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DC-Based Immunotherapy Using Vascular Endothelial Cells Cultured in Conditioned Medium as a Vaccine Antigen Exerts an Anti-Tumor Effect by Inhibiting Angiogenesis

Makie Yamakawa,[1,a] Tetsuya Nomura,[1,a,b] Mariko Yamagata,[a] Takamasa Hirai,[a] Naoya Koizumi,[a] Kazuo Maruyama,[b] and Naoki Utoguchi*[,a,b]

[a]*Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University; Higashitamagawagakuen 3-3165, Machida, Tokyo 194-8543, Japan;* [b]*Faculty of Pharma-Sciences, Teikyo University, Kaga 2-11-1, Itabashi-ku, Tokyo 173-8605, Japan*

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Blood vessels are essential for the maintenance and growth of tumor tissues. Furthermore, tumor angiogenesis promotes metastasis, which greatly affects prognosis. Therefore, tumor blood vessels are considered an important target in cancer therapy. Cancer immunotherapy has been developed recently as a new cancer therapeutic. Notably, vaccine therapy with dendritic cells (DCs), which possess potent antigen-presenting capacities, is expected to induce tumor-associated, antigen-specific immunity. In this regard, as tumor endothelial cells (TECs) constituting tumor blood vessels are derived from endothelial cells (ECs) in the host, DC vaccine therapy targeting tumor blood vessels may have applicability in several cancer types. Thus, we attempted to develop DC vaccine therapy that targeted TEC. In our previous studies, *in vitro* TEC models were created by culturing normal ECs in the culture supernatants of tumor cells. Moreover, we demonstrated that the molecules' permeability is enhanced in an *in vitro* TEC model compared with normal ECs. In this study, we examined whether immunotherapy using TEC-extracted proteins as vaccine antigens would be an effective cancer therapy. The results showed that DC vaccine therapy targeting TECs induced anti-tumor effects in a murine Colon-26 solid tumor model and in a lung metastases model using murine B16 melanoma cells. Moreover, anti-angiogenic effects of immunization with TECs were demonstrated. Thus, immunotherapy using the *in vitro* TEC model as an antigen may be an effective cancer therapeutic. In the future, identifying specific TEC antigens will help generate promising new strategies to inhibit angiogenesis.

Key words tumor endothelial cell, angiogenesis, dendritic cell, immunotherapy, metastasis

INTRODUCTION

When tumor cells exhibit abnormal growth, hypoxic and low nutrient conditions are induced in tumor tissues.[1)] Hypoxia-inducible factor (HIF) serves an important function at angiogenesis in hypoxic conditions. HIF induces the expression of multiple angiogenesis promoting factors, such as vascular endothelial growth factor (VEGF) and angiopoietin 2.[2)] Thus, new blood vessels to supply oxygen and nutrients are formed from existing blood vessels in the tumor tissues. Unlike normal blood vessels, the newly formed tumor blood vessels repeatedly run in irregular meandering and branching pathways. Furthermore, adhesion between vascular endothelial cells (ECs) is incomplete, and there is insufficient coverage of pericytes, which are mural cells.[3)] Therefore, vascular permeability in tumor blood vessels is enhanced compared with normal blood vessels. Furthermore, such immature vasculature facilitates tumor cell invasion of blood vessels and promotes tumor metastasis.[1)] As stated above, tumor blood vessels play a major role in tumor growth as well as in metastasis. Therefore, tumor blood vessels are important targets in cancer therapy.[4)]

Cancer immunotherapy has been developed as the fourth therapeutic modality, in addition to surgery, chemotherapy, and radiotherapy.[5)] Notably, vaccine therapy with dendritic cells (DCs) possessing potent antigen-presenting capacities is expected to induce antigen-specific immunity.[6)] Anti-tumor effects have been exerted in vaccine therapy using DCs loaded with proteins extracted from tumor tissue.[7)] However, in DC vaccine therapy targeting tumor cells, it is essential to use an appropriate antigen depending on the type of cancer. On the other hand, tumor endothelial cells (TECs) constituting tumor blood vessels are derived from ECs of the host. Therefore we thought that TEC can be a common target for several cancer types. Thus, immunotherapy targeting TEC would be an effective cancer therapy. In our previous studies, *in vitro* TEC models were created by culturing normal ECs in the culture supernatants of tumor cells (conditioned medium: CM) (Fig. 1A). In addition, molecules' permeability is enhanced in the *in vitro* TEC model compared with normal ECs.[8)] Then, we developed DC-based immunotherapy with *in vitro* TECs as the vaccine antigen. The purpose of our therapy is to destroy tumor blood vessels by immunization with DCs pulsed with proteins extracted from the *in vitro* TEC model (Fig. 1B). Therefore, this study examined whether immunotherapy using proteins

*To whom correspondence should be addressed. e-mail: utoguchi@ac.shoyaku.ac.jp
[1] These authors contributed equally to the work.

extracted from TECs as vaccine antigens would be an effective cancer therapy.

MATERIALS AND METHODS

Experimental Animals Female 6- to 9-week-old C57BL/6 and BALB/c mice were obtained from Sankyo Labo (Tokyo, Japan). All mice were held under specific pathogen-free conditions and were treated according to the Showa Pharmaceutical University animal experimental stipulation or the Teikyo University animal experimental guidelines.

Cells Mouse melanoma B16/BL6 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A), and cultured at 37°C under a humidified atmosphere of 5% CO_2 in minimum essential medium (MEM), supplemented with 10% fetal bovine serum (FBS), streptomycin (0.5 mg/mL), and penicillin G (500 U/mL). Colon-26 murine colorectal adenocarcinoma cells were provided by Dr. S. Unezaki (University of Tokyo Medical, Japan) and were cultured at 37°C under a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM,) supplemented with 10% FBS, streptomycin (0.5 mg/mL), and penicillin G (500 U/mL). Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka, Japan), and maintained at 37°C under a humidified atmosphere of 5% CO_2 in a half volume of Medium199 and RPMI1640, supplemented with 15% FBS, endothelial cell growth supplement (ECGS; 20 μg/mL), heparin (25 μg/mL), streptomycin (0.5 mg/mL), and penicillin G (500 U/mL). Murine dendritic cell line DC2.4 cells were provided by Dr. N. Okada (University of Osaka, Japan) and cultured at 37°C under a humidified atmosphere of 5% CO_2 in RPMI1640, supplemented with 10% FBS, 2-mercapto ethanol (50 μM), non-essential amino acids (100 μM), streptomycin (0.5 mg/mL), and penicillin G (500 U/mL).

Construction of the *In Vitro* TEC Model Colon-26 cells or B16/BL6 cells were cultured until reaching confluence. The cell supernatants were aspirated, and then the ECs' culture medium was added. After 48 h incubation, the supernatants were collected to serve as the tumor CM. HUVECs (1.5×10^3 cells/cm^2) were seeded on a culture dish and cultured at 37°C under a humidified atmosphere of 5% CO_2. After 24 h incubation, the cell culture medium was replaced with a half volume of Medium199 and CM, and the cells were cultured at 37°C for 48 h.

Preparation of Cell Membrane Proteins After the culture medium was removed from the dish, HUVECs or the *in vitro* TEC model were washed with PBS three times. We added 2.5% 1-butanol in PBS to the dish. After incubation for 5 min, the supernatants were collected. Following dialysis, freeze dehydration was performed. The powder was re-suspended using PBS.

Generation of Bone Marrow-Derived DCs Bone marrow-derived DCs (BMDCs) were prepared as previously reported.[9] Briefly, the femur and cnemis were removed from the sacrificed mice. Bone marrow cells were flushed from these bones and cultured on low detached dishes in RPMI1640 supplemented with 10% FBS, 2-mercaptoethanol (50 μM) (Sigma-Aldrich, St. Louis, MO, U.S.A.), and mouse granulocyte-macrophage colony-stimulating factor (20 μg/mL) (Peprotech Inc., Rocky Hill, NJ, U.S.A.). On day 2, fresh medium was added, and half the culture medium volume was exchanged every fourth day. On day 10, floating cells were collected as BMDCs.

Loading of Extracts Derived from Cells into DCs A mixture of cell extracts and lipofectin (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was incubated at room temperature for 15 min. The mixture was added to BMDCs or DC2.4 cells, and then those cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 in Opti-MEM for 5 h.

Flow Cytometric Analysis The BMDCs (1.0×10^5 cells) were harvested, washed with 2% FBS in PBS, and re-suspended. The cells were then stained with one of the follow-

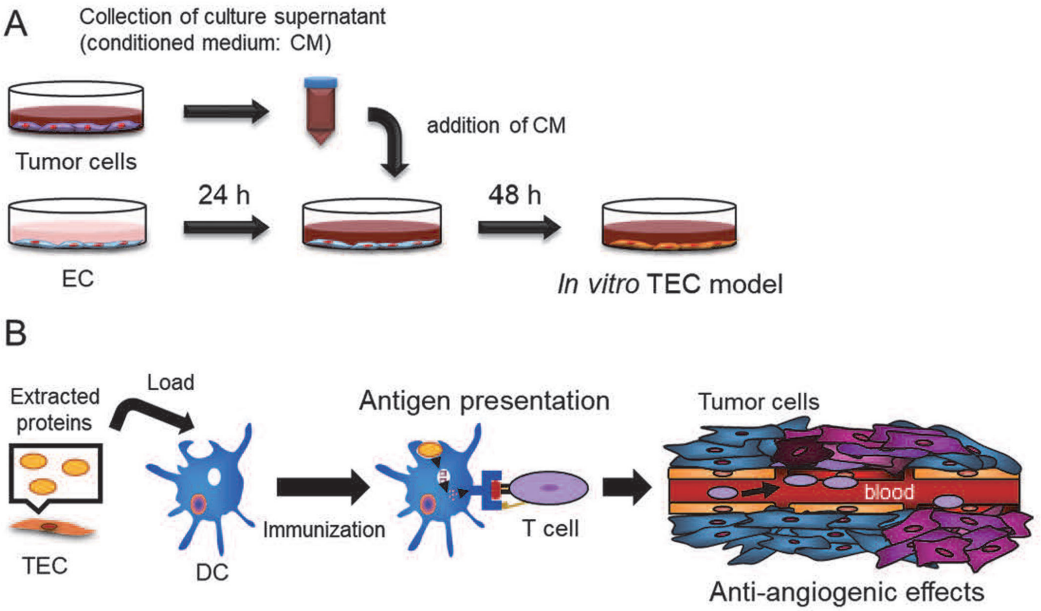

Fig. 1. Method of Preparing the *In Vitro* TEC Model and Strategy of DC Vaccine Therapy Targeting TECs.

(A), After HUVECs (normal endothelial cell models: EC) were cultured for 24 h, the cell culture medium was replaced with a half volume of medium and CM, and then the cells were cultured for 48 h. (B), Anti-angiogenic effects are supposed to be exerted through immunization with DCs, loaded with protein extracted from TECs, to induce immunity against TECs.

ing FITC-conjugated mAbs: anti-CD11c (HL3), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL1), or anti-I-A/I-E mAbs (2G9). Each antibody was applied at a concentration of 1:100 for 30 min at 4°C. The antibodies for CD11c, CD40, CD86, and I-A/I-E were purchased from Becton Dickinson Biosciences (Franklin Lakes, NJ, U.S.A.). The antibodies for CD80 were purchased from BioLegend (San Diego, CA, U.S.A.). The stained cells were analyzed in a FACS Calibur (Becton Dickinson). The data were analyzed using CellQuest software (Becton Dickinson).

Cytokine Assays The quantities of IL-6, IL-12p70, and TNF-α in the BMDCs' culture supernatants were determined by a sandwich ELISA using mAbs specific for each cytokine. They were plated in a 96-well plate (Thermo Fisher Scientific) and analyzed with an ELISA kit (IL-6 and TNF-α: BioLegend, IL-12p70: Thermo Fisher Scientific) according to the manufacturer's instructions.

***In Vivo* Experiments** The *in vivo* anti-tumor effects by DC immunization were evaluated in mice with lung metastases of B16/BL6 or B16F10 cells and in Colon-26 cells solid tumor-model mice. In the lung metastasis model, B16/BL6 or B16F10 cells (1.0×10^6 cells/100 μL) were injected intravenously (iv) in the tail veins of C57BL/6J mice. In the spontaneous lung metastasis model, B16/BL6 (5.0×10^5 cells/100 μL) were injected into the intra-footpads in C57BL/6J mice. When the tumor diameter reached 6–7 mm, the tumor-bearing feet were amputated below the groin. After the mice were sacrificed, the lung tissues were removed and fixed for 24 h with 10% neutral formalin. The number of metastatic colonies in the lungs was counted using a stereomicroscope. In a solid tumor model, Colon-26 cells (1.0×10^6 cells/100 μL) were injected intracutaneously in the flanks of the Balb/c mice. Tumor volume was calculated as 1/2 (length × width^2). Antigenic protein-loaded BMDCs or DC2.4 cells were washed three times and then re-suspended in PBS. DCs (1.0×10^6 cells/100 μL) were injected subcutaneously (sc) into the flanks of the mice.

Histopathological Analysis The mice were sacrificed 14 d after tumor implantation and their lung tissues were removed. After the lung tissues were fixed with 4% neutral buffered formalin, their tissues were washed with PBS three times and then dehydrated with 70% ethanol, 90% ethanol, 99% ethanol, and xylene. Subsequently, the lung tissues were embedded in paraffin, and the paraffin blocks were sectioned. Following deparaffinization with xylene, 99% ethanol, 90% ethanol, and 70% ethanol, the lung tissues were stained with rat anti-mouse CD34 antibodies (MEC14.7) (1:50) (Abcam, Cambridge, MA, U.S.A.) and HRP conjugated-anti-rat IgG (1:200) (Abcam). The staining was developed in diaminobenzidine-H_2O_2.

Evaluation of Angiogenesis by the Dorsal Air Sac Chamber Method The dorsal air sac method was performed as previously described.[10] Briefly, 7 d after the last immunization, a chamber ring filled with B16/BL6 cells (1.0×10^6 cells/150 μL) was implanted sc into the dorsal sides of the mice. In the model, angiogenesis occurred on the murine skin attached to the ring. 5 d after implantation, the mice were sacrificed. Tumor-induced angiogenesis was observed under a stereoscopic microscope.

Statistical Analyses The Student's t-test was used for the statistical analyses of the *in vitro* experiments. An analysis of variance followed by Dunnett's test was used for the *in vivo* experiments. A p value of < 0.05 was considered significant.

RESULTS

Analysis of TEC Membrane Protein-Induced DC Maturation DC maturation, induced by a loading antigen, is an important process leading to anti-tumor immunity.[11] Thus, DC maturation, pulsed with the proteins extracted from the *in vitro* TEC model (TEC/DCs), was evaluated by the expressions of molecular markers and cytokine production.

First, the expression of cell surface markers in TEC/DCs

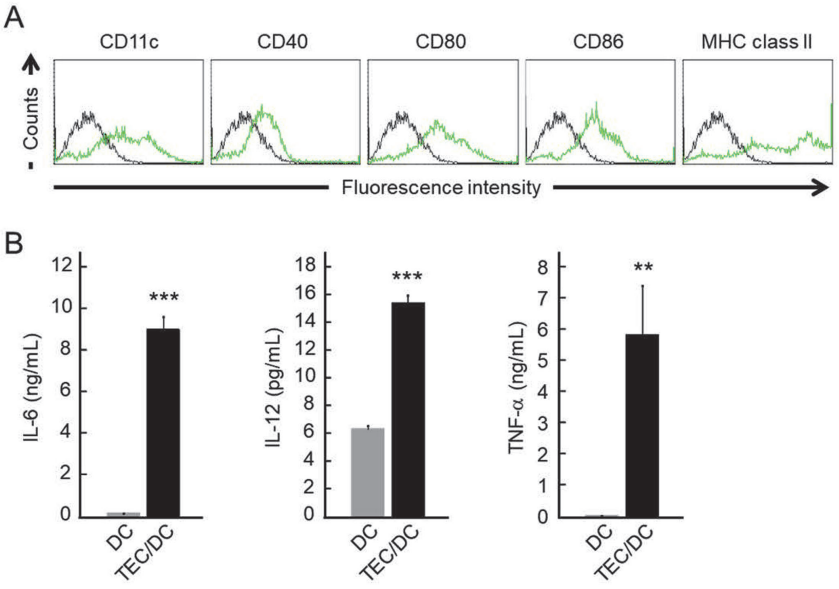

Fig. 2. Loading of Proteins Extracted from the *In Vitro* TEC Model to DCs to Induce DC Maturation.

(A), BMDCs were pulsed with proteins extracted from TECs for 5 h at 37°C. TEC/DCs were stained for CD11c, CD40, CD80, CD86, and MHC class II, and their expressions were analyzed via flow cytometry. One representative experiment of two immunizations is shown. The black line shows non-stained areas. The green line shows each fluorescent-antibody stain. (B), BMDCs were pulsed (or not) with proteins extracted from TECs for 5 h at 37°C. ELISAs were performed to test the IL-6, IL-12, and TNF-α production in TEC/DCs or antigen-unloaded DCs (DCs). Data are the mean and SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$.

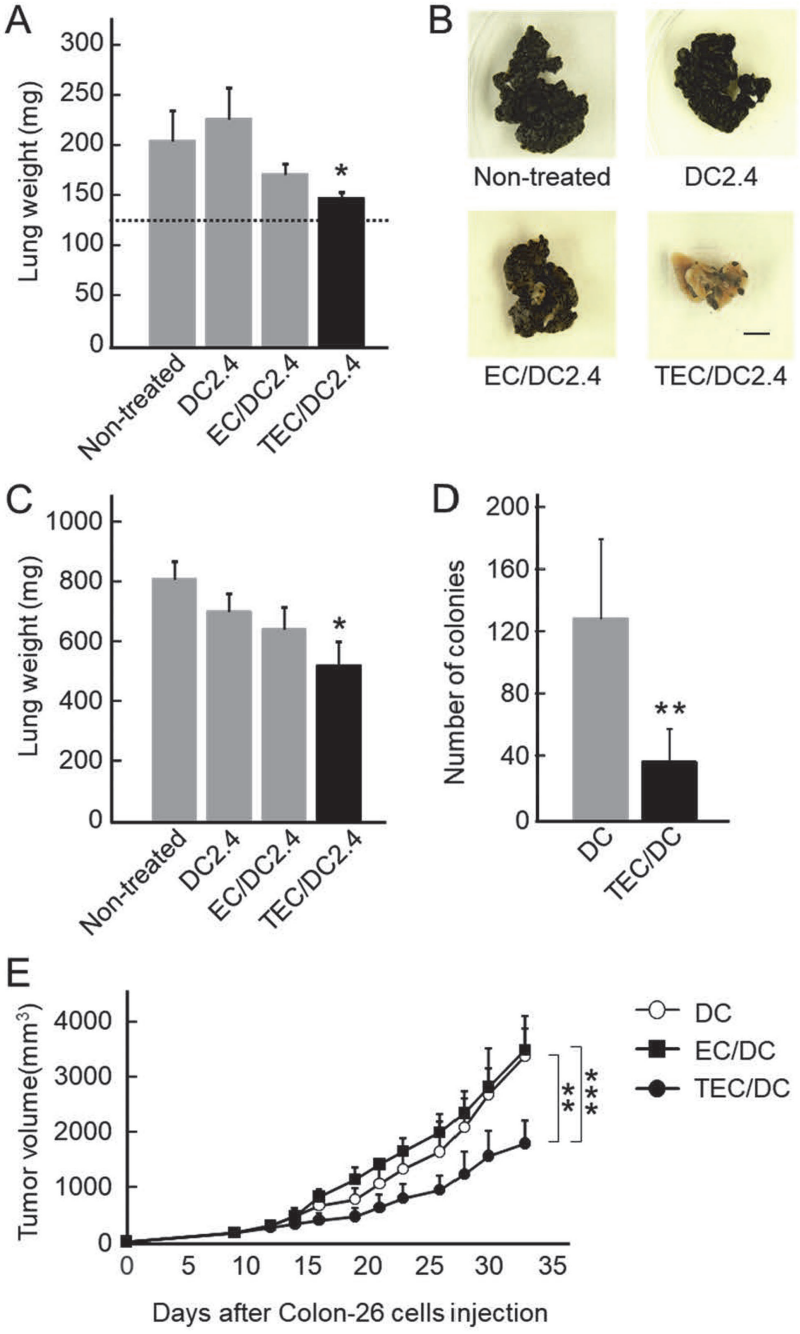

Fig. 3. *In Vivo* Anti-Tumor Effects of Immunization with TEC/DCs.

(A), Mice were immunized with DC2.4 (n = 6), EC/DC2.4 (n = 3), TEC/DC2.4 (n = 6), or without DC2.4 (non-treated, n = 6) (1.0×10^6 cells/mouse) on days 0 and 7. The mice were challenged iv with B16 (1.0×10^5 cells/mouse) on day 14. The mice were sacrificed 14 d after injection with B16. Data are the mean lung weight and SE. The dotted line shows the lung weight of a normal 6-week-old mouse. *$p < 0.05$ compared with the group immunized with DC2.4. (B), Image of a representative lung sample from each group in (A). Bars denote 5.0 mm. (C), Mice were immunized with DC2.4, EC/DC2.4, TEC/DC2.4, or without DC2.4 (1.0×10^6 cells/mouse) on days 0 and 7. The mice were challenged iv with B16 (1.0×10^5 cells/mouse) on day 0. The mice were sacrificed 31 d after challenge with B16. Data are the mean lung weight and SE (n = 6). *$p < 0.05$ compared with the non-treated group. (D), Mice were immunized with DCs or TEC/DCs (1.0×10^6 cells/mouse) three times every 7 d in the spontaneous lung metastasis model. The mice were sacrificed 7 d after the last immunization. Data are the mean number of B16 colonies on the lung and SD (n = 5). **$p < 0.01$. (E), Mice were challenged sc with Colon-26 (2.0×10^6 cells/mouse) on day 0. The mice were immunized with DCs, EC/DCs, or TEC/DCs (1.0×10^6 cells/mouse) on days 9 and 16. Data are the mean tumor volume and SD (n = 4). **$p < 0.01$, ***$p < 0.001$.

was analyzed by flow cytometry. The expression of CD11c was confirmed on the cell surface of DCs generated from bone marrow cells. The DC maturation markers CD40, CD80, CD86, and MHC class II were expressed on the membrane surface of TEC/DCs (Fig. 2A).

Next, the quantity of cytokines in the supernatant of the TEC/DC culture was measured with an ELISA. IL-6, IL-12, and TNF-α are produced by DC maturation. The production of these cytokines increased in the TEC/DC culture supernatant compared with antigen-unloaded DCs (Fig. 2B).

Therefore, it was suggested that DC maturation occurred by loading the *in vitro* TEC model-extracted proteins into the

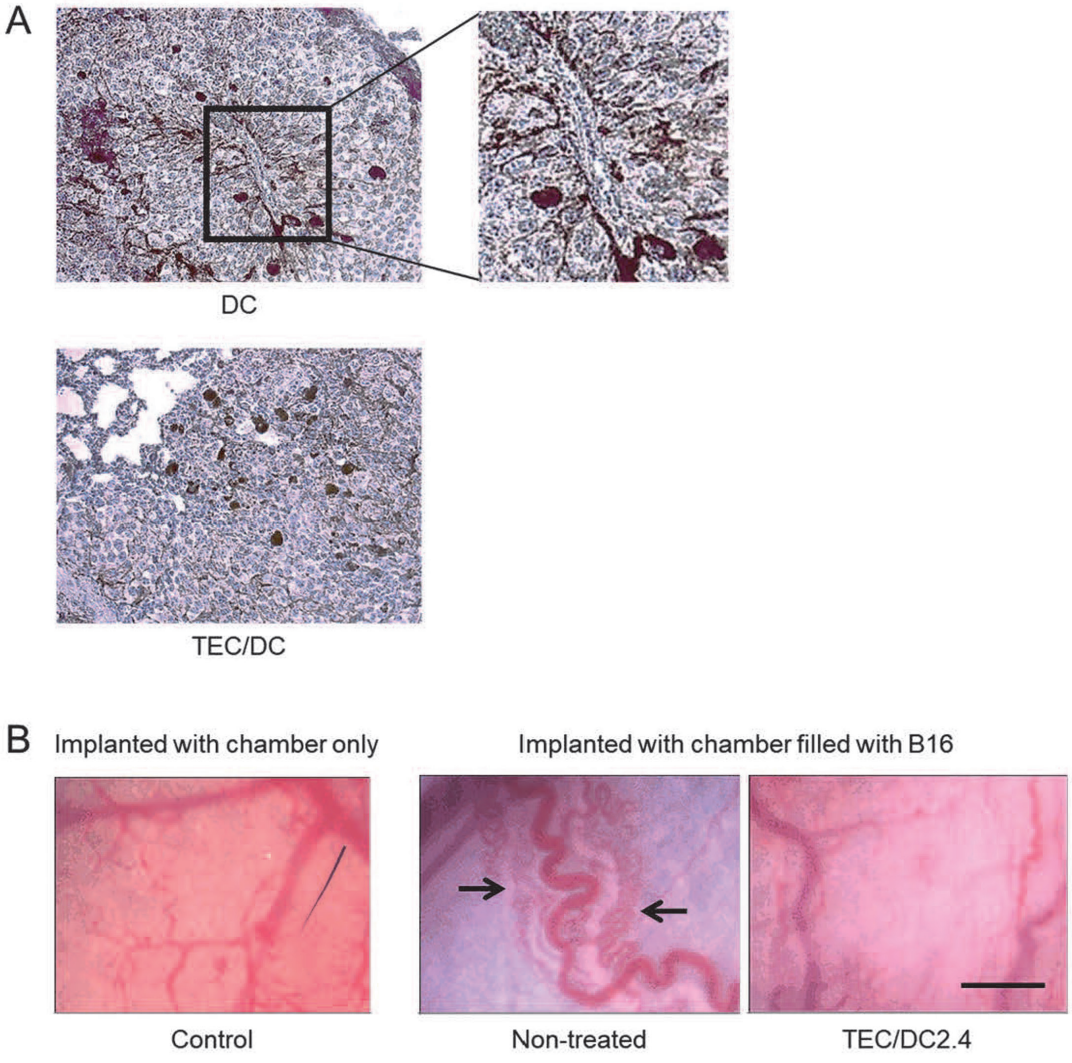

Fig. 4. Inhibition of Tumor-Induced Angiogenesis by TEC/DC Immunization.

(A), Mice were immunized with DC2.4 or TEC/DC2.4 (1.0×10^6 cells/mouse) on days 0 and 7. The mice were challenged iv with B16 (1.0×10^5 cells/mouse) on day 14. The mice were sacrificed 14 d after injection with B16. In CD34 immunohistochemical staining, tumor sections were stained using antibodies against CD34. The panel magnified in right side on the top shows one of CD34-positive tumor blood vessels. (B), Mice were immunized with TEC/DCs (1.0×10^6 cells/mouse) on days 0 and 7 or without DCs and were divided into three groups. The angiogenic potential was determined using the dorsal air sac assay method. The mice were implanted sc with chambers filled with B16 (1.0×10^5 cells/mouse) (control: chamber only). Neovascularization was followed by microscopy. Bars denote 1.0 mm. The black arrow shows tortuous new blood vessels.

DCs.

Anti-Tumor Effects by DC Immunization Using Proteins Extracted from the *In Vitro* TEC Model as an Antigenic Protein The anti-metastatic effects exerted by the immunization of DC2.4 cells, with proteins extracted from the *in vitro* TEC model (TEC/DC2.4), were evaluated using a B16 lung metastatic mouse model. Firstly, the mice were challenged with melanoma 7 d after the final DC immunization. In untreated mice, severe melanoma metastasis to the lung was observed. The immunization of antigen-unloaded DC2.4 (DC2.4) or EC-extracted proteins loaded DC2.4 (EC/DC2.4) barely suppressed metastasis to the lungs. However, TEC/DC2.4 immunization showed significant anti-metastatic effects compared with the group immunized with DC2.4 (Fig. 3A and B). Next, B16 was injected at the same time as the first immunization. The immunization of DC2.4 or EC/DC2.4 showed almost no anti-metastatic effects. However, the TEC/DC2.4 immunization significantly suppressed lung metastasis compared with the non-treated group (Fig. 3C). The group immunized with EC/DC2.4 was not significant compared with the non-treated group and the group immunized with DC2.4 (Fig. 3A and C). Subsequently, to evaluate tumor angiogenesis actions related to metastasis from the primary tumor site, we investigated the anti-metastatic effects in spontaneous metastasis models of B16. In the group immunized with TEC-extracted protein-loaded BMDCs (TEC/DCs), metastasis was significantly suppressed compared with that in the group immunized with antigen-unloaded BMDCs (DCs) (Fig. 3D).

In addition, to elucidate the anti-tumor effects, induced by immunization with TEC/DCs, on the growth of solid tumors, we examined therapeutic effects in mouse models of Colon-26. In mice immunized with TEC/DCs, tumor growth was dominantly inhibited compared to mice immunized with DCs or EC-extracted protein-loaded BMDCs (EC/DCs) (Fig. 3E).

Influence on the Formation of Tumor Blood Vessels by Immunization with TEC/DCs To evaluate the effect of immunization with TEC/DCs on the formation of tumor blood vessels, immunohistochemical staining was conducted using lung tissue in a B16 metastasis model. CD34 is a surface molecular marker of ECs. In tumor sections of the group

immunized with DCs, CD34-positive cells were extensively observed. Conversely, tumor blood vessels were decreased in the tumor tissues of the group immunized with TEC/DCs (Fig. 4A).

Subsequently, the anti-angiogenic effects of immunization with TEC/DC2.4 were examined using the dorsal air sac chamber method. In the non-treated group, many tortuous new blood vessels were formed around the tumor tissue. Conversely, in the group immunized with TEC/DC2.4, angiogenesis around the tumor tissue was suppressed; for example, the group transplanted with the chamber only did not contain tumor cells (Fig. 4B).

DISCUSSION

In this study, we invented DC vaccine therapy using a TEC model prepared *in vitro* as a vaccine antigen and demonstrated the usefulness of our strategy. DC vaccine therapy targeting TECs displayed anti-tumor effects by inhibiting the formation of tumor blood vessels. Moreover, in mice immunized with proteins extracted from *in vitro* TEC models, tumor cell growth, and metastasis from the primary lesion were suppressed. Furthermore, we demonstrated that anti-tumor effects were exerted by DC immunization with the *in vitro* TEC models constructed from mouse ECs (data not shown). Therefore, considering there was no distinction between DC2.4[12] and BMDC, DC immunization targeting TECs was effective not only in solid tumors but also in metastatic tumors. We confirmed that anti-tumor effect of DC vaccination with *in vitro* TEC models is not induced by immunity against tumor cells. In addition, as preliminary data, we elucidated that DC vaccination targeting TECs introduced CTLs against not ECs but also TECs. Thus, DC vaccine therapy targeting TECs could also be an effective cancer therapeutic.

In the past, many anti-angiogenic agents have been developed. Most of the existing anti-angiogenic agents, such as bevacizumab[13] and sorafenib,[14] are designed to block VEGF signaling. VEGF is involved in promoting angiogenesis and increasing vessel permeability, and inhibiting VEGF exerts anti-tumor effects.[15] However, there has been a concern that anti-VEGF agents infrequently cause serious adverse reactions, including hypertension, hemorrhage, gastrointestinal perforation, and hemoptysis, because VEGF signaling is essential for maintaining vascular homeostasis and physiological vascularization.

In previous studies, many researchers reported differences in the expression patterns of genes and proteins in TECs and ECs. The expression of molecules such as CD31, VE-cadherin, and tumor endothelial marker 8 are elevated in TECs.[16,17] However, these molecules are not TEC-specific markers because they are expressed in both TECs and ECs. In this study, DC vaccination, using *in vitro* TEC models as vaccine antigens, exerted anti-tumor effects, but DC vaccination using ECs did not. Our previous studies elucidated that DC immunization, using the TEC-rich-containing fraction separated from tumor tissues as antigenic proteins, showed anti-tumor effects by inhibiting angiogenesis in tumor tissues.[18,19] In addition, we demonstrated that DC immunization with the TEC-rich-containing fraction did not affect physiological angiogenesis during wound healing.[18] These results indicate that a specific tumor-associated antigen exists in the *in vitro* TEC model. In the future, identification of specific antigen expressed in TECs derived from human is promising for the development of tumor blood vessel selective therapeutics such as TEC-specific antibodies and vaccine therapy for clinical trial.

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Conflict of interest The authors declare no conflict of interest.

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