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Synergism of biochemical and mechanical stimuli in the differentiation of human placenta-derived multipotent cells into endothelial cells

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Abstract

There have been intensive studies on the differentiation of endothelial progenitor cells (EPCs) into endothelial cells. We investigated the endothelial differentiation of placenta-derived multipotent cells (PDMCs), a population of $CD34^-/CD133^-/Flk\text{-}1^-$ cells. PDMCs were cultured in basal media or media containing endothelial growth factors (EGM), including vascular endothelial growth factor (VEGF), for 3 days and then subjected to shear stress of 6 or 12 dyn/cm^2 for 24 h. Culture of PDMCs in EGM under static conditions resulted in significant increases in VEGF receptor-1 (Flt-1) and receptor-2 (Flk-1) expression. Application of shear stress at 12 dyn/cm^2 to these cells led to significant increases in their expression of von Willebrand Factor and platelet-endothelial cell adhesion molecule-1 at both the gene and protein levels. Shear stress at 6 dyn/cm^2 had lesser effects. Uptakes of acetylated low-density lipoproteins as well as formation of tube-like structures on Matrigel were significantly increased after subjecting to shear stress of 12 dyn/cm^2 for 24 h. Our findings suggest that the combined use of endothelial growth factors and high shear stress is synergistic for the endothelial differentiation of PDMCs.

Keywords: Endothelial differentiation; Placenta; Shear flow; Stem cell

1. Introduction

Angiogenesis is a critical process in tumor growth and metastasis, as well as in healing after ischemic injury (Rivard et al., 1999a, b). In addition to vessel wall-associated endothelial cells (ECs), endothelial progenitor cells (EPCs) have been shown to contribute to these processes. Recent evidence demonstrate that $CD34^+/CD133^+/Flk\text{-}1^+$ cells are one type of EPCs that can migrate from the peripheral circulation to the vessel wall, where they differentiate into mature ECs (Hristov et al., 2003). In addition to EPCs, recent studies have demonstrated that some types of progenitor cells that do not express traditional EPC markers may also be able to differentiate into ECs under certain circumstances. These non-EPCs include populations of cells isolated from embryonic stem cells (ESCs) (Yamashita et al., 2000; Yamamoto et al., 2005) and adult stem cells (El-Marsafy et al., 1996; Oswald et al., 2004; Martinez-Estrada et al., 2005). However, the factors that mediate the differentiation of non-EPCs to ECs remain unclear.

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It is now known that several endothelial growth factors, including vascular endothelial growth factor (VEGF), and mechanical forces, including flow-induced shear stress, are important factors that can mediate endothelial differentiation and vascular development (Hirashima et al., 1999; Yamamoto et al., 2003; Wang et al., 2005). We hypothesized that the interplay of biochemical and mechanical stimuli may be able to promote the endothelial differentiation of different types of progenitor cells, including non-EPCs. To test this, we used placenta-derived multipotent cells (PDMCs), a population of $CD34^-/CD133^-/Flk\text{-}1^-$ cells at the time of isolation from the human term placenta (Yen et al., 2005), to investigate the synergistic effects of endothelial growth factors and shear stress on the endothelial differentiation. Our previous studies showed that PDMCs are a population of multipotent cells capable of multilineage differentiation (Yen et al., 2005; Chien et al., 2006). In the present study, we found that exposure of PDMCs cultured in media containing endothelial growth factors to a shear stress of 12 dyn/cm^2 can induce endothelial differentiation of these cells. Our findings demonstrate the effectiveness of combined application of biochemical and mechanical factors in inducing endothelial differentiation of multipotent progenitor cells, which do not express traditional EPC markers.

2. Materials and methods

2.1. Cell culture

Term (38–40 weeks' gestation) placentas from healthy donor mothers were obtained with informed consent and approved in accordance with the procedures of the institutional review board. Briefly, PDMCs were isolated from term placenta after mechanical and 0.25% trypsin-EDTA dissociation (Invitrogen Inc., Carlsbad, CA), and cultured in basal media (BM) consisting of Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal bovine serum (FBS; HyClone, Logan, UT), and 1% penicillin/streptomycin without pre-coating (Yen et al., 2005). The cells were then seeded onto glass slides pre-coated with fibronectin (30 μg/mL; Sigma-Aldrich, St. Louis, MO) to reach confluence (~1–2 × 10^5 cells/cm^2). All cells used in the experiments were obtained between passages 4 and 8.

2.2. Shear stress experiments

PDMCs were kept in BM as controls or cultured in endothelial growth media (EGM) consisting of medium 199 (M199; Invitrogen Inc.) supplemented with 20% FBS, 20% EGM-2 (contents hEGF, hydrocortisone, gentamicin, amphotericin-B, hFGF-B, R3-IGF-1, ascorbic acid, and heparin, but no bovine brain extract; Cell Application Inc., San Diego, CA, USA), VEGF (50 ng/mL; Sigma-Aldrich), and 1% penicillin/streptomycin under static condition for 3 days. The cells were then subjected to a physiologic shear stress of 12 dyn/cm^2 or a lower shear stress of 6 dyn/cm^2 using a parallel-plate flow chamber, as previously described (Chiu et al., 2004). Briefly, the shear stress was created by sandwiching a silicon gasket between an acrylic plate and a cell-seeded glass slide. The total area subjected to shearing composed of 1.5 cm in width (W) and 5.0 cm in length (L). The channel height (h) is 0.025 cm, and the flow in this narrow gap is laminar with a parabolic velocity profile. The wall shear stress (τ_{wall}) can be calculated as $\tau_{wall} = \Delta P(h/2L) = 6Q\mu/Wh^2$, where ΔP is the pressure difference between the inlet and the outlet of the flow channel, Q is the volume flow rate, and μ is the fluid viscosity. The pH of the medium was maintained constant by gassing with a mixture of 5% CO_2 in 95% air, and the temperature was maintained at 37 °C. The medium was changed to fresh medium simultaneously in different conditions with or without shear stress. The cells kept under static condition were used as static controls.

2.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously reported (Chiu et al., 2004). The sequences and annealing temperatures of primers used for the target genes are listed in Table 1.

2.4. Immunofluorescence staining and image analysis

The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized in 0.1% Triton X-100 (Sigma-Aldrich), and then were labeled with mouse monoclonal antibodies against von Willebrand Factor (vWF) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) (1:200 for each; Santa Cruz Biotech. Inc.), followed by FITC-conjugated secondary antibodies (1:200; Jackson ImmunoResearch Lab. Inc., West Grove, PA). F-actin filaments were stained with rhodamine phalloidin (1:200; Molecular Probe Inc., Eugene, OR) and cell nuclei were visualized with 4′,6-diamidino-2-phenylindole staining (DAPI, 1:1000; Molecular Probe Inc.). The samples were mounted in a mounting medium (Sigma-Aldrich) and visualized with a fluorescent microscope (Axiovert 200M, Zeiss, Berlin, Germany) connected to a cooled-CCD camera (Photometrics, Tucson, AZ). The fluorescent intensities of labeled proteins were analyzed by MetaMorph Software (MetaMorph, Downingtown, PA). In some experiments, immunostaining was visualized by inverted confocal microscopy (FV300, Olympus, Tokyo, Japan) under 60 × -oil objectives, with images of 1-μm thick optical sections collected from bottom to top of the cell for each field. At least 30 cells were randomly selected and analyzed in each experiment. Cell boundaries were traced manually from phase contrast images. For measurement of cell orientation, cell morphology was analyzed by fitting to an ellipse to determine long/short axes of individual cell. The angle (θ) of cell orientation was determined by the direction of flow and the major axis of the ellipse, with ranges from 0° to 90°, as described by Chiu et al. (1998). At least three random image fields were measured for each condition. The percentage of cell orientation for each condition was summarized in a polar histogram, with the intervals of 15°.

2.5. Western blot

PDMCs were treated with different chemical and/or mechanical stimuli and then lysed with RIPA buffer containing protease inhibitors. Total cell

Table 1
List of primer sequences and annealing temperatures for RT-PCR

Name	Primer sequence	Temperature
Flt-1	F: ATTTGTGATTTTGGCCTTGC R: CAGGCTCATGAACTTGAAAGC	65
Flk-1	F: GTGACCAACATGGAGTCGTG R: CCAGAGATTCCATGCCACTT	60
VEGF	F: TGTGAATGCAGACCAAAGAAAGA R: GCTTTCTCCGCTCTGAGCAA	65
vWF	F: GAGGCTGAGTTTGAAGTGC R: CTGCTCCAGCTCATCCAC	60
PECAM-1	F: CCCGAACTGGAATCTTCCTT R: GGGTTTGCCCTCTTTTTCTC	65
a-SMA	F: GTGTGTGACAATGGCTCTGG R: TGAAGGATGGCTGGAACAGG	60
GAPDH	F: CCACCCATGGCAAATTCCATGGCA R: TCTAGACGGCAGGTCAGGTCCACC	60