Antitumor Effects of Granulocyte-Macrophage Colony-Stimulating Factor Production by Melanoma Cells

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ABSTRACT

The use of immunomodulating gene therapy in the treatment of malignant disease is under intensive investigation. In this study, we examined the potential of melanoma-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) to inhibit melanoma progression in a murine model. The HFH18 murine melanoma cell line was transfected with the murine GM-CSF gene in a SV40 expression vector that resulted in melanoma clones that produced varying amounts of GM-CSF. Syngeneic mice inoculated s.c. with HFH18 parental melanoma cells or HFH18 cells transfected with the GM-CSF gene in the noncoding 3′-S′ orientation (HFH18/GM-CSF S′ S′ cells) develop large tumors that reach a mean tumor volume of 3300 mm3 by day 30. In contrast, animals inoculated with two melanoma clones producing high levels of GM-CSF (HFH18/GM-CSF S′ S′ and HFH18/GM-CSF S′ S′ S′ S′) either completely reject the tumor cells or develop tumors with a mean volume of only 40 mm3. In comparison, animals inoculated with a melanoma clone producing low levels of GM-CSF (HFH18/GM-CSF S′ S′ S′) develop large tumors averaging 2000 mm3, thus demonstrating a dose-response effect of tumor inhibition by melanoma-derived GM-CSF. Additionally, vaccination with irradiated GM-CSF-producing melanoma cells conferred optimal immunogenicity against a subsequent challenge with HFH18 cells. Tissue sections from excised GM-CSF-producing tumor cell inoculation sites but not from HFH18 parental or HFH18/GM-CSF S′ S′ inoculation sites demonstrate a dense inflammatory infiltrate composed of neutrophils, tissue macrophages, and numerous CD4 and CD8-positive lymphocytes but few melanoma cells. Large numbers of dendritic cells and cells expressing the B7–2 costimulatory molecule are detected only within HFH18/GM-CSF S′ S′ S′ melanoma inoculation sites. Our results lend further support to clinical trials of GM-CSF gene therapy in the treatment of advanced malignant melanoma, possibly by the recruitment of dendritic antigen-presenting cells.

INTRODUCTION

Malignant melanoma is responsible for the majority of skin cancer deaths. Surgical excision of thin primary cutaneous melanomas (<1 mm) is associated with greater than 95% survival rate. However, deep primary malignant melanoma (>4 mm) and melanoma metastatic to regional draining lymph nodes continue to have a 50–90% relapse and mortality rate. Once metastases have developed, standard chemotherapy has little effect on the course of the disease. Recent advances in the field of immunotherapy using active, adoptive, antigen-specific, and gene therapy approaches have generated great expectations to improve the treatment of advanced malignancies, including melanoma (1, 2). Immunologically mediated gene therapy approaches generally use the strategy of transfecting cDNAs encoding genetic material to the malignant cells to encode a particular gene product that is capable of augmenting the host immune responses against the developing tumor. Because of their immunomodulating activities, a number of cytokine genes have been transfected into various tumor cells and evaluated both in animal studies and more recently in human trials for antitumor effects (3–5). The advantage of this approach is the production of high levels of specific cytokines secreted in the vicinity of the tumor without the associated toxicities observed with systemic cytokine administration. These locally high concentrations of cytokines in the tumor environment may modulate host immune responses in a number of ways, including the augmentation of tumor antigen presentation by professional APCs and the activation of tumor-specific lymphocytes. Recently, our group studied the effect of tumor-derived IL-6 on the pathogenesis of malignant melanoma in an immunocompetent syngeneic murine host. The results of this study indicated that IL-6 exerts a significant inhibitory effect on cutaneous melanoma growth and progression (6). In the present study, we evaluated the effect of melanoma-derived GM-CSF on the pathogenesis of cutaneous melanoma in a syngeneic immunocompetent murine model system. Our results show that melanoma-derived GM-CSF significantly inhibits the growth of tumor cells and immunizes the host against a subsequent challenge with the parental melanoma cells to a much greater extent than IL-6. Immunohistochemical studies suggest that tumor-derived GM-CSF may be acting in part by the recruitment of large numbers of dendritic cells in the vicinity of tumor.

MATERIALS AND METHODS

Melanoma Cell Lines. The murine B16-derived malignant melanoma cell line HFH18, obtained from Dr. Funan Hu (Oregon Primate Center, Portland, OR: Refs. 7 and 8), was grown and maintained in DMEM cell culture media (Life Technologies, Inc., Grand Island, NY) supplemented with 5% calf serum. Transfected HFH18 cells were cultured in selective tissue culture media supplemented with 0.7 μg/ml geneticin (G418). Transfection of Melanoma Cells. The pEMBL plasmid containing murine GM-CSF cDNA was generously provided by Nicholas Gough (Melbourne, Australia; Ref. 9). A 558-bp BamHI fragment of pEMBL containing the entire murine GM-CSF coding sequence was subcloned into the BamHI site of the pZipNeo SV(X) expression vector (10). An asymmetric Pst I restriction site within the BamHI fragment was used to orient the plasmid. Plasmids were then constructed with: (a) the full coding sequence of GM-CSF cDNA in the sense 5′-3′ orientation, allowing the expression of GM-CSF protein; and (b) the non-GM-CSF-producing HFH18 parental melanoma cell line by electroporation, as described previously (6). Cells were then plated in nonselective media for 48 h and replated in media containing 0.7 μg/ml G418. Neomycin-resistant clones were selected with glass cloning rings after 10–14 days, expanded in cell culture, and screened for GM-CSF production as described below.

Measurement of GM-CSF Secretion. Secreted GM-CSF was measured in the cell culture supernatant by the FDCP-1 proliferation bioassay and ELISA (Endogen). The FDCP-1 cell line is a myeloid progenitor cell line that proliferates in response to IL-3 and GM-CSF (11). Cell proliferation was measured as a function of incorporated [3H]thymidine counts/min (± SE) in the presence of various concentrations of GM-CSF.
triplicate wells at dilutions ranging from 1:2 to 1:256, as described previously (11). The specificity of this bioassay for GM-CSF was determined by neutralization studies with antimurine GM-CSF and IL-3 antibodies (Genzyme, Cambridge, MA).

Melanoma Cell Proliferation Assays. To measure the in vitro proliferation of the parental melanoma cell line (HFH18) and of the transfected cell lines, cells were plated at a concentration of 1000 cells/cm² in triplicate wells of 6-well plates in DMEM with 5% calf serum. Cells were trypsinized and counted with a hemocytometer and Coulter counter (Coulter Electronics) at days 1, 3, and 6 to determine the number of cells/well. Separate experiments were performed to determine the effect of GM-CSF or anti-GM-CSF antibody on the in vitro growth of the parental and transfected melanoma cell lines. In addition, the effect of supernatants from transfected cells on parental HFH18 proliferation was determined. In these studies, cells were treated with recombinant murine GM-CSF (100 units/ml; R&D Systems, Inc., Minneapolis, MN) or media alone every 24 h; with neutralizing antimurine GM-CSF antibody (10 μg/ml; Genzyme) or media alone every 48 h; or with dilutions (ranging from 1:4 to 1:64) of supernatants collected from HFH18/GM-CSF*+* and HFH18/GM-CSF*+* cells or media alone every 24 h. The dose of anti-GM-CSF antibody was in excess of the concentration demonstrated to neutralize GM-CSF activity by the transfected cells in prior studies.

Flow Cytometric Analysis. The parental melanoma HFH18 cells and the GM-CSF-transfected HFH18 melanoma cells were analyzed for expression of class I (H-2Kb) and class II (I-Ab) antigens using the monoclonal antibodies: monoclonal rat antimurine CD-4 (PharMingen); monoclonal rat antimurine CD-8 (PharMingen); monoclonal rat antimurine dendritic cell (NLDC-145, Bachem, Bioscience Inc.); and monoclonal rat antimurine B7-1 (PharMingen). Control cells were treated with FITC-mouse IgG (secondary antibody only) to determine background fluorescence or a FITC-mouse IgG irrelevant antibody. Flow cytometric analysis was determined by a FACScan flow cytometer (Becton Dickinson).

Animals. Specific pathogen-free 5- to 6-week-old female C57BL/6 mice were obtained from The Jackson Laboratory (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 (HFH18 and transfected HFH18 cells) were trypsinized when they approached log-phase growth, were washed twice in PBS, and resuspended in PBS for injection. The number of viable cells was determined by the trypsin blue exclusion method. Suspensions of 1 x 10⁶ cells in 0.2 ml PBS were inoculated s.c. into the anterior right flank of mice. Tumor volume was determined by measuring tumors in methoxyflurane-anesthetized animals in three perpendicular diameters with calipers with tumor volume calculated as ½ x (length x width x height) (Ref. 12). Differences in tumor size between groups were determined by a two-way ANOVA. Differences in survival between groups were determined by life table analysis.

Vaccination Experiments. Melanoma cells (HFH18- and HFH18-transfected cells) were cultured and prepared for injection as described above. Before injection, the cells were irradiated with 3500 rads from a 137Cs source (150 rads/min) and injected at a concentration of 1 x 10⁶ in 0.2 ml PBS into the right anterior flank of recipient mice. Fourteen days later, animals were challenged in the left anterior flank with 1 x 10⁶ nonirradiated HFH18 parental melanoma cells. To study in vitro GM-CSF production after irradiation, 2 x 10⁶ HFH18- and HFH18-transfected cells were plated and maintained in culture for 12 days with cell supernatants collected and new culture media added every 24 h. GM-CSF production was measured in the supernatants with the FDCP-1 bioassay and ELISA.

Histology and Immunohistochemistry. For histological studies, sections of melanoma tissue were fixed in 10% formalin, embedded in paraffin, stained with H&E, and examined by light microscopy. Immunohistochemical studies were performed with sections of melanoma tissue or excised inoculation sites snap frozen and sectioned in 4-μm sections on a cryostat (Minotone; Minot Custom Microtome, International Equipment Company), fixed in cold 100% acetone for 5 min, washed three times in PBS, and incubated in blocking sera for 30 min. Tissue sections were incubated for 60 min with the following antibodies: monoclonal rat antimurine CD-4 (PharMingen); monoclonal rat antimurine CD-8 (PharMingen); monoclonal rat antimurine dendritic cell (NLDC-145, Bachem, Bioscience Inc.); and monoclonal rat antimurine B7-1 (PharMingen). After two washes with PBS, sections were stained according to the avidin-biotin complex immunoperoxidase method (ABC Vectastain; Vector Laboratories). In this procedure, sections were incubated for 60 min in an antirat biotinylated antibody. After two washes, quenching of endogenous peroxidase was performed by incubating the sections for 30 min in a solution of 3% hydrogen peroxide and 10% sodium azide (1 m) in methanol. After four washes in PBS (20 min), there was a 30-min incubation in the avidin- immunoperoxidase conjugate. The slides were then washed and incubated in a 3,3′-dimethylaminobenzene solution for 12 min, counterstained with Gill’s hematoxylin, and mounted. As a negative control, an additional slide of each specimen received nonimmune serum in place of the primary antibody. Spleen sections incubated with the anti-CD4, anti-CD8, NLDC-145, and anti-B7-2 antibodies served as positive controls.

In a separate set of experiments, immunohistochemistry was performed on monolayers of cultured parental and GM-CSF-transfected HFH18 melanoma cells using monoclonal rat antimurine GM-CSF antibody (Genzyme) to evaluate GM-CSF production. In these studies, 1% paraformaldehyde in PBS was used as a fixative, and permeabilization of the cells was accomplished with 0.1% saponin added in each step (13).

RESULTS

The Establishment and Characterization of GM-CSF-producing Melanoma Cell Lines. To generate GM-CSF-producing murine melanoma cells, a murine GM-CSF cDNA expression vector was transfected into the non-GM-CSF-producing parental HFH18 melanoma cell line. A transfected clone designated HFH18/GM-CSF*++* secretes constitutively the highest levels of GM-CSF, as determined in the FDCP-1 bioassay, and thus was selected for further in vivo studies (Fig. 1). Two additional clones that secrete high and low GM-CSF levels in this bioassay were also selected for in vivo studies and designated HFH18/GM-CSF*++* and HFH18/GM-CSF*++*, respectively (Fig. 1). The GM-CSF activity secreted by the tumor cells can be specifically blocked by the addition of anti-GM-CSF antibody but not by anti-IL-3 antibody in the FDCP-1 bioassay (data not shown). Parental HFH18 cells or HFH18 cells transfected with the vector in which the GM-CSF gene is in the antisense orientation [HFH18/GM-CSF*−−*] secrete no constitutive GM-CSF (Fig. 1). Immunohistochemistry studies performed on monolayers of HFH18 parental, HFH18/GM-CSF*++*, and HFH18/GM-CSF*−−* cells using an anti-GM-CSF antibody demonstrate positive staining only in HFH18/GM-CSF*++* cells (data not shown). GM-CSF secretion by the transfected clones was quantitated further in 48-h supernatants by ELISA. HFH18/GM-CSF*+++ cells secrete approximately 50 ng/ml/10⁶ cells.

Fig. 1. GM-CSF secretion by GM-CSF-transfected murine melanoma cell lines. Supernatants were collected from the HFH18 parental cell line; three HFH18 clones transfected with the pEMBL plasmid with GM-CSF cDNA in the sense orientation HFH18/GM-CSF*++*, and an HFH18 clone transfected with pEMBL with GM-CSF cDNA in the antisense orientation [HFH18/GM-CSF*−−*]. Serial dilutions of 48-h melanoma cell supernatants were assayed for secreted GM-CSF bioactivity in the FDCP-1 proliferation bioassay. These data represent triplicate experiments.

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cells, HFH18/GM-CSF*++ cells secrete approximately 30 ng/ml/10⁶ cells, HFH18/GM-CSF*+ cells secrete approximately 2 ng/ml/10⁶ cells, and HFH18/GM-CSF*− cells secrete no GM-CSF.

To assess whether the transfection process itself or GM-CSF production by the HFH18/GM-CSF*++ cells had a direct effect on cell growth in vitro, the proliferation rate of the transfected cells was compared with that of the parental melanoma line. In these studies, the in vitro proliferation rate of the GM-CSF-producing melanoma cells does not differ significantly from that of the parental HFH18 cell line or the HFH18/GM-CSF*− line (data not shown). In addition, the in vitro proliferation of the HFH18 parental cell line is not affected by the addition of recombinant murine GM-CSF as measured by direct cell counts. Furthermore, the in vitro proliferation of the transfected HFH18/GM-CSF*++ cell line is not altered by the addition of neutralizing antimume GM-CSF antibody to the cultured cells. Thus, GM-CSF does not have a direct inhibitory effect on the proliferation of these melanoma cell lines. To determine whether other factors secreted by the GM-CSF-transfected melanoma cells could directly affect parental HFH18 melanoma cell proliferation, dilutions of 48-h supernatant collected from HFH18/GM-CSF*++ and HFH18/GM-CSF*− cells were added to cultured parental HFH18 cells. These conditioned cell supernatants have no effect on parental HFH18 cell proliferation. Therefore, neither GM-CSF nor a secondarily induced melanoma-derived factor directly inhibits tumor cell growth.

Tumor cell class I and II MHC molecule expression may have a profound effect on host immune responses. Thus, we examined the effect of GM-CSF transfection on the expression of these cell surface glycoproteins. Flow cytometric analysis demonstrates that the parental HFH18 melanoma cell line and the GM-CSF-transfected cell line [HFH18/GM-CSF*+++] express low levels of class I (H-2K*) and class II (I-A*) antigens that are not altered by transfection of GM-CSF (data not shown).

**Effect of Tumor-derived GM-CSF on Melanoma Growth.** The role of GM-CSF in modulating HFH18 melanoma growth in syngeneic C57BL mice was studied. Mice inoculated with either the HFH18 parental melanoma cell line or HFH18 cells with the GM-CSF gene in the antisense orientation [HFH18/GM-CSF*− cells] develop tumors that reach a mean tumor volume of 3250 mm³ at day 30 after tumor cell injection, as determined in three separate experiments with eight mice inoculated with each cell type per study (Fig. 2A). In contrast, mice injected with the HFH18 melanoma cells producing high levels of GM-CSF [HFH18/GM-CSF*+++] develop tumors that reach a mean volume of only 40 mm³ at this 30-day time point (Fig. 2A). Significantly, more than 40% (survivors/total) of these animals completely reject the tumor cells and develop no tumors when followed up to 100 days after melanoma cell inoculation.

The effect of different levels of GM-CSF production by the tumor cells on tumor growth was then examined by injecting mice with GM-CSF-transfected HFH18 melanoma cells that secrete different amounts of GM-CSF. Only mice inoculated with cells from clones with high GM-CSF-secreting capacity [HFH18/GM-CSF*+++] and HFH18/GM-CSF*+++] were able to completely reject the tumor cells or developed small tumors with an average volume of less than 100 mm³ at day 30 (Fig. 2B). In contrast, mice injected with cells from a transfected HFH18 clone secreting lower amounts of GM-CSF [HFH18/GM-CSF*− cells] develop tumors comparable in size with those produced by the HFH18 parental melanoma cells or the cells transfected with GM-CSF gene in the antisense orientation [HFH18/GM-CSF*−− cells]. Thus, GM-CSF-transfected melanoma cells that secrete low GM-CSF levels behave biologically like HFH18 parental melanoma cells or HFH18/GM-CSF*−− cells. These results suggest a threshold level of GM-CSF secretion by the tumor cells to elicit an effective host response capable of inhibition or abrogation of the growth of melanoma cells.

Survival studies were then undertaken to compare mice inoculated with either GM-CSF-secreting or -nonsecreting tumor cells. Differences in survival between mice injected with GM-CSF-secreting melanoma cells and those injected with non-GM-CSF-secreting HFH18 cells [parental HFH18 melanoma cells and HFH18/GM-CSF*− cells] are illustrated in Fig. 3. Whereas mice injected with the non-GM-CSF-secreting HFH18 cell lines have a median survival of only 32 days, those injected with the HFH18/GM-CSF*++ cells line that develop tumors have a median survival of 60 days, and those that did not develop tumors (42% of mice) are alive and healthy after 100 days. We are currently studying factors that may contribute to differences in the immune response in these two groups of mice. These studies indicate that GM-CSF secretion by the HFH18 melanoma cell line dramatically inhibits tumor growth and progression in recipient syngeneic mice.

**Vaccination with GM-CSF-producing Melanoma Cells.** Experiments were conducted to test the ability of GM-CSF-producing melanoma cells to protect mice from a subsequent challenge with parental melanoma cells. Continued high levels of GM-CSF production by irradiated HFH18/GM-CSF*++ cells were verified by the FDCP-1 bioassay and ELISA of supernatants from replated cells for
more than 14 days after irradiation (data not shown). As expected, HFH18 parental cells produce no GM-CSF after irradiation. Sixty % of the mice vaccinated with irradiated HFH18/GM-CSF<sup>+++</sup> cells are able to completely reject the challenge injection of nonirradiated parental HFH18 cells, whereas 20% of those mice vaccinated with irradiated HFH18 parental melanoma cells survive without tumors (Fig. 4). As expected from prior immunogenicity studies with HFH18 parental cells, this study demonstrates that vaccination with irradiated HFH18 parental melanoma cells confers some protection to the mice. However, vaccination with HFH18 melanoma cells that secrete GM-CSF increases significantly the ability of mice to reject a subsequent challenge with the parental melanoma cells. Furthermore, the tumor-free survivors were able to completely reject a rechallenge with nonirradiated parental HFH18 cells at day 100, suggesting that immunological memory persists long term.

**Histological Examination of Melanomas.** To evaluate the inflammatory cell infiltrate of the cutaneous tumors, H&E-stained tissue sections from excised tumor cell inoculation sites were examined at different time points (days 2, 6, 10, 15, and 25) after s.c. injection of HFH18 parental, HFH18/GM-CSF<sup>+++</sup>, and HFH18/GM-CSF<sup>---</sup> melanoma cells. By day 2, injected HFH18/GM-CSF<sup>+++</sup> melanoma cells were mixed with a very dense inflammatory cell infiltrate com-
Fig. 5. Histological examination of GM-CSF-producing melanomas. Tumor cell inoculation sites excised at days 2 and 6 from C57BL/6 mice inoculated with HFH18/GM-CSF<sup>+++</sup> melanoma cells (A and B) or HFH18 melanoma cells (C and D) were examined histologically after H&E staining. These photomicrographs represent six mice injected with each tumor cell type.

posed of numerous neutrophils, which in some areas constitute microabscesses (Fig. 5A). There are also numerous eosinophils, lymphocytes, and tissue macrophages. At days 6 and 10, an increasing number of lymphocytes and tissue macrophages are detected infiltrating the GM-CSF-secreting tumor tissue with complete replacement of tumor cells by inflammatory infiltrate in some mice (Fig. 5B). In mice inoculated with HFH18/GM-CSF<sup>+++</sup> cells that do develop small palpable tumors between days 15 and 30, the histological examination reveals a solid collection of neoplastic cells with a sparse inflammatory infiltrate arranged at the periphery of the tumor (data not shown). In contrast, the injected HFH18 parental and HFH18/GM-CSF<sup>−−</sup> cells are associated with a sparse inflammatory infiltrate at days 2 and 6 (Fig. 5, C and D). Only a sparse infiltrate is observed in tissue examined through day 25 (data not shown). The inflammatory cells in these infiltrates consist of only lymphocytes and tissue macrophages at the periphery of the enlarging tumor mass and are not interspersed within the tumor tissue.

Immunophenotyping within Melanoma Cell Inoculation Sites. To characterize further the inflammatory cell reaction at sites of melanoma cell inoculation, immunophenotyping was performed. Excised HFH18/GM-CSF<sup>+++</sup> cell inoculation sites show a dense infiltrate of CD4<sup>+</sup> cells intermingled with the neoplastic cells by immunohistochemistry at day 10. These CD4<sup>+</sup> lymphocytes are observed infiltrating all areas of the tumor mass. Fewer positive CD8<sup>+</sup> cells are observed within GM-CSF-producing melanomas. In contrast, tissue staining from excised parental HFH18 parental and HFH18/GM-CSF<sup>−−</sup> cell inoculation sites shows rare CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes at the periphery of the tumors (data not shown). Immunostaining with a dendritic cell antibody (NLDC-145) shows numerous dendritic cells in sections from HFH18/GM-CSF<sup>+++</sup> cell inoculation sites at early time points (Fig. 6A). These dendritic cells are arranged mainly at the periphery of the tumor in the areas where the CD4<sup>+</sup> lymphocytes are most numerous. Additionally, immunostaining with an antibody against the costimulatory molecule B7<sup>−2</sup> shows numerous positive cells in those areas with a dense infiltrate of CD4<sup>+</sup> lymphocytes and dendritic cells (Fig. 6B). In contrast, only occasional dendritic cells were observed in sections from parental HFH18 and cell inoculation sites, and the B7<sup>−2</sup> costimulatory molecule was rarely expressed by the inflammatory cells located around these tumors (Fig. 6, C and D). These same low numbers of dendritic cells and cells expressing the B7<sup>−2</sup> ligand are observed in HFH18/GM-CSF<sup>−−</sup> cell inoculation sites (data not shown).

DISCUSSION

A number of recent studies demonstrate that GM-CSF has a potentially important antineoplastic activity that appears to be mediated through stimulation of the immune system (14–18). Melanoma is one of the malignancies in which the effects of this cytokine have been studied. In a preclinical study, GM-CSF has been shown recently by Dranoff et al. (14) to induce the most potent systemic antitumor response using murine melanoma cells transfected with genes for a number of different cytokines. Several human studies are currently in progress to evaluate the antitumor effects of GM-CSF delivered both systemically and by gene transduction against melanoma, renal cell carcinoma, and prostate carcinoma (19). Additionally, in a recent
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Phase I study, systemically delivered GM-CSF combined with the murine monoclonal antibody R24 in patients with metastatic melanoma led to a statistically significant enhancement of monocyte and granulocyte direct cytotoxicity and antibody-dependent cellular cytotoxicity (20). Our results demonstrating the dose-dependent inhibition of melanoma growth and progression and induction of systemic immunity that is clearly superior to the inherent immunogenicity of the melanoma cells by GM-CSF-transfected murine melanoma cells expand the current understanding of the role of GM-CSF in immunomodulating neoplastic therapy. Our data suggest that the GM-CSF-mediated antitumor effect may be due in part to the recruitment of dendritic APCs within the first 6 days after melanoma cell inoculation.

GM-CSF has multiple biological activities, including the ability to stimulate the proliferation and differentiation of multipotential bone marrow progenitor cells into mature granulocytes and macrophages. There is evidence that GM-CSF stimulates the phagocytic and cytotoxic activity of macrophages (21, 22) and promotes monocyte and neutrophil antibody-dependent cellular cytotoxicity (23, 24). GM-CSF has been shown to be a major factor in the maturation of cells of dendritic cell/Langerhans cell lineage into potent activators of resting T cells (25–27). The ability of dendritic APCs to stimulate naïve T cells in the primary mixed lymphocyte reaction is increased greatly after culture in GM-CSF (28–30). Kaplan et al. (31) have shown that the intradermal injection of the skin with recombinant GM-CSF leads to a selective recruitment of Langerhans cells into the dermis. The accumulation of Langerhans cells in response to the local production of GM-CSF has been demonstrated in other organs, such as the lungs (32). Some studies suggest a correlation between the number of tumor-infiltrating APCs and clinical prognosis (33, 34). A close correlation has been observed between the number of Langerhans cells infiltrating lung carcinoma and the local production of GM-CSF (32). In several experimental systems, dendritic cells are capable of presenting tumor antigen for the generation of tumor-specific immunity (35–37). Grabbe et al. (35) have demonstrated that short-term culture with GM-CSF of freshly isolated epidermal Langerhans cells exposed to soluble tumor fragments led to the capacity of these cells to induce protective tumor immunity in vivo. This T-cell immunostimulatory capacity may also be related to the observed up-regulation in the expression of B7–1 and B7–2 costimulatory molecules on dendritic cells that have been cultured in GM-CSF (38). Thus, there is considerable evidence that GM-CSF plays a crucial role in T-cell-mediated immune response by recruiting and activating functional APCs.

In the current study, our results confirm and expand the potent antimelanoma effects of GM-CSF observed previously by Dranoff et al. (14) in a similar murine melanoma model system with some important differences. In that study, the B16F10 murine melanoma cell line was transfected with a number of different cytokines and other immunomodulators to compare their ability to stimulate a systemic antitumor immunity. Irradiated GM-CSF-secreting B16F10 melanoma cells provided the most potent antitumor response (14). However, in contrast to the results of our study, the authors reported that nonirradiated melanoma cells transfected with GM-CSF grew progressively in vivo and induced a fatal toxicity manifested by profound leukocytosis, hepatosplenomegaly, and pulmonary hemorrhage. We observed no systemic toxicities in mice bearing live GM-CSF-transfected melanomas producing comparable levels of GM-CSF. This difference may be explained in part by the use of a different murine melanoma B16 derivative cell line. Because mice bearing live GM-CSF-transfected melanoma cells did not suffer from systemic...
toxicity in our system, we were able to use this model to demonstrate a dose-response to melanoma-derived GM-CSF and the need for certain minimal levels of GM-CSF, below which the growth of the transfected melanoma cells is indistinguishable from that of parental melanoma cells. We have demonstrated further that neither GM-CSF nor a secondarily induced melanoma-derived factor directly inhibits melanoma cell growth. The observation in our model system that GM-CSF transfection can significantly decrease tumor growth in nonirradiated melanoma cells and stimulate a systemic antitumor response using irradiated melanoma cells provides even greater support for the use of this cytokine in immunotherapeutic trials.

Dendritic APCs were observed at inoculation sites of GM-CSF-producing melanoma cells by immunohistochemistry using the anti-dendritic cell antibody NLDC-145 and anti-B7-2 antibody. Whereas other investigators have speculated on the potential role of professional APCs in the mechanism of GM-CSF antitumor immune reaction, the actual observation of these cells surrounding GM-CSF-producing melanoma cells has not been reported previously (3, 14, 39). The early influx of neutrophils and eosinophils mediated by the high local concentration of GM-CSF may induce a nonspecific inflammatory reaction and result in the lysis of tumor cells with the release of tumor antigens that can be picked up by the host dendritic APCs recruited to the tumor cell inoculation site by GM-CSF. The NLDC-145 antibody used in our studies to identify dendritic cells has been reported recently to recognize the DEC-205 endocytotic receptor on dendritic cells that directs captured antigens from the extracellular space to a specialized antigen-processing compartment (40). The observation that the B7-2 costimulatory molecule is also expressed on dendritic cells in the inoculation sites of GM-CSF-producing melanoma cells suggests that these APCs may stimulate the activation and proliferation of T-effector cells. Because HFH18 melanoma cells do not themselves express high levels of class I or II MHC molecules that can present tumor antigens, the presence of a minimum number of professional APCs may be necessary to mount an efficient immune response against the tumor. We are currently testing directly the functional activities of these dendritic cells in the tumor-bearing animals.

Recent immunotherapy approaches have attempted to improve tumor antigen presentation by genetic modification of the tumor cells based on the hypothesis that tumor cell antigens are not presented efficiently to lymphocytes, which leads to an ineffective tumor-specific immune response (41, 42). These genetically engineered tumor cells may lead directly or indirectly to more efficient presentation of tumor antigens. Several studies have demonstrated direct antigen presentation by tumor cells transfected with costimulatory molecules (e.g., B7) or MHC molecules (42-44), although host APCs may still be required (45). Our results provide evidence in support of the hypothesis that tumor-secreted GM-CSF is acting through the indirect mechanism of host APC activation and recruitment (3, 14, 39). The success of these approaches in achieving induction of a potent systemic immunity against tumor cells in an experimental animal model awaits validation in a human context.

REFERENCES

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