Vaccination with autologous endothelium inhibits angiogenesis and metastasis of colon cancer through autoimmunity

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Overcoming immune tolerance of tumor angiogenesis should be useful for adjuvant therapy of cancer. We hypothesized that vaccination with autologous endothelium would induce an autoimmune response targeting tumor angiogenesis. To test this concept, we immunized BALB/c mice with a vaccine of glutaraldehyde-fixed murine hepatic sinusoidal endothelial cells (HSEs) in a lung metastasis model of Colon-26 cancer. Vaccination with autologous HSEs induced both preventive and therapeutic anti-tumor immunity that significantly inhibited the development of metastases. ELISA revealed an immunoglobulin response involving IgM and IgG subclasses. These antibodies had a strong affinity for antigens of both murine and human endothelium, and lyzed endothelial cells in the CDC assay. Flow-cytometry and chromium-release cytotoxicity assay revealed a specific CTL response against endothelial cells, which were lyzed in an effector: target ratio-dependent manner. Neither antibodies nor CTLs reacted with Colon-26. The effect of autologous HSEs was more pronounced than that of xenogeneic human umbilical vein endothelial cells (HUVECs), which were tested in the same experimental setting. Our results suggest that vaccination with autologous endothelium can overcome peripheral tolerance of self-angiogenic antigens and therefore should be useful for adjuvant immunotherapy of cancer. (Cancer Sci 2004; 95: 85–90)

Angiogenesis, the growth of new blood vessels, is essential for tumor growth and metastasis.[1–3] In adults, angiogenesis is infrequent due to the balance between inhibitors and stimulators of angiogenesis. But under conditions of hypoxia, tumor cells turn-on the angiogenic switch by secretion of angiogenic stimulators that activate endothelial cells to proliferate and form new blood vessels.[4,5] Proliferation and expression of antigens critical to angiogenesis are two specific characteristics that distinguish angiogenic endothelial cells from quiescent endothelium of normal vasculature. Monoclonal antibodies and synthetic molecules targeting self-angiogenic antigens have been shown to inhibit tumor growth in animal models and were recently used in clinical trials.[6–10] However, relatively high doses of these therapeutics have to be administered for long periods of time due to their short biological half-lives. Furthermore, antigens highly specific for angiogenic endothelium still remain to be isolated. These drawbacks should be overcome by active immunization with whole endothelial cells. Recently, active immunization with xenogeneic, but not autologous, endothelial cells was shown to inhibit the growth of experimental tumors in mice.[11] However, from the viewpoint of clinical applicability of endothelial vaccines, the use of autologous endothelial cells would be preferable, since xenogeneic immunization may cause a species-specific immune reaction, with undesirable consequences. In the present study, therefore, we aimed to test the usefulness of autologous endothelial vaccine in the treatment of metastatic lesions that are highly dependent on tumor angiogenesis. For this purpose, we immunized BALB/c mice with a vaccine of glutaraldehyde-fixed murine hepatic sinusoidal endothelial cells (HSEs) in a lung metastasis model of colon cancer. We demonstrated that vaccination with autologous HSEs significantly inhibited the development of metastasis through an autoimmune response that was mediated by antibodies and cytotoxic T lymphocytes specifically reactive with endothelial, but not tumor cells. In addition, a vaccine of xenogeneic human umbilical vein endothelial cells (HUVECs) was tested in the same experimental setting, and the effects of both vaccines were compared, both in the animals and in *in vitro* immunological assays.

Materials and Methods

Cell culture. Hepatic sinusoidal endothelial cells (HSEs), a cell line isolated from BALB/c mouse,[12] were kindly provided by Dr. Tatsuro Irimura (Faculty of Pharmacology, the University of Tokyo). HSEs were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic (i.e., 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin B; Life Technologies, Grand Island, NY), in an atmosphere containing 5% CO_2 at 37°C. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords (obtained with patients' informed consent, from the Department of Gynecology and Obstetrics, the University of Tokyo) in sterile conditions by treatment with 0.25% trypsin and 0.1% EDTA in PBS for 15 min in an atmosphere containing 5% CO_2 at 37°C, and cultured on 0.1% gelatin-coated dishes in MCDB-151 medium (Sigma Chemical Co., Saint Louis, MO) supplemented with 15% FCS, 1% antibiotic/antimycotic, acidic fibroblast growth factor (aFGF, Pepro Tech, Inc., Rocky Hill, NJ, 2 ng/ml) and heparin (Sigma, 5 μg/ml). A fibroblast cell line of BALB/c mouse, Balb/3T3, clone A31 was purchased from Riken Cell Bank (Tsukuba). These cells were grown under the same conditions used for HSE culture. A colon cancer cell line of BALB/c mouse, Colon-26, was provided by Nippon Roche Co., Ltd. (Tokyo). Tumor cells were cultured in RPMI-1640 medium containing 5% FCS and 1% antibiotic/antimycotic, in an atmosphere containing 5% CO_2 at 37°C.

Animals. Specific pathogen-free female BALB/c mice were purchased from Oriental Yeast Co., Ltd. (Tokyo). Seven-week-old mice weighing 20 g and acclimatized for 1 week were used

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Abbreviations: HSEs, hepatic sinusoidal endothelial cells; HUVECs, human umbilical vein endothelial cells; CTLs, cytotoxic T lymphocytes; CDC, complement-dependent cytotoxicity; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt.

in all experiments. Experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Tokyo.

Flow-cytometric analysis of cell surface markers on endothelial cells. Endothelial cells (HSEs and HUVECs) were harvested by trypsinization, washed with PBS, and suspended in a sample buffer (0.1% BSA-0.1% NaN_3-PBS). Monoclonal antibodies against murine or human CD31, CD51, and CD105 (FITC-labeled) were added at a final concentration of 2 μg/ml. Samples were incubated at 4°C for 30 min, washed with washing buffer (0.2% BSA-0.1% NaN_3 -PBS), re-suspended in sample buffer, and analyzed in a flow-cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA).

Vaccine preparation. Proliferating endothelial cells and fibroblasts of up to ten passages were recovered with a cell scraper at subconfluency. The absence of endotoxin in cell cultures was confirmed with the Toxicolor test (Seikagaku Co., Tokyo). Next, cells were washed with PBS and fixed with 0.025% glutaraldehyde-PBS for 20 min at room temperature. Finally, fixed cells were washed three times with PBS and stored at −80°C.

Vaccination protocols in mouse models of lung metastasis. We used two treatment protocols in a lung metastasis model of colon cancer, i.e., preventive and therapeutic ones. In the preventive protocol (Fig. 1H), mice were divided into four treatment groups (Fig. 1G): control (PBS only), HSE (autologous endothelial vaccine), HUVEC (xenogeneic endothelial vaccine), and Balb/3T3 (autologous fibroblast vaccine) groups (n=9 each), which were given weekly subcutaneous injections of the respective vaccines (5×10^6 fixed cells/0.25 ml PBS/dose) for 5 consecutive weeks. One week after the last vaccination, a single cell suspension of Colon-26 cells in Hanks' Balanced Salt Solution was injected into the tail veins of mice (1×10^5 cells/0.25 ml/mouse). One day later, the mice were boosted with the same dose of vaccine. Ten days after the injection of tumor cells, mice were sacrificed by cervical dislocation; the lungs were removed, flushed with 15% India ink and fixed with Fekete's solution. Lung metastasis was evaluated macroscopically by counting metastatic nodules that were clearly visible on the lung surface. In the therapeutic protocol (Fig. 1I), mice were divided into control (PBS only), HSE (autologous endothelial vaccine) and HUVEC (xenogeneic endothelial vaccine) groups (n=4 each), and injected with tumor cells (day 0) prior to vaccination on days 1, 3, 5, 7, 9, and 11. Mice were sacrificed at day 14 and examined in the same way as for mice in the prevention protocol. Mice were observed daily for clinical signs of therapeutic efficacy, as well as for possible complications of vaccination.

Histological analysis. For microscopic evaluation, formalin-fixed and paraffin-embedded lung tissues were cut into 3 μm thick sections, deparaffinized with xylene, and dehydrated with 98% ethanol. Sections were stained with hematoxylin-eosin and Elastica Van Gieson, then washed and mounted.

Determination of antibody reactivity with membrane proteins by ELISA. Membrane proteins extracted from HSEs, HUVECs, and Colon-26 cells were coated overnight onto the bottom of 96-well immunoplates, at a concentration of 2 μg/ml, at 4°C. Murine sera diluted 100 times with assay buffer were used as the primary antibody, and biotinylated anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA), diluted 2000 times with 10% FCS-PBS, was used as the secondary antibody. After four washes with assay buffer, streptavidin-horseradish peroxidase conjugate (Amersham, Inc., Buckinghamshire, United Kingdom) diluted 2000 times with PBS was added at 100 μl/well. Four washes with assay buffer were followed by ABTS development, and the absorbance was measured at 405 nm.

Complement-dependent cytotoxicity (CDC) assay. To evaluate the CDC activity of the antibodies in murine sera, endothelial or tumor cells were suspended in media routinely used in our lab for their culture, and placed 5×10^4/100 μl/well in 96-well plates. The plates were pre-coated with 0.1% gelatin for use with endothelial cells. Centrifugation of the plates at 500 rpm for 5 min to allow the cells to sediment was followed by culture at 37°C in an atmosphere containing 5% CO_2 for 1 h. Cells that adhered to and spread on the bottom of the wells were washed twice with medium containing 0.2% BSA, and then incubated with 50 μl of murine sera (two-fold diluted with medium-0.2% BSA) for 1 h. Rabbit complement was added at 100 μl/well and the plates were incubated for another 1 h. Finally, the wells were washed twice with medium, and viable cells were detected by MTS assay (Promega, Madison, WI). The % target vi-

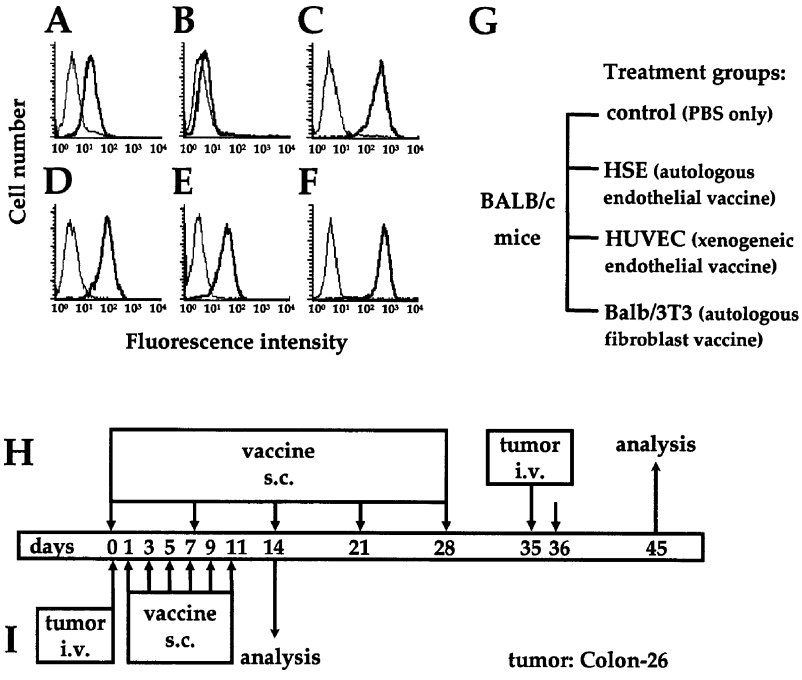

Fig. 1. Flow-cytometric analysis of cell surface markers on endothelial cells, and the *in vivo* study design. CD 31 (A), CD 51 (B), and CD 105 (C) expressions on murine hepatic sinusoidal endothelial cells (HSEs). CD31 (D), CD51 (E), and CD105 (F) expressions on human umbilical vein endothelial cells (HUVECs). For *in vivo* studies, BALB/c mice were divided into groups treated with PBS only (control), endothelial cells (HSE or HUVEC), and fibroblasts (Balb/3T3) (G). The preventive (H) and the therapeutic (I) protocols of the lung metastasis model are shown.

ability was calculated as the ratio to the value in the control group (taken as 100%).

Cytotoxic T lymphocytes' culture and cytotoxicity assay. ^{51}Cr-release assay was used to assess the cytotoxicity of cellular effectors against endothelial and tumor cells. Briefly, splenocytes were re-stimulated in a short-term activation culture on 10 cm dishes coated with fixed endothelial cells, HSEs, or HUVECs (fixation was performed under the same conditions as described for vaccine preparation). The activation medium used was as described,[13] i.e., RPMI-1640 supplemented with 10% FCS, 1.5 μg/ml anti-mouse CD3 (supernatant of 2C11 hybridoma culture; the concentration of immunoglobulin was determined by ELISA), 10 U/ml recombinant human IL-2 (Sigma) and 1% antibiotic/antimycotic. After 5 days of culture, the effectors were washed with PBS, and the cells were re-suspended in RPMI-1640 supplemented with 10% FCS and 1% antibiotic/antimycotic, and placed on 96-well plates in triplicates, to give the desired effector:target ratios of 200, 100, 50, or 25 to 1 in a total volume of 100 μl/well. Target cells (HSEs, HUVECs, Colon-26 cells) were washed with PBS, and labeled with ^{51}Cr (2×10^6 cells/100 μl ^{51}Cr in PBS and 10 μl of FCS) for 1 h. After three washes with PBS, target cells were suspended in RPMI-1640-10% FCS-1% antibiotic/antimycotic, and added to CTLs in the amount of 2×10^4/100 μl/well. The plates were centrifuged at 500 rpm for 5 min to allow cells to sediment, incubated for 4 h at 37°C in an atmosphere containing 5% CO_2, and centrifuged again. Radioactivity of supernatants was measured with a γ counter. The % specific lysis was calculated using the formula: (experimental release−spontaneous release)/(maximum release−spontaneous release)×100. The plates were pre-coated with 0.1% gelatin for use with endothelial cells.

Statistical analysis. Three independent experiments were performed for each evaluation with similar results. The results were analyzed with the two-tailed Student's *t* test or by ANOVA in combination with Scheffe's post hoc procedure (results including three or more means), and expressed as mean±SD. Differences were considered statistically significant at $P\leq0.05$ or $P\leq0.01$.

Results

Expression of cell surface markers on murine and human endothelial cells. First, we examined the expression of surface markers CD31 (PECAM-1), CD51 (αv-integrin) and CD105 (endoglin) on HSEs and HUVECs by flow-cytometry. Except for CD31 on HSEs (Fig. 1A; weak expression of CD31 on cultured HSEs was also reported by other authors),[14] all of the markers were strongly expressed on both HSEs (Fig. 1, B and C) and HUVECs (Fig. 1, D, E, and F), suggesting activated endothelial cells.

Inhibition of lung metastasis by autologous endothelial vaccination. Endothelial and fibroblast cell vaccines were prepared as described above. BALB/c mice, divided into control and immunized groups (Fig. 1G), received vaccination in the preventive (Fig. 1H) or therapeutic (Fig. 1I) protocols of lung metastasis model, and the antitumor effect was evaluated. In the preventive protocol, endothelial vaccination resulted in a significant inhibition of lung metastasis (Fig. 2A). The number of visible metastases was significantly decreased in the HSE (14±10, $P<0.01$) and HUVEC (25±11, $P<0.01$) groups compared to the control (112±9). In contrast, autologous fibroblast immunization had no inhibitory effect (100±15, $P=0.6$). In accordance with the results of the preventive protocol, endothelial vaccination was also effective in the therapeutic setting (Fig. 2B). The number of metastatic nodules was significantly reduced in the HSE (101±23, $P<0.01$) and HUVEC (121±11, $P<0.01$) groups, compared to the control (197±16). Microscopic evaluation of lung tissues gave results that correlated with the macroscopic findings, i.e., the number of microscopic metastatic focuses was lower in the endothelial vaccination groups than in the control (Fig. 3).

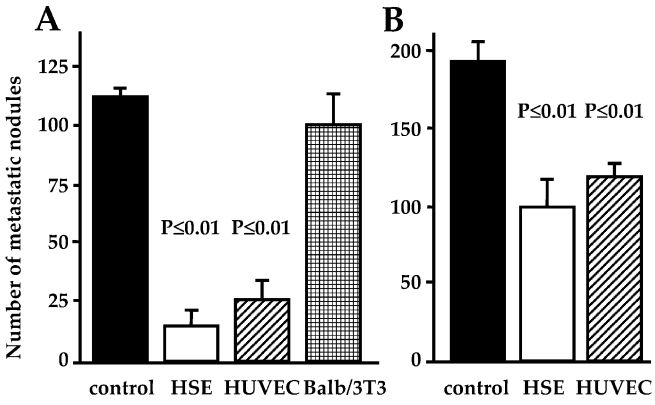

Fig. 2. The numbers of pulmonary metastatic nodules in the preventive (A) and therapeutic (B) protocols. Columns indicate mean (n=9 in A, n=4 in B), and bars indicate SD. Representative results of three independent experiments are shown.

In preliminary experiments, tumor-free mice were given endothelial vaccines and monitored daily for clinical signs of possible complications. All mice in both the HSE and HUVEC vaccination groups appeared generally healthy, without any noteworthy changes in appearance, body weight, habits or life span compared to unvaccinated mice (data not shown). No signs of spontaneous bleeding were observed in the immunized mice.

Antibodies reactive with endothelial but not tumor cells. Next, the serum levels of immunoglobulin subclasses were evaluated by means of sandwich ELISA assay. Small increases in the total levels of IgM, IgG1, IgG2a, and IgG2b were found in the sera of mice immunized with endothelial cells of both murine and human origin (data not shown).

To determine the specificity of antibodies induced by endothelial vaccination, murine sera were reacted with endothelial and tumor cells, and analyzed in a flow-cytometer. Sera of control mice showed no reactivity with HSEs, HUVECs, or Colon-26. In contrast, sera of mice receiving either HSE or HUVEC vaccinations showed a specific reactivity with the respective endothelial cells, but not tumor cells (data not shown).

In immunoprecipitation experiments, sera of mice receiving HSE or HUVEC vaccinations precipitated membrane proteins of the respective endothelial cells, which appeared as specific bands of approximately 140 kDa (data not shown).

Cross-reactivity of antibodies with human and murine endothelium. Although the increase in serum concentration of immunoglobulins was small, in the ELISA assay, the antibodies showed a strong affinity for endothelial membrane antigens. In addition, the antibodies present in the sera of HSE-vaccinated mice strongly bound not only proteins of HSE membranes, but also those of HUVEC membranes (Fig. 4A), and vice versa (Fig. 4B). Thus, the antibodies induced by endothelial vaccines were cross-reactive with both murine and human antigens. Furthermore, the sera of mice in both groups did not react with membrane proteins of tumor cells (Fig. 4C), in accordance with the flow-cytometric analysis.

Cytotoxic activity of antibodies against endothelial cells. To test the cytotoxic activity of antibodies induced by endothelial vaccination, they were reacted with endothelial and tumor cells in the presence of complement. Sera of mice receiving HSE vaccination could lyze HSEs (Fig. 5A), and those of mice receiving HUVEC vaccination could lyze HUVECs (Fig. 5B), but neither serum could lyze tumor cells (Fig. 5C). The specific cytotoxic

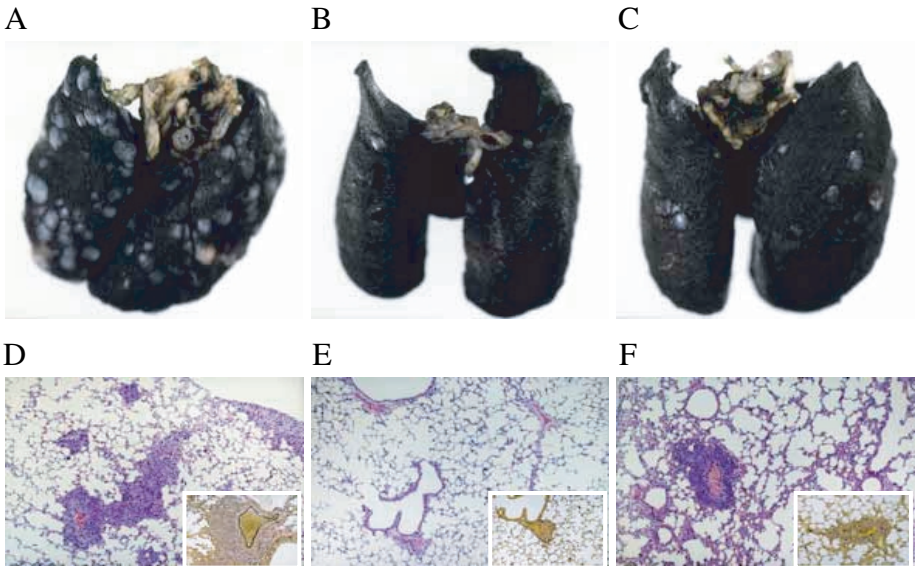

Fig. 3. Representative lungs of mice in the preventive protocol. Macroscopic appearance of lungs of mice in the control (A), HSE (B), and HUVEC (C) groups. Lungs were removed and flushed with India ink. Normal lung parenchyma is stained black and, by contrast, white metastatic nodules are clearly visible. Microscopic appearance of lungs of mice in the control (D), HSE (E), and HUVEC (F) groups. Sections of lung tissue were stained with hematoxylin-eosin (100× magnification) or Elastica Van Gieson (200× magnification).

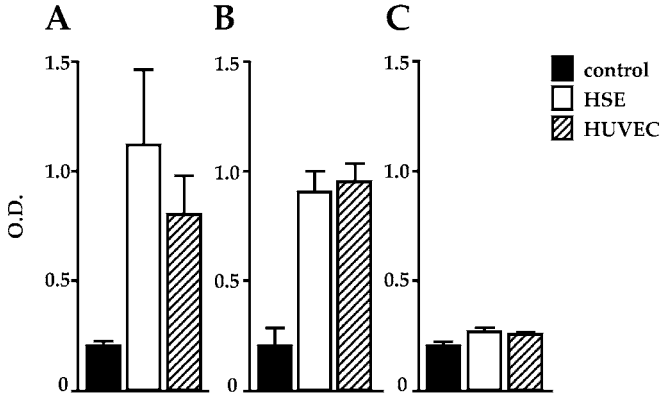

Fig. 4. Determination of antibody reactivity with membrane proteins by ELISA. Membrane proteins extracted from HSEs (A), HUVECs (B), and Colon-26 cells (C) were coated onto the bottom of 96-well immunoplates, and allowed to react with immunoglobulins present in murine sera. Sera of HSE-immunized mice strongly reacted with the proteins of not only HSE, but also HUVEC, and vice versa. However, neither serum reacted with membrane proteins of Colon-26.

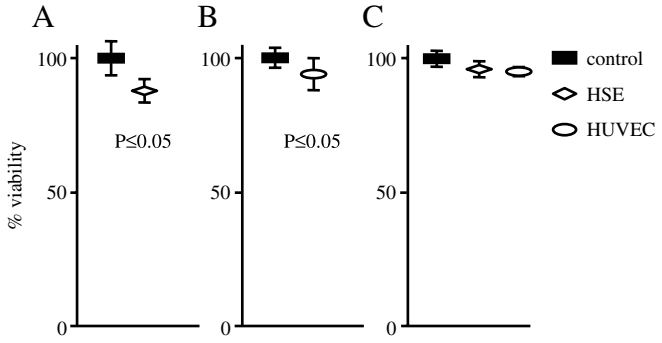

Fig. 5. Complement-dependent cytotoxicity assay. HSEs (A), HUVECs (B), and Colon-26 targets (C) were cultured and incubated with murine sera in the presence of complement. Sera of mice receiving endothelial vaccination were able to lyze HSEs or HUVECs but not Colon-26 cells.

activity was approximately 10% for both groups (P=0.0483 and 0.0527 for HSE-sera and HUVEC-sera, respectively).

Cytotoxic activity of CTLs against endothelial cells. To examine whether endothelial vaccines activated cellular immunity, fresh splenocytes were isolated from mice and analyzed in the flow-cytometer. Increases were observed in the CD8+ T cell and NK cell subpopulations after HSE as well as HUVEC vaccination (data not shown).

To confirm the functional activity and specificity of the cellular immunity activated by endothelial vaccination, splenocytes isolated from immunized mice were re-stimulated with the respective endothelial cells in a short-term re-activation culture and then used as effectors in a chromium-release CTL assay against endothelial or tumor targets. HSE-induced CTLs, at a 200:1 ratio, showed a significant lytic activity (69±7%, P<0.01) against HSEs when compared to the CTLs of control mice (24±12%) (Fig. 6A). HUVEC-induced CTLs, at a 200:1 ratio, also showed a significant lytic activity against HUVECs (46±22%, P<0.01) when compared to the CTLs of control mice (6±4%) (Fig. 6B). However, immune effectors of both groups showed no significant lytic activity against Colon-26 cells when tested under the same conditions (Fig. 6C). Preliminary experiments on CD8+ -depleted splenocytes, using MACS, suggested a crucial role of the CD8+ subpopulation in the effector phase of CTL assays (data not shown).

Discussion

We hypothesized that a vaccine of autologous endothelium would induce an autoimmune response targeting tumor angiogenesis and metastasis. To test this concept, we immunized BALB/c mice with murine hepatic sinusoidal endothelial cells (HSEs) in a lung metastasis model of colon cancer. The three major findings of this study were as follows. First, vaccination with autologous HSEs induced production of functional antibodies showing a strong affinity for antigens of both murine and human endothelium, but not tumor cells. Second, a strong cellular immune response of cytotoxic T lymphocytes was induced, with cytolytic activity against endothelial, but not tumor cells. Finally, this autoimmune response afforded both preventive and therapeutic immunity, significantly inhibiting lung metastasis of colon cancer.

The endothelial cells, HSEs and HUVECs, were chosen for our experiments, since when activated to proliferate, they share some morphological and functional properties with angiogenic

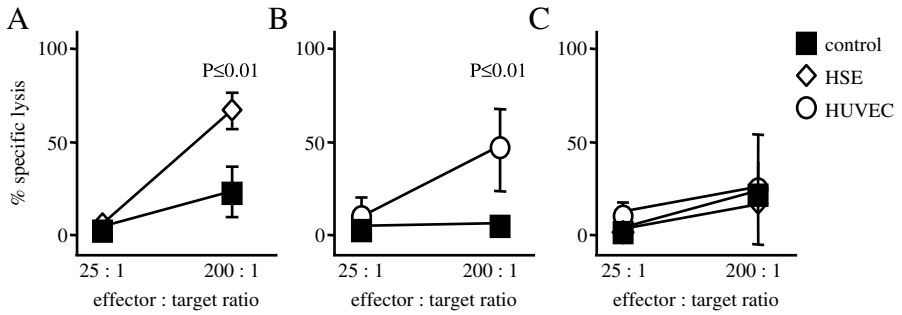

Fig. 6. Cytotoxicity assay of cytotoxic T lymphocytes. Splenocytes re-stimulated in a short-term re-activation culture were used as effectors in a chromium-release CTL assay against ^{51}Cr-labeled HSEs (A), HUVECs (B), or Colon-26 cells (C). Strong cytotoxicity directed against endothelial targets of CTLs from the HSE and HUVEC groups was found in an effector: target ratio-dependent manner. Neither CTL could specifically lyze Colon-26 cells. The graphs represent summaries of the results of multiple experiments performed at ratios from 25:1 to 200:1.

endothelium. Furthermore, HSEs play an active and direct role in tumor angiogenesis,[15, 16] and therefore should be a good model for autologous antiangiogenic vaccination. Interestingly, in this study, autologous HSEs were more potent than the xenogeneic human umbilical vein endothelial cells (HUVECs) in animals as well as in *in vitro* immunological assays. The reason for this is not clear yet. One possible explanation could be that HSEs are 'more homologous' with murine tumor endothelium because they are autologous and derived from microvasculature, in contrast with HUVECs that are xenogeneic cells derived from macrovasculature. Another possible reason could be that 'non-self' antigens of HUVECs might decrease the immune response against angiogenic molecules, because when two antigenic stimuli are given simultaneously, the response to one of these antigens is often decreased.[17] Administration of 'non-self' antigens, in cases such as allogeneic blood transfusion, has also been shown to impair immunity in mice,[18] as well as colorectal cancer patients,[19] resulting in increased tumor growth and decreased survival rates of the recipients, while autologous transfusion does not have such adverse effects.

The present results are consistent with those of other studies that have used autologous antigens to target tumor angiogenesis. Recently, Plum *et al*. have shown that administration of murine liposomal FGF-2 induced antibodies that abrogated FGF-mediated angiogenesis and tumor growth in mice.[20] In this work, however, the involvement of cellular immunity in the antitumor effect was not determined. More recently, dendritic cells pulsed with flk-1 (murine VEGFR-II)[21] as well as a DNA vaccine of flk-1[22] have been shown to induce CTLs with lytic activity against endothelial cells, leading to inhibition of tumor growth in mice. The efficacy of our vaccines was comparable with the reported data, not only in *in vitro* CTL assays, but also in *in vivo* models. In addition, we detected involvement of both cellular and humoral components in the autoimmune response against angiogenic antigens.

Though there is growing evidence that autologous vaccines are effective for inhibition of tumor angiogenesis, some reports have indicated that xenogeneic vaccines are superior. Wei *et al*. have reported that immunization with xenogeneic, but not autologous, endothelial cells, was effective in inducing an antiangiogenic immunity and protecting mice from tumor growth.[11] VEGFR-2 and αv integrin were identified as possible antigens responsible for the xenogeneic immune response. More recently, xenogeneic vaccines of VEGF (DNA vaccine),[23] HP59 and SP55 (synthetic peptides),[24] integrin β3 (DNA vaccine),[25] MMP-2 (DNA vaccine),[26] FGFR-1 (DNA vaccine),[27] and VEGFR-2 (synthetic peptide)[28] have also been reported to be effective in inhibiting tumor progression in mice. In these studies, specific antibodies, but not CTLs, were responsible for the antitumor immunity. Contrary to our results as well as those of others, autologous correlates of these antigens failed to induce either an immune response or protection against the tumor. In the present study, however, autologous endothelial vaccines were more effective than the xenogeneic ones in inducing specific humoral and cellular immunities against tumor endothelium, and consequently provided greater tumor inhibition. The reason for this discrepancy is not known, but it might be dependent on the differences of target antigens, methods of vaccine preparation, and routes of immunization. Such differences might be reduced or eliminated by increasing the amounts of antigens administered.

Specific genes expressed in tumor endothelium have been isolated and characterized, and high homologies between human and murine species (from 77 to 96%) have been reported.[29, 30] In an ELISA system, we found that sera of HSE-immunized mice strongly reacted with the membrane proteins of not only HSEs, but also HUVECs, and vice versa. This cross-reactivity of antibodies may explain why not only autologous, but also xenogeneic endothelial vaccines were effective in our animal models. In addition, the antibodies in sera of immunized mice were able to immunoprecipitate proteins of the endothelial membrane fraction with a molecular weight of approximately 140 kDa. Characterization of the candidate antigens is now ongoing.

Vaccination based on tumor antigens seems to be a promising approach for cancer therapy,[31, 32] but nevertheless targeting tumor endothelium with endothelial vaccines has many advantages over targeting tumor cells. Firstly, tumor endothelium is a homogeneous population of genetically stable cells with a low probability of losing antigen expression or developing acquired resistance.[33] Secondly, endothelial cells exist overlying the lumen of vessels and consequently are easily accessed by antibodies and immune cells. Thirdly, given that the ratio of endothelial and tumor cells in a tumor varies between 1:10 and 1:100, it is clear that the number of cells to be targeted is much smaller.[34] Fourthly, destruction of a small number of endothelial cells leads to activation of the coagulation system, with consequent vascular obstruction. Fifthly, endothelial cells are similar in different tumor types. Thus, the same antiangiogenic vaccine should be effective against tumors of various origins. Sixthly, antiangiogenesis is also effective on slowly growing and poorly vascularized tumors.[35] Finally, antiangiogenic therapy increases tumor sensitivity to chemotherapy and radiotherapy, and consequently should be useful in combination therapies. Additionally, vaccination with endothelial cells offers advantages over other angiogenic agents, including a sustained effect under conditions of chronic disease, and targeting of antigens that remain unknown and unisolated.

There is no consensus yet as to whether antiangiogenic therapy can cause deleterious effects on the host. There is certainly a possibility that antiangiogenesis can cause spontaneous bleeding under specific experimental conditions,[7, 21] as well as interfering with physiologic angiogenesis, impairing wound healing[22] or pregnancy.[21] On the other hand, however, most authors have reported only mild or no side effects during long-term follow-up of treated animals.[6–8, 11, 21–27] In this study, we found no evidence of complications due to endothelial vaccination in mice, but further studies on the safety of endothelial

vaccines, as well as other antiangiogenic therapeutics, are needed.

In summary, this study has demonstrated that an autologous vaccine of murine hepatic sinusoidal endothelial cells can significantly inhibit metastasis of colon cancer in mice, through humoral and cellular autoimmune responses targeting angiogenic endothelium. The results indicate that a vaccine of autologous endothelium can overcome peripheral tolerance of self-angiogenic antigens, and thus is valid for targeting tumor angiogenesis. As regards the clinical applicability of such vaccines, xenogeneic ones may be of limited value due to the development of adverse reactions dependent on species-specific immunity. Autologous vaccines are clearly preferable in this respect, and we should continue looking for an autologous vaccine able to induce a potent and specific immune reaction that eradicates tumors. Testing of other autologous endothelial cells as well as the development of strategies to increase the efficacy and to minimize the side effects of vaccination will help to provide solid basis for adjuvant therapy of cancer.

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