Macrophage Colony-stimulating Factor plus Interleukin 4

Hiroaki Wakimoto, Junko Abe, Rikiya Tsunoda, Masaru Aoyagi, Kimiyoshi Hirakawa, and Hirofumi Hamada

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 vaccine 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 immunopotentiation 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 (1, 2), IL-4 (3, 4), IL-6 (5), IL-7 (6), IFN-γ (7, 8), TNF-α (9), B7-1 (CD80) (10–12), or GM-CSF (13). These gene-modified cancer vaccines have been proposed for intensifying the treatment effect of GM-CSF (13, 23, 25). Instead of simply increasing the cytokine dosage, several strategies are supposed to enhance the functional activity of antigen-presenting cells, resulting in the stimulation of antigen-specific T lymphocytes

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. sIL-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 the virus was 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]thymidine uptake bioassay using CTL2 cells (ATCC TIB 214); murine IL-7, by a [³H]thymidine uptake bioassay using murine thymocytes; and murine IL-12, by a [³H]thymidine uptake bioassay using phytohemagglutinin-activated human lymphoblasts. The recombinant retroviruses used in this study are listed in Table 1.

Received 6/28/95; accepted 2/15/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a grant from the Ministry of Education, Science, and Culture, Japan and by a grant from the Vehicle Racing Commemorative Foundation.

2 To whom requests for reprints should be addressed. Fax: 81-3-3918-3716.
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 × 10^6 B16 tumor cells on day 0, followed 3 days later by s.c. injection of the GM-CSF gene-modified 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.
MIF, LIF, and G-CSF (data not shown). The tumor vaccines producing only IL-4 or TNF-α in the absence of GM-CSF failed to induce significant antitumor immunity in the intracerebral or the s.c. model (data not shown).

Next, we examined the therapeutic potential of the tumor vaccines producing double cytokines. Mice were first challenged with \(1 \times 10^7\) 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 eliminated 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.
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⁺, 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

**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 brainstems. 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

---

Fig. 5. Histology of the s.c. tissues inoculated with tumor vaccines. Three days after the s.c. injection of either control nontransduced parent tumor vaccine (a) or the GM-CSF + IL-4 gene-modified tumor vaccine (b), s.c. vaccination sites were subjected to histological examinations. H&E; ×200.

Fig. 6. Treatment effect of the GM-CSF + IL-4 tumor vaccine on the histology of the challenged tumors. Mice were challenged with an intracerebral injection of parental B16 cells on day 0, followed 3 days later by s.c. injection of either control nontransduced parent tumor vaccine (a) or the GM-CSF + IL-4 gene-modified tumor vaccine (b; day 3 treatment model). Mice were sacrificed on day 14, and the excised brains were subjected to histological examinations. H&E; ×200.
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 another 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 efficacy 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 tumor 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. Mulligan 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.

ACKNOWLEDGMENTS

We thank Kimie Nomura and Hironori Murayama for their help in preparing histological samples, and Dr. Ken-Ichi Hanada for helpful discussions and critical reading of the manuscript.

REFERENCES

12. Townsend, S. E., Su, F. W., Atherton, J. M., and Allison, I. P. Specificity and
11. Townsend, S. E., and Allison, J. P. Tumor rejection after direct costimulation of
10. Chen, L., Ashe, S., Brady, W. A., Hellström, I., Hellström, K. E., Ledbetter, J. A.,
16. Connor, J., Bannerji, R., Saito, S., Heston, W., Fair, W., and Gilboa, E. Regression of
Intensified Antitumor Immunity by a Cancer Vaccine That Produces Granulocyte-Macrophage Colony-stimulating Factor plus Interleukin 4

Hiroaki Wakimoto, Junko Abe, Rikiya Tsunoda, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/8/1828

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.