Interleukin 10 Production by Human Melanoma

Takami Sato, Peter McCue, Kazuhiro Masuoka, Sarah Salwen, Edmund C. Lattime, Michael J. Mastrangelo, and David Berd
Department of Medicine, Division of Neoplastic Diseases, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

ABSTRACT
Interleukin 10 (IL-10) has the physiological role of down-regulating cell-mediated immunity. We have recently reported that mRNA for IL-10 was present in most metastatic melanoma tissues. The purpose of this investigation was to determine whether melanoma metastases produce IL-10 protein. Single-cell suspensions were prepared by enzymatic dissociation of 28 lymph node metastases and 7 s.c. metastases and cryopreserved. Of these 35 samples, 30 produced IL-10 after a 24-h incubation (median, 125.1 pg/ml). IL-10 production was slightly diminished after 25 Gy irradiation but almost completely abrogated after modification with the hapten dinitrophenyl. After 7 or 14 days in tissue culture, melanoma cells continued to produce IL-10 but only at 10% of the levels of freshly dissociated tissues. Moreover, of eight melanoma cell lines established from these cultures, only one produced IL-10 protein. To determine whether IL-10 was produced by melanoma cells or tumor-associated leukocytes, single-cell suspensions were fractionated with anti-CD45 antibody-conjugated magnetic beads. In four of five samples, IL-10 production was increased by depletion of leukocytes, suggesting that the primary source was the melanoma cells themselves. This was confirmed by immunohistochemical staining of cytopsin preparations and frozen tissue sections. Finally, 10 of 55 patients with clinically evident metastases showed elevations of circulating IL-10; three patients who had been melanoma-free developed high serum IL-10 levels, concurrent with the appearance of distant metastases. These data indicate that production of IL-10 is characteristic of metastatic melanomas and raise the possibility that this cytokine allows tumors to avoid or to modulate immunological attack.

INTRODUCTION
IL-10, originally described as “cytokine synthesis inhibitory factor,” has the physiological role of down-regulating cell-mediated immunity and up-regulating antibody responses (1). IL-10 is produced not only by T cells but also by macrophages, B lymphocytes, and keratinocytes (2). When cultured with T cells, IL-10 inhibits their proliferation and suppresses production of IL-2 and IFN-γ. However, its primary physiological target may be the macrophage; it inhibits synthesis of a variety of cytokines, including IL-1, and prevents cytotoxic activity. Recent work suggests that production of IL-10 by activated macrophages constitutes a negative feedback mechanism for those cells (3).

There is accumulating evidence that these in vitro studies reflect a suppressive role of IL-10 on cell-mediated immunity in vivo. Systemic administration of IL-10 inhibits the development and expression of delayed-type hypersensitivity in mice (4, 5). Admixture of IL-10 with antigen at the time of sensitization not only abrogated the development of contact sensitivity to that antigen but also induced long-lasting, antigen-specific tolerance (6). IL-10 knockout mice develop fatal autoimmune inflammatory bowel disease (7). IL-10 suppresses the immune response to pathogenic microorganisms in mice, whereas anti-IL-10 antibody reverses the suppression (8, 9). Finally, Chernoff et al. (10) recently reported a clinical trial of human IL-10 in normal volunteers. Peripheral blood lymphocytes obtained after IL-10 administration were inhibited in their ability to proliferate and to produce cytokines.

Chen et al. (11) made the surprising observation that IL-10 mRNA is expressed and IL-10 protein is made by some human melanoma cell lines. Our group first showed that IL-10 mRNA was present in metastatic melanoma tissues as well (12, 13), and a similar finding was subsequently reported by Krüger-Krasagakes et al. (14). In this report, we demonstrate that cells dissociated from most metastatic melanomas produce IL-10 protein and that melanoma cells, rather than tumor-associated leukocytes, are the primary source. IL-10 production actually diminishes when the cells are adapted to tissue culture. Finally, in patients with advanced metastatic melanoma, IL-10 is sometimes produced in great enough quantity to be detectable in the circulation.

MATERIALS AND METHODS
Clinical Materials. Tissue and serum samples were collected from patients with cutaneous melanoma who were stage III (clinically evident regional lymph node metastasis) or IV (disseminated metastases). Some patients subsequently received postsurgical adjuvant treatment with autologous DNP-modified melanoma vaccine after resection of metastases (15, 16). Serum samples were also collected from 12 randomly selected patients with metastatic adenocarcinoma (6 colon, 3 breast, 1 lung, 1 rectum, and 1 prostate). None of the patients had active infections at the time of serum collections.

Preparation of Melanoma Cell Suspensions. Metastatic masses were obtained immediately after excision; 28 were lymph node metastases and 7 were s.c. metastases. The tissues were dissociated with collagenase and DNase as described previously (17). Then the cell suspensions were aliquoted, frozen in
a controlled rate freezing machine, and stored in liquid nitrogen. For the modification of single-cell suspensions, the cells were irradiated (25 Gy) and modified with DNP as described previously (15).

**IL-10 Production by Melanoma Cell Suspensions.** Tumor-derived cells were incubated in flat-bottomed microtiter plates (2 × 10^5 cells/well) in RPMI (Mediatech, Washington, DC) supplemented with no serum, 10% FCS, or 10% autologous serum. Supernatants were collected at various times points and stored at −20°C. Autologous sera were prescreened to be certain that IL-10 was not detectable. IL-10 was measured by a commercially available ELISA kit for human IL-10 (Endogen, Cambridge, MA). The sensitivity is 3 pg/ml; there is no cross-reactivity with other human cytokines. The mean ± SD concentration of human IL-10 found in 15 normal serum samples was 3.57 ± 3.70 pg/ml (provided by Endogen).

**Cell Separation with Antibody-coated Magnetic Beads.** Single-cell suspensions from metastatic melanomas were fractionated by use of magnetic beads coated with monoclonal antibody, anti-CD45 (pan leukocyte; Immunotech, Westbrook, ME). Beads (1.0 mg; 100 μl) were added to 10^7 cells suspended in 1 ml PBS with 30% FCS, and the mixture was incubated for 10 min at room temperature. Then the cells were placed in a magnetic field for 10 min, and the supernatant containing uncoated cells was removed. The negatively selected cells (leukocyte-depleted) were repeatedly (two to four times) applied to the magnetic field until magnetic bead-coated cells were reduced to <5% by microscopic examination. Morphologically, negatively selected cells were melanoma cells with <5% leukocytes (mainly lymphocytes and macrophages). In contrast, the positively selected cells were more than 95% bead coated and were mainly lymphocytes. These anatomic observations were corroborated by flow cytometry using monoclonal antibodies to CD45 (HB45; American Type Culture Collection) and to the melanoma-associated proteoglycan (antibody 9.2.27, a gift of the Biological Response Modifiers Program, Frederick, MD; Ref. 18).

**Immunohistochemical Staining of IL-10.** Cells (50,000) in 0.1 ml of PBS with 50% FCS were collected on poly-L-lysine-coated slides by cytospin centrifugation (Cytospin 2; Shandon). The slides were fixed with 70% ethanol for 30 min. Immunohistochemical staining was done with mouse monoclonal anti-human IL-10 antibody (R & D Systems, Minneapolis, MN). Biotinylated rabbit antimouse antibody and the avidin-peroxidase complex method (19) were used for chromogen localization of IL-10 protein. The negative control was biotinylated rabbit anti-mouse antibody plus avidin-peroxidase.

**RESULTS**

**IL-10 Production by Cryopreserved Melanoma Cell Suspensions.** We have previously shown that most metastatic melanoma tissues contain IL-10 mRNA (13). To determine whether these tissues produced IL-10 protein, we studied cryopreserved cell suspensions prepared from them. As shown in Fig. 1, 30 of 35 melanoma cell suspensions release IL-10 into the supernatant after an overnight incubation. These results were not due to a FCS-induced artifact, since 26 of 35 tumor cell samples produced IL-10 when incubated with autologous serum. The majority of samples (19 of 35) were capable of producing IL-10 even in the absence of a serum supplement, although the viability of tumor cells was markedly reduced in serum-free medium. It appears that IL-10 was produced under these conditions and not merely released from cytoplasmic stores, because supernatants obtained by lysis (repeated freeze-thaw) of...
The adherent cells were mainly (>95%) melanoma cells, as tumor-associated leukocytes rather than melanoma cells themselves. The possibility that in some specimens the source of IL-10 was cell suspensions that had not been incubated did not contain IL-10. IL-10 was first detected in supernatants after 8 h incubation, and the levels reached a plateau after 24 h (data not shown).

IL-10 was produced by both lymph node metastases (24 of 28) and s.c. metastases (6/7) and by mechanically dissociated as well as enzymatically dissociated cells (data not shown). Since we are conducting clinical trials of an autologous DNP-modified melanoma vaccine (16), we considered it important to evaluate the effects on IL-10 production of irradiation and DNP modification. IL-10 production was slightly diminished after 25 Gy irradiation but almost completely abrogated by irradiation followed by DNP modification (P < 0.001; paired t test; Fig. 2).

**IL-10 Production by Short-Term Cultured Melanoma Cells.** Melanoma cell suspensions that produced IL-10 after overnight incubation were incubated in tissue culture flasks for 7 or 14 days. Then nonadherent cells were removed by washing. The adherent cells were mainly (>95%) melanoma cells, as indicated by binding of the melanoma-associated monoclonal antibody, 9.2.27, and lack of binding of antibody to CD45. They were detached with EDTA and reseeded into microtiter plates. After an overnight incubation, the supernatants were assayed for IL-10.

As shown in Fig. 3, melanoma cells from 11 of 14 specimens produced IL-10 after short-term in vitro propagation, but in 10 of 11, IL-10 production was markedly reduced compared to that of freshly dissociated cell suspensions. From eight of these short-term cultures, we were able to establish long-term melanoma cell lines; only one of eight continued to produce IL-10 after 8 weeks in tissue culture.

**Localization of IL-10 Production to Melanoma Cells.** The decline of IL-10 production with in vitro propagation raised the possibility that in some specimens the source of IL-10 was tumor-associated leukocytes rather than melanoma cells themselves. To test this possibility, we fractionated five cryopreserved cell suspensions using CD45-conjugated magnetic beads and measured IL-10 production in the CD45(+) (leukocyte-enriched) and CD45(−) (leukocyte-depleted, melanoma-enriched) fractions. IL-10 production was measured after overnight incubation of 2 × 10⁶ cells/microwell well. As shown in Fig. 4, in all five specimens the CD45(−) fraction produced more IL-10 than the CD45(+) fraction (P = 0.035). Moreover, in four of five samples, IL-10 production was increased by depletion of leukocytes.

Next, cryopreserved melanoma cell suspensions were thawed and then collected on cytopsin slides. Cytoplasmic IL-10 was determined by staining with mouse monoclonal anti-human IL-10 antibody using an immunoperoxidase method. A representative sample is shown in Fig. 5. IL-10 protein was readily detected in the cytoplasm of melanoma cells, although not all cells were positive. In contrast, IL-10 protein was not seen in tumor-associated lymphocytes or macrophages. We also stained frozen tissue sections from a s.c. melanoma metastasis. As shown in Fig. 6, IL-10 protein was detected in the cytoplasm of melanoma cells but was not apparent in leukocytes or stromal cells.

**Detection of IL-10 in Serum.** Sera from patients with stage III (resected lymph node metastases) and stage IV (unresectable bloodborne metastases) were assayed for IL-10. The results are shown in Fig. 7. Based on data obtained from healthy volunteers (mean IL-10 level, 3.6 ± 3.7 pg/ml; a serum IL-10 level >15 pg/ml (mean + 3 SD) was defined as abnormal. As shown in Fig. 7, 10 of 55 patients with clinically evident metastases showed elevations of circulating IL-10. All of these positive patients had bulky metastases (nine in viscera, one in skin). In contrast, only 2 of 31 patients who were clinically disease-free after resection of lymph node metastases showed slight elevation of serum IL-10. One of these two patients subsequently developed systemic metastases. Moreover, three patients who had been melanoma-free following resection of metastases developed high serum IL-10 levels concurrent with the appearance of distant metastases (Fig. 8). The sera of 12 patients with metastatic adenocarcinoma were also tested and all were in the normal range (Fig. 7).

In one patient, we were able to measure serum IL-10 levels at several times points and to correlate them with his clinical condition (Fig. 9). The patient had lymph node metastases removed in October 1990. He developed lung and skin metastases in July 1991 and received a DNP-conjugated autologous vaccine. He achieved a complete response and was disease free for 8 months. The patient developed recurrent melanoma, which was treated with debulking surgeries between March 1992 and September 1992, and two serum samples obtained during this period showed no elevation of IL-10 level. However, he subsequently developed new lung metastases and then a bulky stomach metastasis and did not respond to systemic chemotherapy or IL-2 treatment. At this point, the serum level of IL-10 dramatically increased and remained high until the time of his death.

**DISCUSSION.** To our knowledge, this is the first demonstration of the production of IL-10 protein in fresh metastatic melanoma tissues. It appears that melanoma cell suspensions from the vast majority of patients produce IL-10 during a brief in vitro incubation. These findings support our previous observation that mRNA for IL-10 was expressed in almost all metastatic mela-
There is now considerable evidence that mRNA for IL-10 is expressed by human cancer cells. The initial reports showed IL-10 mRNA and production of IL-10 protein in about one-third of established cell lines of a variety of histological types, including melanoma (11, 20). Our group was the first to demonstrate IL-10 message in melanoma tissue (12). Of 25 biopsies of lymph node and s.c. metastases, 24 were positive. Subsequently, a similar finding was reported by Krüger-Krasagakes et al. (14).

IL-10 mRNA has now been demonstrated in biopsies of ovarian cancer (21), adenocarcinoma of the kidney (22), gliomas (23), non-melanoma skin cancers but not benign skin lesions (24), non-small cell lung cancer (25), transitional cell carcinoma of the bladder (26), and lymphomas (27).

Production of IL-10 protein by human tumors has been less well documented, and most of the published studies were limited to established cell lines. For example, Chen et al. (11) and Krüger-Krasagakes et al. (14) demonstrated IL-10 protein production by 4 of 7 and 3 of 13 melanoma cell lines, respectively. Up to now, IL-10 protein had been identified conclusively in tumor tissues only from lung carcinoma (25, 28) and AIDS-related lymphomas (27).

Our data indicate that the high levels of IL-10 protein production by metastatic melanoma tissues are markedly reduced when the cells become adapted to tissue culture. Clearly, IL-10 is not required for melanoma cell proliferation in vitro, and its continued production in a minority of cell lines appears to be a remnant of a phenotype advantageous for growth and/or survival in vivo. This is in contrast to IL-8, an essential autocrine growth factor, which is produced by virtually all melanoma cell lines (29, 30) as well as in vivo.

Why IL-10 production should be advantageous to the growth and/or survival of melanoma metastases is unknown.
Fig. 6 Immunohistochemical localization of IL-10 in metastatic melanoma tissue. Frozen tissue sections prepared from a s.c. metastasis were stained with mouse monoclonal anti-human IL-10 antibody. The avidin-peroxidase complex method was used for chromogenic localization of IL-10 protein. A, anti-human IL-10 antibody (×400). Melanoma cells show intense cytoplasmic staining, while stromal cells appear negative. B, negative control (×400). An adjacent tissue section was stained with biotinylated anti-mouse antibody and avidin-peroxidase only.

However, given the potent suppressive effects of this cytokine on cell-mediated immunity, it is tempting to speculate that IL-10 allows tumor deposits to avoid or to modulate immunological attack. Evidence supporting this hypothesis is beginning to accumulate in experimental tumor systems. Gorelik et al. (31) reported that the MOPC-315 plasmacytoma produced IL-10, which interfered with the generation of tumor-specific cytotoxic T cells. Treatment with phenylalanine mustard caused a marked decrease in the production of IL-10, which preceded the immunological rejection of the tumor. Wang et al. (32) showed that immunization of mice with allogeneic tumor cells mixed with IL-10 led to suppression of immunity and allowed the allogeneic tumor to grow. Finally, Suzuki et al. (33) found that a murine melanoma genetically engineered to be immunogenic could be made to grow progressively by transducing the cells with IL-10 of viral origin; the immunosuppressive effect was reversible with anti-human IL-10 monoclonal antibody.

These models could explain the limited effectiveness of active immunotherapy of advanced human melanoma. For example, our laboratory has developed an autologous melanoma vaccine in which the tumor cells are modified with the hapten, DNP. Administration of DNP-vaccine often induces inflammatory responses in metastatic masses. These responses consist of marked erythema, warmth, and tenderness of superficial tumors and the overlying skin; microscopic examination of inflamed tumors shows melanoma cells infiltrated with T lymphocytes, the majority of which are CD8+ (34). In contrast, biopsies of s.c. melanoma metastases taken before vaccine treatment show,
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Serum IL-10 levels in metastatic melanoma patients. Serum samples were collected from melanoma patients with no evidence of disease after surgical removal of lymph node metastases (n = 31), patients with clinically evident metastases (n = 55), and randomly selected adenocarcinoma patients with systemic metastases (n = 12). Serum IL-10 levels were measured by ELISA assay. Each mark represents an individual patient.

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CD8+ T cells. T cells derived from inflamed tumors exhibit cell surface phenotypes indicative of activation. However, even in the face of this inflammatory response, the metastatic tumors rarely regress. It is possible that IL-10 allows the melanoma cells to minimize immunological damage by suppressing the proliferation and/or activation of T cells infiltrating the tumor site.

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(6). Administration of a vaccine consisting of irradiated, unmodified melanoma cells, especially in the absence of an adjuvant, could result in IL-10 production at the injection site leading to tumor-specific tolerance, rather than tumor-specific immunity. It was somewhat reassuring to find that DNP modification of melanoma cells abrogated IL-10 production in vitro, but the composition of cytokines elicited at tumor vaccination sites is largely unknown and is worthy of study.

The hypothesis that intratumor production of IL-10 down-regulates a potentially lethal antitumor immune response may turn out to be simplistic. Yet this idea has heuristic appeal and, if valid, profound clinical implications. New therapeutic agents that neutralize IL-10 or prevent its production, such as, antibodies, antisense oligonucleotides, or conventional drugs, could reverse tumor-induced immunosuppression and greatly enhance the efficacy of human cancer immunotherapy.

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REFERENCES


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