BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 44 (2) 84-181 February 2011
BIOMEDICAL SCIENCES

doi: 10.1590/S0100-879X2010007500145

Braz J Med Biol Res, February 2011, Volume 44(2) 140-148

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The Brazilian Journal of Medical and Biological Research is partially financed by

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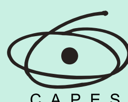
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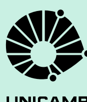

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Brazilian Journal of Medical and Biological Research (2011) 44: 140-148
ISSN 0100-879X

Enhanced inhibition of murine prostatic carcinoma growth by immunization with or administration of viable human umbilical vein endothelial cells and CRM197

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Abstract

Vaccination with xenogeneic and syngeneic endothelial cells is effective for inhibiting tumor growth. Nontoxic diphtheria toxin (CRM197), as an immunogen or as a specific inhibitor of heparin-binding EGF-like growth factor, has shown promising antitumor activity. Therefore, immunization with or administration of viable human umbilical vein endothelial cells (HUVECs) combined with CRM197 could have an enhanced antitumor effect. Six-week-old C57BL/6J male mice were vaccinated with viable HUVECs, 1 x 10^6 viable HUVECs combined with 100 µg CRM197, or 100 µg CRM197 alone by *ip* injections once a week for 4 consecutive weeks. RM-1 cells (5 x 10^5) were inoculated by *sc* injection as a preventive procedure. During the therapeutic procedure, 6-week-old male C57BL/6J mice were challenged with 1 x 10^5 RM-1 cells, then injected *sc* with 1 x 10^6 viable HUVECs, 1 x 10^6 viable HUVECs + 100 µg CRM197, and 100 µg CRM197 alone twice a week for 4 consecutive weeks. Tumor volume and life span were monitored. We also investigated the effects of immunization with HUVECs on the aortic arch wall and on wound healing. Vaccination with or administration of viable HUVECs+CRM197 enhanced the inhibition of RM-1 prostatic carcinoma by 24 and 29%, respectively, and prolonged the life span for 3 and 4 days, respectively, compared with those of only vaccination or administration with viable HUVECs of tumor-bearing C57BL/6J mice. Furthermore, HUVEC immunization caused some damage to the aortic arch wall but did not have remarkable effects on the rate of wound healing; the wounds healed in approximately 13 days. Treatment with CRM197 in combination with viable HUVECs resulted in a marked enhancement of the antitumor effect in the preventive or therapeutic treatment for prostatic carcinoma *in vivo*, suggesting a novel combination for anti-cancer therapy.

Key words: RM-1 prostatic carcinoma; CRM197; Immunization; Human umbilical vein endothelial cells

Introduction

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing ones. In adults, angiogenesis is almost quiescent, except under conditions such as wound healing and the menstrual cycle. However, it is also necessary and required for tumor development and metastasis (1-3). Therefore, anti-angiogenic therapies applied in an attempt to fight cancer and malignancies have been investigated intensively since the 1970's (4-7). The process of angiogenesis is a phenomenon intimately associated with endothelial cell (EC) migration and proliferation. Because tumor immunotherapy is a promising procedure with few side effects (8), it is becoming a new option for cancer patients, in addition to conventional therapies like surgery, radiotherapy, and chemotherapy. Moreover, many studies have successfully used exogenous or allogeneic ECs as vaccines to inhibit the growth of tumors in experimental models (9-12). Recently, a pilot study using human umbili-

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Received December 16, 2009. Accepted December 6, 2010. Available online December 17, 2010. Published February 7, 2011.

cal vein endothelial cells (HUVECs) as a vaccine to treat cancer patients with brain tumors achieved promising results (13), underscoring the clinical importance of such study. However, there are still no reports on immunization with HUVECs to treat prostatic carcinoma. Chen et al. (12) reported that a viable HUVEC immunization can induce a stronger cytotoxic T lymphocyte response than paraformaldehyde-fixed ones. On this basis, we tested whether immunization with viable HUVECs could inhibit the growth of prostatic carcinoma.

CRM197 is the product of a single missense mutation (Gly52 to Glu) within fragment A of the diphtheria toxin (14,15). Recently, CRM197 (58,422 Mr) has been shown to induce very weak toxicity to some cell strains, but it still shares immunological properties with the native diphtheria toxin (16,17). CRM197 commonly acts as an immunological adjuvant or as a carrier protein for vaccination (18,19), or as an inhibitor of heparin-binding epidermal growth factor (HB-EGF). More recently, as an immunogen, CRM197 has also shown promising antitumor activity (20,21). Therefore, it is possible that immunization combining viable HUVECs with CRM197 may enhance the antitumor effects during tumor immunotherapy.

In addition, CRM197 can also bind a specific membrane receptor, the HB-EGF-like growth factor (22,23), which is a member of the superfamily of growth factors that competes for the epidermal growth factor receptor. HB-EGF has been tested especially in cancer and has been shown to play a key role in the acquisition of malignant phenotypes. Moreover, HB-EGF expression is essential for tumor formation in cancer-derived cell lines (24-26).

CRM197 has been used as an inhibitor of HB-EGF to treat cancer in experimental animal studies (20,27,28). Mice bearing xenografted tumors, which were treated with CRM197 showed a remarkable suppression of tumor growth (27), and a clinical trial of CRM197 in patients with advanced cancer also showed a promising antitumor effect (20). Increasing evidence suggests a critical role for HB-EGF against prostatic carcinoma growth and tumor progression (29,30). Thus, blocking the function of HB-EGF expressed on the tumor cell by administering CRM197 and simultaneously blocking the blood supply to prostatic carcinoma using immunization in combination with EC is expected to have a promising therapeutic effect.

The use of endothelial cells as a vaccine to treat cancer has been investigated for almost 10 years. However, there are hardly any reports about their effects on wound healing and on the vascular system. In the present study, we investigated the effect of vaccination or administration of CRM197 by mixing it with viable HUVECs in terms of enhancing the inhibitory ability against RM-1 prostatic carcinoma and prolonging the life span of C57BL/6J tumor-bearing mice. We then compared its effects with those observed in mice vaccinated with or receiving viable HUVEC or CRM197 alone.

Material and Methods

Cell lines and mice

RM-1 cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and cultured in RPMI 1640 supplemented with 10% newborn calf serum and 1% antibiotics (i.e., 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate). HUVECs, endothelial cell medium, and poly-L-lysine were from ScienCell (USA) and were cultured in a poly-L-lysine-coated flask at 37°C in an atmosphere of 95% air and 5% CO_2. For all experiments, 6-week-old C57BL/6J male mice were purchased from the Model Animal Research Center of Nanjing University and housed in our laboratory under specific pathogen-free conditions. All procedures in the animal experiments were approved by the Animal Study Committee of the Institute of China Pharmaceutical University.

Vaccine preparation and effect of vaccination on the growth of murine prostatic carcinoma

Thirty-two C57BL/6J mice were randomly divided into four groups of 8 animals each, which were treated with phosphate-buffered saline (PBS), HUVECs, HUVECs+CRM197, and CRM197, respectively. Subconfluent HUVEC cells were harvested by digesting with 0.25% trypsin and washing three times with PBS. HUVECs (1×10^6) suspended in 200 µL PBS were administered intraperitoneally (*ip*) once a week for 4 consecutive weeks. Also, 1×10^6 HUVECs mixed with 100 µg CRM197 (from Shanghai Institute of Biological Products) in 200 µL PBS, 100 µg CRM197 in 200 µL PBS, and 200 µL PBS were administered in the same way. Sera were sampled at various times for later analyses of antibodies. One week after the last immunization, C57BL/6J mice were inoculated subcutaneously (*sc*) with 5×10^5 RM-1 cells on the back. When tumors became palpable, tumor volumes were measured every day until the mice died.

Tumor dimensions were measured with calipers, and tumor volumes were calculated using the following formula: tumor volume (mm^3) = length x $width^2$ x 0.52. The life span of the animals was also recorded.

Administration of CRM197 and viable HUVECs

Prior to administration, 32 C57BL/6J mice were challenged with 1×10^5 RM-1 cells and randomly divided into the PBS group (control), viable HUVEC group, viable HUVECs+CRM197 group, and CRM197 group (N = 8 in each group). Methods for the preparation of HUVECs, viable HUVECs+CRM197, and CRM197 were the same as those for the preventive procedure. The mice were injected *sc* with PBS, viable HUVECs, viable HUVECs+CRM197, or CRM197 alone around the RM-1 cell injection sites the day after the challenge. Subsequent injections near the tumor were performed twice a week for 4 consecutive weeks. Methods for tumor measurement were the same as those described in the preventive procedure.

Detection of CRM197 antibody by ELISA

An ELISA was performed to detect the anti-CRM197 antibody levels in the immune sera, as described by Yankai et al. (31). Briefly, 96-well flat-bottomed ELISA plates (Costar, USA) were coated with 100 µL/well CRM197 proteins (10 µg/well) and kept overnight at 4°C. Plates were blocked with PBS containing 5% (w/v) bovine serum albumin (BSA, Sigma) and then incubated with 100 µL/well 1:100 dilutions of sera collected from immunized animals in the different vaccinated groups and from the control. A secondary HRP-conjugated goat anti-mouse IgG (Boster Biological Technology, China) was used for substrate reaction. Absorbance was measured at a wavelength of 450 nm. Each measurement was carried out in duplicate.

Effect of immune sera on HUVEC proliferation *in vitro*

Subconfluent HUVECs were seeded in a 96-well plate (10^4 cells, 100 µL/well) and cultured overnight. Thereafter, 10 µL of the immune sera from the three vaccinated groups and the PBS group were added, followed by incubation at 37°C for 24 h in the presence of 10 µL guinea pig serum as the source of complement. At the end of incubation, 20 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added. Cells were cultured for 4 h at 37°C. Finally, the medium was discarded and 100 µL DMSO was added for cell lysis to measure absorbance at 570 nm. Each experiment was performed in triplicate and the mean value was used as the representative value for each experiment.

The inhibition rate (IR) was calculated according to the following formula: IR (%) = [1 - (absorption value of experimental group / absorption value of control group)] x 100%. RM-1 cells were used as the parallel control.

Immunolabeling and confocal laser microscopy

HUVECs (2 x 10^5) were seeded on a poly-L-lysine-coated coverslip and then cultured overnight in 6-well cell culture plates. Cells grown on the coverslip were washed three times with PBS and blocked with 5% BSA/PBS for 1 h at room temperature, followed by incubation for 1 h at 37°C with antisera from the PBS, HUVEC, HUVEC+CRM197, and CRM197 groups, respectively. After washing with 0.1% Tween-20/PBS, 10 µg/mL fluorescein-conjugated goat anti-mouse IgG (Boster Biological Technology, China) diluted in 2% BSA and 0.1% Tween-20/PBS was applied, followed by incubation for 1 h at 37°C. After three additional washes with 0.1% Tween-20/PBS, cells were observed with a confocal laser scanning microscope. One coverslip with HUVEC (but serum-free) served as a negative control.

Wound healing model

Eight mice were vaccinated with viable HUVECs as described above, with eight other mice left untreated and used as control. One week after the last vaccination, mice were sprayed with 75% ethanol to prevent infection and then anesthetized, and about 0.5-cm^2 wounds were made on the hind limbs with eye scissors in a sterile environment. Wounds were then wrapped with a sterile bandage, and rates of wound healing were monitored daily.

Aortic arch

Four groups (N = 3) of mice were vaccinated with PBS, HUVECs, HUVECs+CRM197, and CRM197. The immunization methods were the same as previously described. After four vaccinations, mice were sacrificed and the aortas were collected. The fat surrounding the aortas was removed, the aortas were opened with eye scissor and fixed *in situ* with 2% formaldehyde plus 2% glutaformaldehyde. Samples were subjected to the critical drying point process and gold coated prior to observation by scanning electron microscopy (SEM, Carl Zeiss SMT). Three aortas from each group were evaluated and photographed.

Statistical analysis

The survival data were analyzed using the life tables of survival provided by the SPSS 11.0 software. Further multivariance data were analyzed by one-way ANOVA with the same software and the Student *t*-test was used for all other comparisons. A P value of <0.05 was considered to be statistically significant.

Results

Vaccination with viable HUVECs+CRM197 prevented the development of prostatic carcinoma and prolonged the life span of the tumor-bearing mice

In the preventive procedure, after four vaccinations with viable HUVECs, viable HUVECs+CRM197, and CRM197, 5 x 10^5 RM-1 cells were inoculated *sc* on the animal's back. Tumor measurements were started when the tumor became palpable and continued until the mice died. The results (Figure 1A) show that vaccination with viable HUVECs, HUVECs+CRM197, or CRM197 retarded the development of murine prostatic carcinoma compared to the PBS-treated group. Tumor volumes in mice immunized with viable HUVECs, CRM197, and HUVECs+CRM197 on day 24 after tumor inoculation were only 48, 78, and 24% of those of the PBS-treated group, respectively. HUVEC+CRM197 immunization showed the strongest tumor inhibition among the three vaccinations (Figure 1A). All data were significantly different between groups ($P < 0.05$).

Compared with the PBS group, all three immunization types prolonged the life span of the tumor-bearing mice. Figure 1B shows that all mice in the PBS group died within 30 days. However, those immunized with CRM197, HUVECs, and HUVECs+CRM197 died within 34, 38, and 43 days, respectively. The average survival times for PBS-, CRM197-, HUVEC-, and HUVEC+CRM197-treated groups were 25, 29, 32, and 35 days under preventive treatment.

Induction of therapeutic antitumor activity

Prior to vaccination, C57BL/6J mice were challenged with 1 x 10^5 RM-1 cells, and then injected *sc* with viable HUVECs, CRM197, and HUVECs+CRM197 twice a week for 4 consecutive weeks. Figure 2A shows that administration of viable HUVECs, viable HUVECs+CRM197, or CRM197 could also inhibit the growth of prostatic carcinoma. Tumor volumes of mice treated with viable HUVECs, CRM197, and HUVECs+CRM197 on day 26 after tumor inoculation were 54, 52, and 25% of those of the PBS-treated group, respectively. There were significant differences between the HUVEC+CRM197 group and the two other treatment groups. However, no significant difference was found between the CRM197- and viable HUVEC-treated groups. These two groups differed significantly from the PBS group (P < 0.05; Figure 2A).

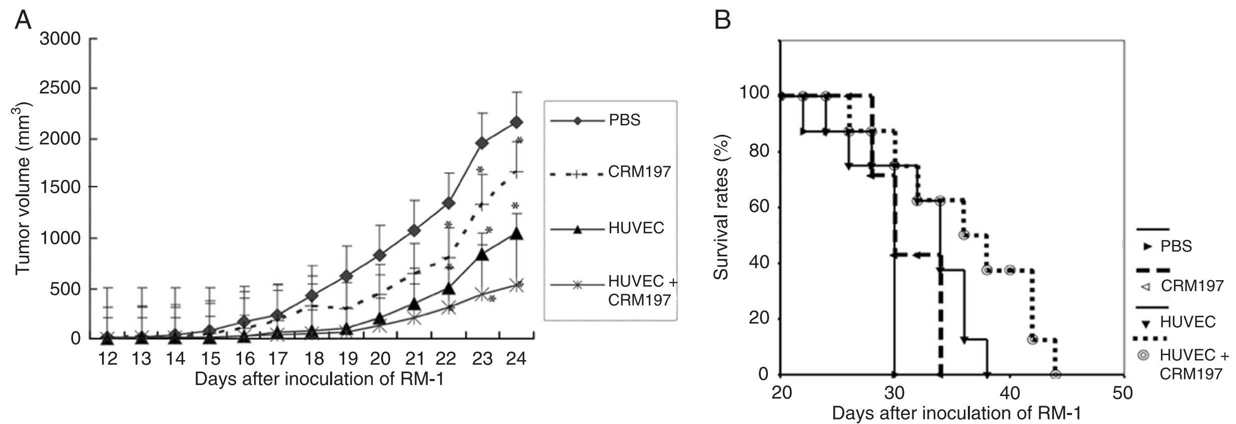

Figure 1. Tumor growth and survival rates in the preventive procedure. *A*, Vaccination with viable HUVECs mixed with CRM197 further retarded tumor growth in the preventive process. C57BL/6J mice were immunized with viable HUVECs, HUVECs combined with CRM197, CRM197, or PBS weekly for four consecutive weeks. Then, they were challenged with 5 x 10^5 RM-1 cells (N = 8). Tumor volumes were monitored until death occurred. The difference in tumor volume was significantly different between the four groups on days 22, 23, and 24 (P < 0.05, Student *t*-test). Data are reported as means ± SD. *B*, Viable HUVECs combined with CRM197-immunized mice survived significantly longer than mice in the other three groups. HUVECs = human umbilical vein endothelial cells; CRM197 = nontoxic diphtheria toxin.

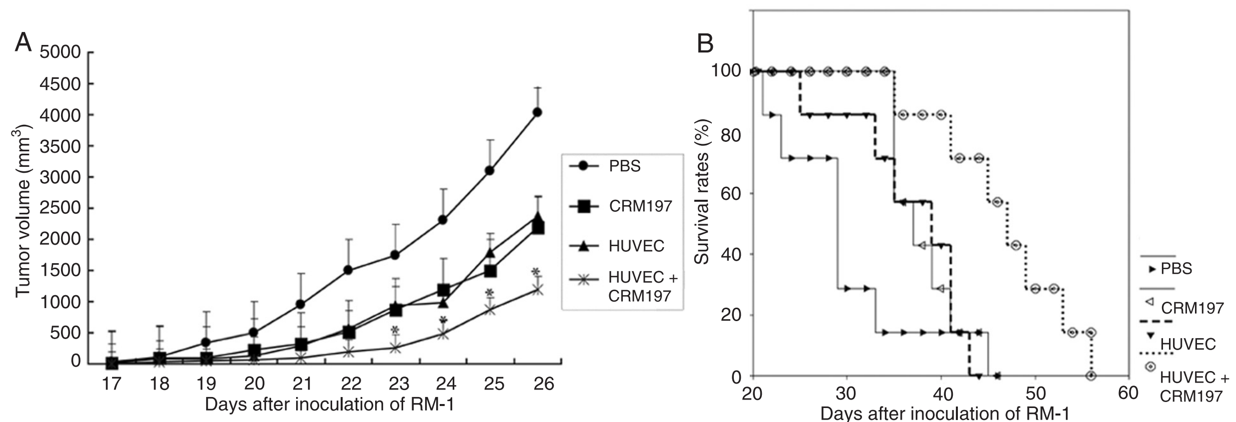

Figure 2. Tumor growth and survival rates in the therapeutic procedure. Prior to administration, C57BL/6J mice were challenged with 1 x 10^5 RM-1 cells and then randomly divided into the PBS (control), viable HUVEC, HUVEC+CRM197, and CRM197 groups (N = 8 in each group). The mice were then injected *sc* with PBS, viable HUVECs, viable HUVECs combined with CRM197, and CRM197, respectively, twice a week for 4 successive weeks. *A*, Administration of viable HUVECs combined with CRM197 further retarded tumor growth in the therapeutic process. There were significant differences between the HUVEC+CRM197 group and the two other treatment groups on days 23, 24, 25, and 26 (P < 0.05, Student *t*-test). Data are reported as means ± SD. *B*, Administration of viable HUVECs combined with CRM197 extended the life span of tumor-bearing mice. HUVECs = human umbilical vein endothelial cells; CRM197 = nontoxic diphtheria toxin.

It can also be seen in Figure 2B that administration of viable HUVECs and CRM197 extended the life span of tumor-bearing mice. The average number of survival days for PBS, CRM197, viable-HUVEC, and HUVEC+CRM197 groups were 27.8, 33, 32.8, and 37, respectively.

Measurement of CRM197 antibody level by ELISA

To investigate the CRM197 antibody level in the preventive procedure, we compared the CRM197-specific immunoglobulin G (IgG) levels in sera collected from immunized mice using ELISA (Figure 3). Serum samples were collected during the first, second, third, and fourth weeks after the initial immunization. The anti-CRM197 IgG levels of the CRM197 group and the viable HUVEC+CRM197 group were greatly increased after the initial immunization compared with the PBS group and viable HUVEC group. However, after additional immunizations, the anti-CRM197 IgG level was the same, although there were three additional immunizations.

Immune sera from the HUVEC+CRM197 group can improve inhibition of HUVEC proliferation compared to the sera from the other two vaccinated groups

To evaluate the inhibitory effect of sera from immunized mice on the proliferation of HUVECs *in vitro*, cultured HUVECs were incubated with pre-immunized serum. Final vaccinated antisera (N = 8) were from mice immunized with HUVECs, HUVECs+CRM197, and CRM197. MTT results showed that sera from mice immunized with HUVECs, HUVECs+CRM197, and CRM197 all inhibited HUVEC proliferation (Figure 4). The rates of inhibition of sera from the HUVEC, HUVEC+CRM197, and CRM197 groups relative to the sera from PBS group were 51.1, 68.2, 32.5% ($P < 0.01$), respectively. However, the anti-HUVEC sera also showed less than 10% inhibition against the proliferation of RM-1 compared to sera from the PBS group.

Antisera reacted with membrane proteins of HUVECs

To determine whether the antisera from the three different vaccinated groups reacted with HUVECs, immunocytochemistry experiments were performed using confocal laser scanning microscopy. The results showed that all antisera, except those from the PBS group, reacted with the HUVEC membrane (Figure 5).

Effect of HUVEC immunization on the aorta's endothelium and wound healing

To study the potential effect of HUVEC immunization on the vascular system, we observed the inner wall of the aorta, isolated from the control and the vaccinated groups, by SEM. The aorta's endothelia from the PBS group and the CRM197-immunized group showed regular aortic plica, whereas HUVEC immunization caused its disappearance in the other two groups (Figure 6). Surprisingly, when the influence of HUVEC on wound healing was monitored,

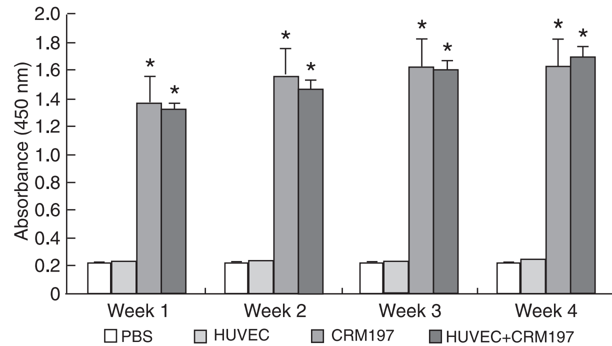

Figure 3. Detection of anti-CRM197 antibodies in immunized mice. Anti-CRM197 antibodies were detected by ELISA as early as 1 week after immunization in the sera of animals immunized with CRM197 and other immunized groups. Antibody levels peaked 2 weeks after immunization. The anti-CRM197 level from the groups vaccinated with CRM197 (CRM197 alone and CRM197+viable HUVECs) showed a significant increase compared with those from the PBS group and the HUVEC group (*$P < 0.05$, Student *t*-test), but there were no differences between these two groups, which were vaccinated with CRM197 or HUVECs+CRM197 (N = 8 animals per group). Data are reported as means ± SD. HUVECs = human umbilical vein endothelial cells; CRM197 = nontoxic diphtheria toxin.

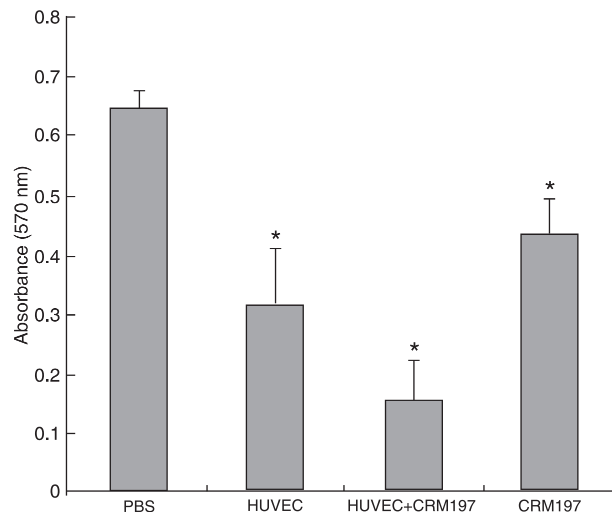

Figure 4. Effect of immune sera on HUVEC proliferation. Immune sera isolated from viable HUVEC+CRM197-immunized mice had the strongest ability to inhibit the proliferation of HUVECs *in vitro*. Immune sera (10 µL) from immunized groups and guinea pig sera (10 µL) were added to the HUVEC culture medium in a final volume of 120 µL/well. Except for the PBS groups, all the immunized sera showed significant inhibition against the proliferation of HUVECs *in vitro*. Moreover, sera from the HUVEC+CRM197 group had the strongest inhibitory ability against the proliferation of HUVECs *in vitro*. The bars denote absorbance values at 570 nm. HUVECs = human umbilical vein endothelial cells; CRM197 = nontoxic diphtheria toxin. *$P < 0.01$ compared to control (Student *t*-test). Data are reported as means ± SD.

HUVEC-vaccinated mice recovered at the same rates as those from the PBS group. The whole healing process lasted approximately 13 days (data not shown).

Discussion

Since angiogenesis is a complex process involving multiple growth factors (32), studies aiming to show that cancer treatment only depends on blocking one specific growth factor have not been very successful (33). Other reports have shown that some proteins, including αvβ3 integrin and receptors of certain angiogenic growth factors, are expressed on proliferating ECs but are not easily detected in the normal quiescent vascular endothelium (2,3,5,34). Proteins expressed on the endothelium of new vessels in mice, humans, and other species are homologous

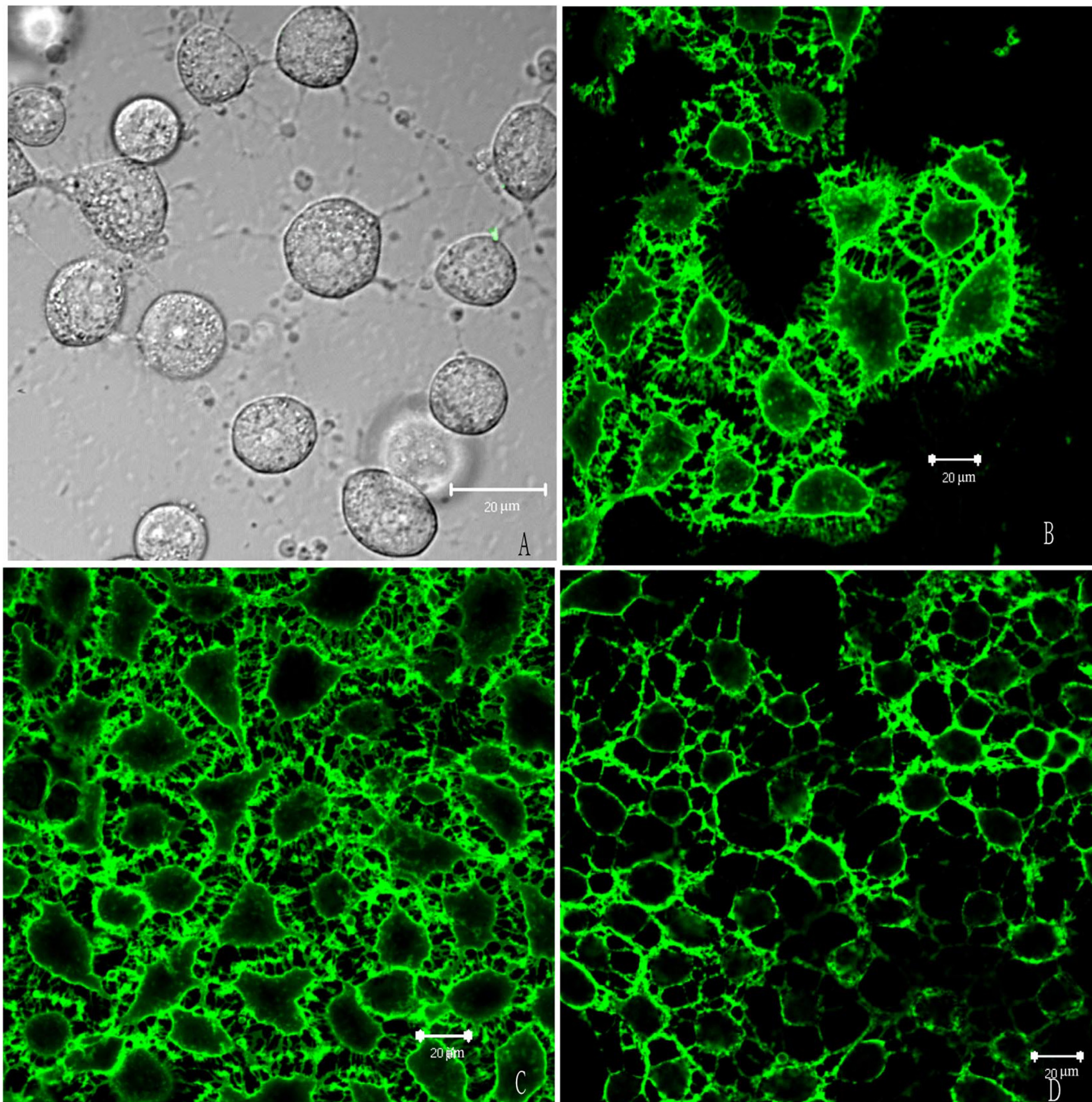

Figure 5. Confocal laser microscopy image of immune sera binding with the membrane of proliferating HUVECs. Immune sera from HUVECs (B), HUVECs+CRM197 (C), and CRM197 (D), but not sera from the PBS group (A), were able to bind to the membrane of HUVECs. HUVECs = human umbilical vein endothelial cells; CRM197 = nontoxic diphtheria toxin.

(11). Chen et al. (12) reported that anti-HUVEC serum can bind tightly to the blood vessels within the tumors. This effect, above all, provides a rational explanation for using HUVECs as a vaccine to inhibit tumor growth. Compared with specific tumor cell vaccines, endothelial cells have a broad spectrum of anti-cancer response.

In the present study, CRM197 in combination with viable HUVECs was first used to inhibit the development of murine prostatic carcinoma. We found that this combination could enhance the inhibitory ability against tumor development *in vivo* compared to the use of each element alone, or when antisera were used against the proliferation of HUVECs. We also found that antisera against HUVECs showed weak inhibition against RM-1 tumor cells (<10%). Presumably, antisera, including various antibodies, and maybe some of antigens, are commonly produced by HUVECs and RM-1 tumor cells. On the other hand, the antisera against CRM197 had little effect on the proliferation of RM-1 tumor cells.

Other researchers have reported that neutrophils and TNF-α might play a role in the antitumor function of CRM197 (20). In the present study, we found that antisera, except that from the PBS group, mainly bound to the membrane of HUVEC. Surprisingly, even antisera from the CRM197-immunized group also reacted with the HUVEC membrane. On the other hand, MTT results showed that antisera from CRM197 also inhibited the proliferation of

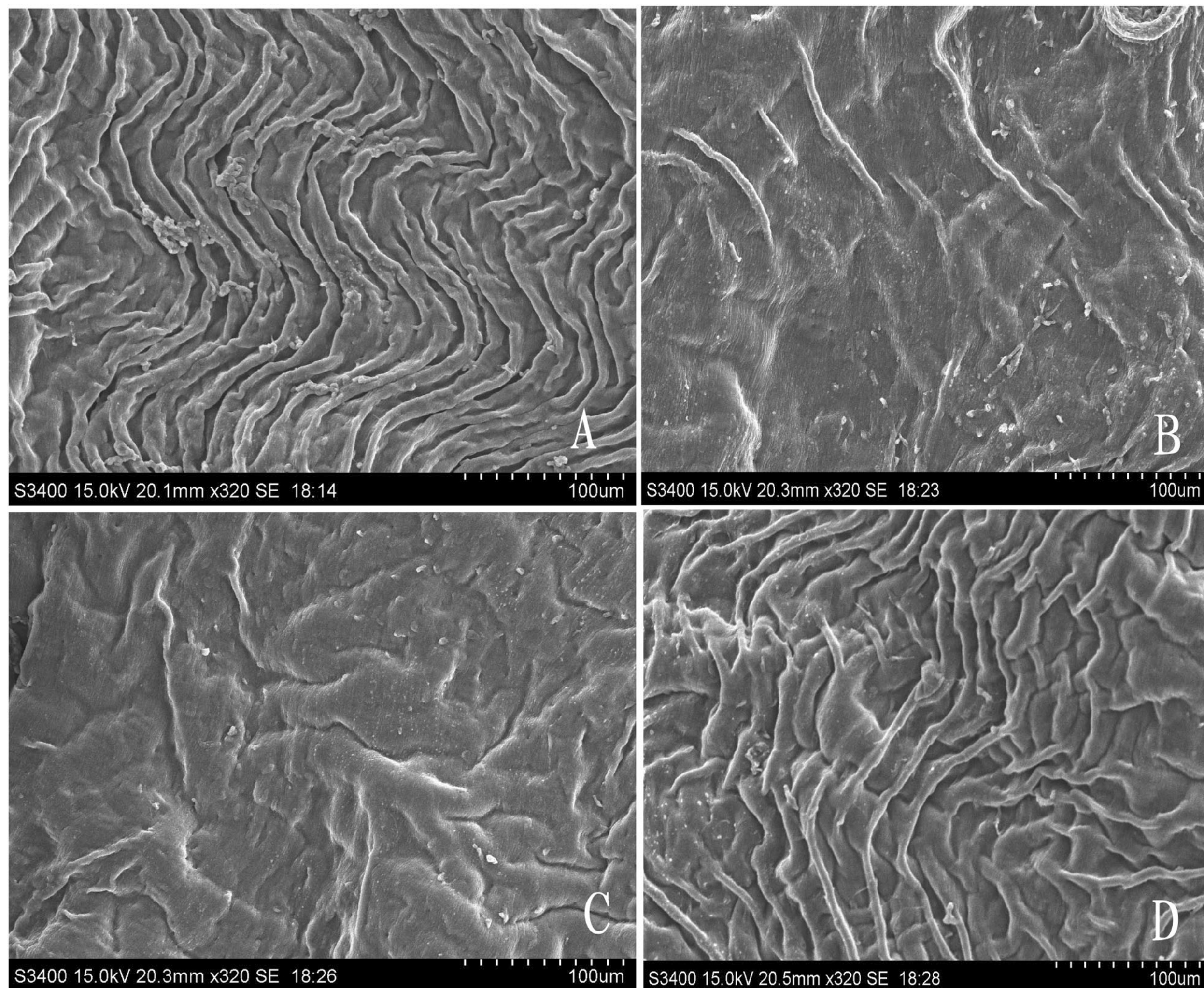

Figure 6. Scanning electron microscopy (SEM) images of the aortic arch wall. Groups of C57BL/6J mice (3 mice per group) were immunized *ip* with PBS, HUVECs, HUVECs+CRM197, and CRM197 over 4 consecutive weeks, 1 week after the last immunization. Mice were then sacrificed and their aortas were harvested and opened. Direct observation of endothelial damage in arterial walls by SEM (original magnification: 450X) showed that vaccination with viable HUVECs (B) and HUVECs+CRM197 (C) caused profound alterations in the aortic tissue structure. However, the aorta from the PBS (A) and CRM197 (D) groups maintained their regular aortic plica. HUVECs = human umbilical vein endothelial cells; CRM197 = nontoxic diphtheria toxin.

HUVECs. Previous study from our laboratory indicated that CRM197 immunization could delay the development of some cancers, like H22 and B16, to a certain extent (data not reported). Therefore, we deduced that sera from CRM197 might cross-react with the proteins expressed on the membrane of HUVECs. Thus, the antitumor activity of CRM197, as an immunogen, originates not only from the function of neutrophils but also from its inhibition against endothelial cell proliferation.

In the preventive procedure illustrated in Figure 1A, the antitumor activity of the HUVEC-immunized group was stronger than that of the CRM197-immunized group. However, in the therapeutic procedure, both the CRM197 and HUVEC groups had similar antitumor effects (Figure 2A). By considering these data in combination with the ELISA results, we can deduce that the antitumor mechanism of CRM197 during the preventive process is different from that occurring during the therapeutic process. In the preventive process, the antitumor effect of CRM197 was mainly attributed to its strong inflammatory-immunological property.

In the therapeutic process, however, because prostatic carcinoma and ECs (30,35) can both express HB-EGF, the main antitumor effects are attributed to the CRM197 competition for the receptor with HB-EGF-like growth factor before the occurrence of humoral immunity. Once humoral immunity occurs, the antitumor effect comes from both functions.

Although EC cells have some promising antitumor properties as vaccines without any visible side effects, the so-called “no visible side effects” feature only denotes that there are no remarkable changes in the animal’s fur, appetite, body weight, etc. (9,12). However, we found that HUVEC immunization could lead to the destruction of the aortic arch in mice, but it is still unknown whether or not this destruction is caused by the xenogeneic EC. Although CRM197 could also inhibit the proliferation of HUVECs, almost no visible changes were observed in the inner wall of the aortas after immunization with CRM197. Presumably, that is because the anti-HUVECs could attack more targets in the EC of the mice, whereas anti-CRM197 has only one or a few targets. This could be the reason why the tissue structure of the aortic arch wall was relatively well-preserved after CRM197 vaccination.

The present results showed that HUVEC immunization did not influence the rates of wound healing. Angiogenesis, however, plays an important role in wound healing (36). The exact effects of HUVEC immunization on wound healing still need further study. Treatment with CRM197 in combination with viable HUVECs resulted in a markedly enhanced antitumor effect on prostatic carcinoma *in vivo* regarding both preventive and therapeutic treatments, suggesting a novel combination for anticancer therapy.

Acknowledgments

Research supported in part by China National Natural Science Fund Committee (#30772570) and Jiangsu Natural Science Fund Committee (#BK 2007170).

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