Effect of a Novel DNA Vaccine on Angiogenesis and Tumor Growth In Vivo

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Objective: To develop a DNA cancer vaccine that targets the vascular endothelial growth factor receptor.

Design: Mice were vaccinated intramuscularly with listeriolysin O–fetal liver kinase 1 (LLO-Flk1) or controls. Mice were also challenged subcutaneously with the tumor cell line TC-1. Tumor sizes were measured after vaccination. At the conclusion of the experiments, the tumors were harvested for immunohistochemical analysis and determination of hemoglobin content.

Setting: Research laboratory.

Subjects: Six- to 8-week-old C57BL/6 mice.

Intervention: Fifty micrograms of each vector was administered 3 times at weekly intervals.

Main Outcome Measures: Tumor size, mean vessel density of tumors, hemoglobin content of tumor.

Results: Mice treated with the LLO-Flk1 vaccine experienced slower tumor growth relative to the other treatment groups. Complete tumor regression was observed in several cases. Immunohistochemical staining of tumors revealed fewer blood vessels in the mice vaccinated with the LLO-Flk1 vaccine relative to the other treatment groups. Finally, colorimetric assessment for hemoglobin suggested decreased vasculature in the tumor bed of these mice relative to the control groups.

Conclusion: The novel DNA cancer vaccine LLO-Flk1 can slow tumor growth in vivo.

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IMMUNOTHERAPY IS A TREATMENT modality for head and neck cancer that holds great promise. Many strategies designed to generate cell-mediated immunity against various malignant neoplasms have been developed during the past 30 years.\(^1,2\) However, despite their recognized therapeutic potential, cancer vaccine strategies have demonstrated little clinical efficacy. Objective tumor regression in patients with cancer is rare when measured using strict Response Evaluation Criteria In Solid Tumors criteria.\(^3\)

Obstacles to successful cancer immunotherapy include (1) the lack of ideal tumor antigens and (2) poor immunogenicity of the vaccines. Regarding tumor antigens, the ideal tumor-associated antigen would be highly expressed by many different tumor types. Among available tumor antigens for cancer, vascular endothelial growth factor receptor 2 (VEGFR2) has received a great deal of attention. The VEGFR2 is a tyrosine kinase receptor for VEGF, and it is the major receptor that mediates the angiogenic effects of VEGF.\(^4\) It is highly expressed in tumor vasculature compared with normal vasculature, and it is expressed on the surface of a variety of tumor cells.\(^4\) High expression of VEGF and VEGFR is associated with worse survival in patients with head and neck cancer.\(^5\) Therefore, this molecule is an ideal target for an antiangiogenic, immunotherapeutic head and neck cancer vaccine strategy.

A second obstacle to successful immunotherapeutic treatments is the lack of immunogenicity of many cancer vaccines. Vaccine strategies that use peptides or naked DNA are safe but require the use of immune adjuvants to elicit strong immune responses. Conversely, vaccine strategies that use infectious agents, such as recombinant bacterial vaccines, do not require immune adjuvants to elicit a response.\(^6\) In particular, Listeria monocytogenes is an intracellular bacterium that has been used with success in preclinical models of cancer. When recombinant Listeria is engineered to secrete tumor antigens, including VEGFR2, regression of sizable tumors is seen in many preclinical studies.\(^6,8\) Immune responses are improved when the tumor antigen is fused to listeriolysin O (LLO), an L monocytogenes virulence factor that has been shown to enhance the antitumor efficacy of cancer vaccines when fused to the tumor antigen of interest.\(^6,10\) It is thought that LLO elicits this effect by
targeting intracellular proteins for degradation in the ubiquitin-proteasome pathway, thus allowing for antigen presentation in the peptide-binding region of major histocompatibility complex class I molecules.9

In the present study, we created a DNA vaccine in which the murine homologue of VEGFR2 (fetal liver kinase 1 [Flk1]) is fused to LLO. DNA vaccines are safe immunotherapeutic agents that have been used previously in preclinical studies that target VEGF and VEGFR.11-14 Using a murine cancer model, we tested the hypothesis that the administration of this fusion construct, LLO-Flk1, will result in greater tumor regression and greater immunologic responses compared with a DNA vaccine that contains Flk1 alone. We found that mice that received the LLO-Flk1 fusion construct experienced slower tumor growth relative to the other treatment groups, and complete tumor regression was observed in several cases. In addition, immunohistochemical staining performed on tumor samples for endothelial cell marker CD31 revealed a decreased preponderance of positively staining vessels in mice vaccinated with the LLO-Flk1 construct relative to the other treatment groups. Finally, colorimetric assessment for intratumoral hemoglobin suggested decreased vascularity in the tumor bed of these mice relative to the control groups. These findings indicate that LLO-Flk1 might be a potentially useful tool for antitumor immunotherapy in human cancers.

**ANIMALS AND CELL LINES**

Female C57BL/6 mice (H-2b) (Charles River Laboratories, Wilmington, Massachusetts) 6 weeks of age were used according to the protocols approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania School of Medicine, Philadelphia.

This squamous epithelial cell line was immortalized with E6 and E7, early genes of human papillomavirus 16 and 18. The TC-1 cells were maintained in RPMI 1640, 10% fetal bovine serum, 1% penicillin-streptomycin, 1% nonessential amino acids, and 1% sodium pyruvate.

**CONSTRUCTION OF VECTORS CONTAINING Flk1, LLO, AND LLO-Flk1**

The experimental vectors were constructed by inserting the genes of interest into the pCDNA 3.1 plasmid (Invitrogen Corp, Carlsbad, California). Fragment 3 of the gene encoding murine VEGFR2 (Flk1) was cloned using the primers 5'-GGGCTCGAGTGATCAGGGGTCCTTACTTAA-3' (forward) and 5'-GGGACTAGTTTTACTTGTCATCGTCGTCTCTTGATGTTGCAATC-3' (reverse) and was amplified via polymerase chain reaction (PCR). The Flk1-Frag 5 was inserted into the pCR2.1-TOPO vector (Invitrogen Corp) between the EcoRI restriction sites. The Flk1 was digested from pCR2.1 at the Kpn-I and Xba-I restriction sites and was inserted into pCDNA3.1 (+) plasmid, thus producing pCDNA3.1-Flk1, or Flk1. The vector was transformed into competent Top-10 Escherichia coli (Invitrogen Corp), amplified by means of bacterial culture, and isolated via bacteriolysis using the Qiagen MaxiPrep Kit (Qiagen, Valencia, California). Insertion of Flk1–Frag 5 was confirmed by means of PCR.

The hly gene encoding LLO was amplified by PCR using the forward primer 5'-GGGGTACCATGTTTTATTAC-3' and the reverse primer 5'-GGGGTACCATCATAATTATCTTCATTCCAGA AATT-3' and was cloned into pCR2.1 between the EcoRI restriction sites. Listeriolysin O was amplified, cut from pCR2.1 at the restriction sites Afl-II and Kpn-I, and inserted into pCDNA3.1 (+) and pCDNA3.1–Flk1, yielding pCDNA3.1-LLO (called LLO) and pCDNA3.1–LLO–Flk1 (called LLO-Flk1), respectively. These constructs were transformed into competent TOPO-10 E. coli, amplified by means of bacterial culture, and isolated via bacteriolysis using the Qiagen MaxiPrep Kit. Insertion of LLO was confirmed by means of PCR.

**METHODS**

**Prophylactic Vaccinations**

Twenty C57BL/6 mice were assigned to 1 of 4 treatment groups: (1) phosphate-buffered saline (PBS), (2) pCDNA3.1 (+), (3) Flk1, or (4) LLO-Flk1. Fifty micrograms of each vector was reconstituted in a total volume of 100 µL of PBS and was administered as an intramuscular injection in the right thighs of the mice on days −21, −14, and −7. On day 0, 5 × 10⁶ TC-1 cells were administered subcutaneously in the right flank. Weekly measurements of tumor volume were performed in 2 dimensions using a digital caliper. Tumor volumes were followed until the death of the mice or until the tumor reached a length of 20 mm, at which point the mice were humanely killed.

**Therapeutic Vaccinations**

Twenty C57BL/6 mice were assigned to 1 of 4 treatment groups: (1) PBS, (2) pCDNA3.1 (+), (3) Flk1, or (4) LLO-Flk1. Each mouse was administered 50 µg of the designated treatment vector in a total volume of 100 µL of PBS and was administered as an intramuscular injection in the right thighs of the mice on days 3, 10, and 17. The presence of tumors was confirmed by palpation, and weekly measurements were performed in 2 dimensions using a digital caliper. Tumor volumes were followed until the death of the mice or until the tumor reached a length of 20 mm, at which point the mouse was humanely killed.

**MATTRigel PLUG ASSAY**

Basement membrane matrix (Matrigel) was obtained from BD Pharmingen (San Diego, California). Twenty-five C57BL/6 mice were randomized into 1 of 5 treatment groups: (1) PBS with basic fibroblast growth factor (bFGF), (2) PBS without bFGF, (3) pCDNA3.1 (+), (4) Flk1, or (5) LLO-Flk1. Each mouse was administered 50 µg of the designated treatment vector in a total volume of 100 µL by intramuscular injection in the right thigh. Vaccination was performed 3 times at weekly intervals on days −21, −14, and −7. For each treatment group (except for the PBS without bFGF group), the Matrigel plugs were fortified with 400 ng of bFGF. On day 0, 500 µL of the Matrigel matrix was implanted subcutaneously in the right flank region of each mouse using a 1-mL syringe and a 24-gauge needle. On day 7, the mice were humanely killed, and the Matrigel plugs were removed. The plugs were weighed and immersed in a shaking water bath for 24 hours (10 mL of distilled water per 1 g of Matrigel plug). On day 8, 100 µL of the water bath solution from each sample was mixed with 100 µL of Drabkin reagent (Sigma-Aldrich Corp, St Louis, Missouri) and plated onto a 96-well plate. This plate was read using a spectrophotometer at 540 nm to quantify the hemoglobin concentration in each sample. The presence of statistically significant differences between samples was determined using the 2-tailed 𝑡 test without logistical transformation.
IMMUNOHISTOCHEMICAL ANALYSIS

Tumor samples harvested 7 days after the final therapeutic vaccination were each snap frozen in liquid nitrogen. Cryosections were prepared for staining with rat anti-mouse CD31 antibody (Abcam, Cambridge, Massachusetts). The slides were first blocked with goat serum and incubated with the primary antibody. They were then stained with a conjugated secondary antibody, goat biotinylated anti-rat IgG (Abcam). Positively staining cells were visualized using the avidin/biotinylated enzyme complex–horseradish peroxidase method, counterstained with hematoxylin, and quantified by a board-certified pathologist (Eugene Einhorn, MD, VA Medical Center, Philadelphia) using a confocal microscope. For each specimen, 10 nonoverlapping high-power fields were evaluated for positively staining vessels and total number of vessels. The average number of staining vessels in these fields was termed mean vessel density (MVD). For positive controls, we used murine liver sections, which contain large numbers of blood vessels. For negative controls, we used sections stained only with the secondary antibody. Statistical comparison between control and treatment groups was made using the unpaired t test with Welch correction.

WESTERN BLOT ANALYSIS

Suspension Chinese hamster ovarian cells (Invitrogen Corp) were transfected with the construct pcDNA3.1–Flk1, pcDNA3.1–LLO, or pcDNA3.1–LLO–Flk1. The supernatants were collected after 6 days and were concentrated 15-fold using a 10 000 nominal molecular weight limit centrifugal filter (Millipore Corp, Billerica, Massachusetts). Fifty microliters of each sample was boiled (at 100°C for 5 minutes) with 10 µL of protein loading buffer containing 5% reducing agent 2-mercaptoethanol (Sigma-Aldrich Corp) and then was allowed to cool at room temperature. The samples were electrophoresed on a 7.5% Tris hydrochloride polyacrylamide gel (Bio-Rad Laboratories, Hercules, California) and then were transferred to a nitrocellulose membrane (GE Healthcare Biological Sciences Corp, Piscataway, New Jersey) at 50 V (4°C for 45 minutes). The membranes were blocked with 5% (wt/vol) skimmed milk in 1% (vol/vol) Tris-buffered saline solution with polysorbate 20 (Tween 20; Promega Corporation, Madison, Wisconsin) for 1 hour followed by probing overnight (at 4°C) with either anti–Flk1 monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, California) or anti-LLO or anti-PEST monoclonal antibody (purchased from Yvonne Paterson, PhD, Department of Microbiology, University of Pennsylvania School of Medicine). The membranes were washed with 1% Tris-buffered saline solution with polysorbate 20 and were incubated with horseradish peroxidase–conjugated antibodies (Santa Cruz Biotechnology Inc and Thermo Fisher Scientific Inc, Rockford, Illinois) for 1 hour at room temperature. The membranes were washed, and protein–antibody binding was visualized using the Western blot system (SuperSignal West Femto system; Thermo Fisher Scientific Inc).

RESULTS

CONSTRUCTION OF LLO–Flk1

To test the hypothesis that the addition of LLO to an anti-VEGFR vaccine improves efficacy, we designed a novel DNA construct. This construct, LLO–Flk1, contains the sequence for hly, the gene that encodes LLO, and the sequence for Flk1, the gene that encodes the murine VEGFR. Two additional constructs were made with the vector pcDNA3.1: Flk1 alone and LLO alone (Figure 1). Protein expression was confirmed by means of Western blot.

LLO–Flk1 PROPHYLAXIS RESULTS IN SMALLER TUMORS

We compared the DNA vaccine LLO–Flk1 with Flk1 regarding their ability to prevent the growth of tumors when administered prophylactically. Twenty C57BL/6 mice were divided into 4 groups. The groups were vaccinated thrice at weekly intervals with (1) PBS, (2) pcDNA3.1, (3) Flk1, or (4) LLO–Flk1. One week after the final vaccination, the mice were challenged with 5 × 10^4 cells from the TC-1 cell line. Mice prophylactically vaccinated with either PBS or the naked plasmid pcDNA3.1 developed palpable 4- to 5-mm tumors within 10 days of tumor challenge. However, mice vaccinated with the experimental vaccines Flk1 and LLO–Flk1 did not develop tumors until days 17 and 24, respectively (Figure 2A). Mice vaccinated with LLO–Flk1 had the smallest average tumor size (2.16 mm) among all treatment groups on day 31 (Figure 2B). In addition, 4 of 5 mice vaccinated with LLO–Flk1 did not develop tumors at all. The 9-fold difference in tumor sizes between both experimental groups (LLO–Flk1 and Flk1) and the negative control groups was significant (P < .001, 1-way analysis of variance). The difference between LLO–Flk1 and Flk1 was not significant (P > .05).

THERAPEUTIC LLO–Flk1 VACCINATION RESULTS IN SMALLER TUMORS THAN DOES Flk1 VACCINATION

We compared LLO–Flk1 with Flk1 regarding the ability to treat tumors in a therapeutic animal model. Twenty C57BL/6 mice were divided into 4 groups. All the mice were challenged with 5 × 10^4 TC-1 cells and then 3 days later were vaccinated with (1) PBS, (2) pcDNA3.1, (3) Flk1, or (4) LLO–Flk1. Mice vaccinated with LLO–Flk1 had significantly smaller tumor sizes than the other groups throughout the experiment (Figure 3A). On day 31, the LLO–Flk1 group had a tumor size of 5.28 mm and the Flk1 group of 10.44 mm (Figure 3B). The difference in tumor sizes between the LLO–Flk1 group and the nega-
TUMORS TREATED WITH LLO-FLK1 CONTAIN FEWER CD31+ BLOOD VESSELS

To test the hypothesis that the effects of LLO-FLK1 are due to the inhibition of tumor vasculature development, we performed immunohistochemical analysis of tumors treated with the various constructs. Tumor slides were stained with antibodies against CD31, a molecule found on most endothelial cells. Cells positive for CD31 staining in 10 high-power fields were quantified by a certified pathologist. The average number of staining vessels per high-power fields, or MVD, was calculated. We found that the MVD was significantly lower in tumors treated with LLO-FLK1 than in tumors treated with any of the other constructs (P < .001). The MVD in the LLO-FLK1 group was 13.3, whereas all the other groups had MVDs of 19 to 23 (Figure 4). There was no statistically significant difference between the mean number of CD31+ cells in the FLK1 group vs the negative control groups.
We hypothesized that the hemoglobin content in tumors would be reduced in mice treated with LLO-Flk1. To test this hypothesis, we performed an assay in which C57BL/6 mice were vaccinated with PBS, pcDNA3.1, Flk1, or LLO-Flk1. After 3 weekly vaccinations, absorbable (Matrigel; BD Biosciences, San Jose, California) collagen basement membrane plugs were subcutaneously implanted in the flanks of the mice. One week later, the plugs were harvested, and the hemoglobin content was assessed using spectrophotometry, as described in the “Methods” section. We found that the plugs from mice treated with LLO-Flk1 had at least a 50.3% decrease in hemoglobin concentration compared with those from the other groups ($P < .03$). The LLO-Flk1 group had a more than 60% reduction compared with the Flk1 group. The Flk1 group had no statistically significant difference in hemoglobin content compared with controls. Error bars represent SD. bFGF Indicates basic fibroblast growth factor.

We showed that the addition of the bacterial protein LLO enhanced the efficacy of an antiangiogenesis vaccine designed to target the murine VEGFR2 Flk1. The Flk1 vaccines have been used in other preclinical models to suppress tumor growth with some success.11-14 In the present study, vaccination with Flk1 DNA alone resulted in slower tumor growth but was no better than controls in assays measuring MVD and hemoglobin content in the tumor. In contrast, the LLO-Flk1 vaccine showed improved therapeutic benefit over Flk1 in the more stringent therapeutic model and demonstrated decreased tumor hemoglobin content and a lower MVD.

Listeriolysin O is a protein expressed by L monocytogenes that facilitates the degradation and perforation of cell membranes as the intracellular bacteria moves from cell to cell.15 In the amino acid sequence of LLO is a region of residues called a PEST (P indicates proline; E, glutamic acid; S, serine; and T, threonine) region, which may be critical to its function. PEST regions are hydrophilic amino acid sequences that reside near the amino or carboxy termini of certain enzymes. PEST regions are thought to target proteins for rapid degradation because they are recognized and bound by components of the ubiquitin proteolytic pathway and are degraded, in part, by the 26S proteasome.16-18 Thus, it is hypothesized that the presence of the PEST region in LLO may enhance vaccine efficacy by causing rapid degradation and presentation of the tumor antigen fused to LLO.7

Of the approved angiogenesis inhibitors in current use, few are designed to generate an immune response against VEGF or VEGFR, and none incorporates the LLO molecule or PEST sequences. Bevacizumab (Avastin; Genen-
tech, San Francisco, California) is among the few that attempt to stimulate an immune response; it is an antibody that binds soluble VEGF, and it has shown efficacy in improving survival and delaying growth in patients with colorectal cancer. This antibody is not yet approved for the treatment of head and neck cancer. Future clinical trials could include a combination of bevacizumab, which would neutralize VEGF, and a DNA vaccine similar to LLO-Flk1, which would target VEGFR. This combination of agents could result in a greatly decreased blood supply to the tumor and, possibly, a survival benefit for patients with head and neck cancer.

In the present study, there was one unexpected finding. The Flk1 vaccine slowed tumor growth but did not demonstrate significant antiangiogenic activity as measured by immunohistochemical staining of the vasculature and the hemoglobin content of the tumors. The LLO-Flk1 vaccine, in contrast, showed superior tumor growth retardation and significant antiangiogenesis activity. This result may imply that, in our hands, the Flk1 vaccine caused tumor growth retardation through a nonspecific mechanism unrelated to angiogenesis, whereas the effects seen with LLO-Flk1 are mediated by a true antiangiogenic mechanism. It is conceivable that LLO-Flk1 also acts in a nonspecific manner. However, the fact that we observed decreased vessel density with LLO-Flk1 seems to indicate specificity in antigen targeting.

Although DNA vaccines have shown promise in many preclinical models of cancer, clinical trials for patients with head and neck cancer are relatively scarce. Trials of DNA vaccines against human papillomavirus–associated head and neck cancer are ongoing, with promising results in terms of safety. The present data show that addition of the protein LLO to a DNA vaccine against the murine VEGFR can result in decreased tumor size in a murine cancer model. The addition of this protein to DNA vaccine strategies for cancer treatment may result in increased efficacy and should be considered in future clinical and preclinical trials.

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