interleukin 6 (IL-6) is a multifunctional cytokine important in the inflammatory response. Its potential role as an antitumor agent has been suggested by its demonstrated activity in a variety of tumor models. The mechanism of antitumor activity has been proposed to be its enhancement of cytotoxic T-cell function. In the current work we demonstrate clear antitumor activity for this cytokine in a nonimmunogenic tumor system. B16 melanoma cells transfected with the human IL-6 complementary DNA demonstrated slower tumor growth in vivo. Tumors that developed from these cells had a prominent stromal matrix, a less prominent inflammatory response, and fewer blood vessels. These in vivo findings corresponded with a greater adhesion of the IL-6-transfected B16 cells to stromal matrix proteins (laminin, fibronectin, and vitronectin) and a less prominent vascular response in an intradermal angiogenesis assay. Therefore, we propose that with weakly antigenic tumors, such as B16 melanoma, IL-6 may mediate important antitumor responses by nonspecific proinflammatory mechanisms.

Introduction

Interest in IL-6 has increased considerably in the last few years as we have learned more about its role in the normal inflammatory response and its involvement in the pathogenesis of a variety of diseases (1). It is a Mr 26,000 protein, produced by a wide variety of cells under varied conditions and, like IL-1, it is a critical factor in the acute phase inflammatory response. Within the immune system, IL-6 appears to serve as an inducer of B-cell maturation and proliferation and of T-cell maturation (2, 3). Its role in host antitumor responses has not been completely elucidated. As a stimulator of B-cell maturation and proliferation, it is not surprising that IL-6 has been shown to enhance B-cell lymphoma and myeloma cell growth directly (4, 5). In fact, one early described activity of this molecule was as a stimulatory factor for hybridoma growth (6). Interestingly, other nonlymphoid tumors have been shown to be directly stimulated by IL-6 and it has been presumed that the common factor among these diverse tumors is the presence of IL-6 receptors. However, the great majority of murine and human tumor lines do not have IL-6 receptors and with these, IL-6 has been shown either to be inhibitory or to have no effect (7).

In light of its demonstrated enhancement of certain T-cell functions, the development of this cytokine for potential anti-cancer therapy has been promoted. Mule et al. (8) have demonstrated that exogenous IL-6 administration to mice bearing fibrosarcomas or colon carcinomas has reduced tumor growth and metastatic colony formation. In light of this and other work, phase I clinical trials have been initiated at the National Cancer Institute.

The mechanisms of IL-6-mediated antitumor responses have not been completely explained. It was the purpose of this research to examine IL-6 effects in a weakly immunogenic tumor system with cells that are devoid of IL-6 receptors. B16 murine melanoma fit these criteria. Others have demonstrated the very weak presence of cell surface antigens on these cells (9, 10) and we had previously shown that IL-6 had no direct effect on B16 cell proliferation (11). We observed that IL-6-transfected B16 cells grew more slowly and in a different histological pattern than mock-transfected controls. Mechanisms that might account for this include the increased expression of receptors for adhesion molecules on the IL-6-transfected cells and the apparent inhibition of neovascularization by these cells.

Materials and Methods

B16 Melanoma and Transfection Procedures. The B16(F10)-IL-6 cell line (kindly provided by Drew Pardoll, Johns Hopkins University) had been prepared by transfection with 5 μg of the plasmid vector pBCMG-Neo-IL-6, a bovine papilloma virus expression vector DNA, by coprecipitation with calcium phosphate (12). The pBCMG-Neo-IL-6 vector contains a human IL-6 complementary DNA under the transcriptional control of a cytomegalovirus promoter with a rabbit β-globin intron and a neomycin resistance gene. The B16-IL6 line was selected by drug resistance and its ability to secrete IL-6 into culture supernatant measured by IL-6 bioassay (as below). All B16 tumor lines were maintained in culture at 37°C in humidified air (5% CO2) in RPMI 1640 (Gibco) supplemented with L-glutamine (2 mm), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (25 mm), gentamicin (50 μg/ml), and 10% fetal calf serum.

Bioassay for IL-6 Activity. A standard IL-6 bioassay with minimal modifications was used for these studies (13). Briefly, 2 × 105 7TD1 cells (IL-6-dependent murine B-cell hybridoma cells, kindly provided for these studies by Diane Norback, University of Wisconsin) were incubated with tumor cell supernatants (100 μl/well) or serial dilutions of recombinant human IL-6 (Sandoz Corp.) to create a standard curve. After 3 days at 37°C, 5% CO2, 10 ml of a 3-mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added to the microtiter wells and incubated for an additional 3 h. The supernatant was then aspirated and 100 ml of dimethyl sulfoxide/well were added to solubilize the formazan salt crystals. Absorbance was measured on a Dynatech MR650 enzyme-linked immunosorbent assay plate reader at a wavelength of 570 nm.

Tumor Growth and Histology. Young (6–8 weeks) female C57BL/6 mice were purchased from Harlan Sprague Dawley and maintained for 2 weeks in our animal care facility under isothermal conditions (23°C) with regular photoperiods and ad libitum access to Purina mouse chow and water. Mice were given s.c. injections into the right flank of either 105 B16 melanoma cells, the B16 mock-transfected cells, or the B16...
IL-6-transfected cells in a total volume of 0.1 ml. Tumor growth was examined daily and palpable tumors were measured in two perpendicular axes with tissue calipers. The tumor volume was calculated assuming spherical growth using the formula

\[ V = \frac{4}{3} \pi r^3 \]

In separate experiments, recombinant human IL-6 was given to the mice s.c. together with B16 melanoma cells. Local injection of recombinant human IL-6 (5 μg daily) was administered daily for up to 20 days and tumor volume was recorded.

Tumors grown in certain animals were excised, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation.

**In Vitro Assessment of IL-6-Transfected B16 Tumor Cells: Attachment Assay.** Tissue culture plates (24-well; Corning) were coated with either fibronectin (10 μg/ml), vitronectin (2 μg/ml), or laminin (10 μg/ml) in PBS, pH 7.4, overnight at 4°C. (These matrix proteins and the RGDS peptides discussed below were kindly provided by Deane Mosher, University of Wisconsin). Tumor cells were harvested by trypsinization using 0.05% trypsin-EDTA (0.53 mm) solution, washed 3 times with PBS, and resuspended in medium (as described above but without added fetal calf serum) at a density of 1 × 10^5/ml. The cell solution (0.5 ml) was added to each well for 30 min at 37°C. The attached cells were fixed by adding 3% paraformaldehyde and washed 3 times with PBS to remove unattached cells. Ten random 1.5-mm^2 fields/well were counted under phase contrast microscopy. The number of attached cells were expressed as the mean ± SEM. Assessment of inhibition of cell attachment was performed by using RGDS peptides or antifibronectin receptor antibodies. RGDS or, for control, RGES peptides were added in the cell attachment assay at a final 40 μM concentration. Polyclonal antibodies (Telio, LaJolla, CA) against fibronectin and vitronectin receptor were also added in some cell attachment assays at a 1:100 dilution. With either competing RGDS peptides or antibody to integrin receptors, inhibition was measured in the cell adhesion assay as described above.

**FACS Analysis.** Cell surface integrin expression on IL-6-transfected B16 melanoma and untransfected cells were compared by FACS analysis. Cells (1 × 10^7) were resuspended in 50 ml of RPMI 1640 containing polyclonal antibodies to either fibronectin or vitronectin receptor (Telio, LaJolla, CA) at a 1:100 dilution. Cells were incubated at 37°C for 1 h, washed 2 times in PBS, resuspended, and incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-rat antibodies. Cells were then washed twice with PBS and analyzed by FACS.

**Angiogenesis Assay.** An i.d. angiogenesis assay was used as described by Kreisle et al. (14). Briefly, tumor cells (10^6 B16, B16-mock, or B16-IL-6) were injected i.d. into metana-anesthetized mice at anterior and posterior sites in a total volume of 0.1 ml with a 27-gauge needle. The animals were sacrificed 72 h later. A midventral incision was made in the skin and the number of tumor-associated blood vessels were scored using a microscopic image analysis system (14).

**Results and Discussion**

**Tumor Growth and Histology.** In our initial experiments, s.c. injection of IL-6-transfected B16 melanoma cells (B16-IL-6) resulted in slower tumor growth than injection of mock-transfected (B16-mock) or nontransfected B16 cells (Fig. 1A). Two more repeats of this experiment revealed virtually identical patterns of growth. The slower growth rate in vivo was of interest because we had previously shown that the transfected cells had virtually the same in vitro growth characteristics (proliferative rate, doubling time, etc.). To determine if the IL-6 itself was the mediator of this reduced tumor growth, additional mice were given injections of wild-type (nontransgenic) B16 cells and subsequently treated with recombinant human IL-6 s.c. at a daily dose of 5 μg/day. The animals in the treatment group showed reduced growth, similar to that observed in mice that received the IL-6-transfected B16 cells (Fig. 1B).

Tumors composed of IL-6-transfected cells were distinctly different when compared histologically to mock-transfected or nontransfected B16 cells. Typically B16 tumors demonstrate diffuse growth with prominent vascularity, a relatively high number of mitotic figures, and a paucity of host inflammatory cells (Fig. 2A). In contrast, the IL-6-B16 tumor grew in a "pseudonodular" pattern created by prominent sheets of stromal matrix (Fig. 2B). Within the stroma there was a striking infiltration of host inflammatory cells (both mononuclear cells and segmented neutrophils; Fig. 2C). Also apparent were fewer vessels and mitotic figures.

**Experimental Metastases.** To further evaluate the differences in these cell types, we injected 5 × 10^4 tumor cells i.v. and examined the lungs for tumor colonies 20 days later. Although an equivalent number of animals in each group were found to have metastases, bulky tumor colonies were found in the two control groups at 20 days, whereas only one of the animals receiving B16-IL-6 cells had extensive metastases at that time (Table 1). We suspect that this observation is another reflection of the IL-6 effect on tumor growth and not particularly on the metastatic process, at least as demonstrated in the i.v. model. Presumably, bulky metastases would eventually occur in the animals receiving the transfected cells.

**Cell Adhesion Assays.** Because of the apparent prominent stromal reaction, we examined the adhesive properties of these tumor cells in vitro. We found that the B16-IL-6 cells adhere more strongly to fibronectin, vitronectin, and laminin-coated plastic surfaces than the controls (Fig. 3A). That this increased adhesion was mediated by binding at the integrin receptor was supported by the near complete inhibition of cell attachment by the RGDS peptides (Fig. 3B). When tumor cells labeled with polyclonal antibodies against either fibronectin or vitronectin receptors were examined by flow cytometry analysis, we found increased labeling which suggested that IL-6 in some way resulted in an up-regulation of both fibronectin and vitronectin receptors on the B16-IL-6 cells. This is particularly noteworthy because others have shown that up-regulation of cell surface integrin receptors by transfection render cells less or even non-malignant in vivo (15, 16). It is possible, therefore, that IL-6 mediates at least some of its tumor-inhibiting effects by influencing adhesive properties of the cells with the tumor stroma.

**Angiogenesis.** In addition to the increased matrix and the presence of inflammatory cells, the B16-IL-6 tumor also appeared histologically to have fewer blood vessels. To strengthen this histological impression we examined the early neovascular...
Table 1 Experimental metastases in different B16 lines

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![Fig. 2. Histological appearance of B16 or B16-IL-6 tumors. Tumors growing s.c. were excised in toto, fixed in formalin, stained with hematoxylin and eosin, and examined by light microscopy. B16 tumors have a diffuse pattern of growth and are highly vascular. × 100 (A). B16-IL-6 tumors are characterized by sheets of fibrous matrix producing a nodular appearance. × 100 (B). Also apparent in the B16-IL-6 tumors was the infiltration of inflammatory cells into the stromal matrix. × 400 (C).](image)

![Fig. 3. A, cell adhesion to matrix protein-coated plastic surfaces. B16-IL-6 cells adhered to a greater extent than mock-transfected B16 cells or the parent, non-transfected (B16 wild-type) cells; ■, B16-IL-6; □, B16-mock; ▲, B16-wt. B, When RGD peptides were added (40 mM), adhesion to each of the proteins by B16-IL-6 cells was dramatically reduced; ■, B16-IL-6; □, B16-IL6 + RGDS.](image)

![Fig. 4. New vessel formation in the intradermal angiogenesis assay. There were fewer tumor-associated vessels in the B16-IL-6 tumors (P < 0.05 by Mann-Whitney log rank analysis).](image)

response in an intradermal assay. We found that there was less tumor-induced vascular sprouting around the B16-IL-6 tumors (Fig. 4).

Although IL-6 has no direct stimulatory or inhibitory effect on B16 melanoma cells grown in vitro (11), we found striking effects on local tumor growth in vivo. Tumors grown in the presence of IL-6 assumed a different histological appearance with prominent tumor stroma, inflammatory cells, less vascularity, and fewer mitotic figures. The prominent stromal reaction may well be important because of the observed increased tumor cell adherence to fibronectin, vitronectin, and laminin. This latter observation is most likely the result of IL-6-induced increased expression of integrin-type receptor. Furthermore, contributing to the reduced tumor growth could also be an IL-6-mediated inhibition of tumor-induced angiogenesis, as demonstrated in the i.d. assay. However, an inhibition of angiogenesis is not what one would expect from a mediator of
inflammation such as IL-6 and further work needs to be done to confirm the role of IL-6 as an inhibitor of tumor-induced angiogenesis. Nevertheless, what can be concluded from this work is that, in this weakly antigenic tumor system, IL-6 clearly alters the growth properties of tumors. We believe that these are mediated by nonspecific inflammatory mechanisms. With more antigenic tumors, such as many of the established fibrosarcomas, one might observe even greater inhibition of tumor growth, because in addition to these nonspecific mechanisms, IL-6 might stimulate specific cytotoxic T-cell killing, as has been suggested by others (8).

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References

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