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Antigenic Differences Between Normal and Malignant Cells as a Basis for Treatment of Intracerebral Neoplasms Using a DNA-Based Vaccine

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Abstract

Antigenic differences between normal and malignant cells of the cancer patient form the rationale for clinical immunotherapeutic strategies. Because the antigenic phenotype of neoplastic cells varies widely among different cells within the same malignant cell-population, immunization with a vaccine that stimulates immunity to the broad array of tumor antigens expressed by the cancer cells is likely to be more efficacious than immunization with a vaccine for a single antigen. A vaccine prepared by transfer of DNA from the tumor into a highly immunogenic cell line can encompass the array of tumor antigens that characterize the patient's neoplasm. Poorly immunogenic tumor antigens, characteristic of malignant cells, can become strongly antigenic if they are expressed by highly immunogenic cells. A DNA-based vaccine was prepared by transfer of genomic DNA from a breast cancer that arose spontaneously in a C3H/He mouse into a highly immunogenic mouse fibroblast cell line, where genes specifying tumor-antigens were expressed. The fibroblasts were modified in advance of DNA-transfer to secrete an immune augmenting cytokine and to express allogeneic MHC class I-determinants. In an animal model of breast cancer metastatic to the brain, introduction of the vaccine directly into the tumor bed stimulated a systemic cellular anti-tumor immune response measured by two independent *in vitro* assays and prolonged the lives of the tumor-bearing mice. Furthermore, using antibodies against the various T-cell subsets, it was determined that the systemic cellular anti-tumor immunity was mediated by CD8⁺, CD4⁺ and NK/LAK cells. The application of DNA-based genomic vaccines for the treatment of a variety of brain tumors is being explored.

Keywords

Gene Therapy; Brain Tumors; Tumor Vaccine; Genomic DNA

Introduction

An emerging strategy in the treatment of cancer involves stimulation of an immune response against the unique antigens expressed by the neoplastic cells. The expectation is that effectively stimulated, the immune system can be called upon to destroy the malignant cells. In most instances, proliferating tumors do not provoke anti-tumor immune responses, which are capable of controlling tumor growth. The neoplastic cells escape recognition by the immune system in spite of the fact that they form weakly immunogenic tumor associated antigens

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(TAA). The successful induction of immunity to TAA could result in tumor cell destruction and prolongation of the survival of cancer patients. A number of different techniques have been designed to increase the host's cellular anti-tumor immune response. One such strategy is to "feed" antigen presenting (dendritic) cells apoptotic bodies from tumor cells or tumor cell lysates. Anti-tumor immune responses followed immunization with such vaccines as well as vaccines prepared by introducing tumor cell-derived RNA into dendritic cells. Immunization with dendritic cells "fed" derivatives of tumor cells or transfected with tumor-RNA can result in the induction of immune responses against the broad array of tumor antigens expressed by the population of malignant cells including tumors of neuroectodermal origin. In one pre-clinical study, intraperitoneal injection of bone marrow-derived dendritic cells pulsed with the RNA derived from the GL261 glioma cells induced a T cell response against intracerebrally implanted GL261 cells [1]. The efficacy of the vaccine was improved further by administration of recombinant interleukin-12 into the vaccine regimen. In patients, immunization with autologous dendritic cells transfected with mRNA from malignant glioma elicited a tumor specific CD8⁺ cytotoxic T-lymphocyte (CTL) response against the patient's malignant cells [2]. However the preparation of dendritic cell-based vaccines can be technically challenging and may not be appropriate for widespread use.

Other strategies have employed the use of modified neoplastic cells in the development of anti-tumor immune responses. In particular many studies have used tumor cells modified to secrete certain immune-augmenting cytokines in an effort to provoke anti-tumor immune responses. In general, however, unmodified, irradiated tumor cells are poorly or not immunogenic, largely because of down-regulation of surface antigen expression and abnormalities in antigen processing machinery (APM), which often occur in tumor cells [3,4]. Thus, tumors are not good APC and most have evolved means to escape from the host immune system either by avoiding immune recognition or by inducing immune suppression. However, immunization of syngeneic mice susceptible to tumor growth with variously modified tumor cells has resulted in immune recognition of TAA expressed by the tumor cells and the development of generalized MHC-restricted anti-tumor immune responses [5–8]. The introduction and expression of genes for various cytokines into tumor cells augments their immunogenic properties in experimental animals and in patients [9–12]. Selective tumor regression was observed in experimental animals and patients receiving immunotherapy alone, suggesting the potential effectiveness of this type of treatment for patients with malignant disease [13].

Antigenic differences between normal and malignant cells form the rationale for clinical immunotherapy protocols. Because the antigenic phenotype varies widely among different cells within the same tumor-cell population, immunization with a vaccine that stimulates immunity to multiple TAA expressed by the entire population of malignant cells is likely to be more effective than immunization with a vaccine for a single antigen. Variants that fail to express the antigen chosen for therapy can avoid destruction. Here, in a mouse model, we describe the application of a novel immunotherapeutic strategy to intracerebral breast cancer. The vaccine was prepared by transfer of genomic DNA from breast cancer cells into a highly immunogenic fibroblast cell line, where genes specifying breast cancer antigens were expressed [14]. The vaccine encompassed the array of TAA that defined the patient's neoplasm. Poorly immunogenic TAA, characteristic of malignant cells, can become strongly antigenic if they are expressed by highly immunogenic cells. In animal tumor models, immunization with DNA-based vaccine was sufficient to deter tumor growth and to prolong the lives of tumor-bearing mice [14,15].

Previous studies indicated that transfection of genomic DNA from the malignant cells into the cell line resulted in stable integration and expression of the transferred DNA altering both the genotype and the phenotype of the cells that took up the exogenous DNA. The genetically engineered cells were effective stimulators of the anti-tumor immune response. Immunization

of tumor-bearing mice with the DNA-based vaccine resulted in the induction of cell mediated immunity directed toward the type of tumors from which the DNA was obtained, and prolongation of survival. This was the case for mice with melanoma, squamous cell carcinoma and in mice with breast cancer [16]. Multiple undefined genes specifying TAA that characterize the malignant cell population were expressed by cells that took up DNA from the tumor. Among other advantages, only microgram quantities of DNA from small amounts of tumor tissue were required to prepare the vaccine. As the transferred DNA is integrated into the genome of the recipient cells, replicated as the cells divide and is expressed, the number of vaccine cells can be expanded as required for multiple immunizations. The recipient cells can also be modified before DNA-transfer to increase their immunogenic properties, as for example, to secrete immune-augmenting cytokines or to express allogeneic MHC-determinants which act as strong immune adjuvants. In animal models, injection of cytokine-secreting allogeneic fibroblasts into the tumor bed of intracerebral neoplasms was also effective in the treatment of mice with established brain tumors [17].

Materials and Methods

Preparation of a vaccine for use in the treatment of intracerebral breast cancer by transfection of cytokine-secreting syngeneic/allogeneic fibroblasts with DNA from a breast carcinoma that arose spontaneously in a C3H/He mouse (SB-5b cells)

Cytokine-secreting syngeneic/allogeneic fibroblasts were prepared as described previously [17]. The cells were further modified by transferring DNA from SB-5b cells, a breast cancer cell line, into the fibroblasts (Figure 1). Sheared, unfractionated DNA isolated (Qiagen, Chatsworth, CA) from a spontaneous mammary adenocarcinoma (SB-5b) that arose in a C3H/He mouse taken directly from *in vitro* cultured cells, was used to transfect mouse fibroblasts modified to express allogeneic H-2K^b-determinants and to secrete IL-2 (LM-IL-2K^b cells), IL-18 (LM-IL-18K^b cells) or GM-CSF (LM-GMCSFK^b cells) or to express H-2K^b-determinants alone (LMK^b cells) using the methods described in [18] as modified. Briefly, high molecular weight DNA from each cell type was sheared by passage through the DNA isolation column. The approximate size of the DNA at the time it was used in the experiments was 25 kb. Afterward, 100 µg of sheared DNA was mixed with 10 µg pCDNA6/V5-HisA, a plasmid that confers resistance to the antibiotic Blasticidin, for use in selection. The sheared DNA and plasmid (DNA: plasmid ratio = 10 : 1) were then mixed with Lipofectamine 2000, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). The DNA/Lipofectamine mixture was added to a population of 1×10^7 actively proliferating LM-IL-2K^b, LM-IL18K^b, LMGMCSFK^b cells, or non-cytokine secreting LMK^b cells divided into ten dishes containing an original inoculum of 1×10^6 cells. Eighteen hours afterward, the medium was replaced with fresh growth medium. The fibroblasts were maintained for 14 days in growth medium containing 2–5 µg/ml Blasticidin HCl (Invitrogen, Carlsbad, CA). One hundred percent of the cells transfected with tumor-DNA alone maintained in the Blasticidin growth medium died within this period. The surviving colonies in each of the plates (a total of at least 2.5×10^4) were pooled and maintained as a cell line for use in the experiments.

Intracerebral injection of C3H/He mice with SB-5b breast cancer cells

As a model of intracerebral metastatic breast cancer in patients, C3H/He mice were injected intracerebrally with SB-5b breast cancer cells. Anesthetized mice were placed into a stereotactic frame. A 1 mm burr hole was introduced into the right frontal lobe in the region of the coronal suture using a D#60 drill bit (Plastics One, Roanoke, VA). A Hamilton syringe containing a 26 gauge needle with a small (2–3 mm) piece of solder placed 3–4 mm from the tip of the needle was used to maintain a uniform depth of injection of the breast cancer cells, and vaccine, into the brain. The total injection volume was 5–10 µl. After injection, the incision over the burr hole was closed with a single 5-0 Dexon absorbable suture.

In the experiments where the animals received multiple intracerebral injections, a small cannula was initially inserted as described previously [17]. In brief, small screws (0–80 × 1/16; 1.6 mm in length) were obtained from Plastics One (Roanoke, VA) and a .022 diameter hole was subsequently drilled through the center of the screw. Anesthetized mice were placed into a stereotactic frame and a small burr hole was placed with a D#60 drill bit (Plastics One, Roanoke, VA) over the right frontal lobe in the region of the coronal suture. The screws bearing a central hole were subsequently secured into the small burr hole using Elmer's Super Glue Gel. The mice were allowed to recover and on specified days injections were made using a Hamilton syringe containing a 26 gauge needle with a small piece of solder placed 5 mm from the tip of the needle to maintain a uniform depth of injection. The total injection volume was 5–10 µl.

T cell mediated cytotoxicity toward breast cancer cells in mice immunized with the transfected fibroblasts

A CellTiter 96 aqueous non-radioactive cell proliferation assay kit (Promega, Madison WI) was used to measure T cell mediated cytotoxicity toward the breast cancer cells in mice immunized with the transfected fibroblasts. Effector T cell [recovered from the spleens of immunized mice by Histopaque (Sigma) density gradient [19] and mitomycin C-treated (50 µg/ml/10⁷ cells for 45 min) SB-5b target cells were co-cultured at 37° C for 18 hrs at a 30:1 effector:target cell ratio. Afterward, the non-adherent cells were removed, washed and viable SB-5b cells were added at various E:T ratios for 4 hrs at 37° C in a 7% CO₂/air atmosphere. The number of remaining viable cells was measured by MTS, which is bioreduced by viable cells into a formazan product that can be detected at 490 nm. Negative control wells were treated with 2% Triton-100 to cause total lysis of the cells. Positive control wells did not receive effector cells. Next 20 µl of MTS and 1 µl of phenazine methosulfate (PMS), an electron coupling reagent, were mixed and added to each well, followed by incubation at 37°C for 1–4 hrs in a 7% CO₂/air atmosphere after which the absorbance was read. The percent specific lysis was calculated from the absorbance using the formula as follows:

$$\frac{\text{Experimental Group} - \text{Negative Control}}{\text{Positive Control} - \text{Negative Control}} \times 100$$

ELISPOT IFN-γ Assays

Spleen cells from C3H/He mice injected i.c. with the various cell constructs were analyzed for T cells reactive with SB-5b cells in ELISPOT IFN-γ assays. T cells recovered by Histopaque density gradient from the spleens of mice injected i.c. with the transfected cells were co-incubated with SB-5b tumor cells (the ratio of spleen cells : SB-5b cells = 10:1) for 16 hours at 37°C in wells precoated with a high-affinity monoclonal antibody for IFN-γ according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). After incubation, the cells were washed before the addition of biotinylated anti-IFN-γ detection antibody and horseradish peroxidase labeled streptavidin (Streptavidin-HRP). In some instances, mAbs for T-cell subsets or NK/LAK cells were added to the mixed cell cultures before the ELISPOT IFN-γ assays were performed as a means of identifying the cell types activated for anti-tumor immunity in the immunized mice, as described previously [19]. The spots were counted using computer-assisted image analysis (ImmunoSpot Series 2 analyzer: Cellular Technology Limited, Cleveland, OH).

Statistical Analysis

Student's *t* test was used to determine the statistical differences between the survival of mice in various experimental and control groups. A *p* value less than 0.05 was considered significant.

Results

Treatment of mice bearing an intracerebral breast cancer with DNA-transfected syngeneic/allogeneic fibroblasts modified to secrete immune augmenting cytokines

The immunotherapeutic properties of the modified fibroblasts transfected with DNA from a breast cancer cell line derived from a tumor that arose spontaneously in a C3H/He mouse were determined in mice with intracerebral breast cancer. C3H/He mice were injected intracerebrally (i.c.) with a mixture of 1.0×10^4 SB-5b breast carcinoma cells and 1.0×10^6 cytokine-secreting syngeneic/allogeneic fibroblasts transfected with DNA from the breast cancer cells. The results (Figure 2) indicated that mice injected i.c. with a mixture of breast cancer cells and the transfected syngeneic/allogeneic fibroblasts modified to secrete IL-2 survived significantly longer than mice injected i.c. with a mixture of breast cancer cells and non cytokine-secreting, transfected fibroblasts ($p < 0.005$). Analogous results were obtained for mice injected i.c. with a mixture of breast cancer cells and transfected fibroblasts modified to secrete GM-CSF ($p < 0.05$). The survival of mice injected i.c. with SB-5b cells and transfected fibroblasts modified to secrete IL-18 was not significantly different than that of mice injected with SB-5b cells and non-secreting transfected cells. The experiment was repeated twice with equivalent results.

Thus, syngenic/allogeneic fibroblasts modified to secrete IL-2 or GM-CSF that were transfected with DNA from breast cancer cells were effective in prolonging the survival of mice with intracerebral breast cancer. Transfected fibroblasts modified to secrete IL-18 were not effective.

T cell mediated toxicity toward breast cancer in mice injected intracerebrally with syngeneic/allogeneic transfected fibroblasts modified to secrete IL-2, GM-CSF or IL-18

An MTS cytotoxicity assay was used to detect the presence of cytotoxic T lymphocytes towards breast cancer in mice injected i.c. with the mixture of SB-5b breast cancer cells and the modified DNA-transfected fibroblasts. The T cells, obtained from the spleens of the injected mice, were analyzed two weeks after the i.c. injection of the cell mixture. The results (Figure 3) indicated that, like the survival of mice with i.c. breast cancer treated with the cytokine-secreting fibroblasts, the cytotoxic response of greatest magnitude was in mice injected i.c. with the mixture of SB-5b cells and transfected fibroblasts modified to secrete IL-2 or GM-CSF. Lesser cytotoxic effects were present in mice injected i.c. with SB-5b cells and transfected fibroblasts modified to secrete IL-18.

An ELISPOT-IFN- γ assay was used to determine the proportion of T cells in the spleen that were reactive with SB-5b cells in mice immunized with transfected fibroblasts modified to secrete IL-2 or GM-CSF. The assay was performed six weeks after the i.c. injection of the mixture of SB-5b cells and the transfected fibroblasts. The results indicated that the highest proportion of T cells reactive with SB-5b cells was in surviving mice injected with fibroblasts modified to secrete IL-2 (Figure 4). Lesser numbers of spots were found in T cells from mice injected with SB-5b cells and non-secreting transfected fibroblasts or SB-5b cells and transfected fibroblasts modified to secrete GM-CSF. The analysis of cells from mice injected i.c. with SB-5b cells and transfected fibroblasts modified to secrete IL-18 was not performed because there were no surviving mice.

The inhibitory effects of antibodies against various T-cell subsets were used to determine the types of cells activated for anti-tumor immunity in mice injected i.c. with the transfected fibroblasts. The assay was performed two weeks after the cells were first injected. As indicated (Figure 5) the greatest inhibitory effects were found in mixed cell cultures carried out in the presence of CD4⁺ antibodies. Lesser inhibitory effects were observed in the presence of CD8⁺ and NK/LAK antibodies.

Treatment of mice bearing an established intracerebral breast cancer with transfected fibroblasts modified to secrete IL-2 and/or paclitaxel

The therapeutic properties of intratumoral injections of the DNA-based vaccine were compared with those of the chemotherapeutic agent paclitaxel in the treatment of mice with an established intracerebral breast cancer. A cannula was placed into the right frontal lobe of C3H/He mice. One day afterward the animals received an injection intracerebrally (i.c.) through the cannula with 1.0×10^4 SB-5b breast carcinoma cells. On the following day, those animals treated with paclitaxel received a single intraperitoneal (i.p.) injection of 2.25 mg/kg paclitaxel. On days two and nine following tumor injection, animals treated with the vaccine received 1.0×10^6 transfected fibroblasts introduced through the cannula into the tumor region. The results (Figure 6) indicated that mice with an established i.c. breast cancer injected either i.c. with fibroblasts transfected with tumor DNA and modified to secrete IL-2 or i.p. with paclitaxel survived significantly longer than untreated mice ($p < 0.025$). In addition, mice with an established i.c. breast cancer that received a combination of i.p. paclitaxel followed by immunization with the transfected fibroblasts survived significantly longer ($p < 0.05$) than untreated mice. However, there was no difference in survival in the mice treated with a combination of paclitaxel and transfected fibroblasts in comparison to those animals treated with either therapy alone. Finally, it should be noted that those animals treated with paclitaxel exhibited significant lethargy and cachexia that was not observed in either the controls or those animals treated only with DNA-transfected fibroblasts modified to secrete IL-2.

T cell mediated toxicity toward breast cancer in mice immunized with transfected fibroblasts modified to secrete IL-2 and/or paclitaxel

To determine if the immunity in mice injected i.c. with the transfected fibroblasts was systemic, spleen cells from mice injected i.c. with the transfected cells were analyzed for cytotoxic effects toward the breast cancer cells. An MTS based cytotoxicity assay was used for this purpose. The analysis was performed two weeks after the i.c. injection of breast cancer cells. The results (Figure 7) indicated that the spleen cell-mediated cytotoxic responses of greatest magnitude were in mice injected i.c. with breast cancer cells and transfected fibroblasts modified to secrete IL-2. Lesser cytotoxic effects were present in mice with an established i.c. breast cancer treated with paclitaxel with or without transfected fibroblasts modified to secrete IL-2. Thus, systemic immune responses directed toward the breast cancer cells were induced in mice injected i.c. with the IL-2 secreting transfected cells.

ELISPOT-IFN- γ assays were used as an additional means of determining if T cells directed toward the breast cancer cells were present in the spleens of mice in the various treatment groups. T cells were recovered by Hypaque density gradient centrifugation from the spleens of mice at two weeks following the i.c. injection of the breast cancer cells. The cells were co-incubated with the breast cancer cells for 16 hours at 37°C in wells before the non-adherent cells were transferred to the ELISPOT IFN- γ plates containing wells precoated with a high-affinity monoclonal antibody for INF- γ . After further steps, the number of spots was determined by a computer aided spot counter. The results indicated that the highest number of spots was present in spleen cells from mice with an established i.c. breast cancer treated with fibroblasts transfected with tumor DNA and modified to secrete IL-2 (Figure 8).

Discussion

Anti-tumor immune responses were generated in C3H/He mice injected i.c. with a mixture of breast cancer cells and cytokine-secreting mouse fibroblasts transfected with unfractionated genomic DNA from the breast cancer cells. The immunity was sufficient to prolong survival, although the mice eventually died of the disease. A major advantage of this type of vaccine is that the fibroblasts could be genetically modified in advance of DNA-transfer to augment their

immunogenic properties. In this instance, the cells were modified to express allogeneic class I MHC-determinants (allogeneic MHC-determinants stimulate uptake of the vaccine by dendritic cells and ensure that the cells will be rejected) and to secrete one of three Th1 cytokines, IL-2, IL-18 or GM-CSF. The prolonged survival of mice with i.c. breast cancer treated solely by immunization with the cytokine-secreting cells points toward the potential of this form of therapy in patients with breast cancer metastatic to the brain. The therapeutic benefits of transfected fibroblasts modified to secrete IL-2 exceeded those of fibroblasts modified to secrete IL-18 or GM-CSF. Although there was variability in the amount of respective cytokine secreted by the various cell constructs, the amount of local cytokine delivery in all cases is likely to have been in great excess in order to stimulate an anti-tumor immune response. The capabilities of various immune augmenting cytokines are variable in this tumor model. This may represent a peculiar attribute of the breast carcinoma cells grown i.c. in this study. We made no effort to compare the benefits of fibroblasts modified to secrete various other immune augmenting cytokines. Conceivably, IL-2 is not most efficacious in this regard. Greater benefits might result if the cells used as DNA recipients were modified to secrete different cytokines, either singly or in combination.

Because the tumor cell population is known to be heterogeneous and includes cells that are resistant to cellular immune mechanisms, a subpopulation of malignant breast cancer cells, resistant to host immune mechanisms must have survived. For further control, a combination of therapeutic strategies will be required. In this study we also compared the benefits of combining immunotherapy with paclitaxel, a standard chemotherapeutic agent. The dose and dosing schedule of paclitaxel used in this study is identical to that used in similar studies with this animal model [20]. Although paclitaxel suppresses the peripheral white cell population for several days, an anti-tumor immune response was found in the spleen cells taken from those animals treated with both paclitaxel and the cellular vaccine. However, the best systemic immunologic effect was detected in those animals treated with vaccine alone. A statistically significant prolongation of survival ($p < 0.025$) was found in mice receiving either form of treatment. Combination therapy did not provide synergy.

The successful construction of a tumor vaccine by transfer of DNA from the neoplasm into a highly immunogenic cell line is an extension of two seminal findings. The first is that the transfer of genomic DNA can stably alter the genome of the recipient cells. This approach was used in the classic studies that resulted in the identification of oncogenes [21,22]. The second is that the genetic composition of tumor cells differs from the genetic composition of non-neoplastic cells of the same individual. Genetic instability is a well-known characteristic of malignant cells.

Transfer of tumor-DNA into recipient cells such as syngeneic/allogeneic fibroblasts results in integration and expression of the transferred genes, including genes specifying TAA. Sufficient quantities of tumor-DNA to prepare the vaccine can be obtained from small surgical specimens. There is no need to specify or select any particular gene or gene segment. The fibroblasts can be readily transfected with tumor-derived native DNA. These cells are autologous i.e., they share identity with the patient's MHC class I-determinants (to allow for direct antigen presentation of TAA to the immune system of the host) and they express allogeneic MHC class I-determinants, to stimulate antigen up take and expression of TAA by dendritic cells of the host.

While vaccination based on transfer of tumor-derived DNA into a highly characterized, fibroblast cell line has a number of advantages, there are potential concerns as well. Since only a small proportion of tumor-DNA specifies TAA, it is possible that the vast majority of the transfected fibroblasts express normal cellular constituents that are unrelated to stimulating tumor rejection or express TAA at low levels. These concerns are minimized, however, by our

findings, which indicate that the proportion of cells that take-up tumor DNA and express TAA is sufficient to induce effective anti-tumor immune responses. Another concern related to therapy with DNA-based vaccines is that genes specifying normal “self” antigens are likely to be expressed by the DNA-transfected fibroblasts, thus creating a danger that autoimmune disease might develop. Evidence for autoimmunity has not been observed thus far following extensive immunization with the tumor-DNA-transfected fibroblasts. The immune system is normally tolerant to “self” antigens. Mice immunized with DNA-based vaccines have not exhibited adverse effects; they lived their anticipated life spans without evidence of disease. Cellular infiltrates into normal organs or tissues have not been detected. It is also conceivable that the vaccine itself may grow in the recipient, forming a tumor or provoking a neoplasm. However in multiple studies, tumor growth at the vaccination site or elsewhere in the body has not been observed.

The ultimate goal of cancer therapy is the elimination of every remaining tumor cell from the patient. It is unlikely that a single form of therapy is capable of achieving this goal. However immunotherapy in combination with surgery, radiation therapy and chemotherapy will likely find a place as a new and important means of treatment for patients with brain tumors. A major advantage of DNA-based vaccines is that they do not require protein purification or its production and yet they are able to elicit robust and long-lasting activation of the immune response, which results in tumor rejection. From a practical point of view, these vaccines are easy to prepare and they are relatively inexpensive. Only a limited quantity of tumor-derived DNA is required, which can be obtained from small surgical specimens. Overall, the disadvantages of DNA-based vaccines are few and are certainly no more difficult to overcome than those associated with other types of vaccines. Thus, DNA-based vaccines offer a number of advantages, which greatly encourage their further development for cancer immunotherapy.

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Figure 1.

Preparation of the DNA-based vaccine. DNA-based vaccines were prepared by transfection of the fibroblast cell line LM with DNA from mouse breast carcinoma. Briefly, high-molecular weight DNA from SB-5b cells was sheared by passage through the DNA isolation column. Next, 100 μg of the sheared DNA was mixed with 10 μg pCDNA6/V5-HisA, a plasmid that confers resistance to Blasticidin. The sheared DNA and the plasmid were then mixed with lipofectamine to facilitate DNA uptake. The DNA-lipofectamine mixture was added to a population of 1×10^7 LM fibroblasts modified previously by retroviral transduction to secrete IL-2 and to express H-2K^b-determinants (LM-IL-2K^b cells). The transfected fibroblasts were grown on a tissue culture plate, and Blasticidin was added to the medium to select for cells that had taken up the foreign plasmid DNA.

Figure 2.

Treatment of C3H/He mice with intracerebral SB-5b breast carcinoma with cytokine-secreting allogeneic fibroblasts transfected with DNA from a spontaneous breast neoplasm (SB-5b). C3H/He mice (nine animals/group) were injected with a mixture of 1.0×10^4 SB-5b cells and 1.0×10^6 cytokine secreting fibroblasts transfected with tumor DNA or with an equivalent number of non-secreting cells transfected with tumor DNA (LMK^b/SB5b). Mean survival time (MST) in days: Media control, 23.0 ± 1.9 ; LMK^b/SB5b, 27.3 ± 6.3 ; LMK^bGMCSF/SB5b, 30.0 ± 9.5 ; LMK^bIL-2/SB5b, 36.6 ± 7.0 ; LMK^bIL-18/SB5b, 28.4 ± 4.8 . Probability values were as follows: LMK^bIL-2/SB5b vs LMK^b/SB5b or media control, $p < 0.005$; LMK^bIL-2/SB5b vs LMK^bIL-18/SB5b, $p < 0.025$; LMK^bIL-2/SB5b vs LMK^bGMCSF/SB5b, $p < 0.05$; LMK^bGMCSF/SB5b vs media control, $p < 0.05$.

Figure 3.

MTS proliferation assay from spleen cells taken from the animals two weeks following a single intracerebral injection of a mixture of tumor and treatment cells. The target cells used in this study were SB-5b breast cancer cells, and the effector (spleen cell) to target cell ratios (E/T) were 50:1 and 100:1. Mononuclear cells from the spleens of the immunized mice obtained through Ficoll-Hypaque centrifugation were used for this assay. The *p* values in % specific lysis between no cytokine vaccine (LMKb/SB-5b) vs. IL-2, IL-18 and GM-CSF vaccines (LMIL-2Kb/SB-5b, LMIL-18Kb/SB-5b, LMGM-CSFKb/SB-5b) are 0.028, 0.017 and 0.07 respectively. The error bars represent one standard deviation.

Figure 4.

ELISPOT assay detecting INF- γ secretion by spleen cells in the animals that have survived for six weeks following the initial injection of SB-5b tumor cells and allogeneic fibroblasts transfected with tumor DNA. Mononuclear cells from the spleens of the immunized mice obtained through Histopaque centrifugation were used in this assay. The assay was done in the presence (SB-5b stimulated) and absence (unstimulated) of SB-5b tumor cells. The error bars represent one standard deviation.

Figure 5.

The effect of mAbs against T-cell subsets or NK/LAK cells on the anti-tumor cytotoxic activities of spleen cells from C3H/He mice injected i.c. with a mixture of SB-5b tumor cells and the cytokine-secreting cells transfected with tumor DNA. The animals were injected with a mixture of 1.0×10^4 SB-5b tumor cells and 1.0×10^6 cytokine-secreting fibroblasts transfected with tumor DNA. Two weeks after the injection, mononuclear cells from the spleens of the immunized mice obtained through Ficoll-Hypaque centrifugation were used for the ELISPOT assay detecting INF- γ producing cells. In this assay the number of INF- γ spots/ 10^6 spleen cells is measured. The frequency of tumor-specific effector cells in the spleen before vaccination was 0.002%. The frequencies of tumor-specific effector cells in the spleen after vaccination with LMKb/SB-5b, LMIL-2Kb/SB-5b, LMIL-18Kb/SB-5b and LMIL-2Kb/SB-5b + LMIL-18Kb/SB-5b are 0.02%, 0.06%, 0.02%, and 0.07% respectively. The over all *p* value between unstimulated vs. tumor cell stimulated group is 0.001. Significantly lowered (*p* = 0.02 *) IFN- γ spots were found in LMIL-2Kb/SB-5b vaccinated, CD8 blocked group. The error bars represent one standard deviation.

Figure 6.

Treatment of an established intracerebral breast cancer with paclitaxel and/or cytokine-secreting allogeneic fibroblasts transfected with a spontaneous breast neoplasm (SB5b). A cannula was inserted into the right frontal lobe of C3H/He mice (ten animals/group). On the following day each animal received through the cannula a single injection of 1.0×10^4 SB5b cells. On the following day those animals treated with paclitaxel received a single intraperitoneal (i.p.) injection of 2.25 mg/kg paclitaxel. On the following day (day two following tumor injection) and one week later (day 9 following tumor injection) those animals treated with the vaccine received 1.0×10^6 syngeneic/allogeneic fibroblasts transfected with DNA from the breast cancer cells and modified to secrete IL-2 introduced through the cannula into the tumor region. Mean survival time (MST) in days: Untreated, 16.6 ± 1.4 ; Paclitaxel, 18.4 ± 1.5 ; Vaccine, 19.2 ± 3.0 ; Paclitaxel + Vaccine; 18.4 ± 2.6 . Probability values were as follows: Paclitaxel vs untreated, $p < 0.005$; Vaccine vs untreated, $p < 0.025$; Paclitaxel + vaccine vs untreated, $p < 0.05$.

Figure 7.

MTS assay for determination of cytotoxicity from spleen cells taken from the animals 2 weeks following the intracerebral injection of tumor cells. The target cells used in this study were SB5b breast cancer cells, and the effector (spleen cell) to target cell ratios (E:T) were 50:1 and 100:1. Mononuclear cells from the spleens of the immunized mice obtained through Ficoll-Hypaque centrifugation were used for this assay. The error bars represent one standard deviation. Probability values were as follows: Paclitaxel vs untreated, $p=0.005$, 0.011 and 0.025 at E: T ratio 25:1, 50:1 and 100:1 respectively; Vaccine vs untreated, $p=0.011$, 0.005 and 0.028 at E: T ratio 25:1, 50:1 and 100:1 respectively; Paclitaxel + vaccine vs untreated, $p=0.020$, 0.001 and 0.029 at E: T ratio 25:1, 50:1 and 100:1 respectively; Paclitaxel + vaccine vs Paclitaxel, $p=0.102$, 0.7 , 0.22 at E: T ratio 25:1, 50:1 and 100:1 respectively.

Figure 8.

ELISPOT assay detecting INF- γ secretion by spleen cells (number of spots/ 10^6 cells) in the animals two weeks following injection of tumor cells. Mononuclear cells from the spleens of the immunized mice obtained through Ficoll-Hypaque centrifugation were used in this assay. The assay was performed in the presence (SB5b stimulated) and absence (unstimulated) of SB5b tumor cells. The frequency of tumor-specific effector cells in the spleen before vaccination was 0.002%. Probability values were as follows: Paclitaxel vs untreated, $p = 0.030$; Vaccine vs untreated, $p = 0.048$; Paclitaxel + vaccine vs untreated, $p = 0.029$; Paclitaxel + vaccine vs Paclitaxel, $p = 0.064$.