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Original research

Establishment of a normalized revascularization mouse model using tumor transplantation

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ABSTRACT

Tumor blood vessels play an important role in tumor progression and metastasis and have recently been shown to differ in structure from normal blood vessels. Thus, normalization of tumor blood vessels using molecular targeted therapy has been attracting attention as a potential new treatment for cancer. However, some studies have suggested that this therapy is unable to cause significant tumor shrinkage and instead promotes drug resistance. Therefore, we hypothesized that regeneration of normal blood vessels in tumors may lead to improvement of hypoxic conditions in tumors. Furthermore, regeneration of normal blood vessels may contribute to potential improvements in the delivery of anticancer drugs to hypoxic tumors. In this report, we sought to characterize whether transplanting normal endothelial cells into tumor-bearing mice would trigger vascular remodeling. Tumor cells (SAS; human tongue squamous cell carcinoma) were injected into the dorsal subcutis of SCID mice. After 2 weeks, the tumor-bearing mice were injected cisplatin intraperitoneally to regress tumors and tumor vessels as a first step. An additional 2 weeks later, to induce normal angiogenesis in the tumor, human endothelial cells (HMVECs) were transplanted into necrotic regions of the tumor as a second step. Microscopic observations revealed that the transplanted human endothelial cells formed anastomoses with the host mouse vasculature, and perfused vessels were detectable after 7 days. Thus, this regenerated blood vessel mouse model is a useful model for the future development of new cancer therapies, construction of reliable drug delivery systems, and improvement of hypoxic conditions.

1. Introduction

Without blood vessels, tumors cannot grow beyond a critical size or metastasize to another organ [1]. Tumor blood vessels have been reported to be histopathologically different from normal vessels in that most tumor vessels have irregular diameters and abnormal branching patterns and do not fit well into the usual classification system of arterioles, capillaries, or venules [2]. Endothelial cells are loosely interconnected and have intercellular openings and abnormal pericytes that may be responsible for much of the vessel leakiness [3]. The structural abnormalities in the basement membrane of tumor vessels are also responsible for their immaturity in comparison with normal vessels [4]. These vascular abnormalities in tumors may be attributed to abnormal tumor microenvironments characterized by interstitial hypertension, acidosis, and hypoxia, which can lead to resistance to conventional chemotherapies and radiotherapies [5–7].

Tumor hypoxia stimulates tumor cells to release angiogenic factors, such as vascular endothelial growth factor (VEGF), a major driver of tumor angiogenesis [8]. In addition, tumor hypoxia, which stimulates angiogenesis, then promotes a vicious cycle of abnormal vessel and tumor growth, followed by enhanced hypoxic regions, repeating the cycle. Although the main purpose of tumor angiogenesis is to maintain a tumor's blood supply, the process occurs in an unmitigated fashion, and the resulting vascular network is highly abnormal. Therefore, unlike normal blood vessels, tumor vasculature has abnormal organization, structure, and function [9]. Because of the importance of angiogenesis, it has been suggested that tumor growth is dependent on angiogenesis, and, accordingly, molecular therapies that target angiogenesis have been studied extensively over the past 2 decades. Indeed, such therapies have attracted significant attention since inhibiting blood vessel formation, thereby preventing the tumor from receiving adequate supplies of oxygen and nutrients, is thought to shrinks the cancer by starvation. However, some studies have suggested that anti-VEGF

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therapy alone is unable to effectively induce sufficient vascular regression to cause significant tumor shrinkage in the clinical setting [9]. Therefore, the hypothesis of vascular normalization has been proposed. The normalization hypothesis suggests that by correcting the abnormalities in the structure and function of tumor vessels (rather than destroying the vessels completely) we can normalize the tumor microenvironment and ultimately control tumor progression and improve responses to other therapies [10]. Normalized tumor vessels are characterized by less leaky, less dilated, and less tortuous vessels with a more normal basement membrane and greater coverage by pericytes [10,11]. However, it is unknown whether normalized vessels in tumors are completely normal; moreover, the functions and structure of these normalized vessels have not been adequately characterized. Thus, in this paper, we sought to determine whether transplanting normal endothelial cells into tumor-bearing mice would trigger vascular reconstruction and vascular normalization.

2. Materials and methods

2.1. Animals

Male SCID mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were maintained in air-filtered clean rooms and fed sterilized standard laboratory chow and water ad libitum. All procedures employed in this study for the handling and care of animals were approved by the Animal Experiment Committee, Tokyo Women's Medical University and were performed in accordance with legislation of the Institute of Laboratory Animals for Animal Experimentation at Tokyo Women's Medical University.

2.2. Cell culture

SAS cells derived from human tongue squamous cell carcinoma were a generous gift from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University SAS cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 g/mL streptomycin (Gibco, Grand Island, NY, USA). Human dermal microvascular endothelial cells (HMVECs) were obtained from Kurabo Industries, Ltd. (Osaka, Japan). HMVECs were cultured according to the manufacturer's protocol.

2.3. Tumor models and tumor transplantation

SAS tumor cells [2.5×10^6 cells in 250 μL phosphate-buffered saline (PBS; Ca^{2+}-, Mg^{2+}-free PBS; pH 7.4)] were injected into the dorsal subcutis (s.c.) of each mouse. After 2 weeks, tumor-bearing mice were injected cisplatin (CDDP; Maruko: 20 mg/kg, Nichi-iko, Toyama, Japan) intraperitoneally to regress tumors. Tumor size was measured with calipers every day, and tumor volumes were calculated as width^2 × length × 0.5. The mice were sacrificed at 7, 14, 21, or 28 days after cell implantation.

2.4. Labeling of blood vessels with tomato lectin for 3D imaging

We used intravascular perfusion of fluorescent tomato lectin to label all blood-circulating vessels. Briefly, under anesthesia, the mice were intravenously (i.v.) injected with 100 μL FITC-conjugated tomato lectin (*Lycopersicon esculentum* lectin; 1 mg/mL; Vector Lab, Burlingame, CA). Tomato lectin binds uniformly to the luminal surface of endothelial cells and can be used to label all blood vessels that have a patent blood supply. After 10 min, the chest was opened, and the aorta was perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) at a pressure of 120 mmHg for 5 min, followed by PBS for 5 min via the left ventricle. After perfusion, the tissues were processed for subsequent analyses as described above.

2.5. General tissue preparation

All mice were anesthetized by intramuscular (i.m.) injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Their chests were opened, and the vasculature was perfused with 4% PFA in PBS for 10 min at a pressure of 120 mmHg using an 18-gauge cannula inserted into the aorta via an incision in the left ventricle. Blood and fixative were removed through an opening in the right atrium. After the perfusion, tissues were removed, cut into small pieces, and rinsed in PBS. Next, the tissues were immersed in PBS containing a graded series of sucrose (up to 30%) at 4 °C overnight. Subsequently, these tissues were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. Cryostat sections (20 μm) were cut and placed on silane-coated glass slides and air dried for at least 2 h. Subsequently, they were either immunostained or stored at −20 °C in a shielded box until further use.

To obtain semithin Epon-embedded sections, various tissues were excised, cut into small blocks, and fixed by immersion in 2% glutaraldehyde in 0.1 M PB (pH 7.2) at 4 °C for 24 h. After washing out the fixatives with 0.1 M PB, the blocks were treated with 1% osmium tetroxide–0.1 M PB (a mixture of 2% OsO_4 4 mL + 0.2 M PB 4 mL). The tissues were dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon. Semithin sections (thickness, 0.5 μm) were made and stained with 1% toluidine blue in PBS.

2.6. Immunohistochemistry

After thawing and air drying, the cryosections were rehydrated in PBS and then incubated with 1% Block Ace blocking solution (Dainippon Seiyaku Ltd., Tokyo, Japan) to reduce nonspecific background staining. The sections were incubated with various primary antibodies at 4 °C overnight in humidified chambers. Immunostaining was performed by using 2 or 3 primary antibodies against a mouse endothelial cell marker: CD31 (PECAM-1; rat monoclonal antibody; dilution, 1:200; BD Pharmingen, San Diego, CA or hamster monoclonal antibody; dilution, 1:500; Chemicon, Temecula, CA) and human endothelial markers: Von Willebrand Factor (vWF; rabbit polyclonal antibody; dilution, 1:100; DakoCytomation, Denmark) and Rhodamine Ulex Europaeus Agglutinin lectin (UEA lectin; dilution, 1:100; Vector Lab). The basement membrane marker type IV collagen (rabbit polyclonal antibody; dilution, 1:1000; CosmBio, Tokyo, Japan) was also used. Pericytes were identified with antibodies to alpha-smooth muscle actin (α-SMA; Cy3-conjugated mouse monoclonal, 1:500; Sigma–Aldrich, St. Louis, MO, USA) and desmin (rabbit polyclonal, 1:200, Abcam, Cambridge, MA, USA). VEGF-A-expressing cells were marked with a goat polyclonal antibody to mouse VEGF (1:100; R&D Systems, MN, USA). After several washes with PBS, the specimens were incubated with fluorescent (FITC, Cy3, or Cy5) secondary antibodies (goat anti-rat, anti-hamster, or anti-rabbit; dilution 1:200 in PBS; Jackson ImmunoResearch, West Grove, PA) for 45 min at room temperature (RT). The proper concentration of each antibody was predetermined. Finally, specimens were mounted in Vectashield (Vector Lab) and analyzed using a Leica TCS-SL confocal laser-scanning microscope (Leica, Wetzlar, Germany).

2.7. Transmission electron microscopy

After perfusion of 4% PFA in PBS, the tumors were removed, cut into small blocks (approximately 1 mm^3 in size), and immersed in 2% glutaraldehyde in 0.1 M PB for at least 2 h. The blocks were