Different Differentiation Kinetics of Vascular Progenitor Cells in Primate and Mouse Embryonic Stem Cells

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Background—We demonstrated that vascular endothelial growth factor receptor 2 (VEGF-R2)-positive cells derived from mouse embryonic stem (ES) cells can differentiate into both endothelial cells and mural cells to suffice as vascular progenitor cells (VPCs). Here we examined whether VPCs occur in primate ES cells and investigated the differences in VPC differentiation kinetics between primate and mouse ES cells.

Methods and Results—In contrast to mouse ES cells, undifferentiated monkey ES cells expressed VEGF-R2. By culturing these undifferentiated ES cells for 4 days on OP9 feeder layer, VEGF-R2 expression disappeared, and then reappeared after 8 days of differentiation. We then isolated these VEGF-R2-positive and vascular endothelial cadherin (VEcadherin)-negative cells by flow cytometry sorting. Additional 5-day reculture of these VEGF-R2+ VEcadherin− cells on OP9 feeder layer resulted in the appearance of platelet endothelial cell adhesion molecule-1 (PECAM1)-positive, VEcadherin-positive, endothelial nitric oxide synthase (eNOS)-positive endothelial cells. On a collagen IV-coated dish in the presence of serum, these cells differentiated into smooth muscle actin (SMA)-positive and calponin-positive mural cells (pericytes or vascular smooth muscle cells). Addition of 50ng/mL VEGF to the culture on a collagen IV-coated dish resulted in the appearance of PECAM1+ cells surrounded by SMA+ cells. In addition, these differentiated VEGF-R2+ cells can form tube-like structures in a 3-dimensional culture.

Conclusion—Our findings indicate that differentiation kinetics of VPCs derived from primate and mouse ES cells were different. Differentiated VEGF-R2+ VEcadherin− cells can act as VPCs in primates. To seek the clinical potential of VPCs for vascular regeneration, investigations of primate ES cells are indispensable. (Circulation. 2003;107:2085-2088.)

Key Words: angiogenesis ■ cells ■ endothelium ■ muscle, smooth ■ vessels

Embryonic stem (ES) cells with pluripotency and self-renewal are now highlighted as promising cell sources for regeneration medicine. Previously we demonstrated that mouse ES cell-derived vascular endothelial growth factor receptor-2 (VEGF-R2)-positive cells can differentiate into both endothelial cells and mural cells (pericytes and vascular smooth muscle cells) and reproduce the vascular organization process.1 Vascular cells derived from VEGF-R2− cells can organize vessel-like structures in a 3-dimensional culture. Mouse ES cell-derived VEGF-R2+ cells can, thus, serve as vascular progenitor cells (VPCs). Furthermore, we have reported that implantation of mouse ES-derived vascular cells into nude mice significantly augmented blood flow in an adult neoangiogenesis model, which suggests the usefulness of ES cell-derived VPCs for vascular regeneration medicine.2 Recently primate embryonic stem cell lines were established from blastocysts of both humans and monkeys.3–6 Primate ES cells possess a number of characteristics distinct from mouse ES cells, such as surface antigens, leukemia inhibitory factor (LIF)-independence, and long doubling times.3–7 Recent study showed that VEGF-R2 was expressed in undifferentiated human ES cells, unlike in mouse ES cells,8,9 and continuously expressed during differentiation in embryoid body (EB) formation. It has also been demonstrated that platelet endothelial cell adhesion molecule-1 (PECAM1)-positive cells can be isolated from human EBs,
and they can act as endothelial cells. However, the vascular differentiation process of primate cells has not been demonstrated, and VPCs that can differentiate into both endothelial cells and mural cells have not been characterized in the primates. To elucidate the vascular differentiation process of primate cells and to seek the clinical potential of VPCs for vascular regeneration therapy with the use of an in vitro 2-dimensional differentiation system of ES cells that we established, we examined whether and how VPCs occur in primate ES cells in comparison to mouse ES cells.

**Methods**

**Cell Culture**

Cynomolgus monkey ES cell lines were established, and their pluripotency was confirmed by teratoma formation in severe combined immunodeficiency mice, as described previously. Undifferentiated ES cells were maintained as described. OP9 feeder cell lines that were established from mouse calvaria were maintained as described previously.

To induce differentiation, undifferentiated ES cells were cultured on OP9 feeder layer in differentiation medium (minimal essential medium [GIBCO] supplemented with 10% fetal calf serum [FCS] and 5×10⁻⁷ M2-mercaptoethanol). Sorted VEGF-R2² cells were re-cultured on an OP9 feeder layer or collagen IV-coated dish with differentiation medium. Three-dimensional culture was performed as described.

**Flow Cytometry and Cell Sorting**

At different time points during the differentiation process, cultured cells were harvested by cell dissociation buffer (GIBCO). Flow cytometry analysis and cell sorting were as described. Monoclonal antibody for VEGF-R2, which we developed, was labeled with Alexa-647 in our laboratory (monoclonal antibody labeling kit, molecular probes). PE-conjugated vascular endothelial cadherin (VEcadherin) antibody and fluorescein isothiocyanate-conjugated PECAM1 antibody were purchased from BD Biosciences. To test the differentiation potential of VEGF-R2² cells, sorted cells were plated into a collagen IV-coated 96-well dish at the density of 2.5×10³ cells per well, or plated on OP9 feeder layer in a 24-well dish at 1×10⁴ cells per well.

**Immunohistochemistry**

Staining of cultures on dishes was as described. Monoclonal antibody for smooth muscle actin (SMA) was purchased from Sigma, those for calponin and smooth muscle myosin heavy chain (SMMHC) were purchased from DAKO, and those for PECAM1, VEcadherin, and endothelial nitric oxide synthase (eNOS) were purchased from BD Biosciences.

**Results**

Although undifferentiated mouse ES cells did not express VEGF-R2, most of undifferentiated monkey ES cells were positive for VEGF-R2 (data not shown). Mouse ES cells differentiated into VEGF-R2² cells during a 4-day differentiation on OP9 feeder layer. These mouse VEGF-R2² cells differentiated into endothelial cells during 4 days of re-culturing on a collagen IV-coated dish or OP9 feeder layer. In contrast, however, we could not induce endothelial cells from VEGF-R2² undifferentiated monkey ES cells in the same condition (data not shown). Thus, we examined VEGF-R2 expression on monkey ES cells during differentiation.

Undifferentiated monkey ES cells were dissociated to single cells and plated on an OP9 feeder layer to induce differentiation (Figure 1A). As shown in Figure 1B, VEGF-R2 was expressed in undifferentiated monkey ES cells (day 0), but disappeared during a 4-day differentiation on OP9 feeder layer, and then reappeared after 8 days of differentiation. VEcadherin² cells appeared at day 10 of differentiation. Alkaline phosphatase activity, which was reported to be detected in undifferentiated ES cells but not in mature vascular cells, was clearly detected in undifferentiated ES cells but not in the cells that were cultured for 8 days on an OP9 feeder layer (Figure 1C and 1D), indicating that VEGF-R2² cells at 8-day differentiation are apparently distinct from those observed in undifferentiated ES cells.

VEGF-R2² VEcadherin² cells were purified by flow cytometry sorting at day 8 (Figure 1A). Additional 5-day culturing of VEGF-R2² VEcadherin² cells on an OP9 feeder layer resulted in the appearance of PECAM1² cells (Figure 2A), which were also positive for VEcadherin and eNOS (Figure 2B and 2C). On the other hand, on a collagen IV-coated dish with 10% FCS, more than 90% of VEGF-R2² VEcadherin² cells became positive for SMA (Figure 2D) and calponin (Figure 2E) after an additional 5 days of culturing. Some were positive for SMMHC (Figure 2F).

In this culturing condition, PECAM1² endothelial cells did not appear. In contrast, addition of 50ng/mL VEGF to culture on a collagen IV-coated dish resulted in the appearance of PECAM1² cells (about 20% of total cells) that were surrounded by SMA² cells (Figure 2G). VEGF-R2² VEcadherin² cells at day 10 also could differentiate into endothelial cells and mural cells similarly to day 8. VEGF-R2² VEcadherin² cells doubled themselves in about 44 hours on a collagen IV-coated dish with VEGF and FCS. Almost all of the VEGF-R2² VEcadherin² cells obtained by flow cytometry sorting at day 10 became positive for PECAM1 after an additional 5 days of culturing on a collagen IV-coated dish with 10% FCS.

We further examined whether VEGF-R2² cells can form vascular structure in vitro. Aggregates of several hundred VEGF-R2² cells were cultivated in collagen I-A gels with 10% FCS, 50ng/mL VEGF, 50ng/mL basic fibroblast growth factor, and 100 pM phorbol myristate acetate. The cells migrated out from the aggregates and formed cord-like structures within 3 days (Figure 2H).

**Discussion**

In the previous study, we showed that VEGF-R2² cells in 4-day differentiation of mouse ES cells can differentiate into 2 major vascular cell types (endothelial cells and mural cells) in vitro and in vivo. Unlike mouse ES cells, undifferentiated monkey ES cells were already expressing VEGF-R2, similar to human ES cells. VEGF-R2 expression on monkey ES cells disappeared during 4-day differentiation on an OP9 feeder layer, and then re-expressed after 8 days of differentiation (Figure 1C). VEGF-R2–expressing cells on day 8 were different from VEGF-R2² undifferentiated monkey ES cells. First, the former did not show alkaline phosphatase activity as the latter did. Second, the former could differentiate into endothelial cells but the latter could not. Thus, the VEGF-R2² cells that re-appeared at 8 days of differentiation in monkey ES cell differentiation seem to possess similar differentiation potentials to those in 4-day differentiation of mouse ES cells,
whereas VEGF-R2 expression in undifferentiated monkey ES cells should be of less functional significance in vascular differentiation.

In the present study, we demonstrated that VEGF-R2+ VEcadherin+ cells that appeared at 8-days' differentiation in monkey ES cells give rise to both endothelial cells and mural cells and form vascular-like structures in a 3-dimensional culture in vitro. Our findings indicate that differentiated VEGF-R2+ cells can act as VPCs in primates, and the differentiation kinetics of VPCs in primate and mouse ES cells were different. Thus, to seek the clinical potential of VPCs for vascular regeneration and to

Figure 1. A, Schematic representation of the differentiation from ES cells to endothelial cells and mural cells. B, Flow cytometric analysis of the time course of differentiation of primate ES cells on an OP9 feeder layer. C, Alkaline phosphatase activity of undifferentiated ES cells on mouse embryonic fibroblast layer. D, Alkaline phosphatase activity of ES cells differentiated for 8 days on an OP9 feeder layer. Flk1 is a VEGF-R2. Scale bars: 100 μM.

Figure 2. Immunohistochemical analysis of differentiation of primate ES cells into vascular cells (A through G). A through C, Immunostaining for endothelial cell markers: PECAM1 (A), VEcadherin (B), and eNOS (C). D through F, Immunostaining for mural cell markers: smooth muscle actin (D), calponin (E), and smooth muscle myosin heavy chain (F). G, Double immunostaining for PECAM1 (brown) and smooth muscle actin (blue). H, Tube formation of VEGF-R2+ cell aggregates in 3-dimensional culture. Scale bars: B through G, 50 μM; A and H, 100 μM.
obtain novel insights in primate vascular development, investigations of primate ES cells are indispensable. Our novel in vitro vascular differentiation system using VPCs derived from primate ES cells is promising for dissecting the molecular and cellular mechanisms in the primate vascular development, to which the knock-out animal research approach is not available.

References
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