Model of Vasculogenesis from Embryonic Stem Cells for Vascular Research and Regenerative Medicine

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Abstract—Embryonic stem (ES) cells are highlighted as promising cell sources for regenerative medicine. Here, we focused on providing the platform that forced ES cells to reproduce the vascular organization process, leading to efficiency and safety evaluation as preclinical testing of biological agents. Murine ES cell–derived embryoid bodies on matrigel, but not collagen or gelatin, could be differentiated into sprouting blood vessels without the addition of growth factors. The expression of endothelial cell marker CD31 and smooth muscle marker α-smooth muscle actin was partially colocalized and started to increase 7 days after culture on matrigel, accompanied by the induction of a number of growth factors, such as vascular endothelial growth factor, fibroblast growth factor-2, hepatocyte growth factor, transforming growth factor-β, and angiopoietin-1. Moreover, notch-related genes, such as Del1 or Del4 (Δ-like 1/4) and hey1 or hey2 (hairy/enhancer of split related TRPW motif 1/2), were upregulated in a similar time course. The treatment of neutralizing antibodies against these growth factors failed to inhibit the differentiation into the sprouting blood vessels, whereas arginine-glycine-aspartic peptide, a selective inhibitor for the αvβ3-integrins, did inhibit differentiation. An anticancer drug to inhibit angiogenesis, TNP-470, also blocked the vascular formation in this model. ES cells could reproduce the vascular organization process on the biosynthetic scaffolds, such as matrigel, without the addition of growth factors. In the future, a human ES-based tissue model would be an optional tool for the screening of pharmaceutical drugs for vascular disease. (Hypertension. 2006;48:112-119.)

Key Words: vasculature ■ endothelial growth factors ■ endothelium

The potential of stem cells and related cell-based therapies to treat disease and injury in humans has generated great excitement in the scientific community as well as among patients and their advocates. Especially, embryonic stem (ES) cells with pluripotency and self-renewal are now highlighted as promising cell sources for regeneration medicine, although ethical and political debates about the use of human embryos in medical research have captured the public eye. When leukemia inhibitory factor (LIF) is removed, ES cells are spontaneously differentiated into cystlike structures, termed embryoid bodies (EBs), which contain derivatives of the 3 primitive germ layers.1 The appearance of blood island-like structures that consist of immature hematopoietic cells surrounded by endothelial cells (ECs) suggest that ES cells produce all the factors necessary for the induction of vasculogenesis.2–5 It has been known that ES cell–derived Flk-1 positive cells could be differentiated into ECs and mural cells (pericytes and smooth muscle cells) and reproduce the vascular organization process.6,7 However, it did not show the sprouting vessel formation colocalized with ECs and mural cells and requires treatment with growth factors such as vascular endothelial growth factor (VEGF).

In this study, we focused on providing the platform that forces ES cells into the blood vessels without growth factors. It is noteworthy to identify the conditions under which ES cells would reproduce the vascular organization process, leading to efficient and safety evaluation instead of animal experiments as preclinical testing of biological agents. Thus, we developed a unique model on the biosynthetic scaffolds to differentiate ES cells into the blood vessels in this study.

Materials and Methods

Cell Culture

Mouse ES cell lines (129/Ola-derived) called h7 (obtained from Hitoshi Niwa, Kobe, Japan) were maintained undifferentiated without feeder layer on gelatin-coated dishes in the presence of 1000 U/mL LIF (ESGRO, Chemicon International, Temecula, Calif) as previously described.8 The hanging drop method was used to produce EBs to induce the differentiation of ES cells. An ES cell suspension containing 600 cells in 20 μL differentiation medium was placed on the lids of Petri dishes so that ES cells could aggregate and...
differentiate spontaneously after putting the lids back on the dishes containing 10 mL PBS. After culturing the ES cells in hanging drops for 3 days, EBs were transferred into bacterial Petri dishes containing 10 mL differentiation medium to continue floating cultivation for 2 days. Then, EBs were transferred onto a matrigel-coated (growth factor–reduced matrigel matrix, BD Biosciences, Bedford, Mass), collagen-coated (Bicocat BD, Franklin, NJ), or 0.1% gelatin-coated (Sigma, St. Louis, Mo) dish, respectively.

RT-PCR
Total RNAs were isolated from EBs on matrigel using Isogen (Wako, Osaka, Japan). For the PCR reaction, first-strand cDNA (the equivalent of 50-ng reverse-transcribed RNA) was amplified in a final volume of 20 μL with 0.5 U LA-Taq (Takara, Otsu, Japan) and 20 pmol of each nucleotide primer. RT-PCR was performed with the specific primers as previously described shown in the Table. All RNA samples were adjusted to yield equal amplification of hypoxanthine phosphoribosyltransferase as an internal standard.

For quantitative real-time PCR analysis, mRNA of ES cells was extracted using the RNeasy Mini kit (QIAGEN, Valencia, Calif). cDNA was synthesized using the Thermo Script RT-PCR system (Invitrogen, Carlsbad, Calif). Relative gene copy numbers of CD31 (platelet-endothelial cell adhesion molecule ([PECAM]) Mm00476702), VE-cadherin (Mm00459467), transgelin ([SM22α] Mm00441660), acidic protein ([GFAP] Mm00546086), bone associated protein 2 ([MAP2] Mm00485230), glial fibrillary acidic protein ([GFAP] Mm00546086), xanthine phosphoribosyltransferase as an initial standard. The absolute number of gene copies was standardized using TaqMan gene expression assays (Applied Biosystems, Foster City, Calif). The absolute number of gene copies was standardized by a sample standard curve. Results are expressed as fold-increase relative to the GAPDH for copy numbers of each mRNA.

Specific Primers for Each Gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Size</th>
</tr>
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<tbody>
<tr>
<td>Tie-2</td>
<td>277 bp</td>
</tr>
<tr>
<td>R: 5'-GAATAGCCATCACCTGTTGCC-3'</td>
<td></td>
</tr>
<tr>
<td>Flik-1</td>
<td>269 bp</td>
</tr>
<tr>
<td>R: 5'-GTAATTTCCACACAAGC-3'</td>
<td></td>
</tr>
<tr>
<td>PECAM</td>
<td>260 bp</td>
</tr>
<tr>
<td>R: 5'-CTCTGCGGCTGAGCTGAGA-3'</td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>240 bp</td>
</tr>
<tr>
<td>R: 5'-CCTGTGCTGCTGAGCTGAGA-3'</td>
<td></td>
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<tr>
<td>SM-MHC</td>
<td>323 bp</td>
</tr>
<tr>
<td>R: 5'-CCCTGACATGTGCTCAATC-3'</td>
<td></td>
</tr>
<tr>
<td>SM22α</td>
<td>327 bp</td>
</tr>
<tr>
<td>R: 5'-GAGTTGAGCCACCTGCTCCATCG-3'</td>
<td></td>
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<tr>
<td>HPRT</td>
<td>248 bp</td>
</tr>
<tr>
<td>R: 5'-CAAGAAGCTAGAACACCTG-3'</td>
<td></td>
</tr>
</tbody>
</table>

SM-MHC indicates SM-myosin heavy chain; HPRT, hypoxanthine phosphoribosyltransferase.

Figure 1. (a) Mouse ES cell lines, ht7, differentiated into vascular cells on matrigel. Typical examples of EBs at 7 days after the culture on gelatin-, collagen-, or matrigel-coated dishes (upper panel: ×40, lower panel: ×100 magnification). (b) Fluorescent immunostaining of EBs 10 days after the culture on a matrigel-coated dish. Upper panel shows the staining with anti-PECAM antibody (×40), anti-α-SMA antibody (×40), and anti-VE-cadherin antibody (×40). Lower panel shows the staining with anti-PECAM antibody (green: ×100), anti-α-SMA (red: ×100), and merged view (×100).

Immunostaining
EBs were stained with PECAM, VE-cadherin, and α-SMA. Briefly, cells were fixed for 30 minutes at room temperature with methanol, permeabilized for 5 minutes with 0.2% Triton X-100, and 1% goat serum. They were then incubated with the antibody for PECAM, VE-cadherin (1:100; Phamingen, San Diego, Calif) and α-SMA (1:100, Sigma) for overnight and Alexa Fluor 488 anti-rat antibody or 688 anti-mouse antibody (1:200, Molecular Probes, Eugene, Ore) for 45 minutes. All pictures were taken after 3 washes in PBS with 1% goat serum with 20 minute incubation time at room temperature.

Fluorescence-Activated Cell Sorting and Immunostaining
A total of 2x10^6 cells were resuspended in 200 μL PBS containing 3% BSA and incubated for 30 minutes with fluorescence-labeled antibodies against CD31-PE and flik-1-fluorescin isothiocyanate or the respective isotype control (BD Biosciences). After washing, the labeled cells were analyzed in the channel: fluorescein isothiocyanate in FL1, and orange fluorescence in FL2 by flow cytometry by means of a fluorescence-activated-cell sorter (FACS) calibur flow cytometer and CellQuest Pro software (BD Biosciences).

Neutralizing Antibody, Arginine-Glycine-Aspartic, and TNP-470
In these culture experiments, the inhibitory effects of VEGF, FGF-2, HGF, and TGF-β activity were examined by neutralizing antibody for VEGF (10 μg/mL, rabbit polyclonal IgG, Neomarkers Co., Fremont, Calif), TGF-β (5 μg/mL, rabbit polyclonal IgG, R&D
Systems, Minneapolis, Minn), FGF-2 (2.5 μg/mL, mouse monoclonal IgG, Upstate Biotechnology, Lake Placid, NY), HGF (10 μg/mL; obtained from Division of Biochemistry, Biomedical Research Center, Osaka University Graduate School of Medicine), and normal rabbit IgG (10 μg/mL). The cyclic integrin αv antagonist [cyclic Arg-Gly-Asp-D-Phe(N-Methyl)Val] and the control peptide [cyclic Arg-β-Ala-Asp-D-Phe(N-Methyl)Val] were synthesized in KURABO (Osaka, Japan) as previously described.10 TNP-470 was obtained from Takeda Pharmaceutical Company, Ltd. (Osaka, Japan).

MTS Assay in Endothelial Cells

Human aortic endothelial cells (HAECs) (passage 3) were purchased from Clonetics Corp (Palo Alto, Calif) and were maintained in endothelial basal medium supplemented with 5% FBS and endothelial growth supplement. Cells were incubated at 37°C in a humidified atmosphere of 95% air–5% CO2 with exchange of medium every 2 days.

Cell viability of HAECs was measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using CellTiter 96 one solution reagent (Promega, Madison, Wis). Absorbance at 490 nm was measured. Recombinant VEGF and FGF were obtained from PeproTec (London, UK), and recombinant HGF was purified from the culture medium of Chinese hamster ovary cells or C-127 cells, which were transfected with an expression plasmid containing human HGF cDNA.11

Statistical Analysis

All values are expressed as mean±SEM. Analysis of variance with ANOVA was used to determine the significance of differences in multiple comparisons. P<0.05 was considered significant.

Results

The hanging drop method was used to produce EBs for the differentiation of ES cells. To further induce the differentiation, we used 3-dimensional matrigel-coated dishes as the suitable scaffold for vascular formation. Seven days after LIF withdrawal, EBs on matrigel-coated dishes showed the pres-
ence of vascular tube–like outgrowths without the addition of growth factors such as VEGF. To explore the role of matrigel, we also applied collagen or gelatin-coated dishes. In contrast, EBs on collagen-coated dish did not exhibit the endothelial sprouts, even though collagen is the well known key factor for angiogenesis through endothelial cell surface interaction. Similarly, EBs on gelatin-coated dishes also did not show the blood vessel–like structures (Figure 1a).

These blood vessel–like structures derived from EBs on matrigel were assessed to be endothelial cells or smooth muscle cells by fluorescent immunostaining using antibodies against PECAM-1, VE-cadherin, and α-SM actin. Entire mount immunostaining with anti-PECAM antibody, anti-VE cadherin, and anti-α-SM actin confirmed that those outgrowing tubelike structures contained endothelial cells and smooth muscle cells. Indeed, these vascular markers, PECAM and α-SM actin, were partially colocalized (Figure 1b). Moreover, these structural changes were accompanied with the induction of angiogenic phenotype mRNA expression such as PECAM, tie-2, Flk-1, α-SM actin, SM-myosin heavy chain, and SM22α, as assessed by RT-PCR (Figure 2a). To quantify the expression of these vascular specific genes, we examined mRNA expression by real-time PCR at every 2 days after culture on matrigel. The endothelial cell markers VE-cadherin and smooth muscle cells markers SM22α, were upregulated at 7 days and further increased at 9 days (Figure 2b). We further quantified the differentiation into endothelial cells by FACS analysis. Flk-1 positive cells increased up to 18% at 5 days after using matrigel and gradually decreased after day 6, whereas PECAM-positive cells gradually increased until day 14 up to 23% (Figure 2c). Hence, we provided a new differentiation method from ES cells into the blood vessels under the growth factors–deprived condition using matrigel, which might offer the 3D assembly of cells in the biological microenvironment.

In the analysis of the specificity for differentiation into vascular cells, we examined the expression of neuron specific marker MAP2, glia specific marker GFAP, adipocyte specific marker PPARY, bone specific marker osteocalcin, and heart specific marker, Nkx2.5. Although the expression of Nkx2.5 was markedly increased at 7 days, the other markers were not elevated (Figure 3a). As it has been known that ES cells would easily differentiate into the heart in the hanging drop conduction, the beating cells were also observed on gelatin-
or collagen-coated dishes. However, the beating cells were usually located in the center of EBs and not colocalized in sprouting vessels in the present model. Of importance, the notch ligands, \( \text{H9254-1} \) and \( \text{H9254-4} \), and the downstream target of notch signaling, \( \text{hey1} \) and \( \text{hey2} \), were also upregulated at 7 days (Figure 3b), whereas the expression of notch 1 and 4, another notch ligand jagged-1 were not increased (data not shown).

To further determine the key factor in the sprouting blood vessels, we examined the expression of angiogenesis-related growth factors by quantitative real-time PCR. Interestingly, a number of growth factors involved in VEGF, HGF, Ang-1, TGF-\( \beta \), and FGF2 were upregulated at 7 days and further increased at 9 days with similar behavior (Figure 4a), even though the increase of FGF2 expression was relatively lower than other growth factors. We further used a series of neutralizing antibodies against vasculogenesis-related genes such as VEGF, FGF2, HGF, and TGF-\( \beta \). Unexpectedly, all of neutralizing antibodies failed to inhibit the differentiation from EBs into the sprouting blood vessels quantified by areas of immunostaining with PECAM and \( \alpha \)-SMA actin (Figure 4b). We also confirmed the effective dose of each antibodies. The addition of anti-VEGF neutralizing antibody completely attenuated the recombinant VEGF-induced increase in cell viability by MTS assay (normal IgG [10 \( \mu \)g/mL], 0.406±0.018; normal IgG [110 \( \mu \)g/mL]+human recombinant [hr] VEGF [150 ng/mL], 0.460±0.025; hrVEGF+ antibody [110 \( \mu \)g/mL], 0.398±0.031; \( P<0.05 \)). Although we previously reported that high glucose treatment induced the release of TGF-\( \beta \), leading to decreased local HGF production, the addition of neutralizing antibody against TGF-\( \beta \) (5 \( \mu \)g/mL) completely attenuated the decrease in HGF concentration induced by high glucose.\(^{12} \) The addition of anti-FGF2 neutralizing antibody completely atten-
uated the recombinant FGF2-induced decrease (normal IgG [10 μg/mL], 0.416±0.022; normal IgG [10 μg/mL]+hrFGF2 [50 ng/mL], 0.498±0.031; hrFGF2+antibody [10 μg/mL], 0.402±0.025; P<0.05). The treatment of anti-rat HGF antibody (5 μg/mL) completely suppressed recombinant rat HGF (5 ng/mL)-induced DNA synthesis in hepatocytes. Moreover, in cell scattering assay with Madin-Darby canine kidney cells, anti-HGF antibody (5 μg/mL) completely blocked rat recombinant HGF (5 ng/mL)-induced cell scattering and in cultured rat cardiomyocytes, this antibody (5 μg/mL) inhibited extracellular signal regulated kinase activation by rat recombinant HGF.11

Of importance, the differentiation into the blood vessels was significantly inhibited by cyclic arginine-glycine-aspartic (RGD) peptide (Figure 5a), which is a selective inhibitors for the αvβ3-integrins.10 These results suggest the importance of cell-matrix recognition and cell-adhesion phenomena in the formation of vascular sprouting. We speculate that this model might be useful for the screening of pharmaceutical drugs to inhibit angiogenesis. As an example, we examined the effect of TNP-470 (AGM-1470), a derivative of fumagillin, which was known as an anticancer drug to inhibit angiogenesis13 and showed evidence of antitumor activity in a human clinical trial.14,15 The treatment of TNP-470 inhibited the differentiation of ES cells into the sprouting blood vessels in a dose-dependent manner (Figure 5b).

**Discussion**

In this study, we demonstrated that cell-matrix interaction using matrigel forced ES cells into the sprouting blood vessels containing endothelial and vascular smooth muscle cells. In general, cell differentiation requires the cooperation of a variety of molecules that regulated cellular processes, such as activation of endothelial cells, proliferation, modulation of ECM, invasion, migration, and vascular remodeling. Several studies have demonstrated the importance of VEGF and other growth factors in vasculogenesis and angiogenesis.16–18 In addition, recent reports suggest that vascular development would be remodeled into a well organized network of large and small vessels through sprouting, branching, and network formation, which are controlled by a variety of growth factors, signaling molecules, and their downstream pathways.7,19 However, the present study
clearly demonstrated that the differentiation into the sprouting blood vessels did not require the specific angiogenic growth factor under certain cell-matrix interactions such as matrigel. The specificity of blood vessel formation on matrigel was supported by several lines: (1) the appearance of sprouting tubular-like formation could be detected only on matrigel-coated dishes, but not collagen- and gelatin-coated dishes, (2) sprouting blood vessels were readily co-stained with endothelial and smooth muscle specific markers, and (3) induction of a number of angiogenesis-related growth factors were simultaneously upregulated. It is noteworthy that none of the neutralizing antibodies against angiogenesis-related growth factors inhibited the differentiation. We also confirmed that ES cells on matrigel did not differentiate into bone, neuron, or adipose tissue.

Of particular importance, only methylated cyclic RGD peptide as highly active and selective ligand for the αvβ3-integrin receptor inhibited the differentiation into the blood vessels.20,21 The αvβ3-integrins are capable of recognizing a variety of ECM proteins with an exposed RGD sequence, including vitronectin, fibronectin, fibrinogen, thrombospondin, proteolysed collagen, von Willebrand factor, and osteopontin.26 Thus, the present study suggests the importance of cell-matrix recognition and cell-adhesion phenomena in this formation of vascular sprouting. Therapeutic angiogenesis using genes or cells can be fairly close to the clinical application to treat ischemic diseases. However, it partially contradicts data from αv-null ES cells.27 S.E. Francis et al did not observe a significant reduction in early plexus formation and maturation in EBs lacking αv integrin subunit, however they used an EB-derived vasculogenesis model by adding growth factors. Thus, we speculate that αvβ3-integrins might have an important role in vasculogenesis as an initial trigger in our model. Integrin can play adhesive as well as signaling functions at the cell surface and endothelial cell interactions with the extracellular matrix are mediated in large part by the integrin family of adhesion receptors, heterodimeric transmembrane glycoproteins, consisting of α and β subunits.28 Blood vessels in vertebrate embryos can develop through either vasculogenesis of angiogenesis that has been generated from mesodermally derived angioblasts or arisen as sprouts from preexisting vessels by bridging. In the future, we must examine how similar our model is to vasculogenesis or angiogenesis and in what step integrin is really needed.

The present findings might be applicable for the screening of pharmaceutical drugs to stimulate or inhibit angiogenesis. A great deal of work still needs to be done to develop appropriate assays for cell differentiation, migration, and functional integration. Both same-species and interspecies testing should be done to determine the relevance of species-specific signaling and host environment and to select the most appropriate animal models for human disease. Thus, a human ES cell–based model will be useful for a preclinical trial. As an example, we found that TNP-470, an anti-cancer drug that inhibits angiogenesis, completely inhibited the differentiation of ES cells into the sprouting blood vessels. Our present study provides the screening method to identify the candidate drugs to stimulate or inhibit the vasculature for the treatment of various diseases.

Overall, the present study demonstrated that ES cells could be differentiated into the sprouting blood vessels under certain cell conditions using matrigels without the addition of any growth factors. These results suggest that the differentiation of ES cells into the blood vessels might be coorganized with growth factors and cell environment. This novel method to differentiate ES cells into the vasculature might be useful for the regenerative medicine and vascular research.

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**Disclosures**

None.

**References**


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