Evidence indicates that the growth and metastasis of solid tumors are angiogenesis dependent (1, 2). Angiogenesis is a complex multistep process that is regulated by numerous growth factors and molecules. A functional blood vessel network is correctly formed only when all these molecules are perfectly regulated (3). The suppression and regression of solid tumors would be achieved by interruption of growth factors and molecules associated with angiogenesis.

The receptor tyrosine kinase Tie-2 (also called Tek) is essential in tumor angiogenesis as well as in developmental and postnatal angiogenesis (4, 5). The embryos lacking Tie-2 develop a rather abnormal vasculature and die at day 12.5 (6).

Overexpression of Tie-2 is found on activated endothelial cells of tumor vasculature (7). Moreover, it has been reported previously that the soluble extracellular domain of Tie-2 (ExTek) inhibits tumor angiogenesis and tumor growth in rodent models (8, 9). Systemic administration of an adenoviral vector encoding ExTek significantly decreased primary tumor growth, vascularization, and pulmonary metastases (10). These studies suggest that Tie-2 plays a key role in tumor angiogenesis.

Overcoming immune tolerance against Tie-2 on angiogenic endothelial cells may be a useful approach for cancer therapy by active immunity. However, an immune response to the self-molecules on angiogenic vessels is presumably difficult to be initiated by a vaccine based on autologous or syngeneic molecules because the presence of the immune tolerance developed in the early stage.

Many genes are highly conserved during the evolutionary process, which have been characterized by varying degrees of gene similarity among different species (11). Many counterparts of the genes of human and mouse can be identified from the genome sequence of *Drosophila melanogaster* and other species, such as chicken. For example, a sequence comparison analysis indicates that the chicken Tie-2 shares 67% and 71% amino acids sequence identical with mouse Tie-2 (mTie-2) and human Tie-2, respectively. The present study explores the feasibility of immunotherapy of tumors by using a protein vaccine based on chicken Tie-2 as a model antigen to break the immune tolerance against Tie-2 in a cross-reaction between the xenogeneic homologous and self-Tie-2.
To test this hypothesis, we prepared a chicken homologous Tie-2 protein vaccine (chTie-2) and a corresponding mouse Tie-2 vaccine (mTie-2) was used as a control. The ability of these vaccines to induce antitumor immunity was tested in tumor models in mice.

Materials and Methods

**Protein vaccine preparation.** Total RNA was isolated from mouse or chicken embryos using TRIZol reagent (Invitrogen, Grand Island, NY) and subjected to reverse transcription-PCR for the amplification of the encoding sequence comprising the NH2-terminal 46– to 537– amino acid residues of extracellular domains of mTie-2 and the corresponding fragment of chTie-2. The reverse transcription-PCR products were then inserted into expression plasmids pQE30 (Qiagen, Chatsworth, CA). The correct coding sequence was verified by nucleotide sequencing. The expression, purification, and dialysis of the recombinant protein were done as reported previously (12). Purified proteins were confirmed by SDS-PAGE and Western blot analysis and also tested for endotoxin. A chTie-2 and a mTie-2 were constructed, respectively. They were dissolved in PBS and mixed with an equal volume of aluminum hydroxide (Al(OH)3) adjuvant at 4 mg/mL for 30 to 60 minutes as described (13). The fragment of chTie-2. The reverse transcription-PCR products were then dissolved in PBS and mixed with an equal volume of aluminum hydroxide (Al(OH)3) adjuvant at 4 mg/mL for 30 to 60 minutes as described (13).

**Tumor models and immunotherapy.** H22 hepatoma models and B16F10 melanoma models were established in 6- to 8-week-old female BALB/c and in C57BL/6 mice, respectively. Mice were immunized by s.c. injection once weekly for 4 weeks with different doses (1-50 μg/mouse) of protein vaccine. Control animals were immunized with vaccine vehicle (AloH3) alone. Live tumor cells (1 × 106) were then inoculated s.c. after the fourth immunization. To investigate the therapeutic effect against the established tumors, mice (10 in each group) were first inoculated 5 × 105 to 1 × 106 live tumor cells by s.c. injection. Seven days later, mice were treated with s.c. injection of the protein vaccines or ALOH3 once weekly for 4 weeks. Tumor volume was determined by the following formula: tumor volume (mm3) = 0.52 × length (mm) × width (mm) × width (mm). All studies involving mice were approved by the West China Hospital Cancer Center’s Animal Care and Use Committee.

To test the efficacy of protein vaccines to treat tumor metastasis model, 5 × 107/100 μL B16F10 cells were injected into the tail vein of each C57BL/6 mouse on day 7 after the fourth immunization. When mice of control group became moribund after 18 days, the mice were sacrificed to count lung metastasis nodules and to measure the weight of lungs (14).

Western blot analysis. Western blot analysis was done as described previously (15). Briefly, recombinant proteins were separated by SDS-PAGE. Gels were electrotransferred with Sartoblot onto a polyvinylidene difluoride (PVDF) membrane. Membrane blots were blocked at 4°C for 4 hours. IgG bound to the membrane was revealed as spots with alkaline phosphatase–conjugated anti-mouse IgG antibodies.

**Induction of immunoglobulin, its induction of cell apoptosis in vitro, and its 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 Laboratories). To explore induction of cell death by immunoglobulins in vitro, flow cytometric analysis was done to identify sub-G1 cells/apoptotic cells and to measure the percentage of sub-G1 cells after propidium iodide staining in hypotonic buffer as described (17). Briefly, exponentially growing human umbilical vein endothelial cells (HUVEC) were exposed to various concentrations (1-150 μg/mL) of the immunoglobulins for 72 hours of culture. HUVECs were then suspended in 1 mL hypotonic fluorochrome solution containing 50 μg/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100. The cells were analyzed by a flow cytometer (Coulter Elite ESP; Coulter Inc., Fullerton, CA). Apoptotic cells appeared in the cell cycle distribution as cells with DNA content of less than that of G1 cells.

To assess the efficacy of immunoglobulins in antitumor in vivo, purified immunoglobulins (10-300 mg/kg) were adoptively transferred and i.v. transferred 1 day before mice were challenged with 1 × 103 to 1 × 107 tumor cells, and the mice were treated twice weekly for 3 weeks as described previously (15).

In vivo depletion of immune cell subsets. Immune cell subsets were depleted as described (18, 19). Mice were injected i.p. with 500 μg of either anti-CD4 (clone GK1.5) or anti-CD8 (clone 2.43, rat IgG), anti-CD8 (clone 2.43, rat IgG), or anti–natural killer (NK; clone PK136) monoclonal antibody or isotype controls 1 day before the immunization and then immunized with protein vaccines once weekly for 4 weeks. Tumor cells were challenged after the fourth immunization. These hybridomas were obtained from the American Type Culture Collection (Manassas, VA). The depletion of CD4, CD8, and NK cells was consistently >98% as determined by flow cytometry.

**Immunohistochemistry and immunofluorescence staining.** Immunohistochemical staining was done as described (20). Briefly, frozen sections were first stained with an antibody reactive to CD31 and then stained with labeled streptavidin biotin reagents (DAKO LSAB kit, peroxidase; DAKO, Carpinteria, CA). Vessel density was determined by counting the number of microvessels per high-power field in the sections. To identify autoantibodies deposited in tumor vasculature, the sections were stained with an antibody reactive to CD31 and then stained with Cy3-conjugated goat anti-rabbit IgG (second antibody; Sigma, St. Louis, MO) as well as with FITC-conjugated goat anti-mouse IgA, IgM, or IgG (Sigma). Slides were examined by fluorescein microscopy.

**Alginate encapsulation assay.** Alginate-encapsulated tumor cell assays were done as described previously (21, 22). Briefly, H22 or B16F10 cells were resuspended in a 1.5% solution of sodium alginate and added dropwise into swirling 37°C solution of 250 mmol/L CaCl2. Alginate beads containing 1 × 107 tumor cells per bead were formed. Mice immunized with protein vaccines or ALOH3 alone were then anesthetized, and four beads were implanted s.c. into an incision made on the dorsal side. Incisions were closed with surgical clamps. After 12 days, mice were injected i.v. with 100 μL of a 100 mg/kg FITC-dextran solution (Sigma). Beads were surgically removed and FITC-dextran was quantified against a standard curve of FITC-dextran.

**Statistical analysis.** For comparison of individual time points, ANOVA and an unpaired Student’s t test were used. Survival curves were constructed according to the Kaplan-Meier method. Statistical significance was determined by the log-rank test. Ps < 0.05 were considered significant.

Results

**Induction of the antitumor immunity.** To explore the protective antitumor immunity, we immunized mice with protein vaccines (chTie-2 or mTie-2) or vaccine vehicle (ALOH3)
once weekly for 4 weeks and then challenged mice with H22 or B16F10 tumor cells at day 7 after the fourth immunization. Tumors grew progressively in all mice immunized with mTie-2 or ALOH₃, but significant protection from tumor growth was observed in mice immunized with chTie-2 (Fig. 1A and B).

The therapeutic efficacy of chTie-2 protein vaccine was next tested in the established tumors models. The mice were treated at day 7 after the inoculation of tumor cells, when the tumors were palpable. Treatment with chTie-2 once weekly resulted in significant antitumor activity in H22 hepatoma and B16F10 melanoma models (Fig. 1C and D). The survival of the tumor-bearing mice treated with chTie-2 was also significantly longer than that of the controls (Fig. 1E and F).

Treatment with chTie-2 protein vaccine also suppressed the formation and growth of lung metastasis in mouse B16F10 melanoma. The number of surface metastases was significantly less in chTie-2-immunized mice than that of controls (Fig. 2A).

In addition, the average lung weight of chTie-2-immunized mice was also lighter than that of the controls (Fig. 2B).

**Observation of possible side effects.** The mice treated with these vaccines have been investigated in particular for potential toxicities for >10 months. No adverse consequences were detected in gross measures, such as weight loss, ruffling of fur, life span, behavior, and feeding. Furthermore, no pathologic changes in liver, lung, kidney, spleen, brain, and heart were found by microscopic examination (data not shown).

**Characterizations of autoantibodies.** In an attempt to explore the possible mechanism by which antitumor activity was induced with chTie-2 vaccine, we isolated sera from immunized mice and tested whether there existed auto-antibodies against Tie-2 in these mice. Sera from chTie-2-immunized mice recognized not only recombinant protein chTie-2 but also mTie-2 in Western blot analysis (Fig. 3A). However, the sera isolated from controls showed negative staining (Fig. 3B and C). Sera from chTie-2-immunized mice also recognized recombinant protein chTie-2 and mTie-2 by ELISA.

Anti-Tie-2 antibody-producing B cells were detected by ELISPOT. The number of anti-Tie-2 antibody-producing B cells was elevated in the spleens of mice immunized with chTie-2 compared with that in controls (Fig. 3D).

To investigate the autoantibodies are specifically deposited in the tumor vasculature, we investigated the tissues staining with CD31 as well as staining with autoantibodies by different immunofluorescence. There was the endothelial deposition of IgG within tumor vasculature from chTie-2-immunized mice (Fig. 3E and H) but not from controls (Fig. 3F, G, I, and J). In addition, no autoantibodies were found within the major organs, such as kidney, liver, spleen, and brain, in the immunized or control mice.

Furthermore, the immunoglobulins isolated from chTie-2-immunized mice could induce the apoptosis of HUVECs...
in vitro detected by flow cytometric analysis (Fig. 4A). Moreover, adoptive transfer of purified immunoglobulins isolated from chTie-2-immunized mice provided effective protection against tumor growth (Fig. 4D).

Function of T-cell subsets in antitumor activity. To explore the role of immune cell subsets played in this antitumor activity, we depleted CD4+ or CD8+ T lymphocytes or NK cells through injection of the corresponding antibodies. Depletion of CD4+ T lymphocytes completely abrogated the antitumor activity induced by immunization with chTie-2 protein. However, injection with monoclonal antibody against CD8 or NK cells failed to abrogate the antitumor activity (Fig. 5A). Furthermore, sera from mice depleted of CD4+ T lymphocytes and immunized with chTie-2 did not react with recombinant protein mTie-2 and chTie-2 in ELISA. In contrast, sera from mice depleted of CD8+ or NK cells and immunized with chTie-2 still had detectable antibodies against recombinant protein Tie-2 (Fig. 5B). These findings suggest that CD4+ T lymphocytes may be involved in this chTie-2-induced antitumor activity.

Inhibition of angiogenesis. Angiogenesis was apparently suppressed in tumors of mice vaccinated with chTie-2. The number of microvessels on the tumor tissue section in chTie-2-immunized mice was significantly reduced compared with that in control groups (Fig. 6A-C and G). The inhibition of angiogenesis in chTie-2-immunized mice was further confirmed by using alginate encapsulation assay (Fig. 6D-F). Alginate...
Implant angiogenesis was quantitated by measuring the uptake of FITC-dextran into beads. Vascularization of beads and FITC-dextran uptake was apparently decreased in chTie-2-immunized mice against that in control groups (Fig. 6H).

Discussion

Recently, several findings suggest that the vascular endothelial growth factor receptor pathway and the Tie-2 pathway are two independent mediators essential for the process of angiogenesis in vivo (9, 10, 23–25). Balanced and sequential expression of angiopoietins and vascular endothelial growth factor is required for successful angiogenesis (3). Thus, the Tie-2 pathway is an attractive target for antiangiogenic agents from a therapeutic perspective. Several published reports have shown that the inhibition of tumor growth can be achieved by interference with the Tie-2 pathway using soluble or dominant-negative receptor domains (8, 26). Very recently, an adenoviral vector was used to deliver a recombinant intrabody capable of inhibiting Tie-2 surface expression in both mouse and human tumor cells. Their results have shown that the intrabody significantly inhibited the growth of tumors and the antitumor effect of the intrabody was due to the inhibition of tumor angiogenesis in this experiment (27). Therefore, blocking Tie-2 receptor pathway may be a useful approach for cancer therapy.

Active specific immunotherapies with cancer vaccines targeting tumor antigens represent very promising approaches for cancer therapy (28). However, with the few exceptions of melanoma antigen, there is still limited information on the identity and density of antigenic peptides and CTL epitopes presented by human solid tumors (28). Efforts are required to develop new strategies for cancer vaccines. In previous studies from our laboratory, Liu et al. found that immunotherapy with protein vaccine vascular endothelial growth factor receptor-2 was effective at the inhibition of tumor angiogenesis and tumor growth (12). Here, to explore the therapeutic potential of blocking Tie-2 pathway, we used chTie-2 protein vaccine as a model antigen, which could induce autoantibodies to study the antitumor effect.

In the present study, the role of chTie-2 as a model antigen has been investigated in antitumor immunity and angiogenesis. The protein vaccine chTie-2 could induce both protective and therapeutic antitumor immunity. Autoimmune response against Tie-2 may be provoked in a cross-reaction by the immunization with chTie-2 vaccine, and the autoantibodies targeting to Tie-2 are probably responsible for the antitumor activity. Autoantibodies against Tie-2 were identified by Western blot analysis and ELISA. Anti-Tie-2 antibody-producing B cells were detectable by ELISPOT. There was endothelial deposition of immunoglobulins in tumor. The apoptosis of the HUVECs was induced by immunoglobulins from chTie-2-immunized mice in vitro. The antitumor activity was acquired by the adoptive transfer of the purified immunoglobulins. Furthermore, angiogenesis was apparently inhibited within the tumors, and the vascularization of alginate beads was also reduced. However, the antitumor activity and production of autoantibodies against Tie-2 could be abrogated by the depletion of CD4⁺ T lymphocytes. Therefore, we may rule out the possibility that the antitumor activity with chTie-2 may result from the nonspecifically augmented immune response against the tumor growth in host mice.

Inhibition of tumor angiogenesis may contribute to this antitumor effect. This idea is supported by the decreased vascular density in chTie-2-treated tumors and the reduction of vascularization of alginate beads in chTie-2-immunized mice. These findings are consistent with results of Tie-2 knockout experiment that revealed indispensable but distinct function of Tie-2 in vessel maturation and maintenance (29). The molecular mechanism by which autoantibodies against Tie-2 inhibits tumor angiogenesis is under investigation. However, we found that autoantibodies against Tie-2 could induce the apoptosis of endothelial cell in vitro, which is supported by previous studies (30–33). It has been reported that the inhibition of Tie-2 signaling induced endothelial cell apoptosis, decreased Akt signaling, and induced endothelial cell expression of the endogenous antiangiogenic molecule, thrombospondin-1 (32). The interruption of Tie-2 signaling either via RNA interference or overexpression of a kinase-dead Tie-2 also led to loss of endothelial cell viability (33). In addition, we found that there was endothelial deposition of the autoantibodies in tumor. Autoantibodies against Tie-2 were identified by Western blot analysis and ELISA. Furthermore, we found that angiopoietin-1 did not inhibit

Fig. 5. Abrogation of antitumor activity and antibody production by depletion of immune cell subsets. A, abrogation of antitumor activity by depletion of immune cell subsets. Mice were immunized with chTie-2, mTie-2, or AlOH₃ and then challenged with tumor cells after depletion of CD4⁺ or CD8⁺ T lymphocytes or NK cells. Depletion of CD4⁺ T lymphocytes showed complete abrogation of the antitumor activity of the chTie-2 vaccine. However, depletion of CD8⁺ T lymphocytes or NK cells had no effect on antitumor activity. B, abrogation of antibody production by depletion of immune cell subsets. Sera obtained from mice immunized with chTie-2, mTie-2, or AlOH₃ were tested against chTie-2 (●) or mTie-2 (□) by ELISA at day 7 after the fourth immunization. Depletion of CD4⁺ T lymphocytes can abrogate the elevation of antibodies against chTie-2 and mTie-2. However, depletion of CD8⁺ T lymphocytes or NK cells had no effect on antibody production.
the autoantibodies-induced apoptosis in endothelial cells, whereas it inhibited mannitol-induced apoptosis in endothelial cells (data not shown). These findings suggest that the autoantibodies may also block angiopoietin binding.

We also found that the antitumor activity in this study could be CD4+ T lymphocyte dependent but not CD8+ T lymphocyte and NK cell dependent. Mice depleted of CD4+ T lymphocytes by the injection of anti-CD4 monoclonal antibody and vaccinated with chTie-2 were not protected from tumor growth. Moreover, mice depleted of CD4+ T lymphocytes did not develop detectable autoantibodies against Tie-2. In contrast, treatment with anti-CD8 or anti-NK monoclonal antibody failed to abrogate the antitumor activity. These findings suggest that the induction of the autoimmune response to Tie-2, which may be responsible for chTie-2-induced antitumor activity, may depend on CD4+ T lymphocytes. It is known that CD4+ T lymphocytes can initiate and amplify immune responses through the secretion of cytokines and the expression of cell surface molecules (34, 35). CD4+ T lymphocytes can be required at the immunization phase as well as at the effector phase for the antibody-dependent immunity (36). Moreover, it has been reported that CD4+ T lymphocytes could be required for the induction of antitumor immunity by vaccination with a recombinant vaccinia virus encoding self-tyrosinase-related protein-1 in a mouse melanoma mode (37). It has been reported that CD4+ T lymphocytes play a prominent role in classic mouse models of autoimmunity, such as experimental allergic encephalitis, systemic lupus erythematosus, and autoimmune gastritis (38–41). All of these findings may help explain the requirement for CD4+ T lymphocytes in the induction of an autoimmune response against mTie-2 in a cross-reaction in the current study.

We have paid special attention to investigating potential toxicity in the mice immunized with these vaccines and have not found apparent adverse effects in the present study. It has been reported that Tie-2 is specifically overexpressed within tumor vasculature than that within normal tissues (7). In addition, deposition of the autoantibodies is found on the tumor vasculature than that within normal tissues (7). In summary, our finding may provide a new vaccine strategy for cancer therapy through the induction of autoimmune response against the self-molecules for angiogenesis by immunization with the single xenogeneic homologous Tie-2. This vaccine strategy may be useful in targeting other growth factors or their receptors associated with tumor growth. Furthermore, the observation that the blockade of Tie-2 pathway by active immunity was sufficient to inhibit xenograft tumor growth suggests that Tie-2 receptor pathway might be essential for tumor growth and interference with Tie-2 pathway might be useful for cancer therapy.

References

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Fig. 6. Inhibition of angiogenesis in tumor by immunohistochemical analysis and alginate encapsulation assay. Sections of frozen B16F10 tumor tissues obtained from mice immunized with chTie-2 (A), mTie-2 (B), and AlOH3 (C) Vessel density was determined by counting the number of the microvessels per high-power field (magnification, × 200) with an antibody reactive to CD31 as described in Materials and Methods (G). Photographs of immunohistochemical analysis (A–C) and vessel density in tumor tissues (G) showed a significant inhibition of angiogenesis in tumor tissues of chTie-2–immunized mice. Mice were immunized with chTie-2 (D), mTie-2 (E), or AlOH3 (F) once weekly for 4 weeks. Alginate beads containing 1 × 106 tumor cells were implanted s.c. into the backs of mice. Twelve days later, beads were surgically removed and FITC-dextran was quantified as described in Materials and Methods (H). Photographs of alginate implants (D–F) and FITC-dextran uptake (H) showed a significant decrease of vascularization in implants of chTie-2–immunized mice.


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Immunotherapy of Tumors with Protein Vaccine Based on Chicken Homologous Tie-2

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