Effects of serum reduction and VEGF supplementation on angiogenic potential of human adipose stromal cells *in vitro*

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Abstract

Objectives: This study investigated effects of reduced serum condition and vascular endothelial growth factor (VEGF) on angiogenic potential of adipose stromal cells (ASCs) *in vitro*.

Materials and methods: Adipose stromal cells were cultured in three different types of medium: (i) F12/DMEM (FD) supplemented with 10% FBS from passage 0 (P0) to P6; (ii) FD supplemented with 2% FBS at P6; and (iii) FD supplemented with 2% FBS plus 50 ng/ml of VEGF at P6. Morphological changes and growth rate of ASCs were recorded. Changes in stemness, angiogenic and endogenic genes' expressions were analysed using Real-Time PCR.

Results: Adipose stromal cells changed from fibroblast-like shape when cultured in 10% FBS medium to polygonal when cultured in 2% FBS plus VEGF-supplemented medium. Their growth rate was lower in 2% FBS medium, but increased with addition of VEGF. Real-Time PCR showed that ASCs maintained most of their stemness and angiogenic genes' expression in 10% FBS at P1, P5 and P6, but this increased significantly in 2% FBS at P6. Endogenic genes expression such as *PECAM-1*, *VE chaderin* and *VEGFR-2* decreased after serial passage in 10% FBS, but increased significantly at P6 in 2% FBS. Addition of VEGF did not cause any significant change in gene expression level.

Conclusion: Adipose stromal cells had greater angiogenic potential when cultured in reduced serum conditions. VEGF did not enhance their angiogenic potential in 2% FBS-supplemented medium.

Introduction

Human mesenchymal stem cells (MSCs) are of interest due to their ability to differentiate into mesodermal cell types including adipocytes, osteocytes and chondrocytes (1) and transplantation of MSCs derived from bone marrow has improved prognosis for patients with chronic ischaemic heart disease following surgical revascularization (2,3). Recently, angiogenic potential of stem cells has been studied actively in the fields of tissue engineering and regenerative medicine (4–7). Stem cells have been recognized to have potential as angiogenic therapy for treatment of cardiovascular disease particularly for patients with ischaemic heart disease where it is important to repair and regenerate healthy blood vessels to improve perfusion into ischaemic tissues.

Trans-differentiation of MSCs particularly derived from human adipose stroma, into other cell lineages such as cardiogenic (8), angiogenic (9) and neurogenic (10), has been reported by a number of investigators. Furthermore, human adipose stromal cells (ASCs) have become a more attractive option for cell-based therapy compared to bone marrow-derived stem cells (BMSCs) due to their abundance and for being robust in preparation (11). ASCs also have similar cell and molecular characteristics to those of BMSCs (12), and ASCs have been shown to be able to retain stemness properties in serial passage up to P10 (13).

A potential use of ASCs in soft tissue reconstruction has been reported, for cosmetic surgery (14),

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however, to maintain transplanted soft tissue, adequate blood supply is essential to prevent progressive resorption of grafted tissue (15,16). Although angiogenic potential of ASCs has been reported (17,18), understanding effects of growth factors and different culture conditions, including low-serum and serum-free conditions, on ASC angiogenic differentiation ability is not well established. Use of serum-free medium has been found to reduce germline potential, indicated by lower spermatogonial stem cell frequency (19), which may affect self-renewal and differentiation ability of stem cells. Use of growth factors such as vascular endothelial growth factor (VEGF) to encourage angiogenesis has been demonstrated to also stimulate angiogenic potential of stem cells (20). VEGF is the main regulator for physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions (21,22). Several reports have shown that angiogenic potential of ASCs can be stimulated by serum and by co-culture with endothelial cells (23) as well as reduced serum and hypoxic conditions, to stimulate VEGF secretion (18). Use of human serum in xeno-free culture medium has been tested on differentiability of ASCs (24), however, the studies did not provide extensive understanding of changes in differentiation gene expression level of angiogenic and stemness genes in cells after differentiation. As the effect of growth factors such as VEGF (18) and basic fibroblast growth factor (bFGF) (25) on angiogenic potential has been well documented, their effects in conditions such as low serum need to be further investigated, in terms of changes in angiogenic genes' expression. Moreover, stemness genes' expression of differentiated cells needs to be further determined to obtain clearer understanding of the process.

As VEGF is more important for angiogenic differentiation than bFGF, we have chosen to evaluate the effect of reduced serum in culture medium supplemented with VEGF alone, on angiogenic potential of ASCs, using a quantitative gene expression approach. Thus, we have aimed to evaluate the effect of reduced serum conditions in the presence of VEGF, on stemness and angiogenic genes' expression level in serial passage ASCs. This study is a preliminary step in determining the most suitable culture conditions in which to increase ASC ability to undergo angiogenic differentiation, in preparing cells for therapeutic purposes. In this investigation, real-time PCR was used to study changes in angiogenic, endogenous and stemness genes' expression levels of ASCs in reduced serum conditions, and in the presence of VEGF as angiogenic inducer. Evaluation of these genes' expression levels may help in further understanding mechanism of angiogenic differentiation of ASCs.

Materials and methods

Isolation and expansion of ASCs

Human adipose tissues ($n = 6$) were collected after intra-operative lipectomy suction, at Subang Jaya Medical Centre, and adipose tissue was obtained from patients (aged 40–60 years) after receiving informed consent. All procedures performed in this study were reviewed and approved (approval number: 02-01-02-SF290) by the Research and Ethical Board of Universiti Kebangsaan Malaysia. Adipose tissues were digested in 0.3% type I collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. Digested samples were centrifuged at 500 g for 10 min to obtain the adipose stromal cells. ASCs were then suspended in medium consisting of equal volumes of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM/F12; Gibco-Invitrogen, Grand Island, NY, USA), supplemented with 10% foetal bovine serum (FBS; Gibco-Invitrogen), 1% glutamax (Gibco-Invitrogen), 1% antibiotic-antimycotic (Gibco-Invitrogen) and 50 µg/ml vitamin C (Sigma-Aldrich). Cells were then cultured in T25 flasks (Falcon; BD Biosciences, San Jose, CA, USA) and maintained at 37 °C with 5% CO₂. Culture medium was changed every 3 days. When the primary cultured ASCs (P0) reached 80–90% confluence, each specimen was trypsinized with 0.125% trypsin-ethylene diamine tetra acetic acid (EDTA) (Gibco-Invitrogen) and expanded for the next passage by 1:4, under the same culture conditions. Seeding density was 5000 cells/cm² for each of the passage. ASCs were cultured in medium supplemented with 10% FBS up to passage 6 (P6) and total number of cells, growth rate and viability were determined using the trypan blue exclusion technique; they were counted using a haemocytometer. Cell morphology was monitored using inverted light microscopy. Endogenous expression levels of stemness, angiogenic and endogenic genes of the ASCs at P1, P5 and P6 were quantified using real-time PCR.

Effect of reduced serum and VEGF supplementation on ASCs

Adipose stromal cells were divided into 3 groups at passage 6: (i) ASCs cultured in medium supplemented with 2% FBS plus 50 ng/ml VEGF; (ii) ASCs cultured in medium supplemented with 2% FBS without VEGF; and (iii) ASCs culture in medium supplemented with 10% FBS as the control group. Culture seeding density was 5000 cells/cm² for each group and medium was changed every 3 days. Alterations in cell morphology were monitored using an inverted microscope and the

total number of cells was counted at the end of each passage, to determine their growth rate. Stemness, angiogenic and endogenic genes expression levels of ASCs at P6 in these three groups were quantified using real-time PCR.

Total RNA extraction

Total RNA was extracted from the ASCs at P1, P5 and P6 ($n = 6$) in medium supplemented with 10% FBS; ASCs cultured to P6 ($n = 6$) in medium supplemented with 2% FBS plus 50 ng/ml of VEGF; and ASCs cultured to P6 ($n = 6$) in medium supplemented with 2% FBS without VEGF. Total RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) in accordance with manufacturer's instructions. Polyacryl carrier (Molecular Research Center) was added to precipitate total RNA which was then washed in 75% ethanol and air-dried. The total RNA was then dissolved in RNase- and DNase-free distilled water (Invitrogen, Grand Island, NY, USA) and immediately stored at -80°C . Yield and purity of total RNA were determined using a spectrophotometer (Bio-Rad, Hercules, CA, USA).

Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using SuperScript III reverse transcriptase (Invitrogen). The procedure was carried out according to the manufacturer's recommendations and protocol conditions were as follows: 10 min at 23°C

for primer annealing; 60 min at 50°C for reverse transcription; and 5 min at 85°C for reaction termination.

Real-time PCR analysis

Real-time PCR was performed using cDNA as template to evaluate stemness gene (*ABCG-2*, *Oct-4*, *TERT*, *BST-1*, *FGF4*, *SOX-2*, *Rex-1*, *Fzd9* and *Nanog-3*), angiogenic gene (*Hepatocyte Growth Factor (HGF)*, *Placenta Growth Factor (PGF)*, *Vascular Endothelial Growth Factor (VEGF)*, *Angiopoietin-1*, *basic Fibroblast Growth Factor (bFGF)*) and endogenic gene (*CD34*, *Ve-cadherin*, *PECAM-1*, *eNOS*, *vWF*, and *VEGFR-2*) expression level in ASCs cultured to P1, P5 and P6 ($n = 6$) in medium supplemented with 10% FBS; ASCs cultured at P6 ($n = 6$) in medium supplemented with 2% FBS plus 50 ng/ml of VEGF and ASCs cultured at P6 ($n = 6$) in medium supplemented with 2% FBS without VEGF. Real-time PCR was carried out using specific gene primer sequences (Tables 1–3), designed using Primer 3 software (<http://frodo.wi.mit.edu/primer3/>), based on published GeneBank database sequences. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as reference gene. The real-time PCR reaction was carried out using BioRad iCycler (Bio-Rad) with SYBR green as indicator. Each reaction mixture consisted of iQ SYBR Supermix, forward and reverse primers ($5\ \mu\text{M}$ each), deionized water and $1\ \mu\text{l}$ of cDNA template. Reaction conditions were: cycle 1, 95°C for 3 min (1X), cycle 2, step 1 95°C for 10 s and step 2 61°C for 30 s (40X). Specificity of the PCR product was confirmed with melting curve analysis and 2% agarose

Table 1. Stemness gene primer sequences and PCR product sizes

Gene	Accession number	Sequence (5'3') sense and antisense primers	Product size, base pair (bp)
Glyceradehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046	tcc ctg agc tga acg gga ag gga gga gtg ggt gtc gct gt	217
Octamer-binding protein3/4 (Oct-4)	NM_002701	aag gat gtg gtc cga gtg tg gaa gtg agg gct ccc ata gc	180
SRY-related HMG-box 2 (Sox2)	NM_003106	tta cct ctt cct ccc act cca ggt agt gct ggg aca tgt gaa	132
Fibroblast growth factor 4 (FGF4)	NM_002007	gat gag tgc acg ttc aag ga ggt tcc cct tct tgg tct tc	118
Reduced expression-1 (Rex-1)	NM_174900	aaa ggt ttt cga agc aag ctc ctg cga gct gtt tag gat ctg	185
Nanog 3	NM_024865	ctg tga ttt gtg ggc ctg aa tgt ttg cct ttg gga ctg gt	153
Frizzled 9 (FZD9)	NM_003508	cag aga agc tgg aga agc tca agg cgt tgc tag aca tag caa	100
ATP-binding cassette, sub-family G, member 2 (ABCG2)	NM_004827	agc tgc aag gaa aga tcc aa tgc cca tca caa cat cat ct	119
Bone marrow stromal cell antigen 1 (BST-1)	NM_004334	cga tta cca atc ctg ccc ta ttt gat ggg ata ggc tcc tg	154

Table 2. Angiogenic gene primer sequences and PCR product sizes

Gene	Accession number	Sequence (5' 3') sense and antisense primers	Product size, base pair (bp)
Vascular endothelial growth factor (VEGF)	NM_001033756	ccc act gag gag tcc aag at aaa tgc ttt ctc cga tct ga	173
Hepatocyte growth factor (HGF)	NM_001010932	ctg gtt ccc ctt caa tag ca ctc cag ggc tga cat ttg at	168
Placenta growth factor (PGF)	NM_002632	gtt cag ccc atc ctg tgt ct ctt cat ctt ctc ccg cag ag	199
Angiopoietin 1 (Ang-1)	NM_001146	gaa ggg aac cga gcc tat tc gct ctg ttt tcc tgc tgt cc	108
Basic fibroblast growth factor (bFGF)	NM_002006	ccg tta cct ggc tat gaa gg act gcc cag ttc gtt tca cc	158

Table 3. Endogenous gene primer sequences and PCR product sizes

Gene	Accession number	Sequence (5' 3') sense and antisense primers	Product size, base pair (bp)
Endothelial cell adhesion molecule 1 (PECAM-1)	NM_000442	tca aat gat cct gcg gta ttc cca cca cct tac ttg aca gga	169
CD 34	NM_001773	tgc aac atc tcc cac taa acc tct tat ttt gct cca ggc aga	128
Vascular endothelial cadherin (VE Cadherin)	NM_001795	cca cga aac gtg aag ttc aaa gca tcc cat tgt ctg aga tga	163
Endothelial nitric oxide synthase (eNOS)	NM_000603	ttt gcc ctt atg gat gtg aag cgc atc aaa gaa agc tca gtc	139
Von willebrand factor (vWF)	NM_000552	gac ctt gct gaa gag tca tcg gcc agt cag ctt gaa att ctg	184
Vascular endothelial growth factor receptor 2 (VEGFR2)	NM_002253	gca atc cct gtg gat ctg aa act cca tgc cct tag cca ct	193

gel electrophoresis. Expression level of each gene was normalized to GAPDH.

Immunocytochemical staining of ASCs

Adipose stromal cells at P6 were cultured on glass coverslips. At 60–80% confluence, cells were fixed in 4% paraformaldehyde overnight. Samples were then treated with 3% hydrogen peroxide (Sigma-Aldrich) for 5 min, followed by 1% bovine serum albumin (Sigma-Aldrich) incubation for 1 h at room temperature to reduce non-specific antibody binding. Samples were rinsed in Tris buffered saline (TBS; Dako, Glostrup, DA, Denmark) before being incubated in mouse anti-human PECAM-1 (1:100 dilution; Dako) and mouse anti-human vWF (1:400 dilution; Dako) monoclonal antibody, for 30 min at room temperature. Samples were rinsed in TBS to wash out any unbound antibody, before being incubated in secondary, anti-mouse antibody, labelled with horse-radish peroxidase (HRP) polymer (Dako), for 30 min at room temperature. Then, samples were rinsed again in TBS, and freshly prepared 3,3'-diaminobenzidine was applied for 10 min to develop the brownish coloured

final reaction product per positive sample. All slides were rinsed in TBS and counterstained with haematoxylin (Sigma-Aldrich), before being mounted with DPX (Sigma-Aldrich).

Statistical analysis

Numerical data were expressed as mean $\pm$ standard error of mean (SEM). Differences in quantitative RT-PCR ($n = 6$) data between tested groups were analysed for significance using Student's *t*-test. *P*-value < 0.05 was considered to be significant.

Results

Morphology and growth rate of ASCs

Adipose stromal cells at P1 had fibroblast-like morphology when cultured in medium supplemented with 10% FBS (Fig. 1a). Serially passaging the cells to P5, cultured in medium supplemented with 10% FBS revealed no change in morphology and cells maintained the fibroblastic features, however, cell density was lower after

Figure 1. Morphology of adipose stromal cells (ASCs). (a) ASCs at P1 cultured in medium with 10% FBS show fibroblastic and spindle-like morphology (50 $\times$). (b) ASCs at P5 cultured in medium with 10% FBS show similar features to those of ASCs at P1, but have lower cell density on the third-day culture (50 $\times$). (c) ASCs at P6 cultured in medium with 2% FBS are relatively larger and have flatter morphology (50 $\times$). (d) ASCs at P6 cultured in medium with 2% FBS plus vascular endothelial growth factor with flatter morphology, but with some elongation features ($\times 50$).

3 days in culture (Fig. 1b). When ASCs were cultured in medium supplemented with reduced serum (2% FBS) at P6, they had flatter morphology with relatively larger cytoplasmic volume (Fig. 1c). Addition of VEGF into the medium also caused increase in cytoplasmic volume relative to other features, and higher cell density compared to those in culture supplemented with 2% FBS alone (Fig. 1d).

Adipose stromal cells at P1, P5 and P6 cultured in medium supplemented with 10% FBS needed 3 and 4 days, respectively, to reach confluence, whereas ASCs at P6 cultured in both medium supplemented with 2% FBS and medium supplemented with 2% FBS plus VEGF needed 16 and 14 days, respectively, to reach confluence. Growth kinetics (Fig. 2) of ASCs cultured in medium supplemented with 10% FBS at P1 (704.8 ± 30.3 cell/cm²/day), P5 (599.8 ± 33.8 cell/cm²/day) and P6 (521.9 ± 30.6 cell/cm²/day) revealed significantly higher growth rate compared to ASCs at P6 cultured in medium supplemented with 2% FBS (62.5 ± 10.5 cell/cm²/day) and ASCs at P6 cultured in medium supplemented with 2% FBS plus VEGF (101 ± 50.2). ASCs cultured in medium supplemented with VEGF had significantly higher growth rate ($P < 0.05$) compared to ASCs cultured in medium supplemented with only 2% FBS.

Stemness genes' expression levels of ASCs

Expression level of *ABCG2* (Fig. 3a) showed no significant changes between ASCs at P1 and P5 cultured in medium supplemented with 10% FBS. *ABCG2* was expressed significantly higher ($P < 0.05$), 4-fold, in

Figure 2. Growth rate of adipose stromal cells (ASCs) ($n = 6$). ASCs at P1 cultured in medium with 10% FBS have the highest growth rate (704.8 ± 30.3 cell/cm²/day) and at P5; ASC growth rate shows a slight decrease (599.8 ± 33.8 cell/cm²/day). Growth rate of ASCs decreased significantly by P6 ($P < 0.05$) when cultured in medium with 2% FBS (62.5 ± 10.5 cell/cm²/day) and in medium with 2% FBS added plus vascular endothelial growth factor (VEGF). There was significant increase in growth rate when VEGF was added to the medium. Significant difference between groups ($P < 0.05$ and $n = 6$) is labelled as follows: *refers to $P < 0.05$ relative to ASCs at P1 in 10% FBS; #refers to $P < 0.05$ relative to ASCs at P5 in 10% FBS; •refers to $P < 0.05$ relative to ASCs at P6 in 10% FBS; and ○refers to $P < 0.05$ relative to ASCs at P6 in 2% FBS.

ASCs at P6 cultured in medium supplemented with only 2% FBS and in medium supplemented with 2% FBS plus VEGF (3-fold) compared to both ASCs groups at P1, P5 and P6. *BST-1* (Fig. 3b) had a similar pattern to that of *ABCG2*; its expression increased significantly ($P < 0.05$) by P6 when cultured in medium supplemented with only 2% FBS (6-fold) and in medium supplemented with 2% FBS plus VEGF (6-fold) compared to ASCs at P1, P5 and P6 cultured in medium

Figure 3. Stemness genes' expression level (relative to GAPDH expression level) of human adipose stromal stem cells (ASCs) in various media. Significant difference between groups ($P < 0.05$ and $n = 6$) is labelled as follows: *refers to $P < 0.05$ relative to ASCs at P1 in 10% FBS; #refers to $P < 0.05$ relative to ASCs at P5 in 10% FBS; •refers to $P < 0.05$ relative to ASCs at P6 in 10% FBS.

supplemented with 10% FBS. *FGF4* (Fig. 3c) also had a similar pattern to that of *ABCG2* and *BST1*, increased significantly ($P < 0.05$) at P6 when cultured in medium supplemented with only 2% FBS (50-fold) and in medium supplemented with 2% FBS plus VEGF (90-fold). *FZD9* (Fig. 3d) expression level increased significantly ($P < 0.05$) in the cells by P6 in medium supplemented with only 2% FBS (3-fold) and in medium supplemented with 2% FBS plus VEGF (3-fold) compared to ASCs at P1 and P6 cultured in 10% FBS. However, only ASCs at P6 cultured in medium supplemented with 2% FBS plus VEGF showed significantly higher ($P < 0.05$) *FZD9* expression compared to P5 cultures (by 1.5-folds). *Sox2* (Fig. 3e) expression increased significantly ($P < 0.05$) at P6 cultured in medium supplemented with only 2% FBS (4-fold) and in medium supplemented with 2% FBS plus VEGF (3-fold) com-

pared to P1 cultures. However, only ASCs at P6 cultured in medium supplemented with only 2% FBS (2-fold) showed significant change compared to ASCs at P5 and P6 cultured in 10% FBS. *Oct4* (Fig. 3f) expression increased significantly in cells cultured in both media (2% FBS only and 2% FBS supplemented with VEGF) at P6 compared to ASCs cultured at P1 and P6 in 10% FBS. *Rex1* (Fig. 3g) had an increasing pattern of expression at P6 when cultured in medium supplemented with only 2% FBS and in medium supplemented with 2% FBS plus VEGF, but no significant changes were recorded. *Nanog3* expression (Fig. 3h) increased significantly by P6 when ASCs were cultured in medium supplemented with 2% FBS (9-fold) and medium supplemented with 2% FBS plus VEGF (4-fold) compared to ASCs at P1, P5 and P6 cultured in medium supplemented with 10% FBS.

Figure 4. Angiogenic genes' expression level (relative to GAPDH) of human adipose stromal stem cells (ASCs) in various media. Significant difference between groups ($P < 0.05$ and $n = 6$) is labelled as follows: *refers to $P < 0.05$ relative to ASCs at P1 in 10% FBS; #refers to $P < 0.05$ relative to ASCs at P5 in 10% FBS; •refers to $P < 0.05$ relative to ASCs at P6 in 10% FBS; and ○refers to $P < 0.05$ relative to ASCs at P6 in 2% FBS.

Angiogenic genes' expression levels of ASCs

Hepatocyte Growth Factor (Fig. 4a) expression in ASCs did not show any significant changes in any of the treatment groups. *PGF* (Fig. 4b) expression in ASCs cultured in 2% FBS supplemented with VEGF decreased significantly ($P < 0.05$) compared to ASCs cultured in 10% FBS at both P1 and P6. *VEGF* (Fig. 4c) expression increased significantly ($P < 0.05$), approximately 3-fold, in ASCs at P6 cultured in both media (2% FBS only and 2% FBS supplemented with VEGF) compared to ASCs at P1, P5 and P6 cultured in medium supplemented with 10% FBS. However, significant reduction of *VEGF* expression was observed in ASCs at P6 cultured in medium supplemented with 2% FBS plus VEGF compared to ASCs cultured in medium supplemented with only 2% FBS. *Ang-1* expression (Fig. 4d) increased significantly ($P < 0.05$), 2-fold, in ASCs at P6 when cultured in med-

ium supplemented with 2% FBS compared to ASCs at P5 and P6 cultured in medium supplemented with 10% FBS. *Ang-1* expression also increased significantly ($P < 0.05$), 2-fold, in ASCs at P6 cultured in medium supplemented with 2% FBS plus VEGF compared to ASCs at P1 and P6 cultured in medium supplemented with 10% FBS. *BFGF* expression (Fig. 4e) increased significantly ($P < 0.05$), 3-fold, in ASCs at P6 cultured in medium supplemented with 2% serum compared to ASCs at P1, P5 and P6 cultured in medium supplemented in 10% FBS. *BFGF* expression also increased significantly ($P < 0.05$), 2-fold, in ASCs at P6 cultured in medium supplemented with 2% serum plus VEGF compared to ASCs at P1, P5 and P6. However, *BFGF* expression was reduced when ASCs at P6 cultured in medium supplemented with 2% FBS plus VEGF compared to ASCs cultured in medium supplemented with only 2% FBS.

Figure 5. Endogenic genes' expression level (relative to GAPDH) of human adipose stromal stem cells (ASCs) in various media. Significant difference between groups ($P < 0.05$ and $n = 6$) is labelled as follows: *refers to $P < 0.05$ relative to ASCs at P1 in 10% FBS; #refers to $P < 0.05$ relative to ASCs at P5 in 10% FBS; and •refers to $P < 0.05$ relative to ASCs at P6 in 10% FBS.

Endogenic genes' expression levels of ASCs

CD34 expression (Fig. 5a) increased significantly ($P < 0.05$) in ASCs at P6 cultured in medium supplemented with 2% FBS and medium supplemented with 2% serum plus VEGF compared with ASCs at P1, P5 and P6 cultured in medium supplemented in 10% FBS. *VE Cadherin* expression (Fig. 5b) decreased significantly ($P < 0.05$), 25-fold, when ASCs at P5 were compared to P1. *VE Cadherin* expression increased significantly ($P < 0.05$), 17-fold, when ASCs at P6 were cultured in medium supplemented with 2% serum and medium supplemented with 2% serum plus VEGF compared to ASCs at P5 in 10% FBS. However, only *VE Cadherin* in ASCs at P6 cultured in 2% FBS supplemented with VEGF increased significantly ($P < 0.05$) compared to ASCs at P6 in 10% FBS. *PECAM-1* expression (Fig. 5c) decreased significantly ($P < 0.05$), 2-fold, when ASCs at P5 compared with culture at P1. However, *PECAM-1* expression increased significantly ($P < 0.05$), 20-fold, when ASCs at P6 were cultured in medium supplemented with only 2% FBS compared to P1 and P5 cultures. No significant changes were observed in *PECAM-1* expression of ASCs at P6 when compared between 2% FBS and 2% FBS plus VEGF. *eNOS* gene (Fig. 5d) did not show any significant difference in its expression in any of the treatment groups but *vWF* gene (Fig. 5e) expression increased significantly ($P < 0.05$) in ASCs at P6 cultured in both media (2% FBS only and 2% FBS supplemented with VEGF) compared to ASCs at P6 in 10% FBS. *VEG-*

FR-2 (Fig. 5f) was significantly lower ($P < 0.05$), in ASCs at P5 compared to P1 cultured in the same medium. However, *VEGFR-2* expression of ASCs increased significantly ($P < 0.05$) at P6 when cultured in medium supplemented with 2% serum only (17-fold) compared to P5 and P6 in 10% FBS. *VEGFR* expression in 2% serum plus VEGF increased significantly ($P < 0.05$) when compared to P6 in 10% FBS.

Immunostaining of ASCs

Adipose stromal cells cultured in 10% FBS-supplemented medium did not show positive staining with anti-PECAM-1 and only stained lightly with anti-vWF (Fig. 6). In comparison, ASCs cultured in 2% FBS or 2% FBS plus VEGF were stained positively for both anti-PECAM-1 and anti-vWF. ASCs cultured in medium supplemented with 2%FBS only showed stronger positive staining with anti-PECAM-1 and anti-vWF compared to groups with added with VEGF (Fig. 6).

Discussion

Angiogenesis is a unique mechanism involved in development and expansion of blood vessels, as well as the lymphatic vasculature of the body and angiogenesis is important for repair and maintenance of perfusion in ischaemic diseases. The mechanism is particularly important for treatment of ischaemic heart disease, although it could be viewed as a harmful process, in cases of

Figure 6. Immunostaining of PECAM-1 (a–c) and vWF (d–f) on cultured human adipose stromal stem cells (ASCs). ASCs cultured in 10% FBS-supplemented medium did not stain with anti-PECAM-1 and only stained lightly with anti-vWF. ASCs cultured in 2% FBS or 2% FBS plus vascular endothelial growth factor (VEGF) stained positive with both anti-PECAM-1 and anti-vWF. (Magnification = $\times 100$).

tumour development. Use of stem cells in treatment of cardiovascular diseases has been applied clinically to patients in the late stages of the disease although recently, treatment of cardiovascular disease has shifted towards use of stem cells in development of new blood vessels. Thus, in the present study, we explored angiogenic potential of ASCs in different media by evaluating expression levels of stemness, angiogenic and endogenic genes using real-time PCR.

The study described here has focused on ASCs' potential to undergo pro-angiogenetic changes when grown in three types of culture media: (i) medium supplemented with 10% FBS; (ii) medium supplemented with 2% FBS; and (iii) medium supplemented with 2% FBS plus 50 ng/ml VEGF. Morphologically, ASCs at P1, P5 and P6 cultured in medium supplemented with 10% FBS had fibroblast-like phenotypic features, being elongated and spindle-like in form. This is a basic feature of stem cells in culture (26). However, at P6, when serum was reduced to 2%, ASCs assumed an apparently larger and flatten morphology. Reduction in serum concentration caused proliferation rate of ASCs to reduce, hence reduction in the total cell number at counting and growth rate. However, there was significant increase in ASC growth rate when VEGF was added to medium compared to ASCs cultured in medium with 2% serum alone.

Analysis of gene expression level using real-time PCR showed that ASCs expressed all stemness genes including pluripotent stem-cell markers, *Sox2*, *Oct4*, *Rex1* and *Nanog3* up to P6 in medium supplemented with 10% FBS; they maintained steady expression of stemness genes except of *Oct4*, which increased in expression at P5. However, the growth kinetic and differentiation ability of ASC were altered when cultured at later passages (13,27,28). While telomerase reverse transcriptase (*TERT*) was not expressed or at least not expressed at a level to be detected by real-time PCR, in various cultures, Bernardo *et al.* have reported that telomerase activity and *TERT* were not detected in bone marrow MSCs and their telomeres shortened during culture (29). However, Katz *et al.* showed that ASCs did not express telomerase (30). We found that on the contrary, most stemness gene expression levels increased when ASCs were cultured in medium with reduced serum (2% FBS) at P6 compared to ASCs in medium supplemented with 10% FBS at P1, P5 and P6. However, addition of VEGF did not cause any significant changes in terms of this stemness gene expression. These data suggest that reduced serum may induce endogenous mechanisms of ASCs to maintain their stemness and self-renewing properties.

Data from real-time PCR showed that angiogenic genes' expression in the ASCs at P1, P5 and P6 did not

have any difference in expression when cultured in medium supplemented with 10% serum. However, most angiogenic genes' expression increased when ASCs at P6 were cultured in medium with reduced serum (2% FBS). Gene expression levels of *vascular endothelial growth factor (VEGF)*, *angiopoietin-1 (Ang-1)* and *basic fibroblast growth factor (bFGF)* increased significantly, while both *hepatocyte growth factor (HGF)* and *placenta growth factor (PGF)* had steady expressions even in reduced serum medium. These angiogenic factors play important roles in development of angiogenesis. Angiogenesis is triggered by tissue-derived signals such as VEGF, which is controlled by ephrin-B2. Ephrin-B2 is a transmembrane ligand for Eph receptor tyrosine kinases, which induce sprouting behaviour and motility in angiogenic endothelium (31). Ang-1 decreases endothelial cell permeability and increases vascular stabilization through recruitment of pericytes and smooth muscle cells to expanding blood vessels during angiogenesis (32). bFGF induces endothelial cell proliferation and migration as well as the release of proteolytic enzymes during angiogenesis (33). Increase of these factors indicates that ASCs cultured with reduced serum have angiogenic potential superior to ASCs cultured in higher serum concentrations. However, addition of VEGF to medium did not cause a great deal of difference in expression of angiogenic genes of ASCs; expression of VEGF and bFGF mRNA were significantly low. This could be due to the exogenous source of VEGF that reduced the endogenous expression levels of both VEGF and bFGF for cell proliferation.

Most endogenic gene expression levels reduced after serial passage in 10% FBS (from P1 to P5), but when ASCs at P6 were cultured in reduced serum concentration (2% FBS only), endogenic markers such as CD34 (endothelial cell marker), platelet/endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial cadherin (VE Cadherin), von Willebrand factor (vWF) and VEGFR-2 increased significantly. During angiogenesis, PECAM-1 is involved in endothelial cell migration and attachment. It is also involved in organization of endothelial cells into vascular tubes (34). Vascular endothelial cadherin (VE Cadherin) is important in development of angiogenesis during embryonic development. It induces cell proliferation and apoptosis, as well as VEGF synthesis (35). Thus, increase in PECAM-1 and VE-Cadherin expression in ASCs cultured in reduced serum, increased endogenic potential of ASCs. Increased level of VEGFR-2, a receptor for VEGF, may indicate that ASCs at P6 cultured in 2% serum have differentiated or had characteristics of endothelial cells. This is supported by evidence showing that VEGFR-2 is one of the markers in early development

of endothelial and blood cells (36). Furthermore, endothelial nitric oxide synthase (eNOS) and vWF expression levels increased in culture with 2% FBS. Overall, addition of VEGF to culture medium did not show any significant difference in expression of endogenous genes, in ASCs.

Increase in stemness, angiogenic and endogenous genes' expression when ASCs were cultured in medium with reduced concentration of serum could induce adaptation to low growth factor-culture conditions by increasing endogenous production of mitotic agents. Low serum concentration, which leads to reduction in nutrient and growth factors needed by the cells, could potentially cause stressful events in the culture. Gimble *et al.* reported that ASCs increased production of paracrine growth factors in stressful environments (37). Rehman *et al.* demonstrated that VEGF expression and other angiogenic factors such as HGF and bFGF significantly increased when ASCs were cultured in 5% FBS without growth factors, in hypoxic conditions, for 72 h (18). Gimble *et al.* also reported that ASCs had the ability to increase growth factor and cytokines production and to promote new blood vessels from surrounding blood vessels of ischaemic tissue. Reduction in serum could also increase differentiation ability of ASCs (37). Serum increased proliferation of stem cells as it contains many cytokines and growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Other growth factors such as transforming growth factor beta (TGF β) and fibroblast growth factor-2 (FGF-2) increased ASC growth rate, but not their differentiation ability (38).

Addition of VEGF to medium with 2% serum did not increase angiogenic potential of the ASCs. However, they still maintained their stemness characteristics and angiogenic potential, as these genes were expressed. Growth kinetics of the ASCs did not show any significant increase with addition of VEGF compared to the 10% FBS culture. Similar results have been demonstrated where proliferation of ASCs cultured in EGM-2 medium supplemented with VEGF did not increase significantly (39), although the reason for this is still unclear. Perhaps VEGF alone may not be able to induce angiogenic differentiation of ASCs and can only do so in the presence of other growth factors. Brindle *et al.* have shown that VEGF alone is not sufficient to induce formation of stable blood vessels in tissue (40). Other investigators have used different combinations of growth factors in induction medium. Apart from VEGF, Miranville *et al.* used Matrigel to demonstrate that addition of other growth factors such as bFGF, epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and platelet-derived growth factor (PDGF) is able to increase

angiogenic potential of the stem cells (41). Use of VEGF and bFGF in combination has successfully induced endothelial differentiation (42) and use of endothelial growth media (EGM-2) supplemented with 2% FBS, VEGF and other growth factors such as IGF and EGF is also able to induce angiogenesis (6). Although VEGF may be a potent angiogenic factor at the beginning of angiogenesis, other angiogenic factors are needed for cells to proceed to subsequent stages of angiogenesis.

In conclusion, angiogenic potential of ASCs could be induced in medium with reduced concentration of serum after serial passaging. However, addition of VEGF did not cause any significant increase in angiogenic or endogenous genes' expression of ASCs cultured in 2% FBS-supplemented medium. Thus, enhancement of angiogenic potential of ASCs should be investigated in the future by combining VEGF with other growth factors.

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