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Incorporation of adult organ-derived endothelial cells into tumor blood vessel

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Abstract

In this study, we attempted to assess the incorporable potential of vascular endothelial cells derived from adult organ blood vessels into tumor blood vessels. Two kinds of adult organ-derived vascular endothelial cells, human aorta endothelial cells (HAEC) and umbilical vein endothelial cells (HUVEC), were administered into murine tumors inoculated to SCID mice. Many human blood vessel networks were visualized in the murine tumors. These cells in solid tumor not only survived and proliferated, but also incorporated into tumor endothelium. These results suggest that adult organ-derived vascular endothelial cells possess the potential to form the neovascular network in various tissues such as vascular endothelial progenitor-like cells in vivo. We propose that these cells can be regarded as a congenic (autologous) vector for vascular regeneration cell therapy and tumor vascular targeting gene therapy.

Keywords: Vascular endothelial progenitor-like cells; Cell therapy; Vascular regeneration

Angiogenesis is an important physiological property of vascular endothelial cells, typically seen during embryonic development, the adult female reproductive cycle, and wound healing. It is well known that the growth of new blood vessels is also a component of some pathological conditions including tumor growth and metastasis. Continuous tumor angiogenesis is indispensable to the growth of solid tumors, because it provides the tumor's nutrient and oxygen supply [1,2]. Thus, the inhibition and destruction of tumor blood vessels result in effective solid tumor death on the ground that one tumor blood vessel supports 1000 of tumor cells. An attractive target is thought to be vasculature of the tumor rather than of the tumor cells themselves in the therapy of solid tumors [3]. Therefore, we have reported several tumor therapy strategies for vascular targeting [4–8]. For instance, we showed a remarkable anti-tumor effect as a result of introducing the TNF-α gene into the vascular endothelial cells of tumor. This anti-tumor effect seemed to be induced by effective diffusion of TNF-α throughout the entire tumor mass since introduction of the TNF-α gene through tumor blood vessels [8].

A landmark study on angiogenesis reported that vascular endothelial progenitor cells (EPCs) were discovered in adult peripheral blood and bone marrow [9], and that these cells were then involved in the angiogenesis of an ischemic limb and tumor [10]. Therefore, these EPCs can be regarded as a congenic (autologous) vector for cell and gene therapies in order for vascular regeneration and destruction. However, two important problems will arise about clinical application of EPCs. The first is that ex vivo repopulation of EPCs may become essential to obtain a large number of these cells as a congenic (autologous) vector for efficient therapy. The second is that it is a debatable point whether repopulated EPCs have equivalent redifferentiation potential of neovasculature in comparison to the originating EPCs.

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A large number of human vascular endothelial cells derived from adult organ, such as skin, aorta, and umbilical vein, have been easily isolated and cultured in vitro [11–13]. If these endothelial cells have the potential to form the neovascular networks in various tissues as well as EPCs, the effective vascular regeneration cell therapy and tumor vascular targeting gene therapy may be established.

It is widely believed that adult organ-derived vascular endothelial cells, typified by human aorta endothelial cells (HAEC) and umbilical vein endothelial cells (HUVEC) have been already and highly differentiated. In fact, brain capillary endothelial cells have continuous tight junctions and γ-glutamyl transpeptidase (γ-GTP) as differentiated properties of the blood–brain barrier [14,15]. In contrast, hepatic blood vessels known as discontinuous capillaries have abundant fenestrations at their cellular junctions [16]. However, in general, these tissue-specific properties of organ-specific endothelial cells are rapidly lost in vitro. We have found that vascular endothelial cells in vitro would possess and retain properties intrinsic to the tissue by the effects of humoral factors secreted from tissue cells and extracellular matrixes [17–19]. For instance, glial extracellular matrix induced γ-GTP activity, which is the specific cell marker to capillary endothelial cells in vivo, of not only brain-derived endothelial cells but also aorta endothelial cells [17]. In addition, parenchymal and nonparenchymal hepatocytes were observed to elevate aorta endothelial cell monolayer permeability in a coculture system [18]. Therefore, we have suggested that adult organ-derived vascular endothelial cells may have redifferentiation potential ability.

The goal of this study was to develop an indispensable new technology for vascular regeneration cell therapy and tumor vascular targeting gene therapy using adult organ-derived vascular endothelial cells as a congenic (autologous) vector. In this study, we, thus, attempted to assess the incorporation potential ability of HAEC and HUVEC into the capillary endothelial cells of the tumors after their injection into murine solid tumors in SCID mice. We found that these cells had the potential to incorporate into tumor vessels and form the neovascular networks such as vascular endothelial progenitor-like cells in vivo, and suggested that these cells may be a useful vector for cell and gene therapies.

Experimental protocol

Cell culture and mice. Normal human aorta endothelial cells (HAEC) and normal umbilical endothelial cells (HUVEC) were kindly provided by Kurabo Industries (Osaka, Japan). HAEC and HUVEC were maintained in HuMedia-EG2 (Kurabo). Meth-A, a murine fibrosarcoma, was maintained intraperitoneally in BALB/c mice (females, 5 weeks old) purchased from Japan SLC (Hamamatsu, Japan). SCID mice (C.B-17/IcrCrj-scid/scid, females, 5 weeks old) were purchased from Charles River Japan (Kanagawa, Japan) All experimental protocols involving animals complied with our institutional Guidelines for the Care and Use of Laboratory Animals.

Reagents. Anti-human CD34 mouse monoclonal antibody (QBend 10), anti-human Ki-67 antigenic rabbit-polyclonal antibody, dextran polymer conjugated with horseradish peroxidase and goat-anti-mouse immunoglobulin (EnVision+ Mouse/HRP), DAB (3,3′-diaminobenzidine tetrahydrochloride), BCIP/NBT (5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium chloride/indonitrotetrazolium), streptavidin/alkaline phosphatase, and biotinylated human genomic DNA probe were obtained from Dako Japan, (Kyoto).

Intramurine tumor administration of human vascular endothelial cells. Meth-A fibrosarcoma cells (1×10^6 cells/head) were transplanted into the abdomen of SCID mice. HAEC or HUVEC were administered into the tumors (1×10^6 cells/head) on days 1, 3, 5, 7, and 9 after tumor implantation. Fifteen days after tumor implantation, the tumors were excised and fixed with 10% formalin neutral buffer solution (pH 7.47) for 6 h at 4 °C. The tumors were embedded in a paraffin compound. Immunohistochemistry and in situ hybridization were performed on the tumor sections (5 μm).

After immunohistochemistry, the number of human blood vessels in 15 randomly extracted tumor sections was counted.

Immunohistochemical detection of human endothelial cells in murine tissue. Prior to immunohistochemical detection of human endothelial cells, anti-CD34 monoclonal antibody (QBend 10, Dako) and anti-mouse antibody and HRP conjugated dextran polymer (EnVision+, Dako) were allowed to react at room temperature for 60 min, and normal mouse serum was added to the complex of QBend 10 and EnVision+ in order to block the binding sites to mouse tissues. The resulting polymer immuno-complex (PIC) was only capable of labeling human CD 34 antigen in mouse tissues.

After deparaffinization, endogenous peroxidase was quenched by pretreating the tissue sections in a water bath in target retrieval solution (Dako) for 40 min at 95 °C and then cooling for 20 min to room temperature. The sections were incubated with the PIC for 60 min at room temperature and the CD34-positive human endothelial cells were visualized using a substrate such as DAB or AEC.

Human genomic DNA detection by in situ hybridization. In order to label the endothelial cells originating from human tissues, in situ hybridization using a biotinylated human genomic DNA probe (Dako) was performed. After the pretreatment as described above, the tissue sections were denatured for 5 min at 95 °C and then hybridized with the human genomic DNA probe for 120 min at 50 °C. After hybridization, the human DNA was visualized using an In Situ Detection Kit (Dako). After in situ hybridization, the slides were employed for immunohistochemical detection of human endothelial cells by incubating them with the PIC.

Results

Human vascular networks derived from HAEC and HUVEC in murine tumors

HAEC or HUVEC were administered into the Meth-A solid tumors on days 1, 3, 5, 7, and 9 (total 5 shots) after tumor inoculation. Fifteen days after tumor inoculation, the survivals of HAEC and HUVEC in Meth-A solid tumors were assessed by conducting immunohistochemistry on the murine tumors. Mouse monoclonal antibody (QBend 10), which is widely used with human CD34 antigen as a human endothelial cell marker, does not react with mice CD34 antigen. Not scattered human CD34-positive staining cells but clearly human CD34-

positive vascular networks were visualized in murine tumors that were administered HAEC and HUVEC (Figs. 1A and B). In addition, various sizes of human CD34-positive blood vessels, from small to large, were confirmed (Figs. 1C and D). Double negative staining blood vessels constructed by host (mouse)-derived endothelial cells were also observed (Figs. 1C and D). In situ hybridization was performed against human CD34-positive blood vessels using a specific probe that only hybridizes with human genomic DNA. It was confirmed that this probe stained only the nucleus of human CD34-positive endothelial cells (Fig. 2A). In addition, peripheral blood cells were visualized into human CD34-positive blood vessels (Fig. 2B). The results for HUVEC were the same as those for HAEC (data not shown). The results of immunohistochemistry with human CD34 antigen and in situ hybridization with human genomic DNA suggest that HAEC and HUVEC incorporated into the capillary endothelial cells of the tumors and remodeled new human blood vessels in murine tumors. The results also suggest that new human blood vessels are involved in normal function as host tumor blood vessels due to the presence of peripheral blood cells intravascularly.

Optimal dose-schedule for remodeling of human blood vessels in murine tumors

HAEC were administered into Meth-A solid tumors in SCID mice at different schedules (on days 1–3, 4–6,

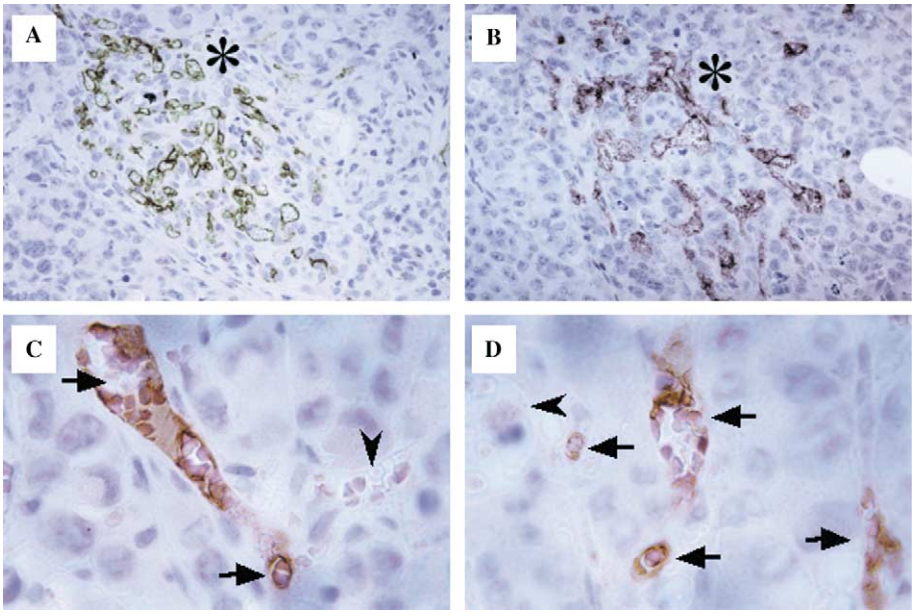

Fig. 1. Immunohistochemical staining of a human CD34-positive blood vessel network derived from HAEC and HUVEC in a murine tumor. Histological sections were prepared from a murine tumor that was administered HAEC (A,C,D) and HUVEC (B). This section was counterstained with human CD34 and HE using the polymer immunocomplex method. A human CD34-positive blood vessel network was observed in the murine tumor (A,B). These vessels were localized at the HAEC and HUVEC administration sites (*). Positive blood vessels from large to small ones (arrow) and double negative staining blood vessels (arrow head) were observed (C,D). Original magnification: 200× (A,B), 400× (C,D).

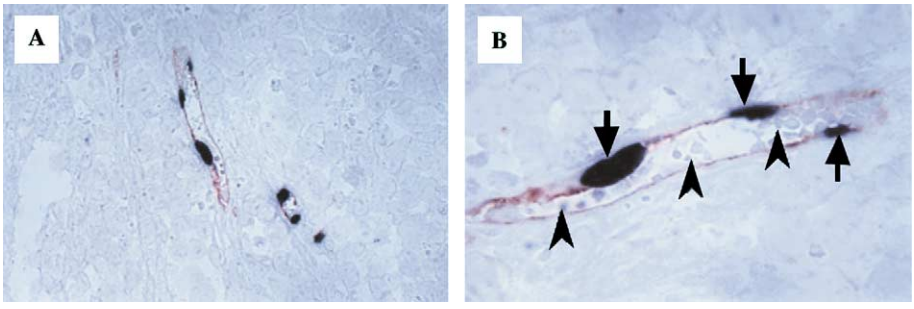

Fig. 2. In situ hybridization of human DNA against human CD34-positive blood vessels. Human CD34-positive blood vessels derived from HAEC underwent in situ hybridization using a specific probe that only hybridizes with human genomic DNA (A,B). Only the nucleus (arrow) of the endothelial cells of these vessels was positive for human genomic DNA. Murine peripheral blood cells (arrow head) were observed in murine tumor vessels derived from HAEC. Original magnification: 200× (A), 400× (B).

Table 1
Angiogenesis assay of human blood vessels during murine tumor angiogenesis

Injection day	Number of human CD34-positive blood vessels
Days 1–3	0
Days 4–6	1127
Days 7–9	621
Days 10–12	15

HAEC were administered into tumors in SCID mice at various times (on days 1–3, 4–6, 7–9, and 10–12 after tumor implantation). Histological sections were counterstained with human CD34 and HE. The number of human CD34-positive blood vessels was counted in 15 randomly extracted tumor sections.

7–9, or 10–12 after tumor inoculation (total 3 shots, respectively)) in order to ascertain whether human blood vessels are remodeled the most rapidly during tumor angiogenesis. Fifteen days after tumor inoculation, human CD34-positive blood vessels were not observed in the murine tumors on days 1, 2, and 3 of HAEC administration. On the other hand, a large number of human CD34-positive blood vessels were observed on days 4, 5, and 6 of HAEC administration in comparison to other time schedules (Table 1). The formation of tumor vascular networks was rapidly initiated from 4 to 6 days after tumor inoculation (data not shown). These results suggest that HAEC and HUVEC remodeled human CD34-positive blood vessels through an effect on murine tumor angiogenesis, and it was optimum to balance between tumor volume (the number of tumor cells) and the number of HAEC administered into tumors on days 4, 5, and 6.

Proliferation of HAEC during remodeling of human blood vessels

We found that these HAEC and HUVEC administered into murine solid tumor not only survived, but also incorporated into tumor endothelium. Additionally, it was proven that the new blood vessels in murine solid tumors were cooperatively formed with human and mouse endothelial cells. We assessed whether the proliferation of HAEC in the murine tumors resulted in a remodeling of new human blood vessels. Human Ki-67 antigen is used as a marker of cell proliferation since this antigen is expressed on human cells in the G1, S, G2, and M cell cycle phases, but not G0. The proliferation of human cells was confirmed by immunohistochemical analysis using human Ki-67 antigen (Fig. 3A). The proliferating human cells were observed in the inner wall of the vessels and they maintained the tumor blood vessels (Fig. 3B). The antibody used in this study only reacts to human Ki-67 antigen. Therefore, these results suggest that new human blood vessels were remodeled as a result of not only the survival but also the proliferation of vascular endothelial cells of normal adult organs in murine tumors.

Discussion

For establishment of vascular regeneration cell therapy and tumor vascular targeting gene therapy, we assessed whether adult organ-derived vascular endothelial cells, such as HAEC and HUVEC, could function as vascular endothelial progenitor cells (EPCs) in vivo. We apprehended the possibility that almost HAEC and HUVEC administered into murine solid tumors only survived, but not proliferated and formed vascular network. But the obtained results overturned our concern. We observed new human vascular networks in murine Meth-A solid tumors that were administered HAEC and HUVEC (Figs. 1A and B). Furthermore, peripheral blood cells were visualized into new human blood vessels and we found that human vascular networks played normal functions as blood vessels (Figs. 2A and B). Human CD34-positive cells were not observed in tumor on days 1–3 of HAEC administration, namely not proliferated (Table 1). However many CD34-positive cells were observed on days 4–6 of administration and

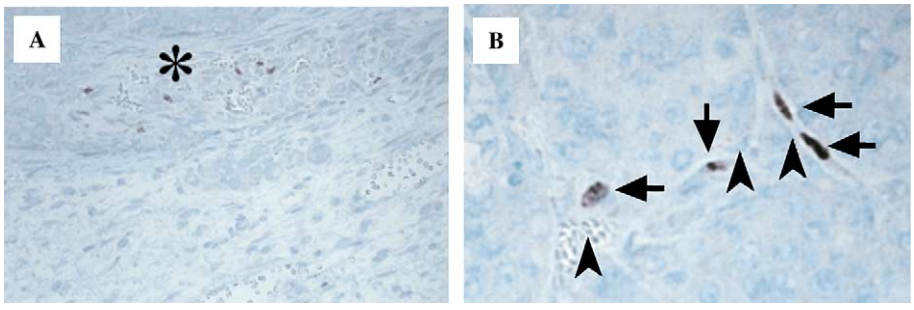

Fig. 3. Immunohistochemical staining of human Ki-67 against a human CD34-positive blood vessel network. Human CD34-positive blood vessel networks were counterstained with human Ki-67 and HE. Human Ki-67-positive cells (arrow) were observed in the area (*) administered HAEC (A). These positive cells (arrow) were involved in normal function as endothelial cells of the tumor blood vessels (arrow head) (B). Original magnification: 200× (A), 400× (B).

the existing HAEC and HUVEC in murine solid tumors expressed human Ki-67 antigen as a marker of cell proliferation (Figs. 3A and B). These data suggested that HAEC administer in optimal time, proliferated and incorporated into tumor vessels. In general, neovasculature in solid tumors is constructed by the migration, proliferation, and redifferentiation of preexisting vascular endothelial cells and EPCs activated by various cytokines such as vascular endothelial cell growth factor (VEGF) [20] and basic fibroblast growth factor (bFGF) [21]. Thus, the inside of tumor mass might be the comfortable soil in which HAEC and HUVEC proliferated and incorporated into tumor vessels.

It is so interesting to note that this tumor vascular network in murine solid tumors is composed of host (SCID mice)-derived endothelial cells and human endothelial cells administered. Mouse and human endothelial cells directly connected and cooperatively formed many blood vessels in murine solid tumors. Additionally, the blood circulated in this chimeric vasculature and solid tumors rapidly proliferated by using this vasculature as life-lines. The detection of an adhesion molecule, which is expressed at the linkage site of human and mouse blood vessels, is currently being attempted.

We strongly suggested that our results and this human vascular network remodeling system offered the following four possibilities. (1) It was confirmed in mice model that ischemic limb was recovered by administration of EPCs [9]. But the pure isolation and large-scale culture of EPCs are difficult. Thus, HAEC and HUVEC may be useful as vascular endothelial progenitor-like cells for vascular regeneration cell therapy. (2) Tumor vascular targeting therapy is expected as one of the most attractive approaches for tumor treatment, because the inhibition of tumor vasculature may result in effective solid tumor death on the ground that one tumor blood vessel supports one thousand tumor cells [1–3]. But there are few vectors and carriers which have the sufficient targeting ability to tumor vasculature. Thus, HAEC and HUVEC transfected the effective gene, such as TNF-α gene, for the tumor treatment may be useful for ex vivo gene therapy targeted to tumor vasculature. (3) In many pathological conditions, wound healing, tumor, and diabetic retinopathy, wound becomes aggravated with the angiogenesis [22]. Consequently, the evaluation systems of angiogenesis in vitro and in vivo were developed and a lot of anti-angiogenic agents have been designed using these systems [23]. Some evaluation systems of tumor angiogenesis in vivo such as CAM (chick embryo chorioallantoic membrane) [24], rabbit cornea [25], and implantation of chamber into mouse back skin method [2] have been used as screening systems of anti-angiogenic agents. However, the question now arises: we cannot evaluate tumor angiogenesis of human but chicken, rabbit, or mouse by the established system above. Frequently, it is observed that anti-angiogenic effects differ in the species. Thus, the human vascular network remodeling system in mice may be useful for the screening and assessing of anti-angiogenic agents. (4) The molecular mechanism of vascular endothelial cells on tumor vascular angiogenesis may be clarified at the DNA and protein levels, because DNA and proteins derived from human endothelial cells can be distinguished from those of the mouse derivation.

In summary, adult organ-derived vascular endothelial cells, such as HAEC and HUVEC, possess the potential to incorporate into tumor vessels such as vascular endothelial progenitor-like cells in vivo. Our results and this human vascular network remodeling system may enable us not only to progress with the vascular regeneration cell therapy and tumor vascular targeting gene therapy, but also to clarify the molecular mechanism of tumor angiogenesis.

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