Intensified Antitumor Immunity by a Cancer Vaccine That Produces Granulocyte-Macrophage Colony-stimulating Factor plus Interleukin 4

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ABSTRACT

Vaccination with irradiated tumor cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF tumor vaccine) induces a potent systemic antitumor immunity. To develop a protocol for cancer therapy to further augment the host immune response, we examined the effects of the GM-CSF tumor vaccines simultaneously producing additional cytokines. We prepared cancer vaccines expressing double cytokines by sequential recombinant retrovirus-mediated genetic transductions. We then used a murine intracerebral tumor model in which the GM-CSF tumor vaccine was less effective in immunopotentiating and evaluated tumor vaccines producing various cytokines in conjunction with GM-CSF. The cytokine combination of GM-CSF and interleukin 4 induced more potent antitumor immunity than GM-CSF alone. An in vivo depletion test showed that CD4+, CD8+, and asialoGM1+ cells were required for the optimum function of the GM-CSF plus interleukin 4 tumor vaccine. Histological examinations revealed infiltration of inflammatory cells at the site of tumor cell challenge as well as at the site of vaccination, indicating the induction of a systemic antitumor immune response which reached the central nervous system. Our findings suggest the feasibility of applying the intensified vaccination strategy to treat human cancers including malignant brain tumors.

INTRODUCTION

Augmentation of host antitumor immunity is observed by immunization with tumor cells transduced to produce certain cytokines such as IL-2, IL-4, IL-6, IL-7, IFN-γ, TNF-α, B7-1(CD80), or GM-CSF (13, 14). These gene-modified cancer vaccines reduce subsequent challenge with parental cells or eradicate small preexisting tumors (15—19) (for reviews, see Refs. 20—22). In our previous reports (23—25), we studied the ability of various cytokines and adhesion molecules to induce host immune responses. C57BL/6 mice were immunized with irradiated, genetically transduced B16 melanoma cells, followed 7 days later by a s.c. challenge with nonirradiated parental B16 cells. Among the 22 molecules examined, GM-CSF was the most potent stimulator of systemic antitumor immunity. Both CD4+ and CD8+ T lymphocytes were important for the GM-CSF tumor vaccination effect (13). GM-CSF is supposed to enhance the functional activity of antigen-presenting cells, resulting in the stimulation of antigen-specific T lymphocytes (13, 23, 25).

Our recent data (26) demonstrated that there was an optimal dose of GM-CSF for cancer vaccines. Very high levels of GM-CSF production by cancer vaccines resulted in suboptimal antitumor immunity. Instead of simply increasing the cytokine dosage, several strategies have been proposed for intensifying the treatment effect of GM-CSF tumor vaccine (27, 28), including adjuvant uses of BRMs (25) and combinations with adoptive transfer of effector T lymphocytes. In a previous report (25), we demonstrated that an associate use of a BRM such as OK432, which in itself is a nonspecific immunopotentiator, could further augment the specific antitumor immunity elicited by the GM-CSF tumor vaccine. We proposed that the BRM could induce particular mediator molecules which were necessary for the maximum immunopotentiating by the GM-CSF tumor vaccine (25, 27). These findings prompted us to investigate the cytokines which could specifically complement the activity of the GM-CSF tumor vaccine.

In this study, we utilized murine intracerebral tumor models. To examine whether genetically modified tumor vaccines can eliminate tumor cells at immunologically privileged sites (e.g., the brain), we introduced B16 cells intracerebrally into the vaccinated mice. The inoculated with the GM-CSF-producing vaccine survived significantly longer than those with the nontransduced vaccine. However, the antitumor effect in this intracerebral model was less satisfactory than that obtained in the s.c. tumor model. To obtain more intensified host immune responses, we tested tumor vaccines with simultaneous expression of other cytokines in combination with GM-CSF. In our intracerebral tumor model, GM-CSF in combination with IL-4 increased the antitumor effect more than GM-CSF alone, leading to the cure of the immunized animals.

MATERIALS AND METHODS

Recombinant Retroviral Vectors. We generated recombinant retroviruses for a wide variety of cytokines according to a method described elsewhere (13). In brief, we cloned the cDNAs for mouse GM-CSF, IL-1α, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12 (p40, p35), TNF-α, lymphotoxin, G-CSF, LIF, and MIF by the reverse transcription-PCR method. An IL-1α was constructed so as to encode a chimeric protein with the signal peptide sequence of mouse IL-2 and the mature form of IL-1α. All of the cDNAs were sequenced using the dideoxynucleotide termination method and confirmed to be identical to the reported sequences in GenBank. Details of the primers for PCRs, cDNAs, and retroviral vectors are available on request. The retroviral MFG plasmid DNA (13) and the amphotrophic retrovirus packaging cell line pCRIP (29) were used to generate recombinant retroviruses. MFG vectors containing the cDNAs were transfected with pPGKneo, which contributes to neomycin resistance, into pCRIP using the calcium phosphate precipitation method. Cells were grown in a medium containing G-418 (1 mg/ml; Life Technologies, Inc.) for 7 days. Clones were expanded, and filtered culture supernatants were used for infecting NIH3T3 fibroblasts in the presence of polybrene (8 μg/ml; Sigma). Genomic DNAs of the infected cells were isolated, and titers of the virus were estimated as copy numbers of integrated provirus, which were determined using Southern blot analysis. The selected high-titer producer clones were used to transduce tumor cells. To verify the expression of transduced genes, we assayed cytokines secreted by infected NIH3T3 cells 48 h after plating 1 × 10⁶ cells in 100-mm dishes containing 10 ml medium. Murine GM-CSF, murine IL-3, murine IL-4, murine IL-6, and murine IL-10 were assayed using an ELISA from Endogen; murine IL-1α and murine TNF-α, using an ELISA from Genzyme; murine IL-2, by a [3H]hymidine uptake bioassay using CTL2 cells, murine IL-7, by a [3H]hymidine uptake bioassay using murine erythrocytes; and murine IL-12, by a [3H]hymidine uptake bioassay using phytohemagglutinin-activated human lymphoblasts. The recombinant retroviruses used in this study are listed in Table 1.
Tumor Cell Line and Tumor Vaccine Preparation. We maintained murine melanoma B16 as described previously (25, 30) and performed retrovirus-mediated gene transfer as described above. Genetically modified tumor cells were treated with trypsin/EDTA and irradiated with 10,000 rads using a Hitachi MBR-l505R X-ray generator. We washed irradiated cells twice in HBSS and resuspended them in HBSS at a concentration of 5 × 10^6 cells/ml. All immunizations were performed s.c. in the left flank of mice with 5 × 10^5 irradiated cells.

Intracerebral Tumor Model. Female C57BL/6 mice, purchased from Charles River Japan (Atsugi, Japan), were used at the age of 6–10 weeks. To prepare an experimental intracerebral tumor, we inoculated parental B16 cells in 100-mm dish with 10 ml medium for 48 h.

In Vivo Depletion Test. We performed in vivo depletion of lymphocyte subsets (13, 31). Monoclonal antibodies used were GK1.5 (ATCC TIB 207) for mouse CD4, 53-6.72 (ATCC TIB 105) for mouse CD8, and PK136 (ATCC HB 191) for mouse NK1.1. These were prepared from hybridoma culture medium using affinity chromatography (32, 33) with protein G-Sepharose (Pharmacia) for GK1.5 and 53-6.72, and protein A-Sepharose (Pharmacia) for PK136. We gave mice i.p. injections of 0.5 mg antibody three times on days −2, −1, and 0 (total, 1.5 mg/mouse). The mice were then challenged intracerebrally with 1 × 10^7 B16 tumor cells on day 0, followed 3 days later by s.c. injection of the GM-CSF plus IL-4 tumor vaccine (day 3 treatment model). A rabbit anti-asialoGM1 polyclonal antibody was purchased from Wako (Osaka, Japan), and a single i.p. injection of 0.75 mg/mouse was given on the day before immunization (i.e., on day 2 in the day 3 treatment model). The survival of five mice in each group was observed as described above.

Histological Examination. s.c. tissues at the vaccination sites were excised 3 days after injection. Brains of the mice that received intracerebral tumor cell challenge 3 days before the immunization (day 3 treatment model) were removed 14 days after the challenge. Tissue samples were fixed in 10% formalin solution, dehydrated, and embedded in paraffin. Thin-sliced sections were stained with H&E.

RESULTS

Intracerebral Tumor Model. In our previous reports, we vaccinated C57BL/6 mice with irradiated gene-transduced B16 melanoma cells, followed 7 days later by a s.c. challenge with nontransduced B16 cells (s.c. prevention model; day −7 vaccine in C57BL/6 mice). In this model, all of the mice immunized with irradiated GM-CSF-secreting B16 cells rejected the subsequent challenge (23, 26). To assess the effect of various cytokines simultaneously expressed in the GM-CSF tumor vaccine, we designed intracerebral tumor models.

First, we conducted a prevention study in which mice were inoculated intracerebrally with viable B16 cells 7 days after receiving s.c. immunization with the GM-CSF tumor vaccine. Experimental conditions were set identical to those in the s.c. prevention model, except for the reduced challenge dose into the brain (5 × 10^3 cells/mouse; the s.c. challenge doses were usually 2–5 × 10^3; Refs. 13 and 23–25). A minimal intracerebral tumorigenic dose was < 1 × 10^3 since all of the mice (25/25) receiving 1 × 10^3 cells died as the result of intracranial tumors. In this model, all of the mice (15/15) receiving a challenge of 5 × 10^3 cells without vaccination died of intracranial tumors by day 22. The vaccination with irradiated parental B16 cells showed virtually no therapeutic effect against the intracerebral challenge with viable B16 cells (Fig. 1).

To obtain more potent antitumor immunity, we tried tumor vaccines which secrete other cytokines in addition to GM-CSF. The panel of highly transmissible high-titer recombinant retroviruses (Table 1) enabled us to compare the effects of a wide variety of cytokines. B16 cells were sequentially transduced with GM-CSF followed by another cytokine gene. As indicated by the transduction efficiencies of retroviral vectors (Table 1), the vaccines in which combinations of cytokines were secreted consisted mainly of a mixed population of B16 tumor cells, some of which had been transfected with GM-CSF, some with another cytokine, and some with both cytokines. We evaluated more than 10 cytokines in combination with GM-CSF to determine their immunopotentiation in the intracerebral prevention model. By this screening, we found that IL-4 and TNF-α were immunostimulatory. Over 80% of the mice (n = 10) immunized with a vaccine producing GM-CSF plus either IL-4 or TNF-α rejected an intracerebral challenge, while only 40% of the mice (n = 10) treated with GM-CSF alone survived without intracerebral tumor (Fig. 2). No significant additional effects over the GM-CSF tumor vaccine were observed for other cytokines examined (n = 10), including IL-1α (secreted form), IL-2, IL-3, IL-6, IL-7, IL-12, lymphotixin (TNF-β), and not done: LT, lymphotoxin.

Table 1 A panel of MFG recombinant retroviruses

<table>
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<tr>
<th>cDNA*</th>
<th>Titer (copy no.)</th>
<th>Level of expression</th>
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</thead>
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<tr>
<td>mGM-CSF</td>
<td>1.5</td>
<td>13.5 ng/ml</td>
</tr>
<tr>
<td>mIL-1α</td>
<td>3.0</td>
<td>20.6 ng/ml</td>
</tr>
<tr>
<td>mIL-2</td>
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<td>6350 IU/ml</td>
</tr>
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<td>mLT</td>
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<td>mIFN</td>
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</tr>
<tr>
<td>mIL-12</td>
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* The initial letters m and h represent the cDNAs of murine and human origin, respectively.

in vitro production of cytokine by 1 × 10^6 cells of gene-transduced NIH3T3 cultured in 100-mm dish with 10 ml medium for 48 h.

nd, not done; LT, lymphotoxin.

Fig. 1. Antitumor effects elicited in C57BL/6 mice immunized with the GM-CSF gene-modified tumor vaccine. Mice were immunized with tumor vaccines on day −7 and challenged with an intracerebral injection of parental B16 cells on day 0. The total results of survival of the mice in the three independent experiments are shown (total n = 15). P < 0.001 for B16/GM-CSF vaccine (O) versus nontransduced B16 vaccine (O). □, no vaccine.

Table 1 A panel of MFG recombinant retroviruses
Mice were immunized with tumor vaccines on day -7 and challenged with an intracerebral injection of parental B16 cells on day 0. The total results of survival of the mice in the two independent experiments are shown (total n = 10). P < 0.001 for B16/GM-CSF (○), B16/GM-CSF + IL-4 (●), and B16/GM-CSF + TNF-α (□) vaccines versus nontransduced B16 vaccine (◇), P < 0.05 for B16/GM-CSF + IL-4 and B16/GM-CSF + TNF-α vaccines versus B16/GM-CSF vaccine. □, no vaccine.

Next, we examined the therapeutic potential of the tumor vaccines producing double cytokines. Mice were first challenged with $1 \times 10^3$ viable B16 cells intracerebrally, followed 3 days later by immunization with irradiated genetically modified B16 cells (day 3 treatment model). Although the results obtained in this model were less satisfactory than those in the prevention model, the cytokine gene-transduced vaccines induced more significant therapeutic effects than the nontransduced vaccine ($P < 0.01$ for GM-CSF, $P < 0.05$ for GM-CSF plus TNF-α, and $P < 0.001$ for GM-CSF plus IL-4; Fig. 3). The combination of IL-4 with GM-CSF led to a high cure rate: 45% (9/20) of the treated mice survived for 90 days without tumor. The combination of TNF-α with GM-CSF demonstrated a similar efficacy to the GM-CSF alone, which allowed 20% (4/20) of the treated mice to survive without tumor.

**In Vivo Depletion Test.** To identify the effector cells required for the effective antitumor immunopotentiation by the tumor vaccine producing GM-CSF plus IL-4, we performed in vivo depletions of lymphocyte subpopulations by administering antibodies in the day 3 treatment model. This test ($n = 10$) revealed that the depletion of CD8+ T lymphocytes completely abrogated the therapeutic effect of the tumor vaccine (Fig. 4). The depletion of CD4+ T cells also abrogated the therapeutic effect of the tumor vaccine ($P < 0.01$ compared with the group treated with the GM-CSF plus IL-4 tumor vaccine without depletion; nonsignificant compared with the control group without treatment). We confirmed successful depletions of more than 90% of the T-cell subpopulations by flow cytometric analysis of the spleen cells from the mice on the same day with the vaccination treatment (day 3; data not shown).

The depletion of natural killer cells by in vivo administration of NK1.1 monoclonal antibody did not significantly decrease the efficacy of the tumor vaccine (Fig. 4). In contrast, the asialoGM1+ cells were essential for the antitumor immunity induced by the GM-CSF plus IL-4 tumor vaccine. Since it has been reported that the anti-asialoGM1 treatment eliminates both effector natural killer cells and precursor cells, including premature CTLs (34, 35), it is likely that an NK1.1+ asialoGM1+ lymphocyte subpopulation is essential for the optimum function of the GM-CSF plus IL-4 tumor vaccine. AsialoGM1+ premature CTLs are among the candidate effector cells. In addition to CD4+ and CD8+ T lymphocytes, which specifically recognize tumor-associated antigens, it may be critical to activate asialoGM1+ lymphocytes for the full potentiation of antitumor immunity induced by tumor vaccines.
Histological Findings. We investigated s.c. tissues at the immunization sites 3 days after the injection of tumor vaccines. Vaccination with irradiated nontransduced B16 cells produced a minimal host reaction; i.e., infiltration of inflammatory cells was not prominent around or within the s.c. B16 tumor vaccine (Fig. 5a). In contrast, a massive, lymphocyte-rich and immature macrophage-rich infiltration was found around and within the GM-CSF plus IL-4 tumor vaccine. In addition, marked tumor necrosis with inflammatory cell infiltration was always observed in the center (Fig. 5b). The marked accumulation of inflammatory cells seems to be the consequence of high local concentrations of the cytokines produced by the injected transduced B16 cells.

Tumors arising from intracerebral injection of B16 cells in nonsensitized control mice developed in white matter of the right cerebral hemispheres and usually invaded the lateral ventricles and the brain stems. Tumor growth sometimes extended into the meninges and through the puncture wound of the skull. To evaluate the effect of gene-modified vaccine treatment, we examined brains of the mice subjected to the day 3 treatment model 14 days after the challenge. The intracerebral tumors of the mice immunized with the GM-CSF plus IL-4 vaccine were smaller than those immunized with nontransduced cells. The average B16 tumor in controls was 3–4 mm in diameter; that in the group treated with the GM-CSF plus IL-4 vaccine was ~1 mm. Microscopical examination revealed that numerous inflammatory cells infiltrated within the intracerebral tumor tissues in the mice treated with the GM-CSF plus IL-4 vaccine, whereas only a few inflammatory cells were found scattered in the transplanted intracerebral tumors of control mice (Fig. 6). We are now investigating the types and differentiation phenotypes of the inflammatory cells infiltrating into tumor lesions in detail. Taken together, the findings indicate that the systemic immune response elicited by the cytokine-producing tumor vaccine reached the central nervous system, resulting in smaller tumors and longer survival of the immunized mice.

DISCUSSION

Several strategies have been proposed for intensifying the effect of GM-CSF tumor vaccine treatment, including the simultaneous expression of cytokines in addition to GM-CSF (27, 28). We utilized the B16 intracerebral tumor model in which the GM-CSF vaccine immunopotentiation was insufficient (Fig. 1), and evaluated the effects of various cytokines in conjunction with GM-CSF. The tumor vaccine expressing GM-CSF and IL-4 exerted more potent antitumor effect than the vaccine expressing GM-CSF alone (Figs. 2 and 3). In vivo depletion of lymphocyte subsets showed that CD4+ and CD8+ T cells, and asialoGM1+ cells were required for antitumor activity induced by the vaccine (Fig. 4). Since the tumor vaccine producing IL-4 alone failed to induce significant antitumor immunity in the intracerebral as well as the s.c. model, it is likely that the locally produced IL-4 complemented the biological activities of GM-CSF. Since GM-CSF has been shown to play important roles in the maturation and/or function of specialized antigen-presenting cells (36, 37; for review, see Ref. 38), localized expression of GM-CSF by irradiated tumor cells is supposed to specifically enhance tumor antigen presentation by host antigen-presenting cells (13, 28). IL-4 could work through further potentiation of antigen-presenting processes, which are initially activated by a
specific stimulation with GM-CSF-secreting tumor vaccination. In accord with this notion, it has been reported that a combination of GM-CSF and IL-4 provided cultured dendritic cells with a sustained capacity of antigen presentation in vitro (39). Our data imply that IL-4 in the presence of GM-CSF primarily activates antigen-presenting cells which mediate activation of CD4+ T cells and lead to activation of effectors including CD8+ and asialoGM1+ cells.

TNF-α was also noticeably effective in intensifying the antitumor immunity induced by the GM-CSF tumor vaccine (Fig. 2). Because TNF-α is also cytokine reported to modify the maturation and activity of dendritic cells (39), it is possible that TNF-α also works through the secondary activation of the dendritic cells which are primarily stimulated by GM-CSF. It is widely accepted among immunologists that there are two differentiation pathways in dendritic cells. CD34+ progenitor cells differentiate into CD1a+, Birbeck granule+ dendritic cells (Langerhans cells) under the influence of GM-CSF and TNF-α, and the other differentiate into CD14+, Birbeck granule- dendritic cells (interdigitating cells) in the presence of GM-CSF and IL-4 (40). This maturation hypothesis, which is proposed from in vitro experiments, also suggests that there may be some subpopulations in the T-cell-associated dendritic cells. In this regard, it is possible that in our cytokine-transduced vaccination system, GM-CSF plus IL-4-producing tumor cells and GM-CSF plus TNF-α-producing cells, respectively, induce growth expansions of phenotypically and functionally different subpopulations of dendritic cells. This might reflect the different efficiency in inducing antitumor immunity between the two cytokines producing B16 cells. Clearly, many more studies are required to clarify the exact cellular and molecular mechanisms of the helper effect of IL-4 and TNF-α involved in the tumor antigen presentation processes.

Brain tumors, arising at so-called immune privileged sites, have been supposed to be resistant to tumor vaccination therapies because the systemic immune response elicited by a vaccine does not adequately reach the central nervous system. The blood-brain barrier is considered to play a major role in preventing immune cells from extravasating into brain parenchyma (for review, see Ref. 41). However, activated lymphocytes have the capacity to enter the brain tumor lesion (42; for review, see Ref. 43). There have been reports describing rejection of intracerebral tumor after s.c. immunizations (44). In this report, we have demonstrated, using intracerebral tumor transplantation models, that specific immune response against tumor cells in the brain, although relatively weak, could be intensified by cytokine gene-modified tumor vaccination. The vaccine producing GM-CSF and IL-4 showed a therapeutic efficacy against the experimental tumor transplanted in the brain, leading to the cure of some of the animals (Fig. 3). Histological examination provided evidence that host immune cells could infiltrate and destroy the intracerebral tumour if the systemic immune system is adequately activated (Fig. 6). Although the intracerebral transplantation model is artificial, it simulates the situation in which human brain tumors are incompletely excised. The data from our murine studies suggest the feasibility of a treatment strategy for malignant brain tumors (e.g., glioblastoma) by intensified vaccinations. Specific activation of the immunity against tumor cells after surgery could be beneficial, hopefully leading to the eradication of residual tumor cells.

Before the clinical application of these findings for treating human brain tumors, many more preclinical studies may be required. Since the antitumor immunity induced in the brain is relatively weak compared with that in the other organs, studies seeking to further intensify the antitumor response will be required (26–28). In addition to vaccination protocols, adoptive immunogene therapy is another promising approach. In a previous report (33), we achieved up to 100% gene transduction of murine lymphocytes using an adenoviral vector. Treatment of mice with the tumor-infiltrating lymphocytes genetically modified to produce IL-2 resulted in remarkable reduction of tumor metastasis and much longer survival until brain tumor death (33). The combination of genetically modified tumor vaccines with adoptive transfer of tumor-specific CTLs induced synergistic immunotherapeutic potentials (28, 45), and could be a useful strategy for treating human cancers.

From a technical point of view, several in vitro studies are required to prepare cytokine gene-modified tumor vaccines. First, large-scaled primary cultures of surgical samples are to be processed for the ex vivo gene transduction procedure. Generally more than 10⁶ cells are required to prepare tumor vaccines for a clinical trial (46), and the vaccination treatment should be started as early as possible (usually in no more than two months after the surgical treatment). Second, efficient and safe genetic transduction should be performed to produce an appropriate quantity of cytokines by irradiated vaccine preparations. The MFG retroviral vector used in this study, which was developed from Moloney murine leukemia virus (47) by R. C. Muligan and his colleagues (13), is highly efficient and widely used for both basic and preclinical studies (13, 25, 26, 46). Recently, we have developed retroviral vectors modified with mutations to the gag sequence of the MFG vector, without sacrificing the highly efficient transduction and expression attained by the original MFG vector. With these vectors, we achieved an efficient introduction of double cytokine genes (i.e., human GM-CSF and IL-4) into fresh human glioma cells cultured from surgical samples, suggesting the technical feasibility of clinical application of autologous tumor vaccines simultaneously secreting double cytokines for eradicating human malignant gliomas.

Finally, it should be noted that it is premature to conclude that a particular combination of cytokines (i.e., GM-CSF plus IL-4) is best for all tumors, since the effect probably depends on different variables including tumor type, vaccine schedule, location of tumor, and levels of cytokines secreted by the cells in the vaccine. Further basic and clinical studies are required before we can determine which cytokine or cytokine combination is best for a particular tumor type in an individual clinical situation.

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