Basic Fibroblast Growth Factor Is Essential to Maintain Endothelial Progenitor Cell Phenotype in TR-BME2 Cells

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Endothelial progenitor cells (EPC) can differentiate into both endothelial cells and contractile smooth muscle cells (SMC). Previously we reported that TR-BME2 cells, a model for EPC, developed contractile SMC-like characteristics in culture medium deprived of endothelial cell growth factors (ECGF). The aim of the present study was to clarify the effect of one of these factors, basic fibroblast growth factor (bFGF) on differentiation of EPC. First it was confirmed that bFGF receptor (FGFR-1) mRNA is expressed in TR-BME2 cultured in both ECGF-rich and ECGF-deprived medium. When TR-BME2 cells were cultured in ECGF-deprived medium, they differentiated into contractile SMC. Expression of an undifferentiated state marker, CD133, and proliferation of TR-BME2 were both reduced by ECGF deprivation, but these changes were diminished in the presence of bFGF. mRNA expression of smooth muscle α-actin (SMA) and smooth muscle protein 22 (SM22), which are contractile SMC markers, was induced by deprivation of ECGF and the induction was suppressed by bFGF. In vascular endothelial cell growth factor (VEGF)-induced tube formation assay, TR-BME2 cells formed tube structures in the presence of bFGF, but not in its absence. Our results indicate that bFGF is essential for the maintenance of EPC phenotype, serving to suppress differentiation to contractile SMC.

Key words endothelial progenitor cell; vasculogenesis; differentiation; vascular smooth muscle cell; proliferation; tube formation

Endothelial progenitor cells (EPC) are derived from bone marrow and differentiation of EPC to endothelial cells (EC) and smooth muscle cells (SMC) contributes to vasculogenesis in adults.1,2) Both EC and mural cells, consisting of SMC and pericytes, contribute to the development and maintenance of the vasculature. The contribution of EPC to vasculogenesis in adults was reported to be around 10%.3) However, the factors controlling differentiation of EPC to EC or SMC remain unclear. Differentiation of stem cells and progenitor cells is regulated by cytokines such as basic fibroblast growth factor (bFGF also known as FGF2). For instance, neural stem cells are maintained in the undifferentiated state by bFGF, and begin to differentiate to neuronal cells or astrocytes if deprived of bFGF.4) bFGF is also involved in survival of angioblasts, which play a role in vasculogenesis.5,6) However, it is not clear whether bFGF acts to maintain the undifferentiation state of EPC.

EPC characteristically express CD133, CD34, and Flk-1 (also known as vascular endothelial growth factor receptor-2 (VEGFR-2)), while mature EC express CD34 and Flk-1.6,7) EPC loses CD133 expression in the systemic circulation, so that CD133 is a marker for early EPC.8,9) CD133, CD34 and Flk-1-positive EPC show chemotactic activity in the presence of vascular endothelial cell growth factor (VEGF).8,10,11) For further study, we have established a conditionally immortalized bone marrow-derived EPC line clone 2, namely TR-BME2, derived from temperature-sensitive SV40 transgenic rats.12) TR-BME2 cells express CD133, CD34, Flk-1 von Willebrand factor and annexin II.2,12) Fluorescence-labeled TR-BME2 cells injected systemically into tumor-bearing rats accumulated into the solid tumors, induced vasculogenesis and promoted tumor growth, suggesting that TR-BME2 cells have potential to differentiate into EC in response to VEGF,13–15) TR-BME2 cells are cultured in endothelial cell growth medium-2 (EGM-2) medium, which contains several endothelial cell growth factors (ECGF) that are required to maintain the characteristics of EPC.

SMC have two phenotypes, i.e., contractile and synthetic.6) Contractile SMC express smooth muscle α-actin (SMA) and smooth muscle protein 22 (SM22) as markers, and have the ability to contract in response to vasoconstricting messages.17,18) We have shown that TR-BME2 cells differentiate to contractile SMC on culture in ECGF-deprived endothelial cell basal medium-2 (EBM-2) medium, and the contractile SMC further differentiate to synthetic SMC in the presence of platelet-derived growth factor (PDGF)-BB.2) Therefore, TR-BME2 cells have the potential to differentiate into either EC or SMC. It is possible that some factor(s) in ECGF is involved in maintaining the undifferentiated state of EPC, but so far it has not been identified. EGM-2 medium contains bFGF, VEGF, epidermal growth factor (EGF) and insulin-like growth factor (IGF) as ECGF. We hypothesized that bFGF is involved in suppression of differentiation of EPC to contractile SMC. Therefore, the aim of this study was to test this idea by examining the effect of bFGF on differentiation of EPC. We used TR-BME2 cells as a model for EPC.

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Cell Culture  Rat bone marrow EPC-derived cell line clone 2, TR-BME2, was used as described below. TR-BME2 cells were maintained on human plasma fibronectin (Life Technologies Inc., Rockville, MD, U.S.A.) and type I collagen-coated dishes (Iwaki, Tokyo, Japan) in EGM-2 medium, i.e., Endothelial Basal Medium-2 (EBM-2; Clontech, San Diego, CA, U.S.A.) supplemented with EGM-2 BulletKit (Clontech Laboratories, Mountain View, CA, U.S.A.) except for hydrocortisone, bFGF, VEGF, EGF, IGF-1, ascorbic acid, heparin, gentamicin and fetal bovine serum (FBS). To investigate the effect of each growth factor, the EBM-2 was supplemented with that given growth factor, i.e., human bFGF (Sigma-Aldrich, St. Louis, MO, U.S.A.), human VEGF (Strathmann Biotec GmbH & Co., KG, Hamburg, Germany), EGF (bundled in the EGM-2 BulletKit) or IGF (bundled in the EGM-2 BulletKit), together with other components mentioned above. Cells were incubated at 37°C in a humidified 5% CO₂ incubator.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis  mRNA expression of rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), rat FGF receptor-1 (FGFR-1) and rat CD133 in TR-BME2 was analyzed by RT-PCR analysis. DNA sequences of the sense and anti-sense primers are shown in Table 1. Total RNA was isolated by the acid phenol procedure using ISOGEN reagent (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to the manufacturer’s protocol. RT was performed from 1 µg of total RNA using oligo (dT)₁₅ as a primer and M-MLV reverse transcriptase (ReverTraAce; Toyobo Co., Ltd., Osaka, Japan). RT-PCR was conducted using Platinum PCR SuperMix (Life Technologies Inc.). The reaction was carried out for 18 to 35 cycles of 94°C, 15 s for denaturing; 58 or 68°C, 20 s for annealing; and 72°C, 1 min for extension. PCR products were visualized by ethidium bromide staining after resolution on 5% acrylamide gel. They were subcloned into a plasmid vector using pGEM-T Easy Vector System I (Promega, Madison, WI, U.S.A.) and then sequenced from both directions using a DNA sequencer (CEQ2000XL DNA Analysis System; Beckman Coulter, Fullerton, CA, U.S.A.). The sequences of PCR products were confirmed to be consistent with the corresponding reference sequence.

Cell Proliferation Assay  TR-BME2 cells were cultured on 96-well plate in EGM-2 or EBM-2 medium in the absence or presence of bFGF at 37°C for 72 h. Cell number was as-

Table 1. Primers Used for RT-PCR Analysis

<table>
<thead>
<tr>
<th>Sense primer (5’–3’)</th>
<th>Anti-sense primer (5’–3’)</th>
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<tbody>
<tr>
<td>G3PDH</td>
<td>GATGCTTCCTCCCAGGACTGTA</td>
</tr>
<tr>
<td>FGFR-1</td>
<td>GTCACCATACCACTGTTGCTGT</td>
</tr>
<tr>
<td>CD133</td>
<td>ACTCTCTCACTGTTGAGTCAGGTA</td>
</tr>
<tr>
<td>SMA</td>
<td>GAGCTTCTCCTGCTCATAG</td>
</tr>
<tr>
<td>SM22</td>
<td>TCTGAGCTTCTGCTCATAG</td>
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Fig. 1. Effects of bFGF on Maintenance of the Undifferentiated State and on Proliferation of TR-BME2 Cells

mRNA expression of FGFR-1 (A), CD133 (B) and G3PDH in TR-BME2 cells cultured by EGM-2 (hatched column) or EBM-2 medium in the presence (closed column) and absence (open column) of 20 ng/mL bFGF or 25 ng/mL VEGF at 37°C for 8 d were analyzed by RT-PCR. The PCR products were electrophoresed on 5% acrylamide gel and then visualized by ethidium bromide staining. (C) TR-BME2 cells were cultured in EGM-2 or EBM-2 medium in the presence of bFGF in the concentration range of 0–10 ng/mL at 37°C for 72 h. Cell number was assessed by WST-1 assay. Each column represents the mean±S.E.M. (n=3). Statistical significance of differences was analyzed by Student’s t-test between EGM-2 (hatched column) and the no-ECGF control (white column) as shown by (*) and by ANOVA with Dunnett’s multiple comparison test compared to the no-ECGF control (open column) as shown by †). A p value of less than 0.05 was regarded as significant.
sessed by measuring mitochondrial reduced nicotinamide adenine dinucleotide (NADH)-dependent dehydrogenase activity using Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan) containing WST-1. Cells were added with 10µL of 5µg/mL WST-1 and incubated for 1 h at 37°C in 5% CO₂ incubator. Then, absorbance at 450nm was measured.

**Quantitative Real-Time PCR Analysis (qPCR)** qPCR analysis was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, U.S.A.) with 2× SYBR Green PCR Master Mix (Life Technologies Inc.) as per the manufacturer’s protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using pGEM-T Easy Vector. Relative expression of a specific gene compared to G3PDH expression was also quantified. The control lacking the RT enzyme was assayed in parallel to check for genomic contamination. PCR was performed through 40 cycles of 95°C for 30s, 60°C for 1 min, and 72°C for 1 min after pre-incubation at 95°C for 10 min using specific primers.

**Tube Formation Assay** TR-BME2 cells were precultured in EGM-2 or EBM-2 medium in the absence or presence of bFGF (20ng/mL) or VEGF (25ng/mL) for 8d. Matrigel (Becton Dickinson, Bedford, MA, U.S.A.) was used to coat a 24-well culture plate (0.25mL/well). After polymerization of the Matrigel at 37°C for 1h, cells suspended in 0.5mL of EBM-2 supplemented with 25ng/mL VEGF (4×10⁴ cells/well) were transferred to the gel and incubated at 37°C for 5h. Tube formation of the cells were observed using a conventional bright-field microscope. Numbers of the tube like structures were counted in randomly selected fields.

**Statistical Analysis** Data are presented as a percentage of the value for untreated control culture and shown as mean±S.E.M. (n=3). The significance of differences was evaluated by means of one-way ANOVA with Dunnett’s multiple comparison test. The criterion of significance was a p value less than 0.05.

**RESULTS**

**FGFR-1 Expression in TR-BME2 Cells** EGM-2 medium is composed of EBM-2 medium with several ECGF supplements. To analyze the effect of bFGF and other growth factors included in EGM-2 medium, we used EBM-2 medium supplemented with a single designated growth factor and the other additives. First, we measured the expression of rat fibroblast growth factor receptor (FGFR-1) in TR-BME2 cells cultured in ECGF-deprived EBM-2 medium and in EGM-2. FGFR-1 mRNA was expressed in TR-BME2 cells in both media, and exposure of TR-BME2 to bFGF did not change the expression level of FGFR-1 (Fig. 1A).

**Effect of bFGF on Undifferentiated State and Proliferation of TR-BME2 Cells** To investigate whether bFGF contributes to maintenance of the undifferentiated state and proliferation of TR-BME2 cells, we measured mRNA expression of CD133 and viability in the presence of bFGF. TR-BME2 cells cultured in ECGF-deprived EBM-2 medium showed a reduction of CD133 expression. The expression of CD133 was maintained in EBM-2 medium supplemented with bFGF, whereas VEGF was ineffective (Fig. 1B). The proliferation rate of TR-BME2 cells cultured in EBM-2 medium was increased approximately 1.5-fold by the addition of bFGF in a concentration-dependent manner (Fig. 1C), and was similar to that of cells cultured in EGM-2 medium.

**Effect of bFGF on a Contractile SMC Marker** To clarify the effect of bFGF on expression of a contractile SMC marker, SMA, we measured mRNA expression of SMA in the presence of various growth factors. Although VEGF, EGF and IGF did not change the expression of SMA in TR-BME2 cells cultured in EBM-2 medium compared to the no-ECGF control, SMA expression in cells cultured in the presence of bFGF was increased approximately 1.5-fold by the addition of bFGF in a concentration-dependent manner (Fig. 1C), and was similar to that of cells cultured in EGM-2 medium.

**Effect of bFGF on Differentiation of TR-BME2 Cells** Quantitative RT-PCR for vascular smooth muscle cell markers, SMA and SM22, was performed. (A) TR-BME2 cells were cultured in EGM-2 (hatched column), or EBM-2 medium in the presence (closed column) and absence (open column) of 20ng/mL bFGF, 25ng/mL VEGF, EGF or IGF at 37°C for 10d. Representative PCR products of SMA and G3PDH as a loading control were electrophoresed (upper panel) and the mRNA copy number per ng total RNA was determined (lower panel). (B, C) TR-BME2 cells were cultured in EBM-2 medium in the presence (closed column) and absence (open column) of bFGF at 37°C for 10d. Relative mRNA expression of SMA and SM22 normalized by G3PDH was shown in panels B and C, respectively. Statistical significance was analyzed by Student’s t-test between EGM-2 (hatched column) and the no-ECGF control (white column) as shown by (†) and by ANOVA with Dunnett’s multiple comparison test compared to the no-ECGF control (open column) as shown by (*). A p value of less than 0.05 was regarded as significant.
significantly lower, being similar to that of cells cultured in EGM-2 (Fig. 2A). SMA mRNA expression and SM22 mRNA expression normalized by G3PDH were more than 5-fold and 2-fold suppressed, respectively, by the addition of bFGF in the concentration range of 5–20 ng/mL (Figs. 2B, C).

**Effect of bFGF on Tube Formation from EPC** To address the effect of bFGF on the differentiation potential of EPC to EC, tube formation by VEGF-exposed cells was examined. TR-BME2 cells precultured in EGM-2 medium formed tubes (Fig. 3A). TR-BME2 cells precultured in EBM-2 medium in the presence or absence of VEGF alone little formed tubes, but they did form tubes if they had been pretreated with bFGF (Fig. 3A). Counting of tube-like structures indicate a significant increase of tube structure by bFGF treatment, which is almost equal to the number pretreated in EGM-2 medium (Fig. 3B).

**DISCUSSION**

TR-BME2 cells are cultured in EGM-2 medium containing ECGF to maintain the characteristics of EPC. TR-BME2 cells cultured in EGM-2 showed expression of CD133 (Fig. 1B) and little expression of SMA (Fig. 2A). However, TR-BME2 cells cultured in ECGF-deprived EBM-2 showed down-regulation of CD133 and up-regulation of SMA (Figs. 1B, 2A). Therefore, TR-BME2 cultured in EGM-2 medium and in ECGF-deprived EBM-2 medium exhibit EPC-like and SMC-like phenotype, respectively. These results confirmed the ability of TR-BME2 to undergo EPC-like differentiation to SMC, as we have reported. Among the ECGF, we found that bFGF increased viability and CD133 expression (Fig. 1). These results are consistent with reports that human umbilical vein endothelial cell (HUVEC) migrate in response to bFGF. EPC express FGFR-1, and two isoforms of FGFR-1 generated by alternative splicing, i.e., FGFR-1b and FGFR-1c, accept bFGF as a ligand. Both EPC-like and SMC-like TR-BME2 showed expression of FGFR-1 (Fig. 1). The effect of ECGF on FGFR-1 expression was minimal (Fig. 1). TR-BME2 cultured in ECGF-deprived medium showed induction of SMA, and addition of bFGF alone suppressed SMA induction (Fig. 2). The suppression of SMA mRNA expression by bFGF was saturated at 5 ng/mL and further suppression was not observed (Fig. 2). The suppression of differentiation from EPC to SMC was also associated with suppression of SM22, but the effect of bFGF on SM22 was smaller than that in the case of SMA (Fig. 2). These findings indicate that bFGF plays a role in maintenance of the undifferentiated state of EPC.

TR-BME2 cells have the ability to differentiate to EC and form tube structure on culture in EGM-2 medium (Fig. 3), like human EPC. Addition of VEGF to TR-BME2 cells cultured in ECGF-deprived medium did not induce CD133 expression, influence SMA expression, or induce tube formation (Figs. 1B, 2A, 3), implying that TR-BME2 cells cultured in ECGF-deprived medium remain as SMC. Supplementation of bFGF enabled VEGF-induced tube formation of TR-BME2 cells (Fig. 3). bFGF is also involved in tube formation of adipose-tissue-derived stem cells. bFGF and PDGF-BB synergistically release VEGF from EPC and promote tumor neovascularization. These results indicate that EPC require bFGF for maintenance of tube-forming potential. bFGF enhances proliferation of retinal vascular endothelial cells by activating extracellular signal-regulated kinase (ERK)1/2 and Akt. ERK1/2 and migration of EC are less activated in bFGF knockout mice compared to wild-type mice. bFGF activates ERK in SMC, and the cells are de-differentiated, promoting proliferation. Taken together, the
above findings suggest that bFGF plays a role in maintenance of undifferentiation and proliferation in EPC via ERK and Akt signaling pathways before activation by VEGF for vasculogenesis.

Therapeutic vasculogenesis is useful for rescue of peripheral and circulatory organs from ischemia. VEGF-transduced EPC transplantation required 30 times fewer cells than cell therapy alone, and contributed to *in vivo* neovascularization. Therefore, EPC are useful for therapeutic vasculogenesis. But, to effectively utilize bone-marrow derived EPC for therapy, it is important to control differentiation of EPC to EC or SMC.

In conclusion, bFGF has roles in maintenance of the undifferentiated state and in proliferation of EPC, allowing EPC to maintain the potential to differentiate to EC. Lack of bFGF exposure leads EPC to differentiate to contractile SMC. Therefore, EPC are useful for therapeutic vasculogenesis. But, controlled exposure of EPC to bFGF could be useful for therapeutic vasculogenesis.

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