Development of Dendritic Cell-Based Immunotherapy Targeting Tumor Blood Vessels in a Mouse Model of Lung Metastasis

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Tumor blood vessels supply cancer tissues with oxygen and nutrients, and it was therefore believed that inhibition of angiogenesis would induce tumor regression. In fact, the situation is complicated by the presence of normal blood vessels in cancer tissues such as carcinomas and sarcomas as well as abnormal vessels. Here, we describe the development of a dendritic cell (DC)-based immunotherapy which targets tumor endothelial cells (TECs) rather than normal endothelial cells (ECs) or cancer cells themselves. After density gradient centrifugation, the TEC-rich fraction from lungs invaded by B16 melanoma cells was separated from the endothelial cell (EC)-rich fraction on the basis of positivity for angiotensin-converting enzyme (ACE) activity. Prophylactic vaccination with DCs pulsed with lysates of TECs isolated from lungs with metastases significantly suppressed lung metastasis in this B16/BL6 mouse melanoma model. This suggests that DC-based vaccine therapy targeting TECs in cancers tissue could show promise as an effective therapy for distant metastasis.

Key words  tumor endothelial cell; lung metastasis; vaccine; angiogenesis; dendritic cell; angiotensin-converting enzyme

INTRODUCTION

The formation of new blood vessels derived from tumor endothelial cells (TECs) plays an important role in tumor progression. 1) In the process of tumor angiogenesis, tumor cells, fibroblasts and epithelial cells secrete vascular endothelial growth factor (VEGF), basic fibroblast growth factor, hepatocyte growth factor, or epidermal growth factor (EGF), and endothelial cells (ECs) are recruited from neighboring, pre-existing vasculatures in the tumor microenvironment. 2–5) In our previous study, we showed that cancer immunotherapy with TECs isolated from solid tumors suppressed tumor growth via the prevention of new blood vessel formation. 6) Tumor blood vessels also have a role as conduits for cancer metastasis. Tumor cells migrate into blood vessels from the primary lesion and may spread to several other organs and tissues. 7–9) Therefore, anti-angiogenesis therapy was expected to be effective against cancer metastasis. However, it proved difficult to develop tumor blood vessel-specific cancer therapy, because there are also many normal blood vessels in carcinomas and sarcomas and a method for isolating TECs from such tumors did not yet exist. Moreover, many investigators have reported the existence of gaps between TECs in tumor blood vessels and enhanced molecular permeability, compared with ECs in normal blood vessels. 10) This suggests that the density of TECs is different from that of ECs.

In the present study, we sought to isolate TECs from lung tissue invaded by metastatic cancer using density gradient centrifugation, based on anticipated differences between EC and TEC densities. Vaccination with dendritic cells (DCs) loaded with lysates of these isolated TECs was then investigated for efficacy in suppressing lung metastasis in a mouse model (Fig. 1).

MATERIALS AND METHODS

Animals Specific pathogen-free C57BL/6 mice were supplied by Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). All animal experiments were conducted in accordance with the guidelines regarding animal experimentation of Teikyo University (Tokyo, Japan).

Cell Culture Mouse melanoma B16/BL6 (B16) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). B16 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 0.5 mg/mL streptomycin, and 500 U/mL penicillin G at 37°C in a humidified atmosphere of 5% CO2.

Lung Metastasis Model 1.0 × 105 cells B16 cells were injected into C57BL/6 mouse tail veins. Mice were sacrificed 14d after tumor injection and the lungs were fixed in 10% formalin. The number of metastatic colonies in the lungs was quantified using a stereomicroscope.

Density Gradient Centrifugation Lung tissues were removed and incubated at 37°C for 1 h in Hank’s Balanced Salt Solution (HBSS; Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 0.75% collagenase (pH = 7.3; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 0.5% bovine serum albumin (BSA; Sigma-Aldrich), and 0.0072% deoxyribonuclease (DNase; FUJIFILM Wako Pure Chemical Corporation). After centrifugation, the cells were resuspended in MEM containing 10% FBS, and then gently layered on the top of a 35% Percoll (Sigma-Aldrich) density gradient. Following centrifugation at 1500 × g for 10min in a swing rotor, the cells were aspirated from the gradient in 8 fractions at 1 mL intervals from the bottom of the tubes.

Angiotensin-Converting Enzyme Activity Each cell fraction was centrifuged twice at 4000rpm for 5min with

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HBSS, and then sonicated in borate buffer solution (pH = 8.3). The angiotensin-converting enzyme (ACE) activity in each extract was measured as described in a previous report.° Protein concentrations were measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA used as the standard protein.

**Cell Lysates** Each cell fraction was lysed over several freeze-thaw cycles in phosphate buffered saline (PBS), followed by centrifugation at 1500 \( \times g \) for 10 min. The supernatants were collected for pulsing DCs prior to their use as vaccines.

**Generation of Mouse Bone Marrow-Derived DCs** As previously reported, DCs were prepared from mouse bone marrow cells.° Briefly, femurs and tibiae of C57BL/6 mice were removed, bone marrow cells were flushed out, and then cultured in RPMI 1640 supplemented with 40 ng/mL mouse granulocyte macrophage colony-stimulating factor (mGM-CSF; Peprotech Inc, Rocky Hill, NJ, U.S.A.), 10% FBS, 50 \( \mu \)M 2-mercaptoethanol (2-ME; Invitrogen, Life Technologies, Carlsbad, CA, U.S.A.), 100 \( \mu \)g/mL streptomycin, and 100 U/mL penicillin G at 37°C in an atmosphere of 5% CO\(_2\). After culture for 10d, loosely adherent cells were harvested for use as DCs.

**Immunization with Lysate-Pulsed DCs** DCs (2.0 \( \times 10^7 \) cells/8 mL) were loaded with lysates prepared as described above (600 \( \mu \)g) using Lipofectin (Invitrogen, Life Technologies) at 37°C under a humidified atmosphere of 5% CO\(_2\) for 5h. After washing three times in PBS, mice were then immunized intradermally (id) on their backs twice at weekly intervals with 1.0 \( \times 10^6 \) lysate-pulsed DCs or DCs treated only with PBS.

**Statistical Analysis** Differences in the number of lung metastatic colonies between experimental groups and controls were compared by one-way ANOVA, and a \( p \) value <0.01 was considered significant. All results are expressed as mean ± standard error of the mean (S.E.M.). All experiments were performed at least twice.

**RESULTS**

**Measurement of ACE Activity in Cell Fractions Containing TECs or ECs Isolated from Lungs with Metastases** Large amounts of ACE are ex-
pressed on the EC surface, where it catalyzes the conversion of angiotensin I to angiotensin II, and inactivates bradykinin. Here, EC-rich cell fractions were identified on the basis of their relative ACE activity. For metastatic lung tissue, the ACE activity per µg protein of lysates from cells collected from Percoll gradient fractions 6–8 was higher than in lysates of cells from the other fractions (Fig. 2A). In particular, the ACE activity in fraction 7 was the highest of all. In contrast, in extracts from normal lung tissue, the ACE activity peaked in fraction 5 (Fig. 2B). In our previous study, we had confirmed that the ACE activity in each fraction was correlated with the proportion of cells positive for the endothelial marker CD34 in each cell fraction derived from solid tumor tissues.\(^6\)

In normal lung tissue, CD34-positive cells are ECs. In lung with metastases, CD34-positive cells are ECs and TECs. Therefore, it may be expected that fraction 5 in normal lung tissue or lung with metastases is EC-rich fraction, and fraction 7 in lung with metastases is TEC-rich fraction. Thus TEC-rich fractions could be distinguished from EC-rich fractions in lung tissues following 35% Percoll density gradient centrifugation. Cells from fraction 5 were then harvested as the richest source of ECs, and cells from fraction 7 were harvested as the richest source of TECs.

**Suppressive Effect of DC Vaccination with TECs on Lung Metastasis** To examine the anti-metastatic effects of DC vaccination with isolated TEC extracts, we tested prophylactic vaccination in a B16 mouse model of lung metastasis. Tumors were implanted one week after the two DC vaccinations (Fig. 3A). In untreated mice, a high degree of lung metastasis was observed (Fig. 3B), which was essentially unchanged by vaccination with DC alone (data not shown). In contrast, in animals receiving DCs pulsed with extracts of B16 cells (B16/DC), vaccination inhibited metastasis (\(p < 0.01\) vs. untreated; Figs. 3B, C). Interestingly, vaccination with DCs pulsed with extracts of the TEC-rich fraction from lungs with metastases (TEC/DC) also significantly suppressed metastasis at least to the same extent as B16/DC vaccination (\(p < 0.01\) vs. untreated; Figs. 3B, C). Tumor rejection without any metastatic colony formation was complete in 33% of the TEC/DC-treated mice (2/6) (data not shown). Indeed, TEC/DC vaccination tended towards effecting a greater inhibition of metastasis than B16/DC vaccination, although this difference did not achieve statistical significance.

**DISCUSSION**

We hypothesized that TECs separated from lungs with metastases may be important target cells for vaccines designed to prevent lung metastasis. Here, we describe the isolation of TECs from lung tissue by density gradient centrifugation.

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**Fig. 3. Anti-metastatic Effect of TEC- or Tumor Cell Lysate-Pulsed DC Vaccination in the B16 Lung Metastasis Model**

(A) Shows the immunization time course. C57BL/6 mice were prophylactically injected twice at weekly intervals in the right flank (d-14 and d-7) with C57BL/6-derived DCs pulsed with lysates of TEC isolated from B16 lung metastatic cancer tissue (TEC/DC; \(n = 6\)), B16 tumor cell lysates (B16/DC; \(n = 6\)), or untreated DCs (untreated; \(n = 7\)). Seven days after the final immunization, B16 cells were injected into the tail vein. After another 14d, B16 metastatic colonies in the lungs were counted. (B) Number of metastatic colonies in lung tissue. Values are expressed as mean ± S.E.M. **\(p < 0.01\) vs. untreated mice (one-way ANOVA). (C) Images of lung by stereomicroscopy.
were recently attempted. We are currently attempting to expressually different proteins from ECs. In recent years, tumor endothelial marker (TEM8) was discovered to be expressed predominantly in TECs. However, this molecule was barely detectable in ECs. We hypothesized that TEC density would be lower than that of ECs, because the structure of tumor blood vessels is more brittle than normal blood vessels. Moreover, vaccination using isolated TEC lysates to pulse DCs for vaccination induced significant anti-metastatic effects. In our previous report, we confirmed that DC vaccination with protein of lysate from cell fraction with low ACE activity hardly suppresses tumor growth. In our preliminary examination, DC vaccination with protein of lysate from EC-rich fraction cells did not show anti-metastasis effect, compared with that of TEC-rich fraction (data not shown). Thus, we may think that anti-metastasis effect in mice vaccinated with TEC-rich fraction was induced by the immunization of TEC contained in TEC-rich fraction derived from lung tissues with metastases. Additionally, we confirmed that DC vaccination using TECs isolated from different solid tumor types showed the same anti-tumor effects as that of TECs derived from the same solid tumor. Therefore, cancer therapy targeting TECs has the potential to be effective for several types of metastatic cancer.

Existing anti-angiogenic agents, such as anti-VEGF antibodies and EGF receptor tyrosine-kinase inhibitors are being applied in the clinical setting for colorectal cancer or lung cancer. However, these agents have many known side effects such as causing perforation of the digestive tract and protracted wound healing, because they also inhibit signaling pathways involved in normal angiogenesis. In this regard, we previously reported that DC vaccination with TECs separated from solid tumors did not induce wound healing. Additionally, DC vaccination with protein of lysate from ECs cultured in tumor conditioned medium suppressed lung metastasis by the induction of not EC- but TEC-specific cytotoxic T lymphocytes (CTLs) (data not shown). Therefore, we may think that DC-based vaccine with TEC does not make an effect on blood vessels in normal tissues. These results show that TECs expressed different proteins from ECs. In recent year, tumor endothelial marker (TEM8) was discovered to be expressed predominantly in TECs. However, this molecule was barely detectable in ECs. We are currently attempting to isolate TEC-specific antibody and to identify TEC-specific molecule with phage display technique. We conclude that cancer vaccine therapy with TEC-lysate pulsed DC is expected to lead to the development of anti-angiogenic therapies for several different types of metastatic cancer free of the side effects of other forms of anti-angiogenic agents.

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Conflict of Interest The authors declare no conflict of interest.

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