# Vaccination with Viable Human Umbilical Vein Endothelial Cells Prevents Metastatic Tumors by Attack on Tumor Vasculature with Both Cellular and Humoral Immunity

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**Abstract**

**Purpose:** Because tumor endothelium is rarely targeted by immunity but is critically important for tumor growth, the immunity against tumor endothelium is to be developed as a novel antitumor strategy.

**Experimental Design:** First, viable human umbilical vein endothelial cells (HUVEC) were immunized to C57BL/6 and BALB/c mice to evoke specific CTLs as well as antibodies against tumor endothelium. Lewis lung carcinoma or myeloma cells were subsequently inoculated to evaluate the effect on tumor growth by vaccination. Second, the effect on tumor metastasis by vaccination was studied using tumor-resected mice receiving HUVEC immunization 3 days after excision. Third, the immune sera and T lymphocytes from HUVEC-immunized mice were transferred to tumor-bearing mice and added to cultured HUVECs to investigate their antiproliferative effect.

**Results:** Viable HUVEC immunization showed potent antitumor effects in Lewis lung carcinoma and myeloma tumor models. Both immune sera and CTL inhibited tumor growth and specifically suppressed proliferation of HUVECs. Particularly, tumors entirely disappeared on day 90 after tumor inoculation in four of six tumor-bearing mice receiving CTL therapy. In a metastatic tumor model, we found that the HUVEC vaccination prolonged life span from 30.9 to 41.5 days after tumor resection compared with PBS-treated mice without apparent side effects.

**Conclusions:** Vaccination with viable HUVECs evoked both humoral and cellular immunity against tumor microvasculature, and therefore significantly inhibited tumor growth and prolonged life span of tumor-resected mice. This may provide with a novel treatment for metastatic tumors. Moreover, we have established a convenient method to evoke specific CTL against tumor angiogenesis.

Because tumor immunotherapy has less side effects and better outcomes (1, 2), it is becoming an important addition to conventional cancer treatments, such as surgical excision, chemotherapy, and radiotherapy. Primitively, it aims to break the immune tolerance and strengthen the immune attack on tumors (3–6). Based on the role of dendritic cells in priming tumor-associated antigens to naïve T cells, an intensive CTL-mediated immunity was successfully elicited after *in vitro* modified dendritic cells were transferred to patients (7, 8). However, emergence of antigen-missing mutants, down-regulation of MHC type I, and lack of expression of costimulatory molecules often occurred in genetically unstable tumor cells (9), which may result in an inefficient antitumor immunity. Because tumor angiogenesis has been shown to be pivotal in tumor development (10–13), immunotherapy against tumor endothelium is developed to attack tumor angiogenesis. This strategy has several obvious advantages. First, tumor endothelial cells can be directly accessed by therapeutics compared with tumor cells that would be affected through diffusion of drugs (14). Second, destroying tumor vascular endothelial cells would indirectly hamper the growth of 100-fold more surrounding tumor cells (15). Third, because surface antigens are relatively similar among different tumor endothelial cells, one type of therapy can be used to treat other tumor types (16, 17).

Although monoclonal antibodies have been developed as angiogenesis inhibitors to prevent tumor growth and metastasis, it is rarely reported to use the strategy of CTL to attack

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tumor endothelium in spite of the success of using CTL against tumor cells (18, 19). Recently, oral DNA vaccine against vascular endothelial growth factor (VEGF) receptor-2 was successfully used to evoke a CTL activity to specifically kill the FLK-1$^+$ endothelial cells in tumor (20). Before that work, two interesting reports targeting tumor vasculature with vaccination of human umbilical vein endothelial cells (HUVEC) had shown a noticeable difference in their immunity, one using 3% paraformaldehyde-fixed HUVECs at 4°C for 24 hours showed mainly the humoral effect (21) and another using 0.025% glutaraldehyde-fixed HUVECs at room temperature for 20 minutes showed both the humoral and CTL effects (22). To explain the discrepancy, we postulated that the difference was related to the cell living status altered by the degree of chemical modification. The prior one was most likely to be dead, whereas the latter one should be alive. To investigate the controversy, we have immunized mice with viable HUVECs with no chemical treatment to induce the antiangiogenic immunity.

## Materials and Methods

### Cell cultures

Lewis lung carcinoma cells (LLC) and a myeloma cell line (FO) were bought from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% newborn calf serum (HyClone, Logan, UT). Human lung tumor cell line SPC-A-1 was purchased from Shanghai Institute of Cell Biology (Shanghai, China) and maintained under the same conditions with LLC and FO.

Primary HUVECs were cultured in Iscove's modified Dulbecco's medium (Life Technologies) containing 10% fetal bovine serum (HyClone) and supplemented with 90 μg/mL heparin sodium (Sigma, St. Louis, MO), 2 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 100 μg/mL penicillin (North China Pharmaceutical, Shijiazhuang, China), and 100 μg/mL streptomycin (Lu-Kang, Jining, China).

### Animals

C57BL/6J, BALB/c, and Nu/Nu BALB/c mice used in the following experiments were raised in our laboratory under specific pathogen-free conditions. All procedures in animal experiments were approved by the Animal Study Committee of Institute of Molecular Medicine, Nanjing University.

### Isolation of primary HUVECs and preparation for vaccination

Primary HUVECs were isolated through collagenase type II (Life Technologies) digestion of fresh umbilical veins using a modified method described previously (23), and cultured on 1% gelatin (Chuangrui, Nanjing, China)–coated dishes in Iscove's modified Dulbecco's medium supplemented with the above-described components.

The confluent primary HUVECs were digested with 0.25% trypsin. After thrice washing with PBS, HUVECs were suspended in PBS for vaccination. The purity of HUVECs was identified >95% by fluorescence-activated cell sorting using anti–factor VIII antibody.

### The effect on tumor growth of LLC and myeloma by vaccination

Six-week-old C57BL/6J and BALB/c mice were i.p. vaccinated with $10^6$ HUVECs or PBS once a week for 4 continuous weeks. One week after the last immunization, C57BL/6J and BALB/c mice were inoculated with $10^6$ LLC and $10^6$ FO cells, respectively. Tumor dimensions were measured with calipers and tumor volumes were calculated using the following formula, tumor volume ($mm^3$) = length × width$^2$ × 0.52.

### VEGF assay

Mouse serum was collected on day 25 after tumor inoculation from both groups for VEGF quantification using a sandwich enzyme immunoassay kit (R&D Systems). The VEGF levels were compared between these two groups.

### The effect on tumor metastasis by vaccination

Six-week-old C57BL/6J mice were s.c. inoculated with 5 × $10^5$ LLC cells. When tumor volumes reached 800 $mm^3$, mice were deep anaesthetized with 10% chloral hydrate. Tumors were carefully excised and wound edges were joined with surgical staples. After 3 days of

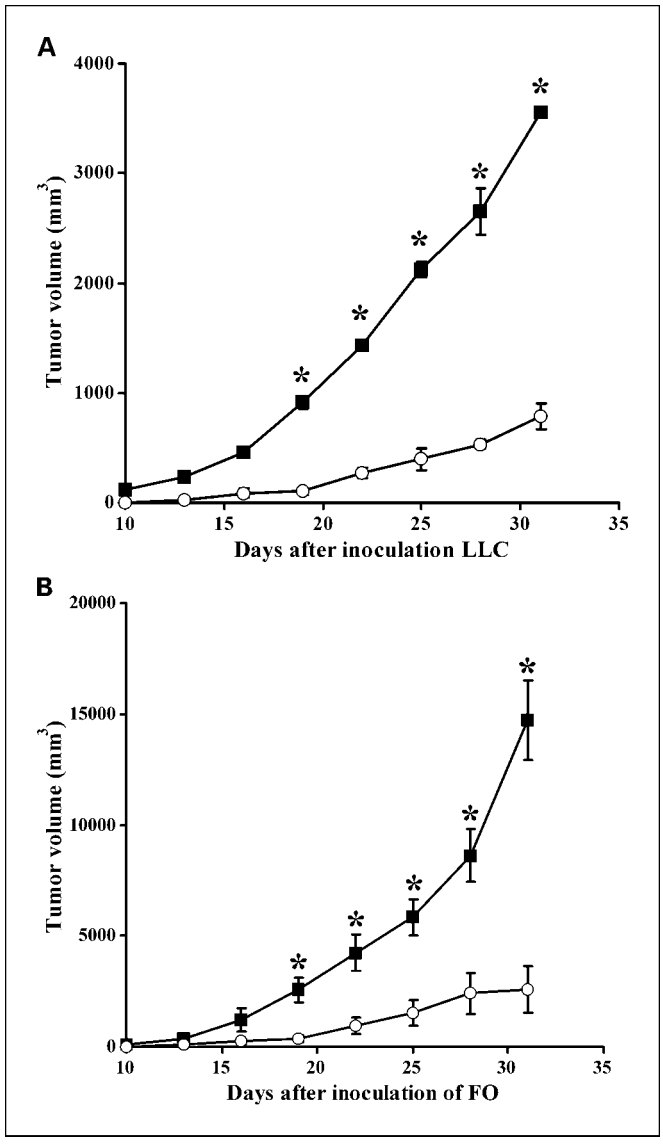


**Fig. 1.** Vaccination with viable HUVECs significantly retarded tumor growth. *A,* C57BL/6J mice were immunized with $10^6$ viable HUVECs (○) or PBS (■) weekly for 4 successive weeks and then challenged with $10^6$ LLC cells ($n$ = 5). Tumor volumes were monitored for ~1 month. *B,* BALB/c mice were immunized with viable HUVECs (○) or PBS (■) weekly for 4 successive weeks and then challenged with $10^6$ myeloma cells ($n$ = 5). Tumor volumes were monitored for ~1 month. *, $P < 0.05$, significant difference in tumor volumes between the two groups.

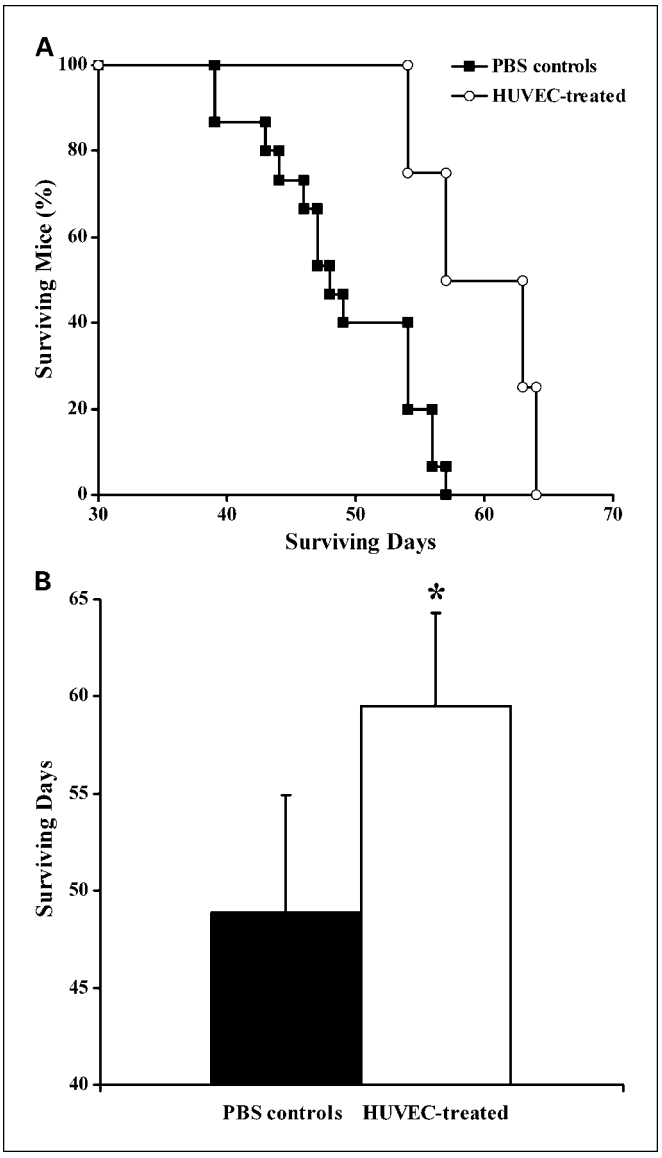


**Fig. 2.** HUVEC-immunized mice survived significantly longer than mice in control groups after tumor resection. *A*, thrice vaccination with HUVECs prolonged life span of tumor-resected mice compared with PBS-treated controls. *B,* average surviving time of the HUVEC-immunized group and PBS-treated group (HUVEC-immunized group: $n = 4$; PBS-treated group: $n = 15$). *, $P < 0.01$, significant difference in surviving days of the two groups through the log-rank test.

recovery, mice were randomly divided into either the PBS control or HUVEC-immunized group. The latter received viable HUVEC immunization ($5 \times 10^5$ cells/mouse) once a week for 3 successive weeks and the controls received PBS instead. Surviving days after tumor cell inoculation were monitored.

### Studies of the immune sera

Six-week-old BALB/c mice were immunized with viable HUVECs ($10^6$ per mouse) every 2 weeks for four times. One week after the last immunization, blood was collected for preparation of immune sera. The antibody titer in immune sera was determined using 96-well-plate coated HUVECs. Most antibodies reacted with HUVECs in the immune sera were identified to be IgG type by ELISA using horseradish peroxidase–linked antibody against mouse IgG, IgM, and IgA (Calbiochem, San Diego, CA). The effects of the immune sera on proliferation of HUVECs *in vitro* and tumor growth *in vivo* were studied.

***The therapeutic effect of the immune sera on tumor growth in nude mice.*** Six-week-old nude mice were s.c. implanted with $10^6$ SPC-A-1 tumor cells. When tumors became easily palpable, mice were randomly divided into two groups (four mice per group). Fifty microliters of immune sera were i.p. injected into nude mice thrice per week for 2 successive weeks, whereas the controls received injection of PBS instead. Tumor dimensions were measured with calipers for calculation of tumor volume.

***The effect of the immune sera on proliferation of HUVECs* in vitro.** Confluent HUVECs were seeded in a 96-well plate ($10^4$ cells, 100 μL/well) and cultured overnight. Different volumes (10, 20, and 30 μL) of the immune sera and control sera were added in triplicate the next day with total volume of 100 μL/well and the cells were allowed to proliferate for another 2 days following the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. SPC-A-1 cells were used as the control to evaluate the specificity of the effect.

### Isolation of T lymphocytes

Nylon wool fiber (Polysciences, Warrington, PA) was used to isolate T lymphocytes from the spleen of HUVEC-immunized mice according to the method described previously (24, 25). Approximately 80% of T cells and ~50% of subset $CD8^+$ T cells were found in the preparation of T lymphocytes, using fluorescence-activated cell sorting with anti-CD3 and anti-CD8 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), respectively.

### The CTL assay

T lymphocytes from viable HUVEC-immunized and control mice were freshly isolated and reactivated with mitomysin-treated HUVECs *in vitro* and then used as an effector to culture with HUVECs preseeded in a 96-well plate in quadruplicate in a ratio of effector/target of 80:1, 40:1, 20:1, 10:1, and 5:1. Supernatants were collected and lactate dehydrogenase released from lysed HUVECs was measured using a nonradioactive cytotoxicity assay (CytoTox 96, Promega, Madison, WI). The FO cells were also assessed as a control under the same conditions as described above. Percentage of lysed cells was calculated according to the following formula: (experimental release − effector spontaneous release − target spontaneous release) / (target maximal release − target spontaneous release) × 100%.

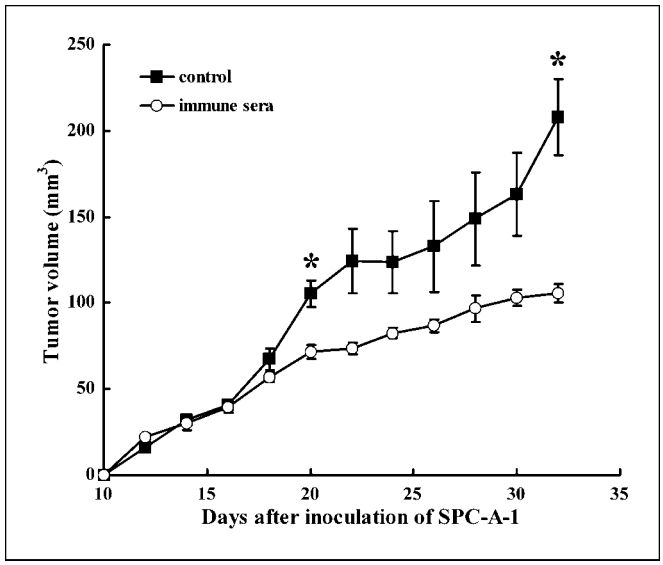


**Fig. 3.** Therapeutic effects of immune sera against SPC-A-1 tumors. SPC-A-1 tumor cells ($10^6$) were s.c. injected into nude mice. Once tumors became palpable, immune sera (○) and PBS (■) were i.p. administered to tumor-bearing mice ($n = 4$). Tumor volumes were monitored for ~1 month. *, $P < 0.05$, significant difference in tumor volumes of the two groups.

### The effect of isolated T lymphocytes on tumor growth

Six-week-old BALB/c mice were s.c. implanted with $10^6$ FO myeloma cells. When tumors became palpable, mice were randomly divided into either the PBS control or T lymphocytes therapeutic group. Mice in T lymphocytes therapeutic group received i.v. injection of $10^7$ T lymphocytes isolated from HUVEC-vaccinated mice thrice a week for 2 successive weeks. In contrast, the PBS controls received PBS instead. Tumor volumes were measured every other day for total 18 days. When the first mouse was going to die, it was killed together with one from the other group. Tumors were dissected and fixed in 10% neutrally buffered formalin for histologic analysis. Life span of the remaining mice was monitored until all control mice were dead.

### Statistical analysis

*P* values were determined through the two-tailed Student's *t* test or the log-rank test. Differences were considered statistically significant when $P < 0.05$.

## Results

***Vaccination with viable HUVECs prevented tumor growth.*** At first, vaccination with viable HUVECs significantly retarded LLC tumor growth (Fig. 1A) as well as FO myeloma growth (Fig. 1B). In the LLC and FO tumor models, tumor volumes of viable HUVEC-immunized mice on day 31 after tumor inoculation were only 22% and 18% of those of PBS-treated group, respectively. Unexpectedly, no significant changes were found in the serum level of VEGF with the HUVEC-immunized tumor mice in comparison with the PBS control mice.

In addition, HUVEC-vaccinated mice had no noticeable changes in fur, body weight, appetite, or life span. No significant pathologic changes were found in heart, liver, lung, spleen, kidney, or brain by histologic examination (data not shown). This suggested that the immunization was apparently harmless to normal mice.

***Vaccination with viable HUVECs prolonged life span of tumor-resected mice.*** In the LLC tumor-resected mice, we found that three times vaccination with viable HUVECs after tumor resection significantly ($P < 0.01$) prolonged the life span of tumor-resected mice (Fig. 2), although it did not arrest metastasis. The average surviving time increased from 48.9 to 59.5 days since tumor inoculation, and from 30.9 to 41.5 days after tumor resection.

***The immune sera inhibited tumor growth in nude mice.*** To investigate the mechanism of antitumor effect of the HUVEC vaccination, the immune sera were isolated from HUVEC-immunized mice and transfused to SPC-A-1 tumor-implanted nude mice. It was found that the HUVEC-immune sera effectively inhibited the growth of SPC-A-1 tumor in nude mice. As shown in Fig. 3, the rate of tumor growth of the treatment group was only a quarter of that of controls treated with vehicles only.

***The immune sera reacted with HUVECs and inhibited their proliferation.*** The antibody titer in the immune sera was 1:51,200, assayed using 96-well-plate coated HUVECs. When up to 30% of sera were included in the culture medium, the

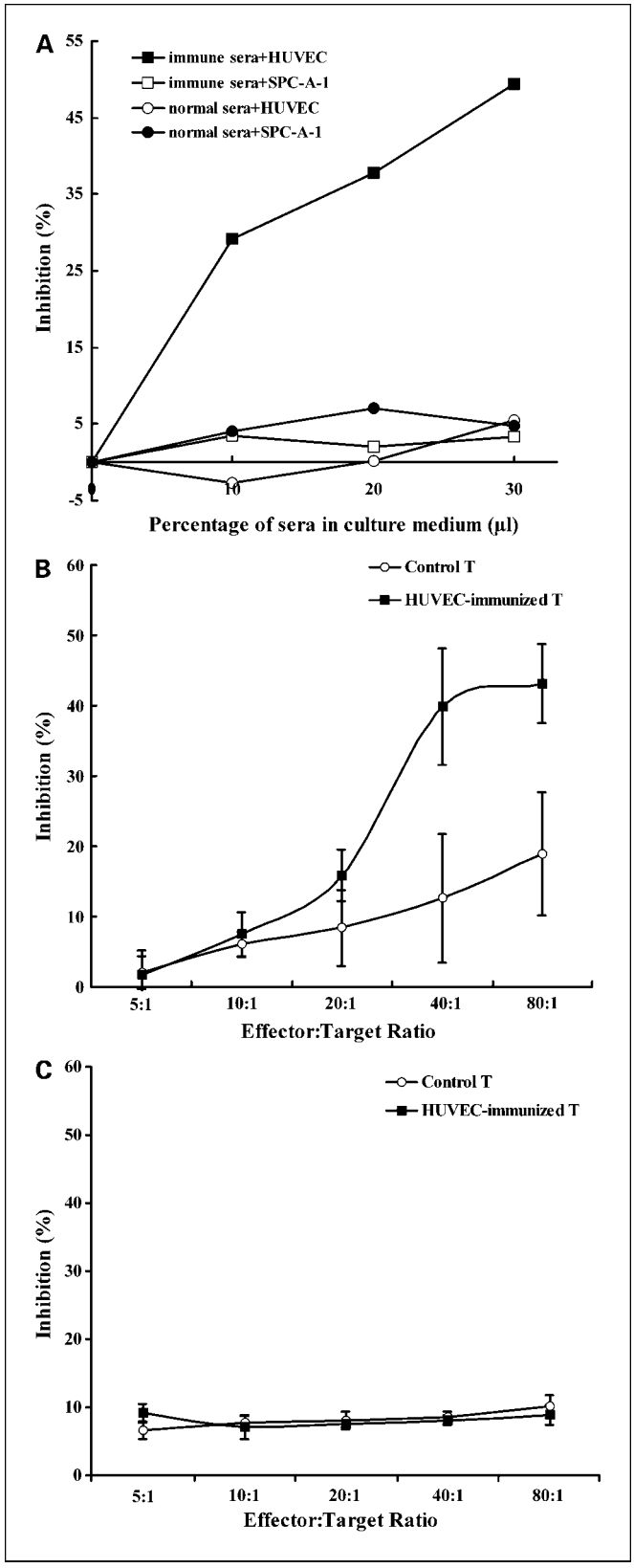


**Fig. 4.** Immune sera and T lymphocytes isolated from viable HUVEC-immunized mice significantly and specifically inhibited the proliferation of HUVECs *in vitro*. *A,* different volumes of immune sera (10, 20, and 30 μL) were added to culture medium of HUVECs with the final volume 100 μL/well and showed significant inhibition on the proliferation of HUVECs *in vitro* (■), but not the normal sera (○). Neither immune sera (□) nor normal sera (●) showed obvious inhibition on the proliferation of SPC-A-1. *B,* T lymphocytes from HUVEC-immunized mice (■) dose dependently killed HUVECs *in vitro* compared with those from PBS-treated mice (○). *C,* T lymphocytes from HUVEC-immunized mice (■) or PBS-treated mice (○) showed no inhibition on the proliferation of FO cells. Data were averaged by quadruplicate. Points, means; bars, SD.

immune sera dose dependently inhibited proliferation of HUVECs *in vitro*, whereas it had little effect on the proliferation of SPC-A-1 tumor cells. As the control, the normal sera caused <5% inhibition on the proliferation of both HUVECs and SPC-A-1 cells (Fig. 4A). Moreover, microvessels in both LLC and FO tumors were strongly stained by the immune sera (data not shown).

***T lymphocytes from HUVEC-immunized mice were cytotoxic to cultured endothelial cells.*** T lymphocytes isolated from HUVEC-immunized mice were found to dose dependently kill HUVECs *in vitro*, whereas a much reduced effect was seen with T lymphocytes from the control mice. When the ratio of effector to target was 40:1, the specific killing of T lymphocytes from HUVEC-immunized mice was 40%, whereas it was only 12% for T lymphocytes from the PBS controls (Fig. 4B). Moreover, neither T lymphocytes were cytotoxic to FO cells (Fig. 4C).

***CTL from HUVEC-immunized mice potently inhibited tumor growth.*** When the CTL isolated from HUVEC-immunized mice was administrated to FO-bearing mice, the myeloma growth was greatly suppressed (Fig. 5A). The tumors in four of six mice receiving CTL remained unchanged in sizes during the period of the treatment (3 weeks) and disappeared when checked on day 90 after tumor inoculation. The tumors of the other two treated mice grew much slower in comparison with those in the PBS-treated mice. The treatment also significantly increased the surviving time of tumor-bearing mice (Fig. 5B). The entire PBS-treated mice died within 45 days after tumor inoculation, whereas only one died in the CTL-treated group. The four CTL-treated mice lived >90 days with no visible tumors. Tumor sections from CTL-treated mice showed severe hemorrhage, necrosis, and inflammatory infiltration (Fig. 6A). In contrast, these phenomena were not present in tumor sections from PBS-treated mice (Fig. 6B).

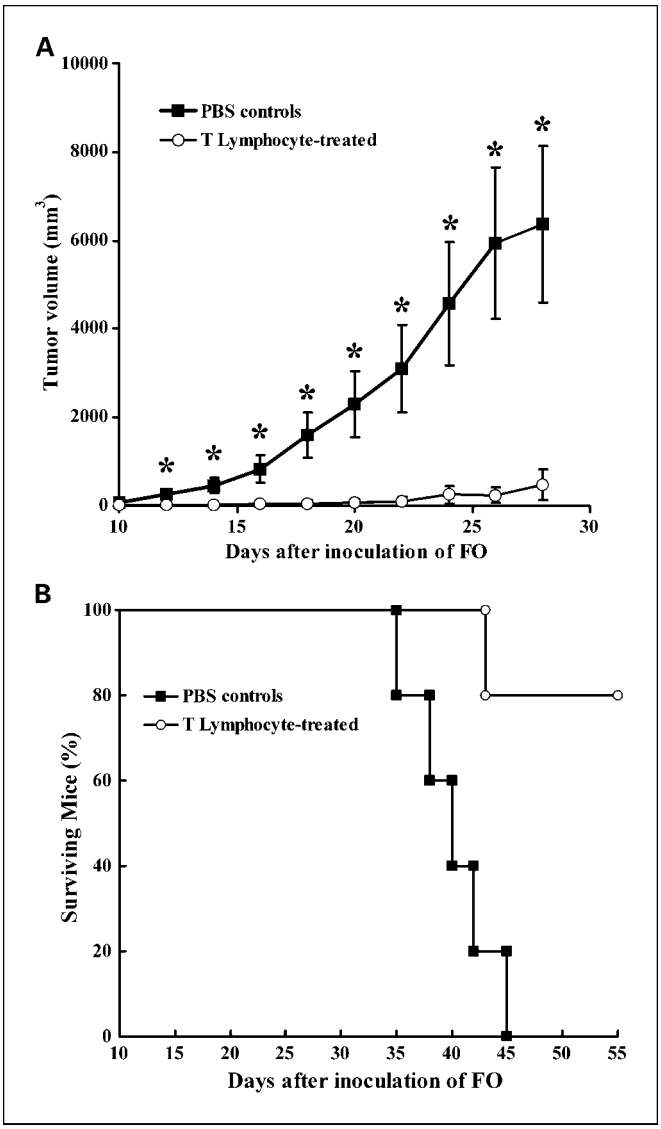


**Fig. 5.** T lymphocytes isolated from viable HUVEC-immunized mice significantly inhibited myeloma growth and prolonged life span of tumor-bearing mice. *A*, $10^6$ FO cells were s.c. injected into BALB/c mice. Once tumors became palpable, $10^7$ T lymphocytes (○) and PBS (■) were i.v. administered to tumor-bearing mice ($n = 6$). Tumor volumes were monitored for ~1 month. *, $P < 0.05$, significant difference in tumor volumes of the two groups. *B*, control mice (■) died within 45 days after inoculation tumor cells but only one mouse in the T lymphocyte–treated group (○) died within this period.

## Discussion

In this study, viable HUVECs were first used as a vaccine to induce antitumor immunity. This type of vaccine evoked both preventive and therapeutic antitumor effects in several tumor models via induction of reactive antibodies and specific CTL against tumor microvasculature. Although both the immune sera and CTL isolated from viable HUVEC-immunized mice actively inhibited tumor growth, CTL appeared to be more potent in our experimental settings. More importantly, this treatment significantly prolonged the life span of tumor-bearing mice after tumor resection (30.9 versus 41.5 days), which was not reported previously. The experiment to prevent metastasis was designed to mimic the clinical condition in which vaccination starts after surgical resection of localized tumors.

Previously, one study using 3% paraformaldehyde-fixed HUVECs as vaccine found that the evoked antitumor immunity was mainly due to the induction of reactive antibodies (21), whereas another using 0.025% glutaraldehyde-fixed HUVECs found that both humoral immunity and CTL were responsible (22). The difference in these two methods was the condition of fixation, which may cause substantial differences in antigenicity and death of the cells. To avoid any structural alterations in surface antigens and cell death, we vaccinated mice directly with viable HUVECs. Our data indicated that CTL was effectively involved as well as the humoral immunity. It is unclear why vaccination with 3% paraformaldehyde-fixed HUVECs induces no cytotoxic T-cell immunity. However, 3% paraformaldehyde-fixed HUVECs were reported to be effective enough to abolish tumor growth (21).

In the present study, we have chosen the primary viable HUVECs mainly because they were derived from new blood vessels with some endothelial markers highly homologous to murine tumor endothelium, such as VEGF receptor-2 and $\alpha_v\beta_3$. Specific antibodies and CTL against HUVECs seemed to directly kill murine tumor endothelium. We believed that the anti-angiogenesis was mainly responsible for the antitumor effect of the HUVEC vaccination. This was evidenced by the following two experimental findings. First, both the immune sera and T lymphocytes isolated from HUVEC-immunized mice dose

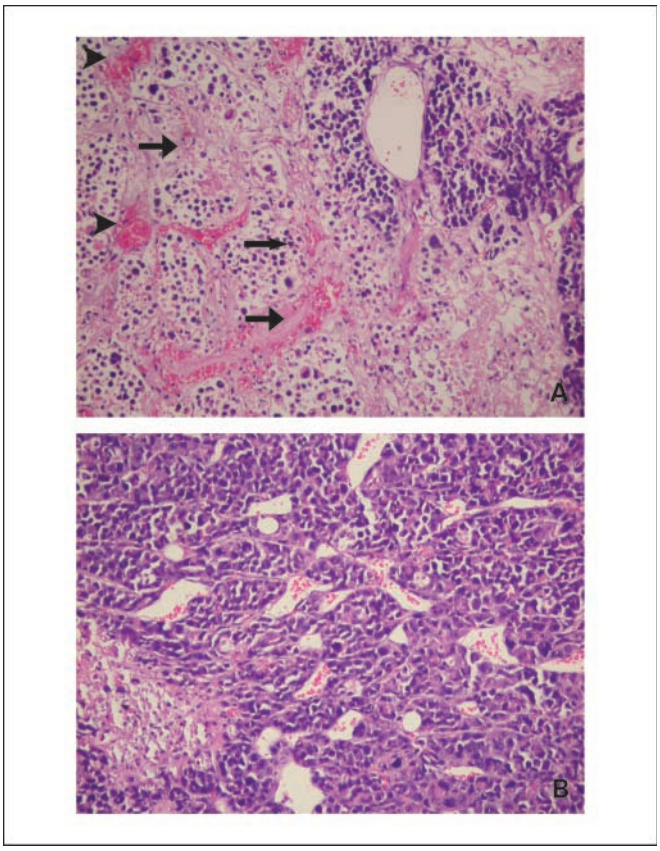


**Fig. 6.** Histologic evidences of tumor sections among different groups. *A,* FO tumor sections from T lymphocyte – treated mice. It showed significant symptoms of tissue injury, in particular, hemorrhage (*arrowheads*), necrosis (*large arrows*), and inflammatory infiltrates (*small arrow*). *B,* FO tumor sections from PBS-treated mice. Tumor cells were highly proliferative and blood vessels were intact, with no symptoms of hemorrhage, necrosis, or inflammatory infiltrates (H&E staining, magnification, ×100).

dependently and specifically harmed cultured HUVECs *in vitro* (Fig. 4). Second, adoptively transferred T lymphocytes from HUVEC-immunized mice caused severe damage to tumor vessels *in vivo* (Fig. 6A). Interestingly, we also found no antitumor effect using ECV304 to vaccinate mice, an endothelial cell line with no expression of VEGF receptor-2 and integrin $\alpha_v\beta_3$ (data not shown).

It is known that antiangiogenesis will increase the chance of spontaneous bleeding, as shown in clinical trial with Bevacizumab, an anti-VEGF antibody. Interestingly, no bleeding events were observed in our study as well as some previous animal studies (26, 27). This may be due to small sample size or specific antiangiogenesis agents. Additionally, the serum level of VEGF was unaffected in our antiangiogenesis therapy using HUVEC immunization. As a matter of fact, several others also found that the serum levels of VEGF were unchanged during the antiangiogenesis therapies (28–30). This could be because antiangiogenesis therapy normalizes tumor vasculature and simply "chokes off the blood supply" (31–33), which may not significantly affect the production of VEGF.

Cancer immunotherapy was originally concentrated on tumor itself. Many strategies were developed to enhance immune attack on tumor cells mostly by CTL (18, 19). After the finding that tumor angiogenesis played a pivotal role in tumor development, many agents, including monoclonal antibodies, were developed and used as angiogenesis inhibitors to prevent tumor growth and metastasis. However, the strategy using CTL against tumor vessels was seldom reported. In the present study, we have established a convenient method to evoke specific CTL against tumor vessels. The CTL produced by vaccination with viable HUVECs specifically killed HUVECs *in vitro* and averted tumor growth *in vivo*.

In conclusion, vaccination with viable HUVECs evoked both humoral and cellular immunity against tumor microvasculature, and therefore significantly inhibited tumor growth and prolonged the life span of tumor-resected mice. This innovative immunotherapeutic approach may have clinical implications.

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