Angiogenesis is important for the growth of solid tumors. The breaking of the immune tolerance against the molecule associated with angiogenesis should be a useful approach for cancer therapy. However, the immunity to self-molecules is difficult to elicit by a vaccine based on autologous or syngeneic molecules due to immune tolerance. Basic fibroblast growth factor (bFGF) is a specific and potent angiogenic factor implicated in tumor growth. The biological activity of bFGF is mediated through interaction with its high-affinity receptor, fibroblast growth factor receptor-1 (FGFR-1). In this study, we selected Xenopus FGFR-1 as a model antigen by the breaking of immune tolerance to explore the feasibility of cancer therapy in murine tumor models. We show here that vaccination with Xenopus FGFR-1 (pxFR1) is effective at antitumor immunity in three murine models. FGFR-1-specific autoantibodies in sera of pxFR1-immunized mice could be found in Western blotting analysis. The purified immunoglobulins were effective at the inhibition of endothelial cell proliferation in vitro and at the antitumor activity in vivo. The antitumor activity and production of FGFR-1-specific autoantibodies could be abrogated by depletion of CD4+ T lymphocytes. Histological examination revealed that the autoantibody was deposited on the endothelial cells within tumor tissues from pxFR1-immunized mice, and intratumoral angiogenesis was significantly suppressed. Furthermore, the inhibition of angiogenesis could also be found in alginate-encapsulate tumor cell assay. These observations may provide a new vaccine strategy for cancer therapy through the induction of autoreactivity against FGFR-1 associated with angiogenesis in a cross-reaction.

Various strategies for cancer vaccines including whole tumor cell vaccines, genetically modified tumor vaccines, dendritic cell vaccines, and peptide and protein vaccines have been developed to induce tumor-specific immune response against autologous malignant cells (1, 2). Active specific immunotherapies with cancer vaccines based on tumor antigens represent very promising approaches for cancer therapy (1, 2). However, to date, with a few exceptions such as the case with melanoma antigens, there is limited information on the identity and density of antigenic peptides and the cytotoxic T lymphocyte (CTL) epitopes presented by human solid tumors (1, 2). In addition, most of the identified tumor antigens are self-molecules (1–3). As expected, the reaction of the host toward these self-molecules may show immune tolerance to them if the host is immunized with the vaccines based on these self-molecules (4–8). Efforts are therefore continuing to develop new strategy for cancer vaccines.

The generation of new blood vessels, or angiogenesis, is a complex multistep process that includes endothelial proliferation, migration and differentiation, degradation of extracellular matrix, etc. (9, 10). The complexity of the angiogenic process suggests the existence of multiple controls in the system that can be temporarily turned on and off (9, 10). Angiogenesis is important for normal embryonic development and the development of pathologic conditions such as cancer, rheumatoid arthritis, retinopathies, etc. (9–11). Several lines of direct and indirect evidence indicate that the growth and persistence of solid tumors and their metastasis are angiogenesis-dependent (9–14). As a strategy for cancer therapy, antiangiogenic therapy attempts to stop new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feed the cancerous mass (15–18).

Endothelial cells in the angiogenic vessels within solid tumors express proteins on their surface that are absent or barely detectable in the normal quiescent vascular endothelium, including certain angiogenic growth factors and their receptors such as basic fibroblast growth factor (bFGF)3 and its high-affinity receptor, fibroblast growth factor receptor-1 (FGFR-1), among others (9–15). The breaking of immune tolerance against important molecules such as some receptors associated with angiogenesis on autologous angiogenic endothelial cells should be a useful approach for cancer therapy by active immunity. However, the immunity to the self-molecules on angiogenic vessels is presumably difficult to elicit by using autologous or syngeneic protein molecules as vaccine because of the
immune tolerance acquired during the development of the immune system (4–8).

Many genes were highly conserved during the evolutionary process, which was characterized by varying degrees of gene similarity among different species (19–21). Many counterparts of the genes of human and mouse can be identified from the genome sequence of *Drosophila melanogaster* and other species such as *Xenopus laevis* (19–21). For example, a comparison analysis made in the present study by searching the Swiss Prot database at the National Center for Biotechnology Information indicates that the *Xenopus* homologue of FGFR-1 (GenBank™ accession number U24491) is 80 and 74% identical in mouse FGFR-1 (GenBank™ accession number M53760) and human FGFR-1 (GenBank™ accession number M34641), respectively, at the amino acid level (22–24). In addition, basic fibroblast growth factor has been shown to be one of the most important angiogenic growth factors for angiogenesis through interaction with its high-affinity receptor, FGFR-1 (25–28). FGFR-1 is markedly expressed both in endothelial cells and in many different forms of tumor (25–28). These findings suggest that FGFR1 plays an important role in tumor angiogenesis and tumor growth, and it may be used as an ideal molecule to explore the feasibility of tumor therapy. The current studies explore the feasibility of immunotherapy of tumors with the plasmid DNA encoding *Xenopus* FGFR-1 as a vaccine by the breaking of the immune tolerance against FGFR-1 in a cross-reaction between the xenogeneic homologous and self-FGFR-1.

To test this concept, we constructed a plasmid DNA encoding *Xenopus* FGFR-1 (pxFR1). At the same time, the plasmid DNA encoding the corresponding mouse FGFR-1 (pmFR1) and empty vector (e-p) were also constructed and used as controls. The plasmid DNA vaccines were tested for the ability to induce antitumor immune response in three tumor models in mice.

**EXPERIMENTAL PROCEDURES**

**Vaccine Preparation**—A cDNA clone encoding *Xenopus* homologous FGFR-1 and mouse FGFR-1 were isolated from by PCR using the *Xenopus laevis* cDNA library (Clontech) and the mouse skeletal muscle cDNA library (Clontech), respectively. The amplified products were inserted into pT-Adv plasmid (Clontech) and then subcloned into pcdNA 3.1(+)(Invitrogen), which contains a cytomegalovirus promoter. FGFR-1 receptors of *Xenopus* and mouse inserted into pcdNA 3.1(+) were named pxFR1 and pmFR1, respectively. As a control, pure pcdNA 3.1(+) was used as empty vector. The full-length sequence of *Xenopus* and mouse FGFR-1 was confirmed by dideoxy method sequence to be identical to those reported previously (22–24). Plasmids for DNA vaccination were purified by using two rounds of passage over Endo-free columns (Qiagen) as described previously (29). The expression of plasmid DNA was confirmed in transfected H22 hepatoma cells by using reverse transcription PCR and a commercially available anti-FGFR-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in Western blotting analysis and ELISA.

**Vaccine Based on Fibroblast Growth Factor Receptor-1**

**Tumor Models and Immunization**—Meth A fibrosarcoma, H22 hepatoma, and MA782/S5 mammary carcinoma models were established in 6-week female BALB/c mice (30). Mice were immunized with different doses (10^6 g per mouse per injection) of DNA vaccine in normal saline by intramuscular injection in both quadriceps once a week for 4 weeks. Additional control animals were injected with normal saline. 1 × 10^6 to 1 × 10^7 live tumor cells were then inoculated subcutaneously into mice after the fourth immunization. For an investigation of the therapeutic effect against the established tumors, ten mice in each group were treated with intramuscular injection of the DNA vaccines or empty vector (e-p) were also constructed and used as controls. The plasmid DNA vaccines were tested for the ability to induce antitumor immune response in three tumor models in mice.

**Immunoglobulin Subclass Response to *Xenopus* FGFR-1 Immunization in ELISA**—The antigen-specific immunoglobulin subclass in the sera was determined by ELISA as described previously (30, 32). Briefly, H22 cells transfected with pxFR1 and pmFR1 were washed with PBS and lysed by three cycles of freezing and thawing. Microtiter plates were coated with 50 μl of cell lysates (1 × 10^6 cell equivalents) at 4 °C overnight. The wells were then washed with PBS and blocked with blocking buffer at 37 °C for 2 h. After washing, experimental mouse sera were serially diluted in blocking buffer and added to the ELISA wells. Plates were incubated at 37 °C for 2 h, washed, and then incubated with a biotinylated horse anti-mouse IgG or IgM, followed by transfer to Vectastain ABC (Vector Laboratories). Also, the lysates of human umbilical vein endothelial cells (HUVECs) expressing FGFR-1 were analyzed.

**Inhibition of Cell Proliferation in Vitro and Adoptive Transfer in Vivo**—Immunoglobulins were purified from the pooled sera derived from the mice at day 7 after the fourth immunization or from control mice by affinity chromatography (CM Affi-Gel blue gel kit; Bio-Rad). The inhibition of bFGF-mediated endothelial cell proliferation was described (28). Briefly, exponentially growing HUVEC and tumor cells (Meth A, H22, and MA782/S5) were treated with various concentrations (0–300 μg/ml) of immunoglobulins isolated from mice immunized with pxFR1, pmFR1, or e-p in the presence of 3 ng/ml bFGF (Sigma). After 72 h of culture, the number of viable cells was determined by a trypan
An alginate-encapsulate tumor cells assay was performed as described (35). Briefly, mice were treated with goat FITC-conjugated antibody against mouse IgA, IgM, or IgG (Sigma), or HUVEC (lane 5) or with FGFR-1-negative cells (Meth A, H22, and MA782/5S). The depletion of immune cell subsets in vivo—Depletion of immune cell subsets in vivo was carried out as described (1, 34). Mice were injected intraperitoneally with 500 µg of either the anti-CD4 (clone GK1.5, rat IgG), anti-CD8 (clone 2.43, rat IgG), anti-NK (clone PK136) mAb, or isotype controls 1 day before the immunization and then immunized with 100 µg of plasmid per mouse per injection) used in Fig. 1 is an optimal one selected for immunization in several preliminary experiments.

**RESULTS**

**Induction of Protective Antitumor Immunity**—Tumors grew progressively in all non-immunized mice (normal saline alone) or in pmFR1 or e-p-immunized mice, but there was significant protection from tumor growth in pmFR1-immunized mice (Fig. 1, A–C). The protective effect was dose-dependent. The dose (100 µg per mouse per injection) used in Fig. 1 is an optimal one selected for immunization in several preliminary experiments.

**Induction of Therapeutic Antitumor Immunity**—The therapeutic efficacy of pxFR1 was next tested in the established tumors. The mice were treated starting at day 7 after the inoculation of Meth A fibrosarcoma cells, H22 hepatoma cells, or MA782/5S mammary cancer cells when the tumor was palpable. Treatment with pxFR1 once weekly resulted in antitumor activity (Fig. 1, D–F). The survival of the tumor-bearing mice treated with pxFR1 was also longer than that of the untreated mice (normal saline alone) or mice treated with pmFR1 or e-p (Fig. 1, G–I).

**Side Effect of Vaccination**—The mice immunized with these vaccines have been investigated in particular for potential toxicity for 10 months. Except for delay of wound healing on the back of pxFR1-immunized mice compared with control groups (data not depicted), no other adverse consequences were indicated in gross measures such as weight loss, ruffling of fur, life span, and behavior. Furthermore, no pathologic changes in liver, lung, kidney, spleens, brain, heart, etc. were found by microscopic examination.
Characterization of Autoantibodies against FGFR-1—Sera from mice immunized with pxFR1 recognized an ~130-kDa band in H22 cells transfected with pxFR1 or pmFR1, but not in H22 cells or H22 cells transfected with empty plasmid in Western blotting analysis (Fig. 2A). Also, the sera show the positive staining for FGFR-1-positive HUVEC (Fig. 2A). The sera isolated from control groups show the negative staining (Fig. 2B). There was endothelial deposition of autoantibody in the tumor tissues from mice immunized with pxFR1 (Fig. 2C), but not in pmFR1-immunized (Fig. 2D), e-p-immunized (Fig. 2E), or non-immunized mice (normal saline alone) (Fig. 2F). In addition, there was no immunoglobulin deposition in the immunized or non-immunized mice in the major organ such as liver, lung, spleens, brain, heart, and kidney.

Treatment with purified immunoglobulins isolated from pxFR1-immunized mice resulted in the apparent inhibition of bFGF-mediated endothelial cell proliferation (Fig. 3A). However, the immunoglobulins had no direct inhibitory effect on the proliferation of tumor cells (Meth A fibrosarcoma cells, H22 hepatoma cells, or MA782/SS mammary cancer cells) (Fig. 3A). Immunoglobulins isolated from pmFR1-immunized mice (Fig. 3B), e-p-immunized mice, or non-immunized mice (normal saline alone) had no inhibitory effect on the proliferation of bFGF-mediated endothelial cells and tumor cells. In addition, adoptive transfer of sera or purified Ig isolated from pxFR1-immunized mice provided effective protection against tumor growth (Fig. 4). Adsorption of sera or Ig with FGFR-1-positive endothelial cells before adoptive transfer could abrogate its antitumor activity (data not shown).

Role of CD4+ T Lymphocytes in Xenopus FGFR-1-induced Antitumor Activity—Sera obtained from mice immunized with pxFR1 showed substantial increases in IgG1 and IgG2b with little increase in IgM or IgA antibody response against the lysate of the cells transfected with pxFR1 or pmFR1 compared with equivalent doses of e-p, or saline (Fig. 5A). The mice depleted of CD4+ T lymphocytes did not develop detectable antibodies against FGFR-1 (Fig. 5A) and were not protected from tumor challenge in pxFR1-immunized mice (Fig. 5B). In contrast, treatment with anti-CD8 or anti-NK mAb or control IgG had no effect (Fig. 5B). These data suggest that the induction of the autoantibody against FGFR-1, which is probably responsible for pxFR1-immunized antitumor activity, may be involved in CD4+ T lymphocytes.

Inhibition of Angiogenesis—Immunohistochemical examination of the tumors treated with pxFR1 or adoptive transfer of immunoglobulins isolated from pxFR1-immunized mice showed decreased microvessel staining (Fig. 6, A and E), compared with control groups (Fig. 6, B–E). Moreover, the inhibi-
tion of angiogenesis could also be found in the alginat-encapsu-
sulate tumor cells assay (Fig. 7). Growth factors produced by
the encapsulated tumor cells induce vascularization of beads over
12 days, which can then be measured by uptake of FITC-
dextran. FITC-dextran uptake was significantly decreased from
mice treated with pxFR1, compared with control groups (Fig. 7).

**DISCUSSION**

Several observations have been made in the present study
concerning the vaccine based on Xenopus homologous FGFR-1
as a model antigen, antitumor immunity, and angiogenesis. The
vaccine based on Xenopus homologous FGFR-1 as a model
antigen could induce both protective and therapeutic antitu-
mor immunity. Autoimmune response against FGFR-1 may be
provoked in a cross-reaction by the immunization of pxFR1,
and the autoantibody targeting to FGFR-1 is probably respon-
sible for the antitumor activity. These suggestions are sup-
ported by our findings in the present study. Autoantibodies
against FGFR-1 were identified by Western blotting analysis.
Endothelial cell proliferation was inhibited in vitro by immu-
noglobulins from pxFR1-immunized mice. The antitumor ac-
tivity and the inhibition of angiogenesis were acquired by the
adoptive transfer of the purified immunoglobulins. IgG1 and
IgG2b were substantially increased in response to pxFR1. There
were the antitumor activity and production of autoanti-
tibodies against FGFR-1 that could be abrogated by the deple-
tion of CD4+ T lymphocytes. Angiogenesis was apparently
inhibited within tumor tissue, and the inhibition of angiogenesis
could also be found in an alginat-encapsulate tumor cell assay.
Based on our findings mentioned above, we may rule out the
possibility that the antitumor activity with Xenopus homolo-
gous FGFR-1 may result from the nonspecifically augmented
immune response against the tumor growth in host mice.

Antitumor immunity depends on CD8+ T lymphocytes in
some mouse models, whereas CD4+ T lymphocytes often have
little, if any, function (1, 2, 36). Some molecular targets of
tumor-specific CD8+ T lymphocytes have been identified in hu-
man and mouse systems (1, 2). CD8+ T lymphocytes have been
the focus of recent efforts in the development of a therapeutic
antitumor vaccine. However, in this study, we found that mice
depleted of CD4+ T lymphocytes by the injection of anti-CD4
mAb and vaccinated with Xenopus FGFR-1 were not protected
from tumor inoculation. At the same time, depleted CD4+ T
lymphocytes did not develop detectable autoantibodies
against FGFR-1. In contrast, treatment with anti-CD8 or an-
ti-NK mAb or control IgG failed to abrogate the antitumor
activity. These findings suggest that the induction of the au-
toantibody response to FGFR-1, which is responsible for Xeno-
opus FGFR-1-induced antitumor activity, may involve CD4+ T
lymphocytes. It is known that CD4+ T lymphocytes can steer
and amplify immune responses through the secretion of cyto-
kines and the expression of surface molecules (36–38). It has
been reported that antitumor immunity could be induced by
DNA immunization against human gp75/tyrosinase-related
protein-1 or tyrosinase-related protein-2 (the slaty locus pro-
tein) and has depended on CD4+ T lymphocytes in melanoma
models (39–41). For the antibody-dependent immunity, CD4+
T lymphocytes can be required at the immunization phase as
well as at the effector phase (42). Furthermore, CD4+ T
lymphocytes have been reported to be required for the induc-
ton immunity by vaccination with a recombinant vacci-
cinia encoding self-tyrosinase-related protein 1 in a mouse melanoma model (42, 43). In addition, it has been re-
ported that CD4+ T lymphocytes play a prominent role in
classic mouse models of autoimmune, such as experimental
allergic encephalitis, systemic lupus erythematosus, and auto-
immune gastritis (44–46). These findings may help explain the
requirement for CD4+ T lymphocytes in the induction of auto-
immune response against mouse FGFR-1 in a cross-reaction.

The mice immunized with these vaccines have been investi-
gated in particular for the potential toxicity in the present
study; except for delay of wound healing, no other marked side
effect was found. It has been reported that FGFR-1 is often
over-expressed and is required for angiogenesis within tumor,
but it does not seem to have a continuous maintenance function
for much of the adult vascular (10, 14, 30, 47, 48). In addition,
deposition of the autoantibodies is found on the microvessels
within tumor tissues but without detectable with normal
tissues. Thus, these findings may help explain why no marked
side effects were observed in the present study.

In conclusion, our findings may provide a new vaccine strat-
ey for cancer therapy through the induction of autoimmune
response against the self-molecules for angiogenesis in a cross-
reaction with the single xenogeneic homologous FGFR-1 gene.
This vaccine strategy may be used in targeting to the other growth factors or their receptors associ-
ated with angiogenesis and growth of tumor. Many counter-
parts of human genes can be identified from the genome se-
quence of the fruit fly D. melanogaster and other animals such as X. laevis and mouse (19–21, 49). Thus, the breaking of
immune tolerance to the self-molecules involving angiogenesis
with xenogeneic counterparts may be of importance to further
explore the applications of xenogeneic homologous genes in
fields such as cancer therapy, autoimmunity, xenogeneic trans-
plantation rejection, human and other animal genome se-
quence projects, and animal evolutionary biology.

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Inhibition of Tumor Growth with a Vaccine Based on Xenogeneic Homologous Fibroblast Growth Factor Receptor-1 in Mice
Qiu-ming He, Yu-quan Wei, Ling Tian, Xia Zhao, Jing-mei Su, Li Yang, You Lu, Bin Kan, Yan-yan Lou, Mei-juan Huang, Fei Xiao, Ji-yan Liu, Bing Hu, Feng Luo, Yu Jiang, Yan-jun Wen, Hong-xin Deng, Jiong Li, Tin Niu and Jin-liang Yang

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