Differentiation of human hair follicle stem cells into endothelial cells induced by vascular endothelial and basic fibroblast growth factors

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Abstract. Hair follicle stem cells (HFSCs) possess powerful expansion and multi-differentiation potential, properties that place them at the forefront of the field of tissue engineering and stem cell-based therapy. The aim of the present study was to investigate the differentiation of human HFSCs (hHFSCs) into cells of an endothelial lineage. hHFSCs were expanded to the second passage in vitro and then induced by the addition of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) to the culture medium. The expression levels of endothelial cell (EC)-related markers, including von Willebrand factor (vWF), vascular endothelial cadherin (VE)-cadherin and cluster of differentiation (CD)31, were detected by immunofluorescence staining, flow cytometric analysis and reverse transcription-polymerase chain reaction. The hHFSCs expressed vWF, VE-cadherin and CD31 when exposed to a differentiation medium, similar to the markers expressed by the human umbilical vein ECs. More significantly, differentiated cells were also able to take up low-density lipoprotein. The data of the present study demonstrated that an efficient strategy may be developed for differentiating hHFSCs into ECs by stimulation with VEGF and bFGF. Thus, hHFSCs represent a novel cell source for vascular tissue engineering and studies regarding the treatment of various forms of ischaemic vascular disease.

Introduction

As the human lifespan increases, cardiovascular diseases are starting to represent a growing health and socioeconomic burden to society (1). Among the therapeutic strategies for such diseases, autologous grafts, including autologous saphenous vein and mammary artery grafts, are the common options (2). However, these approaches are limited by the sources and donor site morbidity, and such synthetic grafts, including expanded polytetrafluoroethylene and Dacron (polyethylene terephthalate fibre) grafts, can often result in immunological and thrombotic complications, particularly in the repair of small-diameter vascular defects (3). Research has been conducted on tissue-engineered, small-diameter vascular grafts or the endothelialisation of the previously mentioned artificial grafts, with endothelial cells (ECs) playing a significant role, particularly with regard to vascular tissue engineering applications (4). However, the limited availability of ECs hampers the development of a suitable vascular graft. Furthermore, the slow expansion rate and limited proliferation capability of fully mature ECs in vitro have presented crucial hurdles in their therapeutic use (5).

Offering the unique advantages of proliferative and growth potential, stem cells, either embryonic or adult, and circulating endothelial progenitors have been widely analysed as possible sources of ECs (6,7). Previous studies have indicated that the hair follicle (HF) is a readily accessible mini-organ within the skin that contains stem cells with notably broad differentiation potential, and that it may be an alternative source of autologous ECs (8,9). HF stem cells (HFSCs) have the broad potential to differentiate into adipogenic, osteogenic, chondrogenic, neurogenic and myogenic lineages under the appropriate conditions (10-13). When compared with other stem cells, including embryonic, bone marrow-derived or adipose stem cells, HFSCs are easier to acquire (less invasive) and have a lower associated risk of donor site morbidity and a higher yield at harvest (9). Thus, HFSCs could be a preferred, novel cell source for blood vessel engineering.

Vascular endothelial growth factor (VEGF) is a signalling protein produced by cells that stimulates vasculogenesis and angiogenesis (14). The VEGF family is composed of at least
seven members, with VEGF-A (normally termed VEGF) being the most significant. The targeted inactivation of the VEGF gene in mice has been shown to cause fatal deficiencies in vascularisation (15), demonstrating the important role of VEGF in this process. VEGF has been shown to affect EC differentiation in vitro (16) and is thus a component of in vitro EC differentiation media (17-19).

Basic fibroblast growth factor (bFGF), also known as FGF2 or FGF-β, is a member of the FGF family (20). bFGF promotes EC proliferation and the physical organisation of ECs into tube-like structures, and is thus critical in mediating the formation of novel blood vessels and promoting angiogenesis (21).

The aim of the present study was to investigate the potential of human HFSCs (hHFSCs) to differentiate into the EC phenotype upon induction with VEGF and bFGF in a low-serum medium. The gene and protein expression of several characteristic EC markers was examined in the resulting hHFSCs. In addition, a low-density lipoprotein (LDL) uptake function of the induced hHFSCs was also demonstrated.

Materials and methods

Isolation and culture of hHFSCs. hHFSCs were isolated from human scalp tissues from healthy adult patients undergoing cosmetic plastic surgery, as described previously (22). All the protocols of human tissue handling were approved by the Research Ethical Committee of the hospital and written informed consent was obtained from all patients. hHFSCs at the second passage were used in the subsequent study. The characterisation of the hHFSCs was determined by their CD marker profile (K15, K19, integrin β1) and their ability to differentiate into osteogenic, adipogenic and chondrogenic lineages (data not shown), as reported previously (10,11).

Induction of EC differentiation. Cells reaching subconfluence were cultured in EC growth medium-2 (EGM-2; Lonza, Walkersville, MD, USA) supplemented with 50 ng/ml recombinant human VEGF (R&D Systems, Minneapolis, MN, USA) and 10 ng/ml recombinant human bFGF (Sigma-Aldrich, St. Louis, MO, USA), with 2% foetal bovine serum (FBS; HyClone, Logan, UT, USA). EGM-2 supplemented with 2% FBS was defined as the basal medium (BM). Human umbilical vein ECs (hUVECs) were used as a positive control. The culture media were changed every 2 days. The cell characterisation and functional evaluation were performed subsequent to 7 days of culture.

Immunofluorescence staining. hHFSCs were harvested, resuspended in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min and permeabilised with 0.1% Triton X-100 (both Sigma-Aldrich) for 10 min. Subsequent to washing with PBS, the cells were blocked with 3% bovine serum albumin (BSA) for 30 min and then incubated with the following primary antibodies: Rabbit polyclonal anti-von Willebrand Factor (vWF, F3520), rabbit polyclonal anti-vascular endothelial cadherin (VE-cadherin, V1514) and mouse monoclonal anti-CD31 (P8590) (all from Sigma-Aldrich). Following incubation with the primary antibodies for 60 min at room temperature, the cultures were washed with PBS three times. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Millipore, Billerica, MA, USA) was used to detect the localisation of the anti-vWF and anti-VE-cadherin antibodies, and FITC-conjugated goat anti-mouse secondary antibody (Millipore) was used to detect the localisation of the anti-CD31 antibodies. The cell nuclei were stained with propidium iodide. The control samples consisted of cells without primary antibodies and were used to assess the background fluorescence. The images were viewed with a fluorescence microscope (Nikon, Tokyo, Japan).

Flow cytometric analysis. The cells were trypsinised, centrifuged at 500 x g for 5 min (Allegra 64R; Beckman Coulter, Brea, CA, USA), resuspended in PBS/1% BSA and incubated with anti-vWF, anti-VE-cadherin and anti-CD31 (all from Sigma-Aldrich) for 30 min at room temperature on a shaking plate (Lab Rotorators, Thermo Scientific, Logan, UT, USA). The cells were then washed, resuspended in PBS/1% BSA with FITC-conjugated secondary antibody and incubated for 30 minutes at room temperature on a shaking plate. The cells were then washed again and fixed, and FITC-conjugated isotype-matching immunoglobulins were used to determine non-specific staining. Fluorescence was determined using a flow cytometer (Becton-Dickinson, San Jose, CA, USA), and the data were analysed using CellQuest software (Becton-Dickinson).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). The expression levels of EC-specific markers (vWF, VE-cadherin and CD31) were identified by isolating the total RNA from the cells using the RNeasy total RNA isolation kit (Qiagen, Inc., Valencia, CA, USA), and cDNA was synthesised using the SuperScript First-strand Synthesis system (Life Technologies, Carlsbad, CA, USA). Specific genes were amplified by PCR using the Fast-Run Taq Master kit (Potech Technology, Taipei, Taiwan). The primer sequences designed using the Primer Express software (Primer Express Software Version 3.0; Applied Biosystems, Foster City, CA, USA) are listed in Table I. The cDNA product was amplified by PCR using standard methods, and electrophoresed through a 2% agarose gel treated with ethidium bromide; the bands were visualised using an ultraviolet light box. hUVECs and human chondrocyte cells (hCs) were used as the positive and negative controls, respectively.

LDL uptake. LDL uptake was assessed by incubating the cells for 4 h at 37°C with acetylated LDL labelled with 1,10-dioctadecyl-3,3',3'-tetramethylinodocarbocyanine (DiI-acLDL; Molecular Probes, Biomedical Technologies, Stoughton, MA, USA) diluted to 10 µg/ml in a complete growth medium. The cells were then washed three times with probe-free medium. The incorporation of fluorochrome-labelled LDL into the cells was analysed with an Eclipse E400 Epi-Fluorescence Microscope (Nikon, Tokyo, Japan).

Statistical analysis. Each experiment was repeated at least three times. Since the original data were normally distributed, the results are presented as the mean ± standard deviation. The comparisons between groups were performed
Results

Culture of hHFSCs. The total number of cells isolated from each scalp tissue sample ranged between 5x10^4 and 1x10^5 cells. In total, 0.5-2% of the isolated cells were found to be adherent stem cells. The hHFSCs elongated subsequent to 1-2 days of culture in plates (Fig. 1A); the cells reached confluency within another 3-4 days and were subsequently passaged onto a novel plate.

VEGF and bFGF induce the differentiation of hHFSCs to ECs. At the second passage, 50 ng/ml VEGF and 10 ng/ml bFGF were used to induce the differentiation of hHFSCs to the EC lineage. The hHFSCs acquired a cobblestone morphology subsequent to treatment with VEGF and bFGF for 7 days, similar to the previous observations in primary isolated hUVECs. No evident change was found in the hHFSCs cultured in BM (Fig. 1B-D). At the fourth passage, the hHFSCs appeared to comprise a relatively homogenous population that exhibited an endothelial lineage morphology.

Expression of EC-specific markers in hHFSCs treated with VEGF and bFGF. To determine whether VEGF and bFGF can induce the differentiation of hHFSCs to the EC phenotype, EC-specific proteins (vWF, VE-cadherin and CD31) were detected by immunofluorescence staining. These three markers were also examined in hUVECs as a positive control. As shown in Fig. 2, there was little expression of vWF, VE-cadherin or CD31 in the undifferentiated hHFSCs cultured in BM. However, when cultured in EGM-2 supplemented with VEGF and bFGF, the expression levels of vWF, VE-cadherin and CD31 were enhanced, reaching a level similar to that of the hUVECs. To determine the percentage of EC-differentiated cells in the hHFSC population, the expression levels of vWF, VE-cadherin and CD31 were also analysed using flow cytometry. As shown
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Figure 2. Expression of EC-specific proteins (vWF, VE-cadherin and CD31) under different conditions by immunofluorescent staining. There was little expression of (A) vWF, (D) VE-cadherin or (G) CD31 in the undifferentiated hHFSCs cultured in BM. The expression of (B) vWF, (E) VE-cadherin and (H) CD31 was enhanced when cultured in BM supplemented with VEGF and bFGF, reaching a level similar to that of (C, F, I) the hUVECs. Scale bar, 100 µm. EC, endothelial cells; vWF, von Willebrand factor; VE-cadherin, vascular endothelial cadherin; hHFSCs, human hair follicle stem cells; BM, basal medium; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; hUVECs, human umbilical vein ECs; CD31, cluster of differentiation 31.

Figure 3. Flow cytometry analysis of EC-specific proteins. Low levels of expression of vWF, VE-cadherin and CD31 were found in the undifferentiated hHFSCs cultured in BM. The expression was enhanced in BM supplemented with VEGF and bFGF, similar to that in hUVECs. EC, endothelial cells; vWF, von Willebrand factor; VE-cadherin, vascular endothelial cadherin; hHFSCs, human hair follicle stem cells; BM, basal medium; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; hUVECs, human umbilical vein ECs; CD31, cluster of differentiation 31.

in Fig. 3, vWF was detected in 3.57±0.47, 85.41±1.42 and 93.62±0.75% of undifferentiated hHFSCs, induced hHFSCs and hUVECs, respectively. By comparison, little expression of VE-cadherin (3.38±0.52%) or CD31 (2.63±0.56%) was found in the undifferentiated hHFSCs, although their expression levels reached 72.36±1.93 and 76.49±1.12%, respectively, in the induced hHFSCs, which is much closer to the expression observed in the hUVECs.
B, was observed at A of rapidly proliferating and ready‑to‑use ECs. Although ECs researchers have urgently been searching for alternative sources, inadequate proliferation capacity and lack of autologous sources.

Regardless of the results obtained from using adult blood vessel‑derived ECs (hUVECs) serving as a positive control (Fig. 4, the undifferentiated hHFSCs did not express vWF, von Willebrand factor; VE‑cadherin, vascular endothelial cadherin; hHFSCs, human hair follicle stem cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; hUVECs, human umbilical vein ECs; CD31, cluster of differentiation 31). The gene expression profile analysed by RT‑PCR further confirmed the EC differentiation of the hHFSCs. As shown in Fig. 4, the undifferentiated hHFSCs did not express vWF, VE‑cadherin or CD31, similar to the observations in the hCs. By contrast, these factors were upregulated in the induced hHFSCs to a similar level as that in the hUVECs. All the data support the hypothesis that hHFSCs can differentiate into ECs upon exposure to VEGF and bFGF.

Differentiated cells can take up Dil‑Ac‑LDL. The cytoplasm of the differentiated cells was fluorescent due to the incorporation of Dil‑Ac‑LDL. (A) Human umbilical vascular endothelial cells (hUVECs) served as a positive control. Scale bar, 100µm. Dil‑Ac‑LDL, 1,10‑diodoacetylcycl‑3,3,3′‑tetramethylindocarbocyanine; ECs, endothelial cells; hHFSCs, human hair follicle stem cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; hUVECs, human umbilical vein ECs.

Figure 4. mRNA levels of vWF, VE‑cadherin, CD31 and β‑actin were determined by reverse transcription polymerase chain reaction (RT‑PCR). The expression of vWF, VE‑cadherin and CD31 was observed in hHFSCs induced by VEGF and bFGF, similar to hUVECs. Human chondrocyte cells (hCs) were used as the negative controls. vWF, von Willebrand factor; VE‑cadherin, vascular endothelial cadherin; hHFSCs, human hair follicle stem cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; hUVECs, human umbilical vein ECs; CD31, cluster of differentiation 31.

The mechanism for EC differentiation induced by the heterozygote knockout mice suffer fatal deficiencies in vascularisation, resulting in embryonic lethality (41,42). VEGF deficiency led to early vascular development disorders, including vascular malformation, angiogenesis and large vessel formation. The role of VEGF in angiogenesis was demonstrated by the observation that heterozygote knockout mice suffer fatal deficiencies in vascularisation, resulting in embryonic lethality (41,42). VEGF deficiency led to early vascular development disorders, including vasculogenesis, angiogenesis and large vessel formation. The role of VEGF in angiogenesis indicates that this protein could be used to augment collateral vessel formation as an alternative to reconstructive surgery (43), as confirmed by the finding that treatment with DNA encoding VEGF augmented collateral vessel formation in acute limb ischaemia through therapeutic in situ neovascularisation and angiogenesis (44‑47). Knowledge of the function of VEGF in endothelial differentiation and blood vessel growth stimulation may be useful for vascular tissue engineering and regenerative medicine, including the treatment of myocardial and lower limb ischaemia, wound healing and skin grafting.

It has been well documented that bFGF is an extracellular matrix component required for supporting EC growth and promoting the formation of differentiated capillary tubes (48,49). bFGF‑mediated signalling is critical for the proliferation of the haemangioblast and thus positively regulates haematopoietic development (50). Several lines of evidence have indicated that bFGF (FGF2) stimulates VEGF expression in ECs (51‑53). In the absence of bFGF signalling, it leads to the loss of adherens and tight junctions, increased vascular leakiness and disassembly of the existing vasculature. The mechanism for EC differentiation induced by the angiogenic growth factors, VEGF and bFGF, has been...
extensively investigated. These factors exert their effects by specifically binding to cell surface-expressed receptors, which are ligand-stimulatable tyrosine kinases. The stimulation of receptor kinase activity allows coupling to the downstream signalling transduction pathways, which results in transcriptional changes and biological responses, thereby regulating the proliferation, migration and differentiation of ECs (54).

Previous studies have also demonstrated that bFGF stimulates VEGF expression in ECs. One key factor in FGF-induced VEGF expression is the Shc protein, an adaptor molecule recruited to FGFRs upon activation, which is crucial in receptor tyrosine kinase-dependent VEGF gene expression (55,56). Therefore, it was speculated that cross-talk between VEGF and bFGF pathways may contribute to the differentiation of hHFSCs to ECs.

To confirm a differentiated EC phenotype, the demonstration of characteristic functional properties is required. In the present study, the hHFSCs differentiated with VEGF and bFGF were shown to gain the capability of transporting LDL as endothelial differentiation progressed. These data indicate that hHFSCs can be induced to differentiate into an EC phenotype with conventional function when stimulated by VEGF and bFGF in combination. Therefore, hHFSCs may be a valuable source of functional ECs for vascular tissue engineering and therapeutic vasculogenesis.

In conclusion, the present study demonstrated that expanded hHFSCs acquire several endothelial-like characteristics when cultured with VEGF and bFGF for 7 days in vitro, as evidenced by the expression of EC-specific transcripts and proteins, including vWF, VE-cadherin and CD31, and the ability to incorporate fluorescent DiI-Ac-LDL molecules. It was hypothesised that this approach has potential applications for using hHFSCs as a candidate in cardiovascular tissue engineering, and that it provides a tool for various clinical studies in which improved vascularisation is desired.

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References