Pathway for Differentiation of Human Embryonic Stem Cells to Vascular Cell Components and Their Potential for Vascular Regeneration


Objective—We demonstrated previously that mouse embryonic stem (ES) cell–derived vascular endothelial growth factor receptor-2 (VEGF-R2)–positive cells can differentiate into both vascular endothelial cells and mural cells. This time, we investigated kinetics of differentiation of human ES cells to vascular cells and examined their potential as a source for vascular regeneration.

Methods and Results—Unlike mouse ES cells, undifferentiated human ES cells already expressed VEGF-R2, but after differentiation, a VEGF-R2-positive but tumor rejection antigen 1-60 (TRA1-60)–negative population emerged. These VEGF-R2-positive but tumor rejection antigen 1-60–negative cells were also positive for platelet-derived growth factor receptor α and β chains and could be effectively differentiated into both VE-cadherin–endothelial cell and α-smooth muscle actin–mural cell. VE-cadherin–cells, which were also CD34+ and VEGF-R2+ and thought to be endothelial cells in the early differentiation stage, could be expanded while maintaining their maturity. Their transplantation to the hindlimb ischemia model of immunodeficient mice contributed to the construction of new blood vessels and improved blood flow.

Conclusions—We could identify the differentiation process from human ES cells to vascular cell components and demonstrate that expansion and transplantation of vascular cells at the appropriate differentiation stage may constitute a novel strategy for vascular regenerative medicine. (Arterioscler Thromb Vasc Biol. 2007;27:2127-2134.)

Key Words: angiogenesis ■ developmental biology ■ embryonic stem cells ■ vascular biology ■ endothelium
from human ES cells to EC or other vascular cell components, such as MC, in the embryoid body differentiation system has so far not been possible.

In the study reported here, we identified the differentiation kinetics of human ES cells to vascular cell components by using our in vitro 2D differentiation system. Furthermore, we succeeded in establishing new human cell sources derived from human ES cells, which may be used for therapeutically effective transplantation in vivo.

Methods

Cell Culture

ES cells were maintained as described. OP9 feeder cell lines were established and maintained as described, whereas their growth was inactivated by mitomycin C.

To induce differentiation, ES cells were dissociated into small colonies with the aid of 0.1% collagenase (Wako) and cultured on an OP9 feeder layer in a differentiation medium (minimal essential medium, GIBCO) supplemented with 5 × 10^{-3} M 2-mercaptoethanol with 10% FCS. Sorted cells were recultured on a collagen IV–coated dish in the differentiation medium with the addition of 10% FCS, VEGF (100 ng/mL; PeproTech EC Ltd.), or platelet-derived growth factor (PDGF)-BB (10 ng/mL) (PeproTech EC Ltd.).

Flow Cytometry and Cell Sorting

Cells were detached by cell dissociation buffer (GIBCO) with or without collagenase and labeled with various fluorescence-conjugated monoclonal antibodies (please see http://atvb.ahajournals.org). Flow cytometry analysis and cell sorting were performed as described.

Immunohistochemistry

Cultured cells were stained with various monoclonal antibodies (please see http://atvb.ahajournals.org) as described. The immunofluorescence photographs were taken with a confocal laser-scanning microscope (LSM5-Pascal, Carl Zeiss).

Hindlimb Ischemia Model

Eight-week–old KSN/Slc and BALB/c Slc nude mice were purchased from SLC Japan. After anesthetization with pentobarbital (80 mg/kg IP), the right femoral vein was ligated. We injected 5 × 10^6 cells in 100 μL of PBS or 100 μL of PBS only into the right femoral artery. Immediately after the cell injection, the right femoral artery was ligated and excised. Experimental procedures were performed in accordance with Kyoto University standards for animal care. Hindlimb blood flow was measured with a laser Doppler perfusion image analyzer (Moor Instruments Ltd), as described. Biotin-conjugated Griffonia simplicifolia I-isolectin B 4 (Vector Laboratories) was injected into the right femoral artery. Immediately after the cell injection, the right femoral artery was ligated and excised.

Statistical Analysis

Results are presented as mean ± SEM. In the hindlimb ischemia model, the statistical significance was evaluated using ANOVA followed by Fisher’s analysis for comparisons between 2 means. P < 0.05 was considered significant.

Results

Analysis of Angiogenic Factor mRNA Expression

Total cellular RNA was isolated from VPCs or ECs in the early differentiation stage (eECs) with RNAeasy Mini kit (Qiagen KK). The mRNA expression was analyzed with the One-Step RNA PCR kit (TaKaRa). Primer pairs were purchased from R&D Systems Inc. PCRs were performed as manufacturers protocols.

Measurement of Angiogenic Factors in VPC- or eEC-Conditioned Media

Cells (1 × 10^6) of VPCs or ECs were plated on 10-cm dishes and incubated with 5 mL of media (minimal essential medium with 0.5% bovine serum) for 72 hours. The concentrations of human VEGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) were measured by SRL, Inc. Human PDGF-BB was measured with the Human PDGF-BB Quantikine ELISA kit (R&D Systems Inc).

Vascular Cell Components

First, we examined the expression of VEGF-R2 and some putative stem cell markers on a human ES cell line, HES3, which was established at Monash University in Australia. Approximately 50% of undifferentiated human ES cells expressed VEGF-R2, whereas these cells were also positive for AC133 and c-Kit but negative for CD34, respectively (Figure 1A). We also analyzed the expression of tumor rejection antigen (TRA) 1-60 on these human ES cells. The TRA1 antigen is expressed on the surface of human tetracarcinoma stem cells, human embryonic germ cells, and human ES cells. Thus, we used it as a marker of the human ES cell. The VEGF-R2* population of human ES cells was also positive for TRA1-60 (Figure 1B).

Next, we induced differentiation of human ES cells in an in vitro 2D culture on an OP9 layer, human ES cells have not survived as single cells. We, therefore, plated small human ES cell colonies on OP9 to induce differentiation. Under these conditions, the TRA1-60* cell population gradually decreased in number during differentiation. On the other hand, a VEGF-R2* TRA1-60* population emerged and accounted for 15% of all of the cells on day 8 (Figure 1C). We confirmed the differentiation kinetics of human ES cells by using another human ES cell line, KhES-1, established by us. Similar to the HES3 cell line, VEGF-R2 was low positive, and the VEGF-R2* cells were also TRA1-60* in undifferentiated KhES-1 (Figure 1D). After differentiation on an OP9 feeder layer, VEGF-R2* TRA1-60* cells decreased, and VEGF-R2* TRA1-60* cells appeared on days 8 (Figure 1E). Next, we analyzed the expression of several cell surface markers on the VEGF-R2* TRA1-60* population on day 8 of HES3. Flt1 was positive, c-Kit and CXCR4 were both negative, PDGFR receptor (PDGFRα and PDGFRβ) were positive, AC133...
was still positive, and CD34 and vascular endothelial cadherin (VE-cadherin) were negative on a large population of the VEGF-R2/TRAP1-60 cells (Figure 1F). Monocyte markers, such as CD45, Cad11b, and CD14, were negative.

The VEGF-R2/TRAP1-60 and VE-cadherin-negative cells were sorted by flow cytometry on day 8 and cultured on a collagen IV-coated dish without a feeder cell layer for an additional 8 days in the presence of 10% FCS and VEGF. This cell culturing condition induced the emergence of CD34+, VE-cadherin+, CD31+, and endothelial NO synthase–positive cells (Figure 2A through 2D), which can be categorized as ECs. The rest of the cells negative for CD31 were polygonal in shape and showed α-smooth

Figure 1. Flow cytometry analysis of differentiation kinetics of human ES cells.
A, Expression of cell surface markers on undifferentiated human ES cells (HES3).
B through E, TRA1-60 and VEGF-R2 expression on 2 human ES cell lines (HES3 and KhES-1) during differentiation on an OP9 feeder layer. B and C, HES3; D and E, KhES-1. F, Cell surface marker expression on VEGF-R2/TRAP1-60 cells on day 8.

Figure 2. Immunocytological analysis of differentiation of vascular progenitor cells into vascular cells. A through D, Immunostaining for endothelial cell markers on VEGF-R2/TRAP1-60 cells recultured with VEGF and FBS. A, CD34; B, VE-cadherin; C, CD31; D, endothelial NO synthase; E, Double immunostaining for CD31 (blue) and α-smooth muscle actin (brown). F and G, Immunostaining for MC markers on VEGF-R2/TRAP1-60 cells recultured with FBS. F, α-Smooth muscle actin; G, Calponin; H, Immunostaining for α-smooth muscle actin with treatment of PDGF-BB on VEGF-R2/TRAP1-60 cells. Scale bars, 50 μm.
muscle actin expression (Figure 2E). In the absence of VEGF, VEGF-R2/TRA1-60 cells did not differentiate into ECs, but almost all of them differentiated into α-smooth muscle actin and calponin-positive cells, which can be categorized as MCs (Figure 2F and 2G). Because these VEGF-R2/TRA1-60 cells expressed PDGFRβ, PDGF-BB with 0.5% FCS induced MC induction in a similar manner (Figure 2H). We, therefore, concluded that these VEGF-R2/TRA1-60 cells could be categorized as human VPCs that can differentiate into both ECs and MCs.

Next we examined whether VEGF-R2/TRA1-60 cells after 8 days of differentiation is immature or not. Before differentiation, VEGF-R2/TRA1-60 cells were positive for flt1, AC133, and c-Kit and negative for CXCR4, PDGFRα, PDGFRβ, CD34, and VE-cadherin. However, after 8 days of differentiation, c-Kit expression decreased, and PDGFRα-positive and/or β-positive cells appeared in VEGF-R2/TRA1-60 cells (data not shown). Thus, VEGF-R2/TRA1-60 cells after 8 days of differentiation were not equivalent to the immature ES cells on day 0.

Isolation and Expansion of Vascular eECs

Next, we focused our attention on VE-cadherin+ ECs that were more differentiated than the VPC. On 10 days of differentiation of HES3 on an OP9 feeder layer, VEGF-R2+ and VE-cadherin+ cells emerged and accounted for ∼1% to 2% of all the cells (Figure 3A). This VE-cadherin+ cell population was almost identical to the CD34+ population (Figure 3A). We sorted these VE-cadherin+ cells, and, because these cells were also VEGF-R2+ and CD34+ (Figure 3A), we used the term “eEC” for these EC in the early differentiation stage. These cells formed a network-like structure on Matrigel in vitro (Figure 3B), showed a cobblestone appearance when they became confluent (Figure 3C), and immunofluorescence staining with CD31 showed a characteristic marginal staining pattern (Figure 3D). These eECs were negative for monocyte makers CD45, CD11b, and CD14 (data not shown) and could be successfully propagated by a factor of ∼1.2×10^2 (from 2×10^5 cells to 2.4×10^7 cells) after 6 passages on collagen IV–coated dishes. They were cultured with a cell density of 1.5×10^4 cells/cm^2 with VEGF because they did not expand when they were more sparsely plated or cultured without VEGF. Flow-cytometric analysis showed that VE-cadherin+ cells were reduced to ∼35% of the total number of cells after 6 passages (Figure 3E), but they were still VEGF-R2+, CD34+, and CD31+ at the sixth passage, indicating that the cell differentiation stage had been maintained (Figure 3E). In another series of experiments, we sorted these VE-cadherin+ cells on day 10 and replated them on an OP9 feeder layer or nonfeeder collagen IV–coated dishes for 1 additional week. The VE-cadherin+ cells in these 2 culture groups were then resorted and plated on nonfeeder collagen IV–coated dishes for reculturing. After an additional 3 weeks of reculturing, VE-cadherin expression was examined. The cells that were cultured for 1 additional week on OP9 were 90% positive for VE-cadherin, but the cells kept on nonfeeder dishes were only 44% positive. This suggests that VE-cadherin+ eECs still retain the potential to differentiate.
into other cell types, but more differentiated VE-cadherin−ECs may lose this ability.

Transplantation of Human ES Cell-Derived Vascular Cells to the Hindlimb Ischemia Model of Immunodeficient Mice

As the next step, we investigated whether human ES cell–derived vascular cells can be used for vascular regeneration in cell transplantation in the hindlimb ischemia model. KSN nude mice received an intra-arterial injection of cells in PBS or PBS only into the right femoral artery, followed by right femoral artery ligation and removal to create hindlimb ischemia. VEGF-R2/TRA1-60 cells, identified as VPCs, were transplanted, but laser Doppler perfusion image analysis on day 14 showed no significant difference in recovery of the blood flow (expressed as the ischemic/normal limb blood perfusion ratio) between the cell-transplanted mice (0.496±0.29; n=10) and the control PBS-injected mice (0.433±0.42; n=14). Next, we used the eECs expanded at passages 4 to 6. As shown in Figure 4A and 4B, the hindlimb blood flows had significantly improved in the cell-injected group 9 and 12 days after injection. For histological analysis, transplanted cells had been labeled with CM-DiI before cell transplantation, and biotin-conjugated isolectin B4 was intravenously injected to stain ECs before sacrifice on day 14. Although some of the transplanted cells were incorporated as isolectin B4+ vascular ECs in the large vessels (Figure 5A), most of the transplanted cells were incorporated as small capillaries (Figure 5B). To quantify the capillary density, sections of the ischemic hindlimbs were stained with anti-mouse and human-specific CD31 antibodies (Figure 5C and 5D). Human CD31+ capillaries were detected in the expanded eEC-transplanted mice. The mouse and/or human CD31+ total capillary number and area significantly increased in the expanded eEC-transplanted group compared with the control PBS-injected group, whereas there was tendency but no significant difference in the mouse CD31+ host capillary number and area (Figure 5E and 5F). On the other hand, there was no significant difference in the capillary length (Figure 5G). Because we cut our sections at right angles with muscle fibers and femoral artery, it might be difficult to estimate the capillary density by vessel length.

Next we performed our transplantation experiments with the same procedure using BALB/c Slc nude mice, in which hindlimb ischemia is more severe than in KSN/Slc nude mice. PBS, VPCs, eECs or human adult aortic ECs were transplanted, and the ischemic hindlimbs were observed on day 14. In the PBS-injected mice, the ischemic hindlimb was autoamputated in 3 of 7 mice, and mild necrosis was observed in 1 of 7. In the VPC-transplanted mice, 3 of 7 were autoamputated and mild necrosis was seen in 2 of 7. In the eEC-transplanted mice, the ischemic hindlimb was not auto-amputated, and only mild necrosis was observed in 2 of 7. In the human adult aortic EC-transplanted mice, 4 of 7 were auto-amputated, and mild necrosis was seen in 1 of 7. Furthermore, sections of the ischemic hindlimb in mice without autoamputation were stained with anti-mouse and human-specific CD31 antibodies. Human CD31+ capillaries were most abundant in the eEC-transplanted mice, although some human CD31+ cells were detected in the VPC or human adult aortic EC-transplanted mice (please see http://atvb.ahajournals.org).

Exclusion of Possible Teratoma Formation by the Expanded eEC

Further experiments were conducted to detect possible teratoma formation by eEC. We conducted long-term follow-ups...
by transplanting expanded eECs or undifferentiated human ES cells into 3 mice each and following them for 5 months. We transplanted $5 \times 10^5$ cells under the dorsal back skin of SCID mice, which are commonly used for teratoma formation for human ES cells. Large tumors had formed after 3 to 5 months in 2 of the 3 mice in the human ES cell–transplanted group, but none had formed in any of the 3 mice in the expanded eEC-transplanted group. In immunohistological analysis, HLA-ABC tumors were not observed in the subcutaneous region of eEC transplanted mice, although only a few HLA-ABC human cells were remaining (data not shown).

Expression of Angiogenic Factors in Human ES Cell–Derived Vascular Cells
In addition, we investigated whether VPC or eEC produced major angiogenic factors such as VEGF, bFGF, human growth factor, and PDGF-BB. RT-PCR analysis detected mRNA expressions of VEGF, bFGF, and human growth factor in VPCs and PDGF-B and bFGF in eECs (please see http://atvb.ahajournals.org). We measured the protein concentration of these angiogenic factors in culture media by ELISA; however, the concentration of VEGF, human growth factor, and PDGF-BB did not reach the detectable level, and the concentration of bFGF was $<30$ pg/mL.

Discussion
In this study, we were able to clarify the differentiation process from human ES cells to mature vascular cell components. In adults, VEGF and PDGF receptors are expressed on EC and MC, respectively, and VEGF and PDGF stimulate the growth of the respective cell types. In this study, human ES cell–derived VPCs expressed both VEGF and PDGF receptors. In addition, stimulation with VEGF and PDGF-BB induced 2 differentiation pathways for EC and MC in this cell population. In mouse embryos, VEGF-R2 and PDGFRα were reported to be expressed in the mesoderm. In whole-mount immunohistochemistry of mouse embryos (E7.5 to 8), VEGF-R2 was expressed predominantly in the extraembryonic and proximal-lateral mesoderm. PDGFRα was detected mainly in the paraxial embryonic mesoderm. Both VEGF-R2 and PDGFRα were
detected in the anterior paraxial mesoderm. It was also reported that vascular endothelial precursors were identified from the cephalic mesoderm of the avian embryo labeled using an antibody against Quek1 (avian homolog of VEGF-R2).12 Our result that VEGF-R2PDGF-R VPCs can differentiate into vascular cells may agree with their reports. In our transplantation examination, some human CD31 ECs were observed in the ischemic hindlimb of VPC-transplanted mice. This suggests that some transplanted VPC (negative for CD31) differentiated into CD31 ECs in vivo.

In addition, we investigated whether human ES-derived vascular cells can be used for vascular regeneration. Transplanted eECs were successfully incorporated into the host circulation and significantly accelerated improvement of local blood flow, whereas VPCs did not. We reported recently that VEGF-R2 cells derived from mouse ES cells could differentiate into not only vascular cells but also cardiomyocytes.13 Thus, VPCs may be too immature to be used directly as the source for vascular regeneration. It has also been reported that ischemia-induced neovascularization did not improve in mice receiving human mature ECs (eg, human microvascular ECs).14 Their result is compatible with our result that human adult aortic EC transplantation had no effect for the prevention of ischemic necrosis. The induction and isolation of the cells at the differentiation stage most appropriate for transplantation seem to be important. Judging from our results obtained from histological analysis and capillary density evaluation, at least some of the therapeutic effect of transplantation of expanded eECs could be attributed to vascular regeneration as a result of incorporation of the transplanted cells into the host vessels. Because RT-PCR analysis detected mRNA expression of PDGF-B not in VPCs but in eECs, PDGF-BB secretion might affect the effect of eEC transplantation, although PDGF-BB did not reach the detectable level in culture media. In adults, endothelial progenitor cells (EPCs) reportedly participate in postnatal angiogenesis,15 whereas other reports suggest that EPCs contribute to neovascularization in tissue ischemia.16 However, the expansion of EPCs in sufficient quantities to improve blood flow in large animals has not yet been achieved. In addition, some recent reports suggest that adult bone marrow-derived cells, such as EPCs, do not transdifferentiate into ECs under physiological conditions.16,17 Although the role of EPCs as a modifier of vascular growth awaits further investigation, our findings may help provide an alternative and novel supply of vascular cells for cell therapy as a contribution to vascular regenerative medicine.

Furthermore, the establishment of an in vitro differentiation system of human vascular cell components from human ES cells in this study should also make it possible to dissect out cellular mechanisms in human vascular development and diseased states for which the knockout animal research approach is not practical. Trials on gene expression profiling using our in vitro differentiation system of human vascular cells from human ES cells could assist in the search for novel gene products to develop new therapeutic approaches for vascular regeneration.

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Disclosures

None.

References


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