Interstitial gene delivery in human xenograft prostate tumors using titanium metal seeds

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Abstract
Gene therapy is a promising approach for the treatment of cancers. Strategies for gene vector delivery include systemic and local-regional approaches. Intratumoral delivery of vectors has generally employed direct injections into single or multiple locations throughout the tumor volume. However, this approach leads to nonuniform distributions of reagents within tumors and becomes cumbersome as the required number of injections is increased. We have investigated the effectiveness of an interstitial plasmid gene delivery based on using tiny metallic seeds (GeneSeeds) analogous to technology used for brachytherapy. Feasibility for interstitial use of GeneSeeds was demonstrated expressing reporter plasmids (green fluorescence protein or β-galactosidase) in human xenograft prostate tumors. Immunohistochemical analysis confirmed effective interstitial delivery, vector expression, and distributions of reporter genes within tumors. Applicability of GeneSeeds for delivery of radiosensitizing cytokines was examined by generating a cytokine [tumor necrosis factor-α (TNF-α)] expressing vector under the cytomegaloviral promoter and interstitially implanting GeneSeeds with this vector into prostate cancer tumors. TNF-α protein expression was observed around the ends of seeds and decreasing in an exponential gradient as a function of distance. The expression of TNF-α resulted in tumor growth delay of a human prostate cancer xenograft. These results demonstrate the feasibility of applying interstitial delivery of gene expressing vectors for the treatment of human cancers. [Mol Cancer Ther 2004;3(6):655–9]

Introduction
The ability to selectively kill cancer cells without damaging normal cells is a therapeutic goal under intense current investigation. Of the various approaches that include chemotherapy, radiation therapy, immunotherapy, and gene therapy, the latter appears to offer this potential, but practical limitations of gene delivery have presented obstacles preventing easy implementation. Systemic administration of genetically engineered vectors offers treatment for primary and metastatic diseases; however, the physiology of tumors presents many of the same hurdles faced by chemotherapeutic approaches—heterogeneously perfused tumors with resultant underdosed regions (1).

For these several reasons, current approaches to gene delivery have included direct injection of genetically engineered vectors (viruses or plasmids) into tumors (2-6). Although the mechanics of such delivery are improving, distributions of vector containing reagents within tumors currently are suboptimal. Injections into tumors using a fine needle and syringe have been observed to follow the paths of least resistance, resulting in unpredictable distributions of injected materials (7-11). Proper evaluation of the potential of gene therapy, in part, rests on future improvements in the delivery of gene containing vectors to tumor cells.

We have developed a novel technology in which tiny metallic seeds (GeneSeeds) containing reagents are used for insertion into tumors. This approach offers the potential to achieve more efficient and uniform interstitial distributions of gene containing vectors. Our preliminary studies using a human prostate cancer xenograft demonstrate effective delivery and expression of genes, suggesting potential value for this technology for clinical translation.

Materials and Methods
Cell Lines and Plasmid Vectors
PC3 human prostate cancer cells are maintained in MEM containing 5% FCS at 37°C in 5% CO2 with penicillin and streptomycin added to all medium. Reporter vectors, pGFP and pβ-gal, were obtained from Stratagene (La Jolla, CA) and used for determination of gene expression. pCMV-TNF-α was constructed by subcloning tumor necrosis factor-α (TNF-α) CDNA (0.6 kb) encoding “mature” protein under the cytomegaloviral promoter (12).

Manufacture of the GeneSeeds
The prototype GeneSeed consists of a metallic tube made of high purity titanium metal suitable for medical applications with a thickness of 0.005 inch. Low weight, high strength titanium is the metal of choice for the majority of implantable devices. Titanium grade metal specified in the American Society for Testing of Materials F67-69 “Standard Specifications for Unalloyed Ti for
Surgical Implant Applications’ was used. The surgical grade titanium tubes (medical grade metal) were cut to required size (0.3 cm). The seeds were washed with an aqueous solution containing a mild detergent followed by acetone and sterile water for injection. The washed seeds were dried in an oven at 110°C for about 2 hours. Autoclaving was performed to assure sterility. The vector solution was loaded into seeds manually for the described studies.

Subcutaneous Tumor Model

The mice (n = 6 per arm of the study; 6 to 7 weeks old male BALB/c nu/nu) were used for human tumor xenografts. Tumors were induced by s.c. flank injections of 5 x 10^6 PC3 cells in 0.1 mL. Tumors are measured by external caliper to the 0.1 mm, and volumes were calculated (V = H x L x W).

Implantation of GeneSeeds Containing Reporter Plasmid DNA in Tumors in Mice

The ability of seeds to release plasmid into tumors in vivo was assessed by implantation of GeneSeeds containing plasmid DNA into the centers of tumors in nu/nu mice. The technical aspects of GeneSeed implantation involve loading of 1 μL of plasmid containing buffer or Lipofectamine solution into seeds. Seeds were loaded into an 18-gauge hollow stainless steel needle applicator (disposable needle designed for prostate brachytherapy, Best Medical Industries, Springfield, VA). The long axis of the tumor (0.5 to 1.0 cm long) was identified and the loaded seed was introduced percutaneously into the tumor center. Implanted tumors were subsequently excised at indicated intervals after implantation and analyzed for expression of green fluorescence protein or β-galactosidase. Animal tumors were implanted with each reporter plasmid DNA (pGFP or pβ-gal). Implanted tumors were excised on days 0, 1, 2, 4, and 6. Tumors were divided into two sections parallel to the implanted seed. Half of the tumor was fixed and embedded in paraffin, while the other half was frozen in liquid nitrogen for biochemical analysis. One piece (5 μm) of the tissue sections mounted on poly-L-lysine-coated slides was stained using H&E to visualize adenocarcinoma. The others were used for immunohistochemical analyses. Quantification of histologic sections was obtained by capturing images at 20× or 40× magnification to a computer for analysis.

Immunohistochemistry

Tumor tissues were fixed in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) and embedded in paraffin. Sections (5 μm thick) were mounted on poly-L-lysine-coated slides. For detection of β-galactosidase expression, slides were deparaffinized, permeabilized using Tris buffer (pH 7.5) containing 1% Triton X-100, and incubated with blocking serum (normal goat) diluted 1:100 in PBS for 20 minutes at room temperature. Slides were washed twice for 5 minutes in PBS and incubated with primary antibody for 1 hour at room temperature according to manufacturer’s instructions (Promega, Madison, WI). Slides were rinsed with PBS and incubated with biotin-conjugated secondary antibody against IgG for 30 minutes at room temperature using the streptavidin-AP system containing biotinylated second antibody (KPL, Gaithersburg, MD) and HistoMark kit (KPL) for color developing. For detection of green fluorescence protein expression, slides were visualized using an epifluorescent microscope (Bx60 universal fluorescent microscope, Olympus, Melville, NY) equipped with FITC filters and a camera (DKC 5000 Sony 3 CCD, Sony, New York, NY).

In vivo Assay of Tumor Response to TNF-α Expression

Male nude mice bearing PC3 xenografts (~100 to 200 mm³ in volume) were implanted with GeneSeeds carrying plasmid DNA, pCMV-TNF-α. The tumor diameters were measured twice weekly with a Vernier caliper following implantation. Growth delay was the primary endpoint to assess the effects of TNF-α expression on tumor growth. Control mice were treated by LipofectAMINE alone loaded in seeds or by direct injection. Tumor sizes were determined until a maximum volume of ~1,200 mm³, at which time the animals were sacrificed.

Statistical Analysis

All statistical analyses were performed on the percentage of volume changed for each tumor rather than the tumor volume to remove variations caused by differences in initial tumor size. Student’s t tests were performed to determine if the differences between the two treatments were significantly different. ANOVA testing was performed to determine if the change in volume during the time course was significant. P < 0.05 was considered statistically significant.

Results

To optimize seed design, we tested several prototypes of varying size, shape, and numbers of holes to provide portals for diffusion. Seeds used for brachytherapy of human tumors typically are 4 to 5 mm long (13, 14). Seeds (5 mm) readily hold 1 μL (10 μg/mL) of plasmid containing solution. To apply this technique to smaller tumors and to be more flexible in seed positioning within the tumors, we have also manufactured 3-mm seeds for animal studies (Fig. 1). The following designs were tested in initial

Figure 1. Proposed designs of GeneSeeds. The prototype GeneSeed consists of a metallic tube made of high purity titanium metal suitable for medical applications with a thickness of 0.005 inch.
studies: (1) titanium tubes with both ends open, with one central hole of 0.5 mm diameter; (2) titanium tubes with both ends open, with two central holes of 0.5 mm diameter; and (3) titanium tubes with both ends open, with no central holes. We observed that one seed (3 mm long) with both ends open, with no central holes, was optimal for use in small animals because 5-mm-long seeds or implanting multiple seeds were limited by desired tumor volumes.

Delivery of Gene Vectors in Human Xenograft Model Tumor System

Human prostate cancer xenograft tumors were grown to \( \sim 100 \) to 200 mm\(^3\) in volume. Following implantation of reporter vector containing seeds, tumors were excised and fixed at indicated intervals and embedded in paraffin. Distributions of reporter expression were examined by performing immunohistochemical analyses. H&E staining of tissues confirmed adenocarcinoma (data not shown). As shown in Fig. 2, the reporter proteins, green fluorescence protein and \( \beta \)-galactosidase, were readily expressed by 48 hours of implantation. The expressed reporter proteins were concentrated around the end of implanted seeds (arrow marks). These results defined optimal conditions for interstitial gene delivery.

Distributions of Cytokine and TNF-\( \alpha \) Protein Expression

To determine the feasibility of using GeneSeeds to deliver radiosensitizing cytokines, we constructed a vector expressing TNF-\( \alpha \) under the control of a constitutive expression promoter (cytomegaloviral promoter). Xenografts were grown to appropriate sizes and were implanted with vector containing seeds. TNF-\( \alpha \) expression was determined at various intervals (days 0, 2, 4, and 6) in tumors. The efficacy of vector delivery was determined in the tissue sections cut proximal to the ends of the seeds by scoring cells interacting with an antibody to TNF-\( \alpha \). As shown in Fig. 3, \( \sim 17\% \) of cells showed TNF-\( \alpha \) expression over the first 2 days. The maximum expression level (\( \sim 67\% \)) was observed on day 4 following implantation and decreased to 26% by day 6.

Next, the distributions of expressed TNF-\( \alpha \) were determined in tumors as a function of distance from the open ends of seeds (Fig. 4). Tissue sections (5 \( \mu \)m thick) were obtained starting from the ends of seeds and probed with TNF antibodies. Eighty-two percent of cells expressed TNF-\( \alpha \) within 50 \( \mu \)m of the ends of seeds by the fourth day. From 5% to 15% of cells showing TNF-\( \alpha \) were detected at distances of 4,000 to 6,000 \( \mu \)m, respectively (Fig. 4A). The transfection rate shown as a function of distance from seeds in a one-dimensional model results in a sigmoidal function (Fig. 4B). Taken together, these data show that the distributions of expressed gene were maximal by 4 days, with high localization around the ends of the seeds, gradually decreasing as a function of distance from the open ends of seeds.

TNF-\( \alpha \) is a therapeutic cytokine capable of inducing tumor regression and radiation sensitization (3, 15-19). Following implantation of seeds containing pCMV-TNF-\( \alpha \), the tumor volumes were measured weekly for 36 days. As shown in Fig. 5, tumor growth delay was observed for up to 36 days. Control seeds with Lipofectin showed no inhibition of tumor growth. Statistical data analysis was performed using the percentage volume changed in tumors rather than the tumor volume because the initial volume of when seeding occurred was variable. Student’s \( t \) tests were performed for each time point comparing the percentage change in tumor volume in TNF-\( \alpha \) treated samples.

Figure 2. Immunohistochemical analyses. (A) \( \beta \)-galactosidase staining in cells and (B) green fluorescence protein expression. Histologic sections were obtained by capturing images at 20 x magnification to a computer. Arrows, areas of the end of seeds and concentrated staining.

Figure 3. Immunohistochemistry of TNF-\( \alpha \) expression. Following implantation of GeneSeeds loaded with Lipofectamine containing pCMV-TNF-\( \alpha \) in PC3 xenografts, tumors were excised at indicated intervals and analyzed for TNF-\( \alpha \) expression as described in Materials and Methods.
(n = 6) with the seed-Lipofectin treated samples (n = 6). The percentage change in tumor volume for the seed-TNF-α treated samples was significantly lower than the seed-Lipofectin treated samples at all the time points from day 6 to day 27 as can be seen in the bar graph plotted in Fig. 5. ANOVA testing was performed first on the seed-Lipofectin treated tumors; as expected, these control samples showed a significant increase (P = 0.0066) in the relative tumor volume. The ANOVA testing on the seed-TNF-α treated tumors showed a significant (P = 0.0145) decrease in the relative tumor volume throughout the time course. These results confirm the suitability of using these seeds interstitially. Seeds (3 mm long) with both ends open, with no central holes, were optimal for small animal studies. Distributions of expressed gene products were localized around the ends of seeds with decreasing gradient as a function of distance. Gene expression as a result of vectors delivered via GeneSeeds was observed to be more locally concentrated than that achieved by direct injection (data not shown). Furthermore, TNF-α expression delayed tumor growth following implantation of seeds, supporting the potential for translational applications.

Discussion
Gene therapy offers promising treatments for genetic diseases and solid tumors (2-6). Current approaches for gene transfer include direct injection of genetic materials by the i.v. route or interstitial placement in tumors (7-11). Improvement in the technology for gene delivery is necessary to optimize therapeutic efficacy. In this study, we have demonstrated an improved delivery method for genetically engineered plasmid vectors by using hollow metallic seeds. Our data show that gene expression by vectors delivered using GeneSeeds is more locally distributed than that achieved by direct injection. Furthermore, the local retention and expression of the cytokine (TNF-α) resulted in tumor growth inhibition for up to 36 days, although immunohistochemical analysis revealed a decrease of TNF-α expression in tumor cells after day 6. We interpret our observation to reflect expression and secretion of TNF-α into the tumor bed resulting in tumor growth inhibition. No mortality nor morbidity was observed due to GeneSeeds or TNF-α expression in these animals, consistent with the known applications of similar metallic seeds in the treatment of cancers in humans. Therefore, these data support this delivery vehicle potentially for use in human gene therapy applications.

The combination of gene therapy and radiation therapy enhances tumor control within normal tissue tolerance limits. With the use of radiation-inducible vectors, a regional cytokine therapy or radiation sensitization may be employed by achieving spatial and temporal control over gene expression by conformal radiotherapy to the inoculated tumor bed (15, 16). Radiosensitization is predicted to enhance local tumor control with lower doses of radiation.

Cytokines such as the interleukins, TNF, and interferons have proven toxic when used systemically but are attractive agents for local use (17). For example, TNF-α...
enhances the tumoricidal activity of ionizing radiation in vitro and in vivo (15-18). The recent combination of TNF-α and radiotherapy in a clinical trial has produced encouraging preliminary results (19). However, toxicity from systemic delivery of TNF-α has resulted in fever, nausea, loss of appetite, fatigue, and hypotension (17).

Viral-mediated transfer of cytotoxic or radiosensitizing genes, such as TNF-α, attached to a radiation response element has been used for control of ex vivo gene expression with ionizing radiation (18, 20). Use of TNF-α as the prototype for gene therapy with radiation has been reported to sensitize tumor cells and has little or no effect on normal cells (3, 17). However, intratumoral delivery of gene therapy vectors involves injection into single or multiple locations throughout the tumor volume. We see this as a slow, poorly controlled process, which leads to non-uniform deposition of the reagents within these tumors. Our data show that interstitially delivered TNF-α was expressed in high local concentrations around the end of seeds, suggesting the feasibility of distributions of these seeds over a tumor volume for use in gene therapy of cancers.

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References

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