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Laboratory Investigation

Effects of combined granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-2, and interleukin-12 based immunotherapy against intracranial glioma in the rat

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Summary

Cytokines play a major role in the regulation of the immune system. Granulocyte–macrophage colony-stimulating factor (GM-CSF) has been shown to be useful for immunotherapy against glioma because it can stimulate dendritic cells to present tumor antigen. Interleukin-2 (IL-2) is involved in T-cell expansion, and interleukin-12 (IL-12) drives the T-helper cell type I response. Previous studies have shown that each of these cytokines alone can induce the regression of tumor cells. In the present study we postulated that peripheral infusion of GM-CSF along with either IL-2 or IL-12 and irradiated tumor cells can lead to increased survival from 9L brain tumors. 9L gliosarcoma cells (10^6) were implanted in the brains of syngeneic Fischer 344 rats. Osmotic minipumps were utilized for subcutaneous, continuous delivery of GM-CSF, either alone or with IL-2 or IL-12. Irradiated 9L cells were injected subcutaneously at various time points during treatment. Delayed-type hypersensitivity (DTH) and immunohistological analysis were used to further characterize the anti-tumor response. Treatment with GM-CSF and irradiated tumor cells led to an increase in survival rate in rats with intracranial 9L tumors when compared to untreated animals. The addition of IL-2 or IL-12 to the GM-CSF/tumor cell therapy further increased the survival rate up to 90%. The anti-tumor response was associated with vigorous DTH against 9L cells and increased infiltration of CD4+ and CD8+ lymphocytes into the tumor. These results suggest that the combined infusion of GM-CSF and other cytokines may be effective adjuvants in treating brain tumors.

Introduction

Brain tumors pose a unique set of problems for any immunotherapeutic strategy. Screened by the blood–brain barrier and situated in an 'immunologically privileged' site, these tumors may be inaccessible to systemic, cellular immune responses. Glial tumors, in particular, are also known to produce soluble factors like transforming growth factor β_2 [1,2] and prostaglandin E_2 [3], which inhibit interleukin-2 (IL-2) mediated T-cell proliferation. Finally, the maturation of the systemic anti-tumor immunity must out-pace the rapidly growing glial tumor cells, as even a relatively small tumor mass in the brain may be sufficiently lethal. Despite these various challenges, immunotherapy for glioma has been explored for many years. Several previous strategies, focusing on the use of lymphokine-activated killer cells and systemic administration of cytokines, such as IL-2, have been met with limited success [4,5].

Many new strategies for immunotherapy against systemic cancers have emerged in the last several years. A recent focus has been on the use of genetically modified tumor cells as vaccines. This strategy utilizes the tumor cells as a source of antigen. Because these cells are also transfected with the genes for various cytokines, they also serve as sources for the paracrine delivery of immunomodulatory molecules [6]. A recent comparative study involving multiple cytokine genes found that cell vaccines transduced

with the gene for granulocyte–macrophage colony-stimulating factor (GM-CSF) were the most potent inducer of long-lasting, specific, systemic anti-tumor immunity [7]. Subsequent studies have confirmed the efficacy of GM-CSF-transduced vaccines for various systemic cancers [8–10] and experimental brain tumors [11–17]. The therapeutic action involves the paracrine, local release of GM-CSF at the vaccine/tumor antigen presentation site, and its subsequent effect in recruiting and activating antigen presenting cells (APCs), such as dendritic cells and macrophages [7,18,8]. These activated APC, in turn, prime CD4+ (helper) and CD8+ (cytotoxic) lymphocytes, which recognize the tumor-associated antigen, infiltrate the tumor, and subsequently lead to tumor regression and systemic anti-tumor immunity [8,10]. The promising results of using GM-CSF in preclinical brain tumor studies have prompted the initiation of Phase I clinical trials. The clinical results by Plautz et al. [19] and Sloan et al. [20] show modest benefit after T-cell adoptive transfer using GM-CSF alone as an adjuvant. These results suggest that other cytokines may be required to enhance the immune response. Pre-clinical studies have examined the combined effect of using IL-2 and interleukin-12 (IL-12) on various neoplasms and have demonstrated efficacy [21–24]. The effects of infusing these cytokines in combination with GM-CSF on brain tumors, however, have yet to be examined.

Previously, we have demonstrated that the strategy for paracrine cytokine infusion utilizing osmotic minipumps can lead to regression of glioma established as flank tumors [25,26]. The subsequent systemic anti-tumor immunity was long-lasting and specific. The use of osmotic minipumps and inoculations of irradiated, non-transfected tumor cells allow the tight and independent control of antigen and cytokine doses. This strategy also circumvents the necessity of genetic modification of tumor cells and the subsequent characterization of these cell vaccines. In the present study, we report our experience with paracrine, subcutaneous infusion of GM-CSF in combination with IL-2 or IL-12 for the treatment of intracranial glioma in a rat model.

Materials and methods

Cell culture and tumor implantation techniques

9L gliosarcoma cells were grown at 37°C in RPMI media supplemented with L-glutamine, penicillin, streptomycin and 10% fetal bovine serum. These cells were then collected, centrifuged at 1000*g* for 10 min and washed with phosphate buffered saline (PBS). A total of three PBS washes were performed, between which the cells were counted with a hemacytometer. Central tumor implantations were performed by injecting 10^6 cells/10 μl stereotactically into the striatum of ketamine (35 mg/kg, i.p.)/xylazine (5 mg/kg, i.p.) anesthetized rats. Cells used as a source of tumor antigen were irradiated with 6000 rad ^{137}Cs, and injected at a concentration of 5×10^6 cells/300 μl of PBS.

Animals and cytokine delivery

Female Fischer 344 rats weighing 75–150 g, were obtained from Harlan (Indianapolis) and cared for according to the University of Minnesota guidelines. Animals weighed ∼75 g when first used in these studies. Vaccinated animals that survived the initial tumor were used for the tumor re-challenge experiments, and weighed ∼150 g at the time of tumor re-implantation. Recombinant murine GM-CSF was obtained from R&D Systems (Minneapolis, MN). Recombinant human IL-2 was obtained from Chiron Therapeutics (Emeryville, CA). Recombinant murine IL-12 was purchased from Genzyme Diagnostics (Cambridge, MA). PBS with 0.1% bovine serum albumin was used to dilute these cytokines to various concentrations used for the experiments. Alzet model 2004 osmotic minipumps (Alza, Palo Alto, CA), which continuously release their contents at a rate of 6 μl/day for 28 days, were filled with the appropriate concentration of cytokine(s) in order to release GM-CSF at 10 ng/day, IL-2 at 10 000 IU/day, and/or IL-12 at 1 ng/day. The concentrations of GM-CSF and IL-12 were chosen based on the results from our previous studies (26, 25, respectively); and the concentration selected for IL-2 was based on preliminary studies in our laboratory (unpublished data). Filled pumps were placed in 37°C saline for 48 h prior to implantation in order to 'prime' their pumping mechanism, as specified by the manufacturer's guidelines. Under sterile conditions, the primed pumps were then implanted into subcutaneous pockets in the right flanks of anesthetized animals.

Recombinant rat tumor necrosis factor alpha (TNF-α) was obtained from R&D systems (Minneapolis, MN). TNF-α (100 ng in 10 μl) was delivered directly to the site of the tumor, concurrent with tumor implantation.

Immunotherapy regimen

Animals were divided into groups receiving varying doses of cytokine(s) or buffer infusion. Unless otherwise specified, immunotherapy was started on the day of brain tumor implantation, when cytokine releasing minipumps were placed subcutaneously in the right flank. Immediately after minipump implantation, the animals received an injection of 5×10^6 irradiated 9L tumor cells for antigen presentation. These injections of irradiated tumor cells were made at the tip of the infusion cannula and were repeated on days 3, 7, 14 and 21 after minipump implantation. All treated animals received the same number of irradiated tumor cells at all times. Control animals received either no treatment at all, or the combination of buffer infusion and irradiated tumor cell injections. Animals were monitored for their hydration and nutritional status, and were euthanized when they were unable to groom or feed themselves.

Some animals received immunotherapeutic treatment before 9L tumor implantation. Specifically, GM-CSF-filled minipumps implantation and the initial irradiated tumor cell injection took place 3 days before intracranial tumor placement, and proceeded as described above. This regimen will be referred to as the 'negative 3 protocol' in the following text.

At the start of the experiments, it was determined that animals which survived beyond 75 days after intracerebral tumor implantation would be considered cured of their brain tumor. For tumor re-challenges, 1 million (10^6) 9L tumor cells were implanted in the left striatum (a site contralateral to the initial tumor placement) of cured animals. Naive animals served as controls.

Delayed-type hypersensivity

Delayed-type hypersensivity (DTH) testing was conducted after the tumor re-challenge experiments. One million (10^6 cells/10 μl) ^{137}Cs irradiated 9L tumor cells were injected into the right pinna of each animal. The left pinna received saline injection only. Forty-eight hours after injection, the resultant swelling in the right pinna was compared to that in the left. This differential swelling was compared between treated animals cured of brain tumors and naïve animals.

Data analysis

Survival analysis was undertaken using the Log Rank (Mantel–Cox) test, which examines the equality of survival time distributions. Data from the DTH experiment were analyzed using the Student t-test. A p-value of less than or equal to 0.05 was considered to be statistically significant.

Immunohistochemistry

Animals were perfused with 4% paraformaldehyde in 0.1 M PBS. The brains were dissected free and post-fixed in the same fixative for 48 h at 4°C. The solution was switched to 30% sucrose/0.1 M PBS for 48 h. Sections were cut at 30 μm with a freezing microtome and collected in phosphate buffer containing 0.02% sodium azide. Before staining, the sections were washed with PBS and quenched in 3% H_2O_2 and 10% methanol/0.1 M PBS. The tissue was then preincubated with 5% normal horse serum and 0.3% Triton in PBS. Incubation in primary antibody took place at 4°C in 2% normal horse serum and 0.3% Triton in PBS, and lasted 16 h. Primary antibodies used included OX-8, OX-62, and W3/25, all obtained from Serotec (Oxford, UK). The tissue was then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) in 1% normal horse serum/0.1 M PBS for 1 h. Detection began with incubation with 'ABC reagent' (Vector Laboratories, Burlingame, CA) for 1 h, followed by 3,3′-diaminobenzidine solution. Tissue sections were then mounted on glass slides for light microscopy.

Results

Survival from intracranial 9L tumors

Treatment with GM-CSF/tumor cell therapy increased the survival rate of animals with intracranial 9L glioma tumors. Treatment effects were studied in three separate experiments with similar results so the data were pooled for final analyses. When GM-CSF infusion via osmotic minipumps (10 ng/day) and irradiated tumor cell injections were initiated on the day of tumor implantation, animals that received 10^6 9L tumor cells ($n = 18$) showed a 22% survival rate through day 75 of the experiment (*pre hoc* designated as end of the study). All of the untreated animals ($n = 16$) died by day 30 (Figure 1). The difference in survival between these two groups was statistically significant ($P < 0.05$). The animals, which received tumor cell injections only ($n = 18$) had an intermediate survival rate of 11%

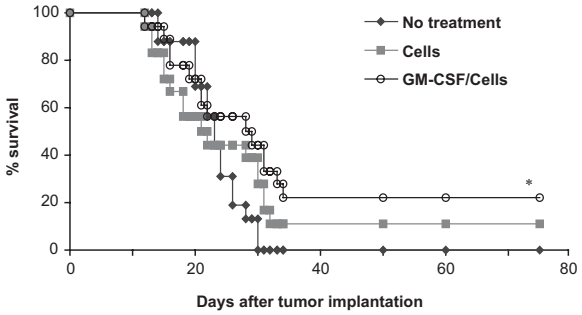

Figure 1. Survival after GM-CSF/tumor cell therapy. Animals were treated with continuous, subcutaneous GM-CSF and tumor cell injections ($n = 18$), cell injections alone ($n = 18$), or received no treatment ($n = 16$). Therapy was started on the day of intracranial 9L tumor implantation. *, statistical difference in survival was significant when compared to untreated animals (Log Rank $P < 0.05$). The difference in survival between the group treated with GM-CSF/cell therapy and the one treated with cell injections alone did not reach statistical significance ($P = 0.24$).

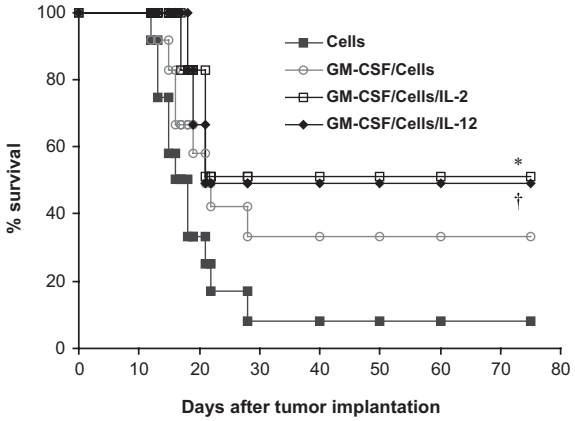

Figure 2. Survival From intracranial 9L tumor after combination GM-CSF/interleukin therapy. Animals were treated with GM-CSF/tumor cell therapy ($n = 12$). Additional animals received adjuvant of IL-2 or IL-12 delivered via osmotic minipump ($n = 6$ per group). *, statistical difference in survival was significant when compared to animals treated with cells alone (Log Rank $P < 0.05$). †, statistical difference in survival was significant when compared to animals treated with cells alone (Log Rank $P = 0.05$).

(Figure 1). There was no significant statistical difference when this was compared to the survival rate of the group which received GM-CSF/tumor cell therapy ($P = 0.24$).

Interleukins, which independently activate the cytotoxic arm of the immune system, were added to the cytokine regimen for possible additive effect. IL-2 (10 000 IU/day) and IL-12 (1 ng/day) were administered through osmotic minipumps along with GM-CSF. The group of animals which received IL-2/GM-CSF/tumor cell therapy ($n = 6$) showed a survival rate of 50%, as did the animals that received IL-12/GM-CSF/tumor cell therapy ($n = 6$). The control group in this experiment received tumor cell injections alone ($n = 12$). The increase in survival with IL-2 or IL-12 enhanced GM-CSF therapy was statistically significant when compared to control ($P < 0.05$) (Figure 2). TNF-α induces a pro-inflammatory state when directly injected into brain parenchyma [15]. To investigate whether TNF-α enhances the immune response generated by GM-CSF and irradiated tumor cells, intratumoral injections of 100 ng TNF-α were undertaken on the day of tumor implantation. The animals, which received this additional TNF-α injection along with GM-CSF therapy ($n = 6$), exhibited a survival rate no different than the group which received GM-CSF/tumor cell therapy alone ($n = 6$) (data not shown).

The modest response to GM-CSF therapy may be related to temporal aspects of the 9L glioma model used. The implantation of a large tumor load, such as 10^6 tumor cells, may kill the animals or render them immunologically incompetent, before adequate antitumor immunity can be mobilized. To investigate this possibility, animals were given a 3-day 'head-start' with GM-CSF/tumor cell therapy before the intracranial implantation of tumor cells. Animals treated with GM-CSF according to the 'negative 3 protocol' (see Materials and methods) exhibited a 90% survival to the end of the study ($n = 10$). The control group in this experiment received tumor cell injections alone, which also began 3 days before tumor implantation. This control group had a 40% survival rate. The comparison between these two groups showed a statistically significant difference attributable to the effect of GM-CSF (Figure 3, $P < 0.05$). Representative coronal sections of brains from animals treated with GM-CSF are shown (Figure 4). Animals shown in Figure 4 were randomly selected from each group.

Immunological memory and DTH

Immunological memory was demonstrated by rechallenging animals from the negative 3 protocol, who had survived past 75 days after initial tumor implantation and treatment with GM-CSF/tumor cell therapy, with a second tumor injection in the contralateral

hemisphere. No further treatments were undertaken. Naïve animals served as controls. The re-challenged subjects showed an 86% survival rate past day 75 ($n = 7$), but naïve animals all died by day 28 ($n = 8$) (Figure 5). One re-challenged subject died, and necropsy confirmed a large tumor in the left (re-challenged) hemisphere, confirming that the tumor re-challenge was the cause of death.

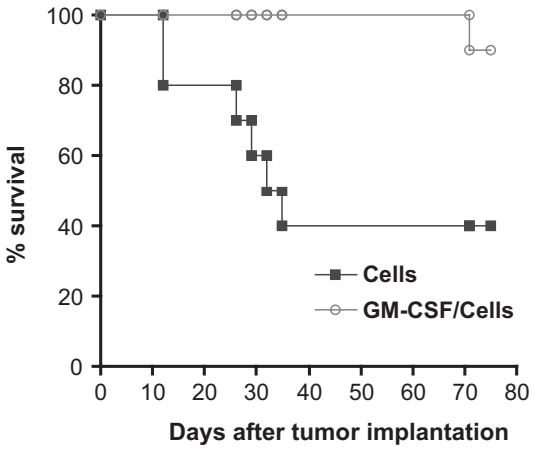

Figure 3. Survival from intracranial 9L tumor after pre-treatment with GM-CSF/cell therapy. Animals received either GM-CSF/tumor cell therapy or cell injections alone beginning 3 days before tumor implantation ('negative 3 protocol', $n = 10$ per group). Pre-treatment with GM-CSF greatly increased the survival rate of the animals (Log Rank $P < 0.05$).

GM-CSF treatment responders from the 'negative 3 protocol' were tested for DTH against irradiated 9L tumor cells. When injected with these cells, the right pinna of the GM-CSF treated group swelled considerably more than the left pinna, which was injected with saline. At 48 h, the differential swelling between the two pinnae in the GM-CSF treatment group ($n = 7$) was 12.9 ± 1.9 (10^{-4} inches $\pm$ SEM) compared with $4.2 \pm 1.1(10^{-4}$ inches $\pm$ SEM) for naïve animals ($n = 4$). The difference was statistically significant ($P < 0.01$) (Figure 6).

Immunohistochemical analysis

Animals used for immunohistochemical analysis received GM-CSF treatment under the 'negative 3 protocol.' At 1 week after tumor implantation, both CD4+ and CD8+ T-cells were found at the periphery of the tumors of untreated animals. Treatment with irradiated cells alone increased the number and density of CD4+ T-cell infiltration into the tumor, but the addition of

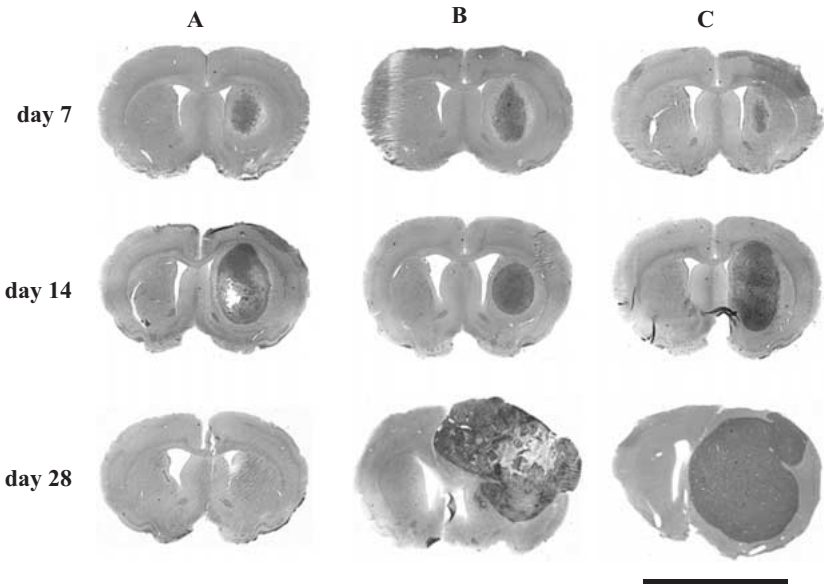

Figure 4. Coronal sections of brains from animals treated with cytokine. All animals were treated according to the 'negative 3 protocol.' The animals were perfused with paraformaldehyde, brains were removed at selected time points after tumor implantation; top row: 7 days; middle row: 14 days; and bottom row: 28 days. All brain sections were stained with hematoxylin and eosin. Column (A): animals treated with GM-CSF/tumor cell therapy; (B) animals injected with irradiated tumor cell only; and (C) untreated animals. Scale bar: 10 mm.

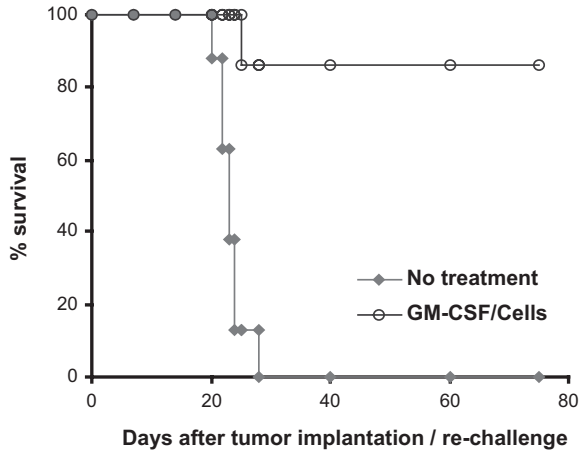

Figure 5. Immunological memory. Animals treated with GM-CSF/tumor cell therapy were re-challenged with intracranial tumors in the contralateral hemisphere after surviving at least 75 days from the initial 9L tumor implantation ($n = 7$). No further treatment was given and naïve animals served as controls ($n = 8$). GM-CSF treated animals had a survival rate of 86%. Control animals all died by day 28 ($P < 0.005$). One GM-CSF treated animal died from a tumor re-challenge, and necropsy revealed a large tumor in the left (re-challenged) hemisphere.

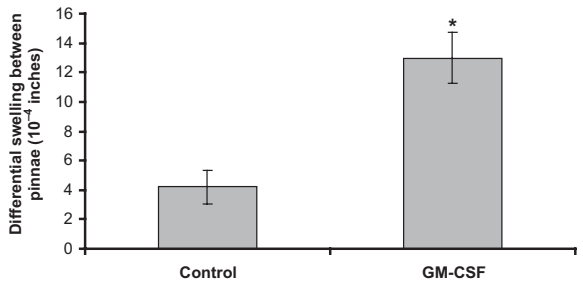

Figure 6. DTH against 9L tumor cells. Animals responding to the 'negative 3 protocol' for GM-CSF treatment were tested for DTH against 9L cells. 10^6 irradiated tumor cells were injected into the right pinna, while saline was injected into the left pinna. The differential swelling of the pinnae at 48 h in the GM-CSF treated animals ($n = 7$) was 12.9 ± 1.9 (10^{-4} inches $\pm$ SEM) compared to 4.2 ± 1.1(10^{-4} inches $\pm$ SEM) for naïve animals ($n = 4$). *, differential swelling was significant when compared to naïve animals ($P < 0.01$).

GM-CSF further increased the density of these lymphocytes (Figure 7). Throughout the remainder of the treatment period, animals treated with irradiated cells or the combination of GM-CSF and irradiated tumor cells continued to display increased tumor infiltration of CD4+ cells over untreated animals. However, the density of CD4+ infiltration continued to decrease within each treatment group as the treatment period proceeded.

Cytotoxic CD8+ T-cells were present within the 9L tumors of all animals. In the untreated animals, these were initially found at the tumor periphery, but then infiltrated throughout the tumor even as it grew to a larger size. Treatment with irradiated cells alone increased the density of CD8+ T-cell tumor infiltration. However, with the combination treatment of GM-CSF and irradiated cells, the CD8+ cytotoxic T-cell presence in the tumor was dramatically increased (Figure 8). Unlike the CD4+ T-cells, this marked CD8+ T-cell infiltration was sustained throughout the treatment period. GM-CSF treated animals, which had rejected their 9L tumors, showed a 'scar' at the tumor implantation site lined by the CD8+ T-cells.

Although their presence was not nearly as dramatic as that of CD4+ or CD8+ T-cells, OX-62+ dendritic cells were scattered throughout the tumors of GM-CSF treated animals during the early stages of tumor growth (day 10). Tumors treated with irradiated cells alone were practically void of these 'professional' APCs, as were tumors that received no treatment. As the treatment period proceeded, the presence of intratumoral dendritic cells became scarce in all tumors.

Discussion

Contrary to the original postulates of Lewis Thomas' Immune Surveillance Hypothesis [27], effective immune responses against endogenous tumor antigens that arise during transformation are seldom observed. In nearly all studies in which active systemic anti-tumor immunity was induced experimentally or therapeutically, T-cells have been found to play a critical role. Several mechanisms have been put forth to explain the failure to develop endogenous T-cell immunity in the typical cancer patient. The shedding of tumor antigens [28], loss of MHC expression [29], down-regulation of antigen processing [30], and production of immunosuppressive molecules [1–3] are some possible explanations. Recently, it has become clear that the generation of T-cell tolerance may be one of the most important mechanisms by which cancer evades the immune system under physiologic conditions. As part of this model, endogenous tumor antigens are presented to T-cells by tolerance-inducing APCs, which lack the appropriate co-stimulatory signals. The engagement of

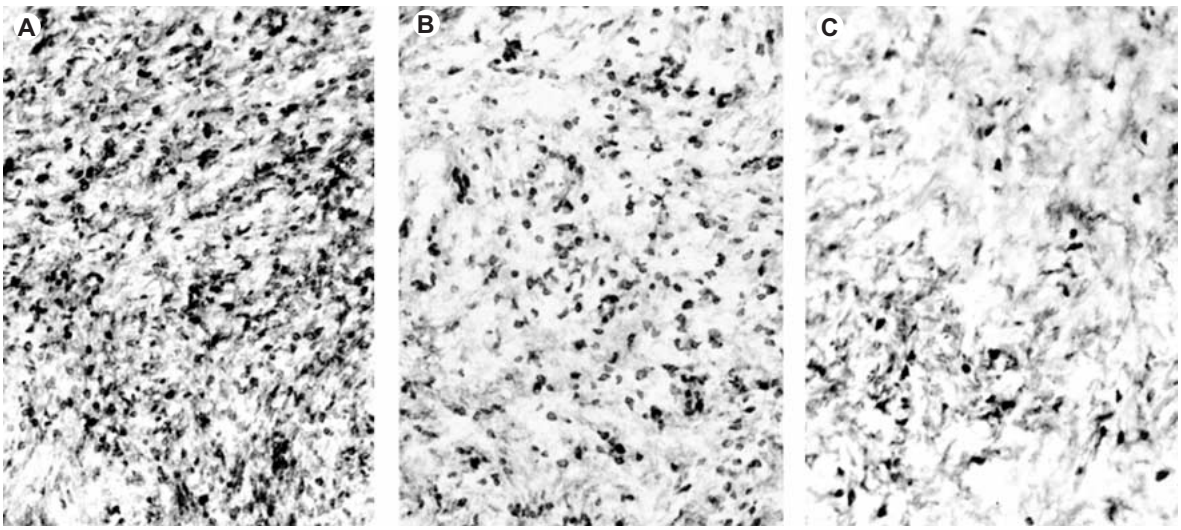

Figure 7. CD4+ T-cell infiltration of intracranial 9L tumor. Seven days after tumor implantation, CD4+ helper T-cells were observed to infiltrate densely into the 9L tumors in GM-CSF/tumor cell treated animals (A). Tumor infiltration by CD4+ T-cells in animals treated with irradiated tumor cell injections alone (B) and untreated animals (C) was much less significant (original magnification, ×100).

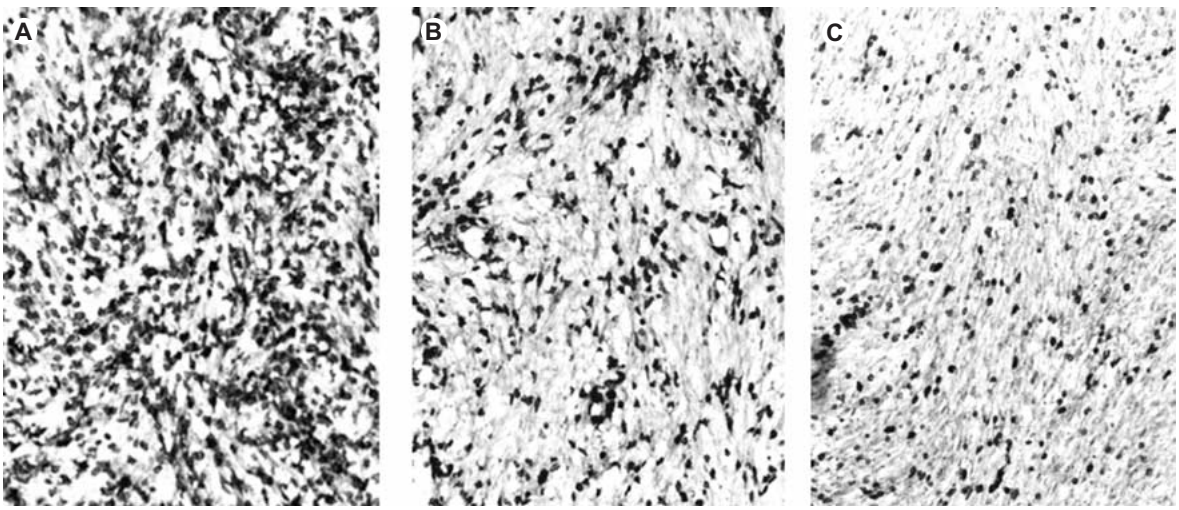

Figure 8. CD8+ T-cell infiltration of intracranial 9L tumor. Twenty-eight days after tumor implantation, CD8+ cytotoxic T-cells were still present throughout the tumors of GM-CSF/tumor cell treated animals (A). The density of CD8+ T-cell presence was dramatically higher in tumors treated with GM-CSF (A), when compared to those treated with irradiated tumor cell injections alone (B), or untreated tumors (C) (original magnification, ×100).

the T-cell receptor with these APCs then leads to anergy and apoptosis of the antigen-specific T-cells [31].

For any cancer immunotherapy to be effective, it must ultimately overcome this T-cell tolerance, and one important strategy towards achieving that goal may be to target the tumor antigens to appropriate APCs. It is not surprising, therefore, that GM-CSF, with the ability to recruit and activate APCs, was shown to be the most potent cytokine in generating systemic anti-tumor immunity [7,31]. GM-CSF has been identified as a critical factor in inducing the differentiation of dendritic cells, which are considered the primary APCs necessary for activating quiescent T-cells [32]. These activated T-cells then, in turn,

become the primary effectors of tumor rejection. Recent efforts in utilizing GM-CSF to enhance tumor immunogenicity have depended on generating transduced tumor cell vaccines, which deliver the cytokine in a continuous, paracrine fashion [7–10]. These studies have confirmed the efficacy of GM-CSF-transduced vaccines for various systemic cancers [8–10]. Specifically regarding brain tumors, Herrlinger et al. [11] were able to show that subcutaneous vaccinations with GM-CSF-transduced glioma cells extended the survival of mice with intracranial GL261 glioma. The safety and immunologic activity of autologous GM-CSF-transduced vaccines for human use were also recently demonstrated in several Phase I clinical trials (for review see Borrello and Pardoll, 2002) [33]. The efficacy of this strategy hinges on the sustained release of GM-CSF, and the promotion of local dendritic cell differentiation [31,34].

In addition to temporal considerations, the site of delivery of cytokine-transduced vaccines may also be important. Thompson et al. showed that a subcutaneous, but not intracranial, delivery of GM-CSF-transduced cell vaccine was able to extend the survival of mice with intracranial melonoma [35]. This may relate to the abundance of dendritic cells in the subcutaneous space and the relative paucity of them in the brain. In direct contradistinction, studies involving brain tumors and IL-2-transduced cell vaccines have demonstrated that a direct intracranial delivery is necessary for therapeutic effects [35,36]. Unlike GM-CSF, a local, sustained release of IL-2 at the site of the intracranial tumor may be crucial for the targeted lymphocyte cell types in this strategy.

The sustained delivery of cytokines can be achieved in ways other than cytokine-transduced cell vaccines. We have previously reported that a continuous delivery of cytokine via osmotic minipumps, combined with inoculations of irradiated tumor cells for tumor antigen presentation, can in fact mimic the paracrine effects of transduced cell vaccines [25,26]. This latter method has several advantages. First, the use of minipumps obviates the need to transfect tumor cells and characterize their cytokine production. Second, it allows for independent and rigorous control over the administration of cytokine and antigen dosages. Unlike our previous experiments involving peripheral tumors, we have applied this treatment method to intracranial 9L glioma in the present study. These tumors may behave differently from those implanted heterotopically in the flank because they may be protected by the blood–brain barrier. Furthermore, intracranial tumor challenges of 1 million tumor cells are lethal to the animals far quicker than the tumor load previously used. We have demonstrated that GM-CSF, in combination with tumor cell injections, had a significant anti-tumor effect. When GM-CSF/tumor cell immunotherapy was started on the day of tumor implantation, the survival of rats with 9L gliosarcoma was lengthened. This improvement of survival reached statistical significance when compared to untreated subjects. When compared to animals treated with irradiated cells alone, the survival of those treated with GM-CSF/tumor cell therapy was only marginally increased. It is commonly known that although 9L tumor cells are syngeneic to Fischer 344 rats, they are at least partially immunogenic [25,37]. It is therefore not surprising that animals treated with 9L tumor cell injections alone experienced a survival benefit intermediate between their untreated and GM-CSF/tumor cell treated counterparts.

In the present study, we also observed that cytokines that directly act on T-cells may increase the efficacy of GM-CSF-based therapy for 9L glioma. The major role of IL-2 is to activate and induce the proliferation of cytotoxic T-cells [38]. IL-12 has the additional ability to enhance the proliferation of natural killer cells and direct the differentiation of uncommitted T-cells towards the T-helper type 1 (Th-1) phenotype crucial for anti-tumor cell-mediated immunity [39,40]. Thus, acting through a parallel pathway independent of dendritic cells, each of these cytokines, when administered through minipumps in combination with GM-CSF, improved the survival of animals with intracranial 9L tumors over treatment with GM-CSF/tumor cell therapy alone. It is also important to note that the addition of intraparenchymal injection of TNF-α did not enhance the efficacy of GM-CSF therapy. Unlike the interleukins chosen for this study, TNF-α induces local inflammation in the brain by activating the vascular endothelium. When directly injected into the brain parenchyma, as was performed in this study, TNF-α was found to increase leukocyte adhesion to local microvessels [41]. It is possible that the dose of TNF-α used was insufficient to sustain an enhanced, local inflammatory state, and that multiple dosing of intraparenchymal TNF-α could improve upon the results of GM-CSF/tumor cell therapy. It is also important to note that IL-2 therapy may have detrimental effects in the central nervous system by the induction of brain edema. A study by Tjuvajev et al. [42] demonstrated that the intraparenchymal implantation of cell lines that secrete IL-2 can result in the disruption of the blood–brain barrier and vasogenic brain edema. Our approach,

however, differs from that of Tjuvajev et al. [42] since the delivery of IL-2 in our study was subcutaneous and not directly into the brain parenchymal.

When the GM-CSF/tumor cell therapy was started 3 days before tumor implantation ('negative 3 protocol'), nearly all the animals treated experienced long-term survival. The improvement of survival over animals pre-treated with cell injections alone was statistically significant. The fact that pre-treatment (or immunization) was necessary to show a dramatic GM-CSF effect may be due to the idiosyncrasies of our tumor model. In our previous reports involving immunotherapy for 9L flank tumors, the size of the tumor increased for 3–4 weeks before regression took place [25,26]. This trend was observed even in the most optimally treated animals, which eventually experience full rejection of their tumors. In our intracranial model, implanting 10^6 tumor cells kills the average animal in ~3–4 weeks. With MRI studies performed on some of the experimental animals, we also have anecdotal evidence that regression of intracranial tumors may begin between the 3rd and 4th week after implantation. Given the limited intracranial volume in the rodent subjects, it is possible that the initial growth of the tumor may have out-paced the maturation of the anti-tumor immune response and led to herniation and death of the animal before the effects of therapy reached fruition and tumor regression occurred. Reducing the number of implanted tumor cells may give the immune system the necessary 'head start,' but we chose pre-treatment over alterations in our tumor model in order to delineate the time-course necessary for the GM-CSF anti-tumor response. Our results showed that a duration as short as 3 days is sufficient for the maturation of this response.

The GM-CSF anti-tumor response was long-lasting as demonstrated by the survival results. In addition, survival from tumor re-challenge is consistent with an immune-mediated mechanism. As in previous studies involving cytokine-mediated immunotherapy, DTH served as a qualitative measurement of T-cell activity in our study [10]. It is important to note that one animal, which survived ~3 months after initial tumor placement, died following a re-challenge with intracranial tumor. Of particular interest, this animal was the only experimental subject that showed a poor DTH response during the re-challenge period, in the range similar to control animals. Thus, all the animals that survived tumor re-challenge without further treatment had significantly positive DTH responses. A positive correlation between vigorous DTH reaction and therapeutic outcome was also seen in the clinical trial in patients with renal cell carcinoma treated with autologous GM-CSF transduced vaccines [10]. In that study, one patient with a significantly positive DTH response had regression of multiple pulmonary metastases following three vaccinations with GM-CSF-transduced cells.

In our study, we also used immunohistochemical analysis to further characterize our anti-tumor response. Tumor infiltrating lymphocytes were of the CD4+ and CD8+ phenotypes, and the density of infiltration of these cells correlated with GM-CSF/tumor cell therapy. This is consistent with data suggesting that the GM-CSF anti-tumor response is dependent on the presence of both CD4+ helper and CD8+ cytotoxic T-cells [8]. The 9L tumors in control animals also showed T-cell infiltration, albeit at a lower density. The continual growth of these tumors implies that these infiltrating T-cells were inactive or non-specific. GM-CSF treatment also led to increased numbers of intratumoral dendritic cells. Although these cells are known to travel throughout the body, they usually come to rest in lymphoid organs, such as the spleen, where they interact with T-cells [32]. Their presence within the intracranial tumor is therefore unsuspected. It is tempting to speculate that they may have a local function within the tumor, perhaps in activating or recruiting T-cells.

In conclusion, we have demonstrated that continuous, subcutaneous infusion of GM-CSF, in combination with IL-2 or IL-12, can lead to increased survival of rats with intracerebral 9L glioma and resistance to future tumor re-challenges. These were long lasting and specific responses. Therefore, immunotherapy based on combined cytokines represents a promising treatment for glioma.

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