Immunotherapy of tumors with xenogeneic endothelial cells as a vaccine

YU-QUAN WEI[1], QING-RU WANG[1], XIA ZHAO[2], LI YANG[1], LING TIAN[1], YOU LU[1], BIN KANG[1], CHONG-JIU LU[1], MEI-JUAN HUANG[1], YAN-YAN LOU[1], FEI XIAO[1], QIU-MING HE[1], JING-MEI SHU[1], XING-JIANG XIE[1], YUN-QIU MAO[1], SHONG LEI[1], FENG LUO[1], LI-QUN ZHOU[1], CHONG-EN LIU[1], HAO ZHOU[1], YU JIANG[1], FENG PENG[1], LIANG-PING YUAN[1], QIU LI[1], YANG WU[1] & JI-YAN LIU[1]

[1]*Center for Biotherapy of Cancer and Cancer Center, First University Hospital, West China University of Medical Sciences (HuaXi Medical School, Sichuan University), Guo Xue Xiang, Number 37, Chengdu, Sichuan, 610041, The People's Republic of China*

[2]*Department of Gynecology and Obstetrics, Second University Hospital, West China University of Medical Sciences (HuaXi Medical School, Sichuan University), Ren Min Nan Road, Number 17, Chengdu, Sichuan, 610041, The People's Republic of China*

Correspondence should be addressed to: Y.-Q. W. email: yuquawei@mail.sc.cninfo.net

The breaking of immune tolerance against autologous angiogenic endothelial cells should be a useful approach for cancer therapy. Here we show that immunotherapy of tumors using fixed xenogeneic whole endothelial cells as a vaccine was effective in affording protection from tumor growth, inducing regression of established tumors and prolonging survival of tumor-bearing mice. Furthermore, autoreactive immunity targeting to microvessels in solid tumors was induced and was probably responsible for the anti-tumor activity. These observations may provide a new vaccine strategy for cancer therapy through the induction of an autoimmune response against the tumor endothelium in a cross-reaction.

The generation of new blood vessels, or angiogenesis, is important for normal embryonic development and for the development of pathologic conditions such as cancer, rheumatoid arthritis and retinopathies[1–5]. Several lines of direct and indirect evidence indicate that the growth and persistence of solid tumors and their metastases are angiogenesis-dependent[1,4–8]. As a strategy for cancer therapy, anti-angiogenic therapy attempts to stop new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass[3,4,9,10].

Endothelial cells in the angiogenic vessels in solid tumors express proteins on their surfaces that are absent or barely detectable in normal quiescent vascular endothelium, including αvβ3 integrin and receptors for certain angiogenic growth factors[2,4,8,9]. The proteins on the endothelium of new vessels in the mouse are homologous to those in humans and in other species, to varying extents[14–16]. The breaking of immune tolerance to autologous angiogenic endothelial cells should be a useful approach for cancer therapy. However, immunity to angiogenic vessels is presumably difficult to elicit by autologous or syngeneic endothelial cells or their proteins as vaccine because of the immune tolerance acquired during the development of the immune system. Here we explored the feasibility of immunotherapy of tumors with xenogeneic endothelial cells as a vaccine by breaking immune tolerance against autologous angiogenic cells in a cross-reaction between the xenogeneic homologs and self molecules.

We prepared vaccines using proliferative endothelial cells cultured *in vitro*, like new vessels with proliferative activity in solid tumors. We tested paraformaldehyde-fixed human and bovine endothelial cells as vaccines for their ability to induce ant-tumor immunity in several tumor models in mice. For this, we used human umbilical vein endothelial cells (HUVECs primary cells and HUV-EC-Cs), human dermal microvascular endothelial cells (HDMVECs), and bovine glomerular endothelial cells (GEN-T). We also used a cell line of transient SV40 infection of mouse endothelial cells derived from lymph node stroma (SVEC4-10), a cell line of human aorta vascular smooth muscle cells (T/G HA-VSMCs) and a human B-lymphoblastoid cell line transformed by Epstein-Barr virus (RPMI 7666 cells) as control cell vaccines.

Induction of protective and therapeutic anti-tumor immunity

We immunized mice intraperitoneally once weekly for 4 weeks with different doses of HUVECs, HDMVECs, HUV-EC-C, GEN-T cells, SVEC4-10 cells, T/G HA-VSMCs and RPMI 7666 cells or treated them with PBS alone (non-immunized mice), and then challenged with 1×10^5–1×10^7 live tumor cells after the fourth immunization. Tumor grew progressively in all non-immunized mice and mice immunized with SVEC4-10 cells, T/G HA-VSMCs or RPMI 7666 cells, but there was complete protection from tumor growth in mice immunized with HUVECs, HDMVECs, HUV-EC-C or GEN-T cells (Fig. 1). The protective effect was long-lasting, as mice of different strains (BALB/c, C57Bl/6 and C3H) challenged up to 12 weeks after the last immunization resisted challenge with several solid tumor cells of different histological origin (data not shown).

We next tested the therapeutic efficacy of xenogeneic endothelial cells as vaccines in established tumors. We began treating the mice on day 7 after injection of Methylcholanthrene A induced (Meth) A-fibrosarcoma, hepatoma or breast cancer cells, when the tumors were visible and palpable. Treatment with HUVECs or GEN-T cells twice weekly for 4 weeks resulted in re-

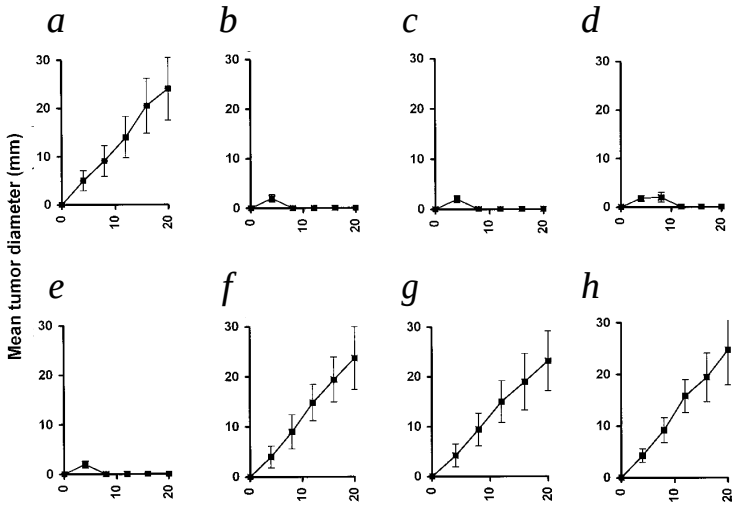


Fig. 1 Induction of protective anti-tumor immunity. BALB/C mice were immunized intraperitoneally with fixed endothelial cells or control cells, and then challenged with live Meth A cells. ***a***, treatment with PBS alone (control). ***b***–***h***, Vaccination with HUVECs (*b*), HUV-EC-Cs (*c*), HDMVECs (*d*), GEN-T cells (*e*), SVEC4-10 cells (*f*), T/G HA-VSMCs (*g*) and RPMI 7666 cells (*h*).

tarded progression and, finally, regression of the established tumors (Fig. 2*a*–*d*). Survival of the tumor-bearing mice treated with GEN-T cells or HUVECs was also significantly longer than that of the untreated mice or mice immunized with SVEC4-10 cells (Fig. 2*e*–*h*). We monitored mice immunized with xenogeneic endothelial cells, using gross measures such as weight loss, ruffling of fur and life span, and found no adverse consequences. In addition, we found no pathologic changes of liver, lung, kidney, spleen or brain by microscopic examination.

Characterization of autoantibodies against endothelial cells

To determine the possible mechanism by which anti-tumor activity was induced with xenogeneic endothelial cells, we treated the endothelial cells and tumor cells with various doses of immunoglobulins isolated from mice immunized with HUVECs or SVEC4-10 cells or from non-immunized mice. Treatment with immunoglobulins from mice immunized with HUVECs resulted in apparent inhibition of proliferation of human, mouse and bovine endothelial cells, compared with those from mice immunized with SVEC4-10 cells or non-immunized mice (Fig. 3). In contrast, the treatment had no effect on proliferation of tumor cells.

Adoptive transfer of immunoglobulins isolated from mice immunized with HUVECs, GEN-T cells or HDMVECs was effective in affording protection from tumor growth (Fig. 4). Adsorption of immunoglobulins with fixed endothelial cells before adoptive transfer abrogated the anti-tumor activity, but T/G HA-VSMCs had no effect (Fig. 4). By flow cytometric analysis, both human and mouse endothelial cell lines showed positive staining with sera isolated from mice immunized with HUVECs, but negative staining with sera from mice immunized with SVEC4-10 cells or non-immunized mice (data not shown). Sera isolated from mice immunized with HUVECs also positively stained microvessels in tumor tissues derived from the non-immunized mice (Fig. 5*a*) and granulation tissues from a healing wound (Fig. 5*b*), but not those in other normal tissues of the body (Fig. 5*c*).

To identify possible endothelial deposition of autoantibodies, we stained microvessels using immunohistochemistry. There was endothelial deposition of immunoglobulins in the tumor tissues from mice immunized with HUVECs (Fig. 5*d*), but not in those from the non-immunized or mice immunized with SVEC4-10 cells or T/G HA-VSMCs (data not shown). In addition, there was no immunoglobulin deposition in the immunized or non-immunized mice in normal quiescent endothelium in the major organs such as kidney, liver, spleen and brain, and the results were similar in appearance to those in Fig. 5*c* (data not shown).

Inhibition of angiogenesis

We sequentially analyzed microvessel density as tumors regressed in response to the vaccine (Fig. 5*e*). Microvessel density gradually decreased as a result of prolongation of the vaccine treatment (Fig. 5*e*). Also, vessel length, 'clock-hours' (the proportion of the circumference that is vascularized if the eye is viewed as a clock) and area of neovascularization (assessed by Corneal micro-pocket assay) were inhibited by 68 ± 6%, 72 ± 5% and 81 ± 7%, respectively, in mice treated by the adoptive transfer of immunoglobulins isolated from mice immunized with HUVECs,

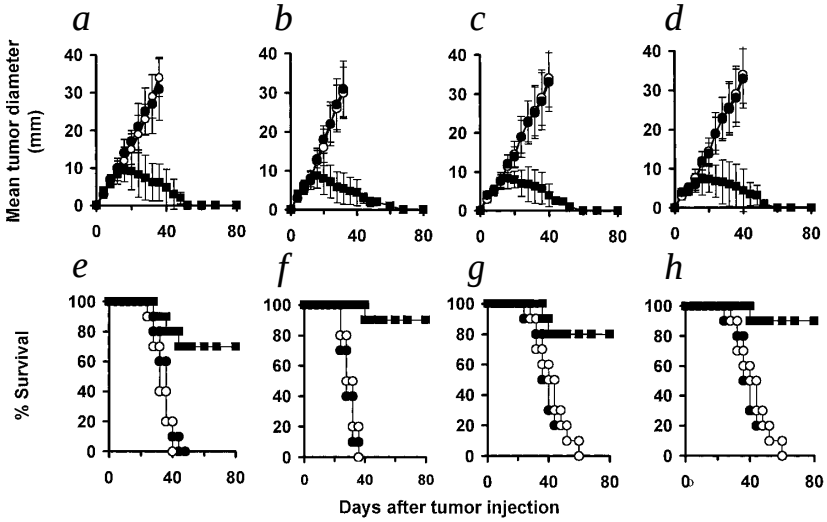


Fig. 2 Induction of the therapeutic anti-tumor immunity. Meth A fibrosarcoma cells (***a*** and ***e***), H22 hepatoma cells (***b*** and ***f***) and MA782/5S mammary carcinoma cells (***c***, ***d***, ***g*** and ***h***) were introduced subcutaneously into mice, and then the mice were treated with fixed SVEC4-10 cells (○), HUVECs or GEN-T cells (■) or PBS alone (●). *a*, *b* and *d*, Tumor sizes in mice treated with HUVECs (■). *e*, *f* and *h*, Survival of mice treated with HUVECs (■). *c* and *g*, Tumor sizes and survival of mice treated with GEN-T cells (■).

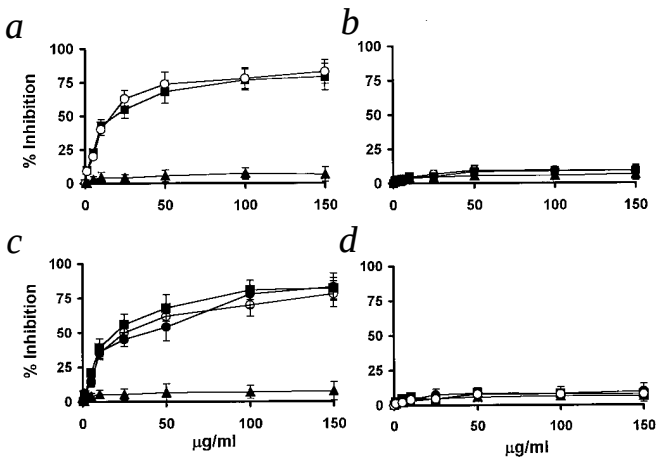


Fig. 3 The inhibition of proliferation of endothelial cells *in vitro* with immunoglobulin. ***a***, Exponentially growing HUVECs (■),SVEC4-10 cells (○) or Meth A cells (▲) were exposed to immunoglobulins isolated from mice immunized with HUVECs, and the percentage inhibition was calculated. ***b***, HUVECs (■),SVEC4-10 cells (○) or Meth A cells (▲) were treated with immunoglobulins isolated from non-immunized mice. ***c***, HDMVECs (■), HUVEC-Cs (○),GEN-T cells (●) and LL/2 cells (▲) were also treated with immunoglobulins isolated from mice immunized with HUVECs. ***d***, As a control of the experiment in *c*, cells were treated with immunoglobulins isolated from non-immunized mice. Horizontal axes, immunoglobulin concentrations.

compared with control samples. Also, we obtained similar results by direct immunization with HUVECs.

Function of CD4⁺ T cell in the anti-tumor activity

There was no anti-tumor activity induced by xenogeneic endothelial cells in nude mice, indicating that T cells may be required for the anti-tumor response. Furthermore, mice depleted of CD4⁺ T lymphocytes by the injection of monoclonal antibody against CD4 and vaccinated with xenogeneic endothelial cells were not protected from tumor challenge. In contrast, treatment with monoclonal antibody against CD8 or natural killer cells or normal rat immunoglobulin (Ig) G failed to abrogate the anti-tumor activity (Fig. 6*a*). We assessed immunoglobulin subclass response to the endothelial cells using enzyme-linked immunosorbent assay (ELISA), and found substantial increases in IgG1, IgG2a and IgG2b with little increase in IgM or IgA in sera obtained from the mice at day 7 after the fourth immunization, compared with that in control samples (Fig. 6*b*). Mice depleted of CD4⁺ T lymphocytes did not develop detectable antibodies against the endothelial cells (Fig. 6*b*).

Identification of the possible peptides for the cross-reaction

The endothelial cell extracts showed multiple positive bands by western blot analysis when probed with sera from mice immunized with HUVECs (Fig. 7*a*) but negative staining with those from non-immunized mice (Fig. 7b). At least two bands with molecular sizes of 220 and 130 kDa had sizes similar to those of the known angiogenesis-associated molecules vascular endothelial growth factor receptor (VEGFR) II and αv integrin. These two molecules were also expressed on the endothelial cells we used, as shown by the use of commercially available antibodies against VEGFR II or αv integrin in flow cytometric and western blot analysis, and immunoglobulins isolated from mice immunized with HUVECs showed positive reactions against the recombinant extracellular parts of VEGFR II and αv integrin by ELISA (data not shown). Sequence comparison analysis using the SwissProt database in NCBI indicated that the primary sequences of VEGFR II and αv integrin of mice and humans were homologs that were 82% and 89% identical, respectively, at the amino-acid level. Next, we selected pairs of peptides for synthesis from the regions that shared the most-identical amino-acid sequences between humans and mice. Each peptide synthesized was 35 amino acids long. We screened the possible peptides responsible for the cross-reaction in two steps. We probed these peptides with immunoglobulins from mice immunized with HUVECs, using ELISA. We immunized mice with pairs of homologous peptides that showed immunoglobulin-positive binding and determined their anti-tumor activity. Three pairs of the homologous peptides showed immunoglobulin-positive binding, and their human homologs showed anti-tumor activity. We identified two pairs within αv integrin and one pair within VEGFR II (Table 1). These immunoglobulin-binding regions were represented by amino-acid residues 330–364 and 545–579 within the extracellular fragment of αv integrin in both human and

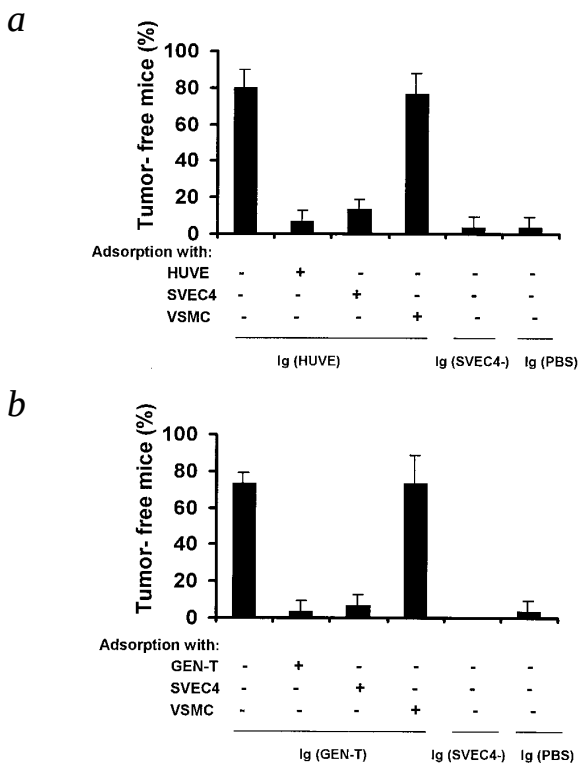


Fig. 4 Adoptive transfer of immunoglobulins *in vivo*. ***a***, Protective anti-tumor effect on H22 hepatoma was tested with purified immunoglobulins from mice immunized with HUVECs and control cells, the immunoglobulins were adsorbed with (+) HUVECs, SVEC4-10 cells and T/G HA-VSMCs before adoptive transfer. ***b***, Protective effect on Lewis lung carcinoma (LL/2) with immunoglobulins from mice immunized with GEN-T cells and control cells, and immunoglobulins adsorbed with (+) GEN-T cells, SVEC4-10 cells and T/G HA-VSMCs.

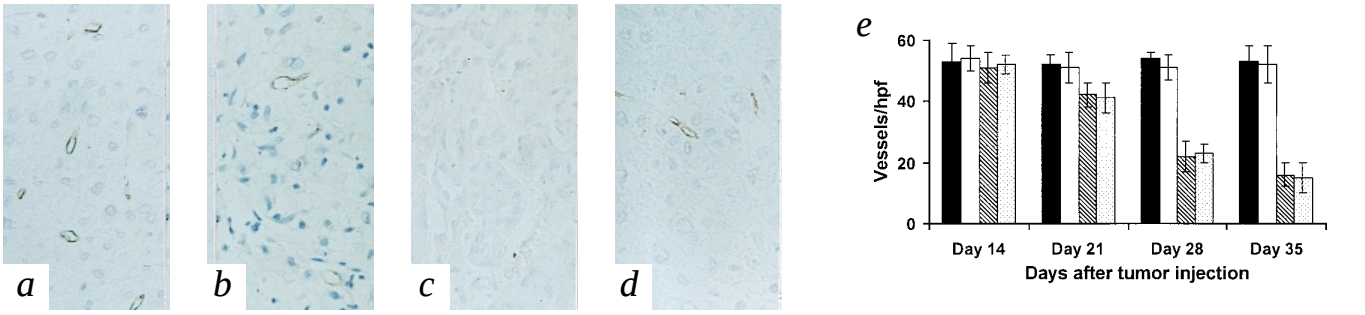


Fig. 5 *In situ* observation of microvessels and inhibition of angiogenesis. *a–d*, Immunohistochemical analysis of microvessels in tumor tissues, stained with immunoperoxidase. ***a*** and ***b***, Microvessels in hepatoma tissues (*a*) and granulation tissues of healing wound (*b*) from non-immunized mice, using serum from the mice immunized with HUVECs. ***c***, Microvessels in kidney (control). ***d***, Endothelial deposition of immunoglobulins in the hepatoma tissue from a mouse immunized with HUVECs. ***e***, Sequential analysis of inhibition of angiogenesis in tumors. Mice were treated with PBS alone (■) or with fixed SVEC4-10 cells (□), HUVECs (▨) or GEN-T cells (□) after H22 hepatoma cells were introduced subcutaneously. Vessel density was determined by counting of the microvessels in each high-power field (hpf) in the sections.

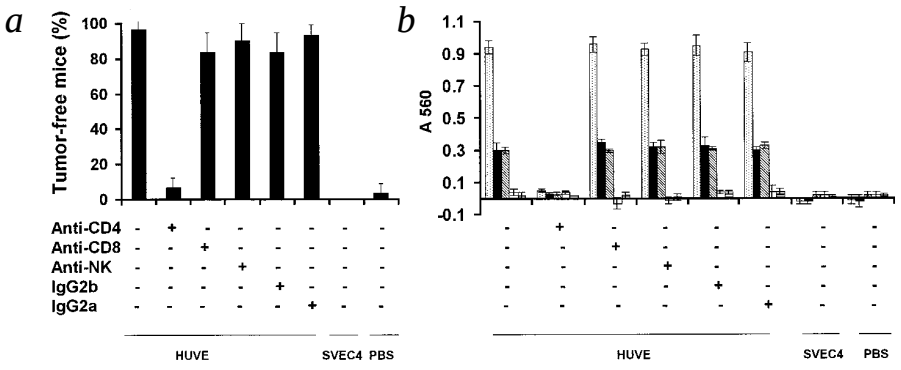
mouse, and by residues 239–273 in human and the corresponding residues 243–277 in mouse within VEGFR II. We identified these immunoglobulin-positive binding peptides from 42 pairs of homologous peptides examined. Furthermore, adoptive transfer of immunoglobulins isolated from these mice immunized with xenogeneic peptide also showed inhibition of tumor growth, but immunoglobulins from mice immunized with mouse peptides or with Freund's adjuvant alone had no effect compared with those from mice treated with PBS alone (Fig. 7*c*).

Discussion

Here we obtained many results regarding xenogeneic endothelial cell vaccines, anti-tumor immunity and angiogenesis. Fixed xenogeneic endothelial cells as vaccine induced both protective and therapeutic anti-tumor immunity. The autoreactive immune response against the microvessels in solid tumors may be provoked in a cross-reaction by immunization of xenogeneic endothelial cells, and the autoreactive immunity targeting to microvessels in solid tumor was probably responsible for the anti-tumor activity. These suggestions were supported by our results. Endothelial cell proliferation was inhibited *in vitro* by purified immunoglobulins from xenogeneic endothelial cell-immunized mice. Anti-tumor activity and inhibition of angiogenesis was acquired by adoptive transfer of purified immunoglobulins. Immunoglobulins present in sera positively stained microvessels in the tumor and endothelial cell lines of both human and mouse. We identified cross-reactive peptides within αv integrin and VEGFR II on endothelial cells, and similar molecules in both human and mouse endothelial cell lines were recognized by western blot analysis. IgG1, IgG2a and IgG2b were substantially increased in response to the endothelial cells. There was endothelial deposition of immunoglobulins in tumor. There was also anti-tumor activity and production of immunoglobulins against the endothelial cells that was abrogated by the depletion of $CD4^+$ T lymphocytes. Angiogenesis was apparently inhibited in tumor (Fig. 5*e*), and corneal angiogenesis induced by basic fibroblast growth factor was inhibited. In addition, the anti-tumor activity with xenogeneic endothelial cells may not result from the nonspecifically augmented immune response against tumor growth in host mice, as we found no increase in natural killer cell activity of spleen cells or in levels of cytokines such as interferons α, β and γ, tumor necrosis factor α and β chemokines in sera from immunized mice (data not shown), and found no anti-tumor activity after immunization with a xenogeneic smooth muscle cell line or B-lymphoblastoid cells.

Anti-tumor immunity depends on $CD8^+$ T lymphocytes in some mouse models, whereas $CD4^+$ T lymphocytes often have little, if any, function[17–20]. Some molecular targets of tumor-specific $CD8^+$ T lymphocytes have been identified in human and mouse systems[18,19]. $CD8^+$ T lymphocytes have been the focus of

Fig. 6 Abrogation of the anti-tumor activity and immunoglobulin subclass response to the endothelial cells by the depletion of $CD4^+$ T lymphocytes. ***a***, Abrogation of anti-tumor activity. Mice were immunized and challenged with MA782/5S mammary carcinoma cells after depletion of immune cell subsets. ***b***, Abrogation of immunoglobulin subclass response to endothelial cells. Sera obtained from mice immunized with HUVECs were tested against lysates of SVEC4-10 cells by ELISA, after depletion of immune cell subsets. Immunoglobulin detected: ⬚, IgG1; ■, IgG2a; ■, IgG2b; □, IgM; ■, IgA. Data represent absorption at 560 nm. Anti-, antibody against; +, molecule used for subset depletion.



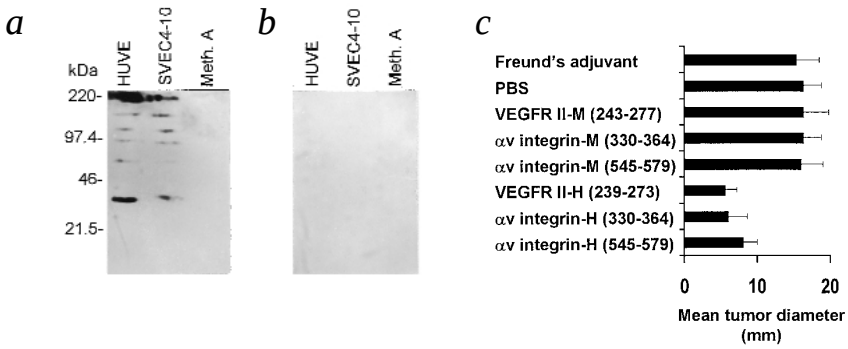


Fig. 7 Identification of the possible antigens responsible for the cross-reaction. ***a*** and ***b***, Western blot analysis for possible antigens of both HUVECs and SVEC4-10 cells recognized by the immune sera and control sera respectively. ***a***, HUVECs, SVEC4-10 cells and Meth A cells, stained with sera isolated from mice immunized with HUVECs. ***b***, The cells in *a,* stained with sera from non-immunized mice (control). Left margin, molecular sizes. ***c***, Inhibition of tumor growth by adoptive transfer of immunoglobulins. There is a protective anti-tumor effect with immunoglobulins isolated from the mice immunized with xenogeneic peptides, but not from mouse peptides, Freund's adjuvant or PBS alone. Data represent day 20 after tumor cell injection.

recent efforts in the development of a therapeutic anti-tumor vaccine[18,19]. However, here we found that mice depleted of $CD4^+$ T lymphocytes by the injection of monoclonal antibody against CD4 and vaccinated with xenogeneic endothelial cells were not protected from tumor challenge. These mice did not develop detectable antibodies against the endothelial cells. In contrast, treatment with monoclonal antibody against CD8 or natural killer cells or control IgG failed to abrogate the anti-tumor activity. These data indicate that the induction of the antibody response to the endothelial cells, which is responsible for anti-tumor activity induced by xenogeneic endothelial cells may involve $CD4^+$ T lymphocytes. $CD4^+$ T lymphocytes can 'steer' and amplify immune responses through the secretion of cytokines and expression of surface molecules[20–22]. Moreover, $CD4^+$ T lymphocytes are prominent in classic mouse models of autoimmunity, such as experimental allergic encephalitis, systemic lupus erythematosus and autoimmune gastritis[22–24]. These findings may help explain the requirement for $CD4^+$ T lymphocytes in the induction of autoimmune response against the tumor endothelium in a cross-reaction.

Here we prepared vaccines using proliferative endothelial cells cultured *in vitro*, like new vessels with proliferative activity in solid tumors. Endothelial cells in culture may be heterogenous and express genes that may not be expressed in the original tissue, whereby they may lose the expression of a number of antigens. However, we found that immunoglobulin or serum isolated from mice immunized with cultured xenogeneic endothelial cells showed positive staining not only for cultured endothelial cells but also for microvessels in tumor tissues or in a healing wound. These findings indicate that there may still be some common antigens or cross-reactive epitopes between cultured endothelial cells and those in tumor tissues, which are responsible for the autoimmune response against the tumor endothelium in a cross-reaction. This is also supported by the findings that the cultured endothelial cells can still express some genes that are expressed in the microvessels in tumor tissues. For example, molecules such as VEGFR II, αvβ3 integrin and endoglin, which are associated with angiogenesis in tumors, can be found in primary culture of HUVECs, HDMVECs and bovine endothelial cells as well as HUV-EC-Cs and some bovine endothelial cell lines[25–32]. Some of these molecules were also present on the endothelial cells used here.

The molecules αv integrin and VEGFR II are important during angiogenesis[2–4,10,12,29,33]. Blockade of the ligand binding domain of these molecules results in the inhibition of angiogenesis *in vivo* or of endothelial cell proliferation *in vitro* and of anti-tumor activity[2–4,10,12,29,33]. Here, at least two bands by western blot analysis showed sizes similar to those of VEGFR II and αv integrin. We also identified these two molecules on the endothelial cells using commercially available antibodies against VEGFR II and αv integrin, by flow cytometric and western blot analysis, and immunoglobulins isolated from mice immunized with HUVECs showed positive reactions against the recombinant extracellular parts of VEGFR II and αv integrin by ELISA (data not shown). We identified three pairs of possible peptides responsible for cross-reaction in the extracellular parts of these molecules. Furthermore, two of three pairs of immunoglobulin-binding sites were located within the regions encompassing the ligand-binding domain (residues 247–261) of VEGFR II (ref. 29) and partially encompassing the ligand-binding domain (residues 139–349) of αv integrin[34]. The other immunoglobulin-binding site was located outside the ligand-binding domain of αv integrin. However, some antibodies against the non-binding domain can block the function of the integrin allosterically as well[35]. Immunoglobulins isolated from the mice immunized with xenogeneic peptides of αv integrin and VEGFR II identified αv integrin and VEGFR II, respectively, on the endothelial cells, and we demonstrated inhibition of endothelial

Table 1 The amino-acid sequence of cross-reactive peptides in αv integrin and VEGFR II

Peptide name	Species	Amino acid sequence
αv integrin-H (545–579)	Human	YSRSPSHSKNMT I SRGGLMQCEEL I AYLRDESEFR
αv integrin-M (545–579)	Mouse	HNRSPVHSKTMTVFRGGQMQCEELVAYLRDESEFR
αv integrin-H (330–364)	Human	FMDRGSDGKLQEVGQVSVSLQRASGDFQTTKLNG
αv integrin-M (330–364)	Mouse	FMDRGSDGKLQEVGQVSVSLQRAVGDFQTTKLNG
VEGFR II-H (239–273)	Human	KLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNR
VEGFR II-M (243–277)	Mouse	KLVLNCTARTELNVGLDFTWHSPPSKSHHKK IVNR

Peptides derived from the αv integrin or VEGFR II were synthesized and then probed by ELISA with immunoglobulins isolated from mice immunized with HUVECs. Mice were immunized with pairs of homologous peptides that showed immunoglobulin-positive binding, and anti-tumor activity was determined. Three pairs of immunoglobulin-positive binding homologous peptides between human and mouse were identified as candidates of the cross-reactive peptides.

cell proliferation *in vitro* (data not shown). Also, the adoptive transfer of immunoglobulins isolated from these mice immunized with xenogeneic peptides showed inhibition of tumor growth. The findings described above indicate that the cross-reaction seen here may involve in part the epitopes within VEGFR II and αv integrin on endothelial cells. The other four bands on the western blot were difficult to match to sizes of known angiogenesis-associated molecules. Whether they belong to new angiogenesis-associated molecules for cross-reaction must be explored further. The findings described above also indicate that the high potency of the polyclonal serum isolated from xenogeneic whole endothelial cells found here may result from the blockade of some important angiogenesis-associated molecules such as αv integrin and VEGFR II, and may involve targeting to multiple sites, as angiogenesis is a complex process involving many molecules on new vessels.

These findings may provide a new vaccine strategy for cancer therapy through the induction of an autoimmune response against the microvessels in solid tumors in a cross-reaction by the immunization with xenogeneic endothelial cells as vaccine. It may be important to explore further the application of xenogeneic cells or single homologous protein between human and other species for cancer therapy.

Methods

Vaccine preparation. The cells cultured *in vitro*, including a variety of endothelial cells or control cells, were collected and were washed three times with PBS. The cells were then fixed with 3% paraformaldehyde in PBS, pH 7.4, for 24 h at 4 °C, then washed three times and incubated at 37 °C for 2 h to remove the residual paraformaldehyde. After being washed three times, the cells were resuspended in PBS for use as vaccines. HUV-EC-Cs, SVEC4-10 cells, T/G HA-VSMCs and RPMI 7666 cells were obtained from American Type Culture Collection (Rockville, Maryland). The GEN-T cell line was from Dainippon Pharmaceutical (Osaka, Japan). HUVECs and HDMVECs were collected and cultured as described[36]. HUVECs and HDMVECs from passages 5–12, inclusively, were used for all experiments.

Immunotherapy and tumor models. Mice were immunized intraperitoneally or subcutaneously once weekly for 4 continuous weeks with different doses (1×10^2–1×10^7 cells/mouse) of a variety of endothelial cell vaccines or T/G HA-VSMCs as well as RPMI 7666 cells (as a control) or were treated with PBS alone (non-immunized). Mice were then challenged with 1×10^5–1×10^7 live tumor cells after the fourth immunization. For investigation of the therapeutic effect against the established tumors, ten mice in each group were treated with intraperitoneal injection of the vaccines or control cells or PBS alone twice weekly for 4 weeks starting a day 7 after subcutaneous introduction of 1×10^6 live tumor cells. The Meth A fibrosarcoma and H22 hepatoma models were established in BALB/c mice. MA782/5S and FM3A mammary carcinoma models were established in BALB/c and C3H mice, respectively. The Lewis lung carcinoma model was in C57Bl/6 mice. All studies involving mice were approved by the institute's Animal Care and Use Committee.

Purification of imunoglobulin, its inhibition of cell proliferation *in vitro* and its adoptive transfer *in vivo*. Immunoglobulins were purified from the pooled sera derived from the mice on day 7 after the fourth immunization or from control mice, by affinity chromatography (CM affi-gel blue gel kit; BioRad, Richmond, California). For determination of the effects of purified immunoglobulins on cell proliferation, exponentially growing endothelial cells or tumor cells, at a concentration of 2×10^5 cels/ml were exposed to various concentrations (1–150μg/ml) of the imunoglobulin for 72 h of culture. The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage inhibition was calculated[37].

For assessment of the efficacy of imunoglobulin in anti-tumor *in vivo*, purified imunoglobulins (10–300 mg/kg) were adoptively transferred intravenously 1 d before mice were challenged with 1×10^5–1×10^7 tumor cells, and then mice were treated twice per week for 3 weeks. As a control, immunoglobulin was adsorbed four times by incubation for 1 h at 4 °C, with rocking, with fixed xenogeneic endothelial cells or VSMCs.

Immunoglobulin subclass response to the endothelial cells and peptide ELISA. Immunoglobulin sublass was determined by ELISA as described[38]. Endothelial cells and tumor cells were washed and lysed by three cycles of freezing and thawing, and then 1×10^4 cell equivalents (50 μl) were plated, dried and blocked. Experimental mouse sera were serially diluted and added to the wells. Plates were incubated for 2 h at 37 °C, washed, and then incubated with serially diluted alkaline phosphatase-conjugated antibody against mouse IgG subclass, IgM or IgA. Enzyme activity was measured with an ELISA reader (BioRad, Richmond, California). For the identification of cross-reactive peptides, the peptides were coupled to 96-well assay plates at a concentration of 1 μg/well, as described[39], then probed with immunoglobulins from mice immunized with HUVECs, by ELISA.

***In vivo* depletion of immune cell subsets**. Immune cell subsets were depleted as described[40]. Mice were injected intraperitoneally with 500 μg monoclonal antibodies against CD4 (clone GK 1.5, rat IgG), CD8 (clone 2.43, rat IgG) or natural killer cells (clone PK136) or isotype controls 1 d before the immunization, and then twice per week for 3 weeks. Tumor cells (1×10^6–1×10^7) were challenged after the fourth immunization. These hybridomas were obtained from American Type Culture Collection (Rockville, Maryland). The depletion of $CD4^+$, $CD8^+$ and natural killer cells was consistently greater than 98%, as determined by flow cytometry (Coulter Elite ESP; Coulter, Hialeah, Florida)[40].

Immunohistochemistry and flow cytometric analysis. Immunohistochemistry was done as described[41]. Frozen sections were fixed in acetone, incubated with serum diluted 1:50-1:2,000, isolated from immunized or non-immunized mice. The sections were then stained with labeled streptavidin biotin reagents (Dako LSAB kit, peroxidase; Dako, Carpinteria, California). To identify the endothelial deposition of autoantibodies, we stained the sections without using primary antibody. For determination of vessel density, microvessels were counted in each high-power field in the sections as described[7]. Wound healing was created by excision of the skin of the mid-dorsal region as described[42]. Granulation tissue at day 5 after wounding was obtained for immunohistochemical analysis. For the flow cytometric analysis, endothelial cells and tumor cells were stained by an indirect method[43], using serum diluted 1:50–1:3,000, and then goat antibody against mouse IgG, IgM and IgA, conjugated to fluorescein isothiocyanate (Sigma).

Western blot analysis. Western blot analysis was done as described[43]. Cells (2×10^7) were lysed in 1 ml lysis buffer. The membrane blots were blocked by incubation at 4 °C in 5% non-fat dry milk, then were washed and probed with mouse sera at a dilution of 1:100. Blots were then washed and incubated with a biotinylated secondary antibody (Biotinylated horse antibody against mouse IgG or IgM, followed by transfer to Vectastain ABC solution (Vector Laboratories, Burlingame, California).

Micropocket assay. The micropocket assay was done as described[5,44]. Five mice in each group were immunized by vaccines for 4 continuous weeks or were treated by adoptive transfer of purified immunoglobulin as described above before the pellets containing basic fibroblast growth factor were implanted into the cornea.

Synthesis of peptides and immunization with the peptides. Peptides were synthesized by FMOC (fluorenyl methoxycarbonyl)-t-butyl-based solid-phase peptides chemistry on an AB433A peptide synthesizer (Perkin Elmer, Norwalk, Connecticut) as described[45]. The crude peptides were purified by reverse-phase high-performance liquid chromatography[44].

Peptides (100 μg antigen per injection) were emulsified 1:1 (volume/volume) with complete Freund's adjuvant for the first immunization, followed by a boost in incomplete Freund's adjuvant at 2 weeks and weekly thereafter, as described[46], then challenged with 1×10^5–1×10^7 live tumor cells after the fourth immunization. Purified immunoglobulins were prepared from the pooled sera derived from the mice at day 7 after the fourth immunization without injection of tumor cells.

Acknowledgments

This work was supported by the National Outstanding Young Scientist

Foundation of China, the National Natural Sciences Foundation of China, the National 973 Project and the Foundation for University Key Teacher.

RECEIVED 28 DECEMBER 1999; ACCEPTED 31 AUGUST 2000

1. Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.* **82**, 4–6 (1990).
2. Ferrara, N. & Alitalo, K. Clinical application of angiogenic growth factors and their inhibitors. *Nature Med.* **5**, 1359–1364 (1999)
3. Risau, W. Mechanism of angiogenesis. *Nature* **386**, 671–674 (1997)
4. Zetter, B.R. Angiogenesis and tumor metastasis. *Annu. Rev. Med.* **49**, 407–424 (1998)
5. Cao, R., *et al*. Suppression of angiogenesis and tumor growth by the inhibitor K1-5 generated by plasmin-mediated proteolysis. *Proc. Natl. Acad. Sci. USA* **96**, 5728–5733 (1999)
6. Bicknell, R. in *Tumor Angiogenesis*. (eds. Bicknell, R., Lewis, C.E. & Ferrara. N.) 19–28 (Oxford University Press, Oxford, 1997).
7. O'Reilly, M.S. *et al*. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88**, 277–285 (1997)
8. Kerbel. R.S. A cancer therapy resistant to resistant. *Nature* **390**, 335 (1997)
9. Fidler. I.J. & Ellis L.M. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* **79**, 185–188 (1994).
10. Arap, W., Pasqualini, R. & Ruoslahti, E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* **279**, 377–379 (1998);
11. Fan, T.P., Jaggar, R. & Bicknell, R. Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. *Trends Pharmacol. Sci.* **16**, 57–66 (1995)
12. Kim, K.J. *et al*. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. *Nature* **362**, 841–844 (1993)
13. Hanahan D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *J. Natl. Cancer Inst.* **88**, 1091–1092 (1996).
14. Quinn, T.P., Peters, K.G., De Vries, C., Ferrara, N. & Williams, L.T. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc. Natl. Acad. Sci. USA* **90**, 7533–7537 (1993)
15. Djaffar, I. *et al*. A new alternative transcript encodes a 60 kDa truncated form of integrin beta 3. *Biochem. J.* **15**, 69–74 (1994)
16. Eichmann, A., Marcelle C., Breant, C. Le Douarin, N.M. Molecular cloning of Quek 1 and 2, two quail vascular endothelial growth factor (VEGF) receptor-like molecules. *Gene* **174**, 3–8 (1996)
17. Barth, R.J.J., Bock, S.N., Mule, J.J. & Rosenberg, S.A. Unique murine tumor-associated antigens identified by tumor infiltrating lymphocytes. *J. Immunol.* **144**, 1531–1537 (1990).
18. Boon, T., Coulie, P.G. & Van den Eynde, B. Tumor antigens recognized by T cells. *Immunol. Today* **18**, 267–268 (1997).
19. Rosenberg, S.A. Cancer vaccines based on the identification of genes encoding cancer regression antigens. *Immunol. Today* **18**, 175–178 (1997).
20. Murray, J.S. How the MHC selects Th1/Th2 immunity. *Immunol. Today* **19**, 157–163 (1998).
21. Romagnani, S. The Th1/Th2 paradigm. *Immunol. Today* **18**, 263–266 (1997).
22. Ohashi, P.S. T cell selection and autoimmunity: flexibility and tuning. *Curr. Opin. Immunol.* **8**, 808–814 (1996).
23. Kumar, V., Stellrecht, K. & Sercarz, E. Inactivation of T cell receptor peptide-specific CD4 regulatory T cells induces chronic experimental autoimmune encephalomyelitis (EAE). *J. Exp. Med.* **184**, 1609–1617 (1996).
24. De Silva, H.D., Van Driel, I.R., La Gruta, N., Toh, B.H. & Gleeson, P.A. CD4+ T cells, but not CD8+ T cells, are required for the development of experimental autoimmune gastritis. *Immunology* **93**, 405–408 (1998).
25. Marconcini, L. *et al*. C-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis *in vivo and in vitro*. *Proc. Natl. Acad. Sci. USA* **96**, 9671–9676 (1999).
26. Mandriota, S.J. *et al*. Transforming growth factor β1 down-regulates vascular endothelial growth factor receptor 2/fik-1 expression in vascular endothelial cells. *J. Biol. Chem.* **271**, 11500-11505 (1996).
27. Yeh, C.H., Peng H.C. & Huang T.F. Cytokines modulate integrin αvβ3-mediated human endothelial cell adhesion and calcium signaling. *Exp. Cell Res.***251**, 57–66 (1999).
28. Soker, S. *et al*. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-endoded domain. *J. Biol. Chem.* **271**, 5761–5767 (1996).
29. Piossek, C. *et al*. Vascular endothelial growth factor (VEGF) receptor II-derived peptides inhibit VEGF. *J. Biol. Chem.* **274**, 5612–5619 (1999).
30. Cheifetz, S. *et al*. Endoglin is a component of the transforming growth factor-b receptor system in human endothelial cells. *J. Biol. Chem.* **267**, 19027–19030 (1992).
31. Jones, N. *et al*. Identification of Tek/Tie 2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. *J. Biol. Chem.* **274**, 30896–30905 (1999).
32. Witzenbichler, B. *et al*. Chemotactic properties of angiopoietin-1 and –2, ligands for the endothelial-specific receptor tyrosine kinase Tie-2. *J. Biol. Chem.* **273**, 18514–18521 (1998).
33. Eliceiri, B.P & Cheresh, D.A. The role αv integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J. Clin. Invest.* **103**, 1227–1230 (1999).
34. Smith, J.W. *et al*. Integrin avb3-ligand interaction. Identification of a heterodimeric RGD binding Site on the vitronectin receptor. *J. Biol. Chem.* **265**, 2168–2172 (1990).
35. Lin, E.C., Carron, C.P., Meyer, D.M. & Smith, J.W. A series of function blocking antibodies against the αvβ3 integrin bind allosteric to the ligand binding site and induce ligand dissociation. *Cell Adhes. Commmun.* **6**, 451–464 (1998).
36. Beekhuizen, H. *et al*. Infection of human vascular endothelial cells with staphylococcus aureus induces hyperadhesiveness for human monocytes and granulocytes. *J. Immunol.* **158**, 774–782 (1997).
37. Wei, Y.-Q. *et al*. Induction of apoptosis by quercetin: involvement of heat shock protein. *Cancer Res.* **54**, 4952–4957 (1994).
38. Pulendran B., *et al*. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc. Natl. Acad. Sci. USA* **96**, 1036–1041 (1999).
39. Schellekens, G. A. *et al*. Citrulline is an essential constitute of antigenic determinants recognised by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest.* **101**, 273–281 (1998).
40. Horton, H. M. *et al*. A gene therapy for cancer using intramuscular injection of plasmid DNA encoding interferon α. *Proc. Natl. Acad. Sci. USA* **96**, 1553–1558 (1999).
41. Pan, Z-K. *et al*. A recombinant *Listeria monocytogenes* vaccine expressing a model tumor antigen protects mice against lethal tumor antigen protects mice against lethal tumor cell challenge and causes regression of established tumours. *Nature Med.* **1**, 471–477 (1995).
42. Ortega, S. *et al*. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc. Natl. Acad. Sci. USA* **95**, 5672–5677 (1998).
43. Wei, Y.-Q. *et al*. Induction of autologous tumor killing by heat treatment of fresh human tumor cells: involvement of γδ T cells and heat shock protein 70. *Cancer Res.* **56**, 1104–1110 (1996).
44. Volpert, O.V. *et al*. A human fibrosarcoma inhibits systemic angiogenesis and the growth of experimental metastases via thrombospondin-1. *Proc. Natl. Acad. Sci. USA* **95**, 6343–6348 (1998).
45. Pummerer, C.L, *et al*. Identification of cardiac myosin peptides capable of inducing autoimmune myocarditis in BALB/c mice. *J. Clin. Invest.* **97**, 2057–2062 (1996).
46. Schenk, D. *et al*. Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**, 173–177 (1999).