ABSTRACT

Murine squamous cell carcinoma (SCCVII) cells were genetically engineered to produce murine granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF immunotherapy, based on the peritumoral injection of lethally irradiated GM-CSF-producing SCCVII cells, was examined as adjuvant to photodynamic therapy (PDT) treatment of this tumor. The GM-CSF immunotherapy administered three times in 48-h intervals, starting 2 days before the light treatment, substantially improved the curative effect of Photofrin-mediated PDT. A comparable effect of GM-CSF immunotherapy was observed in the combination with benzoporphyrin derivative-mediated PDT. The tumor-localized GM-CSF immunotherapy alone had no obvious effect on the growth of parental SCCVII tumors. This treatment did not significantly alter the differential peripheral WBC count and appeared not to affect tumor leukocyte infiltration. However, GM-CSF treatment did increase the cytotoxic activity of tumor-associated macrophages against SCCVII tumor cells. It appears, therefore, that tumor-localized immune stimulation by GM-CSF amplifies a PDT-induced antitumor immune reaction, which has a potentiating effect on tumor control.

INTRODUCTION

Eradication of tumors treated with PDT is a result of combined effects on both tumor cells and various host-derived cell types (1). In addition to direct killing induced by phototoxicative damage to vital cellular structures (2), tumor cells appear to be inactivated not only by ischemia secondary to the damage to tumor vasculature, but also by the integrated tumorical activity of nonspecific and specific immune effector cells (3). The host response triggered by the PDT treatment is dominated by a strong tumor-localized acute inflammatory reaction associated with the functional activation of tumor resident and newly arrived leukocytes (4). Neutrophils, mast cells, monocytes, and macrophages have been suggested to participate in the antitumor activity in this early phase after PDT treatment (3, 4). The inflammation of tumor tissue induced by PDT is accompanied by intense tumor destruction followed by the release and phagocytosis of tumor cell debris. This creates conditions for the processing and presentation of tumor antigens by macrophages and dendritic cells or other professional antigen-presenting cells, resulting in development of tumor-specific immunity (3, 5). Indeed, experimental evidence obtained in our laboratory (6) and elsewhere (7) supports the hypothesis that specific T-cell-mediated immune responses facilitate the eradication of tumors following PDT.

Tumor regression induced by radiotherapy, chemotherapy, or surgical excision is generally not associated with induction of systemic antitumor immunity. In contrast, PDT appears to have the capacity to reset the tumor-host immune relationship from tumor dominated to antitumor oriented. This justifies the development of specific strategies for the potentiation of the antitumor effect of PDT by adjuvant immunotherapy. One obvious type of immunotherapeutic intervention that could be considered is to augment the activity of myeloid effector cells at the treated site. Neutrophils, which massively and rapidly accumulate in PDT-treated tumors (4), have the capacity to inflict substantial damage on tumor tissue by releasing reactive oxygen metabolites and a number of other tissue destructive mediators or by attracting other immune cells to the tumor site. Macrophages isolated from PDT-treated tumors were shown to exhibit elevated tumoricidal activity (4). Increased macrophage activity after PDT in vitro has also been documented (5, 8). The activation of these cells by systemic treatment with a specific macrophage-activating factor generated from vitamin D₃-binding protein was shown to potentiate the curative effect of PDT (9). Macrophages were shown to release TNF-α following PDT treatment (10). In addition, macrophages appear to preferentially recognize and destroy PDT-treated tumor cell targets (11).

Communication among granulocytes, macrophages, and lymphocytes, in which the participants receive and/or deliver regulatory messages through the secretion of cytokines and other regulatory factors, builds up and amplifies the antitumor immune reaction (12). The localized production of some cytokines within the tumor microenvironment can prevent or inhibit tumor growth by a T-cell-independent mechanism, whereas other cytokines can stimulate a potent systemically acting T-cell-mediated antitumor immune response (13). One of the latter is GM-CSF, which is a key regulator controlling the maturation and function of granulocytes and monocytes/macrophages (14). This glycoprotein stimulates the proliferation and differentiation of dendritic and other antigen-presenting cells, and is a potent enhancer of antigen presentation by these cells (15–17). The protection against challenge with parental tumors induced by immunization with a GM-CSF-producing tumor cell vaccine was clearly shown to be mediated by CD4⁺ and CD8⁺T cells (18, 19). This cytokine is one of the immunotherapeutic agents whose effectiveness is currently under intense investigation both clinically and with in vivo tumor models (13, 18). A number of groups are currently examining the therapeutic potential of combining cytokine immunotherapy (including GM-CSF) with conventional modalities such as radiotherapy and chemotherapy. Although an area of active investigation, little has as yet been published, except for several articles showing that systemic interleukin 2 improves tumor radiotherapy (e.g., Ref. 20).

Since systemic treatment with high doses of GM-CSF may lead to profound perturbations in hemopoiesis and induce serious side effects (14) and i.v. injected GM-CSF  has a relatively short half-life (~2 h), it would be preferable if a means could be developed to achieve a sustained local release of this cytokine at the tumor site. This can be accomplished by the constitutive release of GM-CSF from peritumorally injected lethally irradiated tumor cells transfected with the gene encoding this cytokine (18). Using such an approach, we introduced the murine GM-CSF cDNA into SCCVII tumor cells (mouse squamous cell carcinoma), and, in combination with PDT, these genetically engineered cells were used for the GM-CSF immunotherapy of mice bearing the parental noninfected SCCVII tumor. Data observed

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2 To whom requests for reprints should be addressed. Phone: (604) 877-6010, ext. 3044; Fax: (604) 875-6857.
3 The abbreviations used are: PDT, photodynamic therapy; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; TAM, tumor-associated macrophage; TNF-α, tumor necrosis factor α; SFPE, saline-sodium-EDTA; BFD, benzoporphyrin derivative.

Potentiation of Photodynamic Therapy-elicited Antitumor Response by Localized Treatment with Granulocyte-Macrophage Colony-stimulating Factor

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Table 8 Respiratory gas exchanges before and during maximum exercise on treadmill

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<th>Treatment</th>
<th>Follow-up (months)</th>
<th>ANOVA</th>
<th>P02—4</th>
<th>6—5</th>
<th>10—3</th>
<th>30</th>
<th>Oxygen uptake-R (mI/mm)</th>
<th>Pulse rate-R (beats/mm)</th>
<th>Exercise capacity-M (W)</th>
<th>Pulserate-M (beats/mm)</th>
<th>Oxygen uptake-M (mI/mm)</th>
<th>Carbon dioxide production-M (mI/mm)</th>
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ERYTHROPOIETIN AND WEIGHT LOSS IN CANCER PATIENTS

(' Values are mean ± SE. NS, not significant; I, indomethacin; E, indomethacin + erythropoietin; -R, resting condition; -M, maximum exercise; LBM, lean body mass. b Compares all observations between study and control patients. C Number of patients. d I vs. E, P < 0.10. C I vs. E, P < 0.05. '@l vs. E, P < 0.01.

The results in this study may suffer from the limitation that only 108 patients were randomized for intervention and follow-up. Our estimations, based on previous studies on nutritional, biochemical, and functional evaluations, have, however, indicated that the investi...
Macrophage Cytotoxicity. Mice bearing SCCVII tumors received peritumoral injections of $1 \times 10^7$ lethally irradiated SCCVII/JzGM-CSF or SCCVII/Jzneo cells. The mice were sacrificed 48 h later, and the tumors were excised and dissociated into single-cells suspension by an enzymatic digestion procedure used routinely in our laboratory (28). TAMs were isolated from these cell suspensions using a modification of the differential attachment procedure described previously (4, 29). Briefly, the ability of macrophages to attach firmly to a plastic substrate much more rapidly than the other cells present in SCCVII tumors was exploited for obtaining highly purified TAM populations. Tumor cell suspension was resuspended in RPMI 1640 supplemented with 10% FBS, and aliquots containing $6.9 \times 10^7$ cells (of which approximately $2 \times 10^7$ cells were expected to be TAMs) were transferred into the wells of a 24-well plate. After a 30-min incubation at 37°C, the medium was replaced with 0.5 ml trypan-EDTA solution (Sigma Chemical Co., St. Louis, MO) and incubated for 30 s at room temperature. This was followed by washing the attached cells vigorously three times with 1 ml HBSS. The washout containing removed cells was collected and pooled for each sample. The number of TAMs remaining in the wells was determined by subtracting the number of cells removed by washing from the number of plated cells. The TAMs were then incubated with lipopoly saccharide from Escherichia coli 011:B4 (Sigma) at 10 μg/ml in RPMI 1640 +10% FBS (serum decomplemented) for 24 h.

The target cells (SCCVII tumor cells maintained in vitro for 6 to 8 weeks) were added to the wells containing TAMs at a ratio of 20:1. Before that, the target cells were prelabeled by exposure to [methyl-3H]thymidine (2.0 Ci/mmol; New England Nuclear) at 2 μCi/ml for 24 h in cell growth medium and then reincubated for 4 h with actinomycin D (1.5 μg/ml; Sigma). The samples with effector and target cells were incubated for 72 h at 37°C, and the supernatants were then collected for the measurement of radioactivity released from killed SCCVII cells as described in detail earlier (11). All experiments were carried out in quadruplicate (four wells for each TAM population).

RESULTS

GM-CSF Production by SCCVII/JzGM-CSF Cells. Northern blot analysis of total RNA isolated from SCCVII/JzGM-CSF and SCCVII/Jzneo cells is shown in Fig. 1. Upper panel confirms the presence of GM-CSF mRNA in SCCVII cells transduced with the JzGM-CSF retrovirus and shows that this message is not present in control cells infected with JzenhKneo virus. Lower panel shows that in SCCVII/JzGM-CSF-derived RNA, the neo' probe recognized a transcript of approximately 3500 bp initiated within the retroviral long terminal repeat and encoding both GM-CSF and neo', whereas the SCCVII/Jzneo cells expressed a shorter neo' message, initiated by the internal Tk promoter.

The production of GM-CSF protein by SCCVII/JzGM-CSF cells was tested with a bioassay using cytokine-dependent B6SuT-A cells. The conditioned media from SCCVII/JzGM-CSF cells induced a proliferative response of B6SuT-A cells, whereas the conditioned media from both parental SCCVII cells and SCCVII/Jzneo cells showed no support of the growth of B6SuT-A cells (Fig. 2). A comparison to the response achieved with known concentrations of recombinant GM-CSF (Fig. 2, inset) indicates that the SCCVII/JzGM-CSF cell conditioned medium contained approximately 400 ng/ml GM-CSF released/10^6 cells over a 24-h period.

Blood Leukocyte Content in GM-CSF-treated Mice. Blood smears were prepared at 2, 4, and 6 days following single or multiple treatment with $1 \times 10^7$ lethally irradiated GM-CSF-producing SCCVII cells administered s.c. in the dorsal area of C3H/HeN mice (otherwise the site of SCCVII tumor implantation). Following Wright staining, a differential WBC count was performed based on 200 nucleated cells/smear (five mice per group). The only difference noted was an increase in the average monocyte content from 7 ± 3% in control mice to 13 ± 6% in mice examined 6 days after the last of three injections of SCCVII/JzGM-CSF cells that were given in 48-h intervals; this change, however, was not statistically significant. The blood content of neutrophils, other granulocytes, and lymphocytes remained unchanged. Injection of SCCVII/Jzneo cells had no detectable effect on total and differential blood cell counts (data not shown).

Effect of GM-CSF Treatment on Growth of SCCVII Tumors. Mice bearing a parental SCCVII tumor received peritumoral injections of $1 \times 10^7$ lethally irradiated SCCVII/JzGM-CSF cells at the
time when the largest tumor diameter reached 5–6 mm. The tumor volumes were then determined every second day. All treated mice developed edema around the tumor, which persisted for 2 to 4 days after the injection of GM-CSF-releasing cells. However, this treatment, as well as the injection of lethally irradiated SCCVII/Jzneo or parental SCCVII cells, had no significant effect on the rate of growth of the SCCVII tumors (compared to nontreated tumors) and did not affect the survival of the mice (data not shown).

In other experiments, $1 \times 10^7$ lethally irradiated SCCVII/JzGM-CSF cells were injected s.c. into the area of tumor implantation 2 days after the inoculation of parental SCCVII cells. Even in this situation, when the tumor mass is very small (similarly as in the case with the reduced tumor burden after PDT), the GM-CSF treatment had no visible effect on tumor growth (data not shown).

The effect of GM-CSF treatment on the levels of tumor-infiltrating leukocyte populations in the SCCVII tumors was also examined. Mice bearing SCCVII tumors (of a similar size as used for PDT) received peritumoral injections of $1 \times 10^7$ lethally irradiated SCCVII/JzGM-CSF cells, and the tumors were excised 48 h later and analyzed for the content of malignant and other cell populations. This was determined by flow cytometry analysis of cells dissociated from the tumor tissue and stained with monoclonal antibodies against specific membrane markers which serve for the identification of various types of tumor-infiltrating leukocytes. The experimental protocols for such analysis of various tumor models (including SCCVII tumors) were developed in our earlier related studies (30, 31). As reported previously (31), the majority of cells in SCCVII tumors are cancerous (61%), i.e., CD45−, the second largest population is TAMs (29%), i.e., F4/80+, and there is 5% of other myeloid cells (GR1+/F4/80−) and 5% T lymphocytes (CD3+). These average values for the incidence of major cell populations in SCCVII tumors remained unchanged following the above described treatment with GM-CSF-releasing cells.

**Effect of GM-CSF Treatment on Cytotoxic Activity of TAMs.** Mice bearing SCCVII tumors received peritumoral injections of $1 \times 10^7$ lethally irradiated SCCVII/JzGM-CSF or SCCVII/Jzneo cells. The tumors were excised 48 h later, and TAMs were harvested for the analysis of their cytotoxicity against syngeneic in vitro cultured malignant SCCVII cells. The measurement of $[^{3}H]$thymidine release from killed target cells (Fig. 3) revealed that, in comparison to TAMs isolated from nontreated or SCCVII/Jzneo-treated SCCVII tumors, the TAMs obtained following tumor-localized GM-CSF treatment exhibit a 3–4-fold increase in tumoricidal activity.

**Effect of GM-CSF Treatment on the Response of Tumors to PDT.** Three groups of 16 mice were included in the experiments designed to investigate the effect of GM-CSF immunotherapy on Photofrin-mediated PDT. In two groups, $1 \times 10^7$ lethally irradiated SCCVII/JzGM-CSF cells or SCCVII/Jzneo cells were injected peritumorally at 48 h before the photodynamic light delivery, immediately after light treatment, and 48 h later. The third group received PDT only. Complete tumor ablation was observed within 2 days after light delivery in all three groups (Fig. 4). However, the tumors started to regrow in the PDT-only group and the PDT + Jzneo group at 8–10 days after PDT. By day 20, only one mouse (1/16) showed no signs of tumor recurrence in both of these groups. In the PDT + JzGM-CSF group, however, the tumor-free period lasted at least 2 weeks. Although tumor regrowth occurred afterwards in some mice, the overall cure rate for this group was 75%. A prolonged tumor-free period and significantly increased survival of tumor-bearing mice in Photofrin-mediated PDT combined with GM-CSF immunotherapy indicate that the described treatment modality offers increased effectiveness of tumor control.

The same GM-CSF immunotherapy protocol was also examined in combination with BPD-mediated PDT of SCCVII tumors. The results are depicted in Fig. 5. In all three groups, the tumors regressed and became unpalpable within 3–4 days after PDT. However, visible signs of recurrence appeared by days 8 and 10 in the PDT-only and PDT + Jzneo groups, respectively. All mice in these two groups showed tumor regrowth by day 18 after PDT. In the PDT + JzGM-CSF group, there was no tumor recurrence until day 12, with 50% of the animals remaining tumor free at the end of the observation period (96 days).
reduces polyamine contents in the esophagus. Also, DFMO treatment
deficient rats exposed to a single dose of NMBA. These data support
a role for apoptosis in tumor prevention, as well as for DFMO as a
prepared by Teklad (Madison, WI). Zinc levels in these diets were regularly
formulated, egg white-based, zinc-deficient and zinc-sufficient diets were
(San Antonio, TX). NMBA was from Ash Stevens, Inc. (Detroit, MI). Custom
were purchased from Taconic Laboratory (Germantown, NY). The animals
randomly divided into four experimental groups: Zn+/DFMO—, Zn+/DFMO+
libitum either a zinc-sufficient (Zn+, 75 ppm zinc) or a zinc-deficient (Zn—,
ity-controlled room with a 12-h light-dark cycle. On arrival or day 0, they were

tumor incidence analysis 12 weeks after carcinogen dosing. This single dose of
animals (3l).@ isolation of tissues. Whole esophagi were excised and opened longitu-
was filtered through a 0.22 μm filter, and aliquots were applied to a column
(17) 5 μL in 0.01% pepsin in 0.01 N HCl at 37°C. Endogenous peroxidase was
hibited with 3% hydrogen peroxide, and nonspecific binding sites were
bated with strepavidin horseradish peroxidase, and BrdUrd incorporation was
bation with biotinylated rabbit anti-mouse antibody. Slides were then incu-
mouse anti-BrdUrd (Becton Dickinson, 1:40 dilution, 1 h), followed by incu-
localized by a final incubation with DAB.

number of BrdUrd-labeled nuclei by the total number of cells counted per
process, presumably before morphological changes become fully evident (35).
TUNEL assay was performed in serial paraffin-embedded sections to facilitate
randomly occurring DNA fragmentation due to necrosis or other forms of
DNA degradation (35). Therefore, in this study, apoptotic cells were evaluated
method detects cells committed to apoptosis in earlier phases of the dying
identification of apoptotic cells.

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the cytokine production by SCCVII/JzGM-CSF cells is mediated through their regular metabolic activity, there is no basis to
expect an increased release of GM-CSF from these cells by a light-
activated mechanism. On the contrary, photosensitizer accumulation in
the cells subcutaneously injected before its administration and consequent
induction of PDT-mediated photodamage can negatively affect
the metabolism of these cells, resulting in decreased GM-CSF release.
The results presented in this report demonstrate that tumor-localized
GM-CSF treatment substantially improves the curative effect of
PDT in the SCCVII tumor model. This potentiating effect of GM-CSF
was highly pronounced irrespective of whether Photofrin or BPD was
used as photosensitizers for PDT. The enhancement level with both
photosensitizers was equivalent to a ~2.5-fold increase in the light
dose for PDT used alone. The SCCVII cells transfected with the
control retroviral vector encoding for neo only were used to demon-
strate that: (a) peritumoral injections per se do not affect the PDT
response, and (b) localized treatment with lethally irradiated tumor
transfected with the retroviral vector are not introducing an artifact in the evaluation of tumor response to PDT.
Based on available knowledge of the immunomodulating properties of
GM-CSF, several putative mechanisms can be proposed to account
for the observed effects of treatment with this cytokine on the antitumor activity of PDT. The increased local concentration of GM-CSF
attained with the treatment protocol used in the present study may: (a)
up-regulate the expression of leukocyte adhesion molecules and conse-
quentially the extravasation of inflammatory cells into tumor tissue;
(b) potentiate specifically the tumoricidal activity of neutrophils
and/or monocytes-macrophages; (c) induce release of a number of
secondary cytokines and other molecules (including, e.g., TNF-α and
interleukin 2) involved in mediating the antitumor effect; (d) facilitate
the presentation of tumor antigens released following PDT treatment
and thereby activate specific T-cell-mediated immune responses; (e)
promote the inactivation of remaining islets of viable tumor cells,
unhindered by the tumor burden, as PDT effectively debulked the
malignant mass; and (f) diminish the immunosuppressive effect (34)
induced by PDT (35, 36). The ongoing research in our laboratory is
designed to address specifically the contribution of the above listed
mechanisms. The fact that the potentiation of the antitumor effect of
PDT was observed with the SCCVII tumor, which is poorly immu-
nogenic, is particularly encouraging with respect to potential clinical
ramifications. It remains to be determined whether the potentiating
effect of GM-CSF treatment is equally pronounced or different with
PDT-treated immunogenic tumors.

We reported previously that the response of the SCCVII tumor to
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endotoxin (40), as well as with specific immune agents such as the
macrophage-activating factor GeMaf (11) and cytokines TNF-α (41)
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inflammatory/immune character, PDT is highly responsive to adju-
vant immunotherapy. Immunotherapy regimens tailored and opti-
mized for particular malignant lesions may produce outstanding gains
in PDT-mediated tumor control. The treatment with PDT, serving as
a strong acute inflammatory insult associated with debulking of tumor
mass, may emerge as a model system of value for characterization of
different aspects of the immune reaction.

DiscusSion

To investigate the effect of localized GM-CSF treatment on the
control of tumors treated with PDT, we introduced the gene for
murine GM-CSF into SCCVII tumor cells. Northern blot analysis and
the bioassay with GM-CSF-dependent B6SuTA cells confirmed that
GM-CSF-transduced cells (denoted SCCVII/JzGM-CSF) express
GM-CSF mRNA and secrete a biologically active form of this cyto-

Since the cytokine production by SCCVII/JzGM-CSF cells is mediated through their regular metabolic activity, there is no basis to expect an increased release of GM-CSF from these cells by a light-activated mechanism. On the contrary, photosensitizer accumulation in the cells subcutaneously injected before its administration and consequent induction of PDT-mediated photodamage can negatively affect the metabolism of these cells, resulting in decreased GM-CSF release.

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