The ratio of VEGF/PEDF expression in bone marrow mesenchymal stem cells regulates neovascularization

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Abstract

Angiogenesis, or neovascularization, is a finely balanced process controlled by pro- and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is a major pro-angiogenic factor, whereas pigment epithelial-derived factor (PEDF) is the most potent natural angiogenesis inhibitor. In this study, the regulatory role of bone marrow stromal cells (BMSCs) during angiogenesis was assessed by the endothelial differentiation potential, VEGF/PEDF production and responses to pro-angiogenic and hypoxic conditions. The *in vivo* regulation of blood vessel formation by BMSCs was also explored in a SCID mouse model. Results showed that PEDF was expressed more prominently in BMSCs compared to VEGF. This contrasted with human umbilical vein endothelial cells (HUVECs) where the expression of VEGF was higher than that of PEDF. The ratio of VEGF/PEDF gene expression in BMSCs increased when VEGF concentration reached 40 ng/ml in the culture medium, but decreased at 80 ng/ml. Under $CoCl_2$-induced hypoxic conditions, the VEGF/PEDF ratio of BMSCs increased significantly in both normal and angiogenic culture media. There was no expression of endothelial cell markers in BMSCs cultured in either pro-angiogenic or hypoxia culture conditions when compared with HUVECs. The *in vivo* study showed that VEGF/PEDF expression closely correlated with the degree of neovascularization, and that hypoxia significantly induced pro-angiogenic activity in BMSCs. These results indicate that, rather than being progenitors of endothelial cells, BMSCs play an important role in regulating the neovascularization process, and that the ratio of VEGF and PEDF may, in effect, be an indicator of the pro or anti-angiogenic activity of BMSCs.

Introduction

Angiogenesis or neovascularization is thought to be a regulated process between pro- and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factor and can initiate angiogenic differentiation of endothelial progenitor cells (EPCs) (Koch et al., 2006; Asahara and Kawamoto, 2004). VEGF is expressed by a wide range of cells including bone marrow derived mesenchymal stem cells (BMSCs) (Farhadi et al., 2005). Pigment epithelial-derived factor (PEDF) was originally purified from the conditioned media of human fetal retinal pigment epithelial (RPE) cells, and is the most potent natural angiogenesis inhibitor (Ohno-Matsui et al., 2003). PEDF is thought to be a key factor associated with avascularity of the cornea (Ohno-Matsui et al., 2003) and the balance between VEGF and PEDF expression plays a crucial role in retinal vascularization (Zhang et al., 2006). It is therefore clear that the balance between VEGF and PEDF is vital in the processes of angiogenesis and neovascularization. There is, however, little information to be found in the scientific literature concerning VEGF and PEDF expression in BMSCs with respect to angiogenesis and neovascularization, but it is known that BMSCs are involved in a number of pro-angiogenic and anti-angiogenic activities (Chen et al., 2003). It is therefore of interest to explore how VEGF and PEDF expression patterns affect the fate of BMSCs in the regulation of angiogenesis and neovascularization.

The bone marrow comprises two dominant stem cell populations, namely hematopoietic stem cells and mesenchymal stem cells (Bianco et al., 2001; Bianco et al., 2006). When cultured in vitro, the latter rapidly adhere to the tissue culture substrate and is thus easily separated from the non-adherent hematopoietic cells through repeated media changes. Hematopoietic stem cells (HSCs) serve as the reservoir for the various blood cells, such as erythrocytes, leukocytes, macrophages or platelets. They also contain endothelial progenitor cells (EPCs), which are capable of differentiating into mature endothelial cells (Loomans et al., 2006; Schatteman et al., 2007). BMSCs are well characterized and are negative for CD45, CD14, CD31 and CD34, but positive for CD105, CD 44, CD73 and CD90. They are a heterogeneous

mix of multipotent progenitor cells, capable of differentiating into mesodermal cell lineages, such as osteoblasts, chondrocytes, fibroblasts and adipocytes (Reyes et al., 2001). However, whether BMSCs are capable of differentiating into endothelial cell lineages has yet to be definitively proven and therefore remains an open question (Oswald et al., 2004; Zhang et al., 2007).

In this study we investigated, both *in vitro* and *in vivo*, the differentiation potential of BMSCs into endothelial cells and the regulatory role in angiogenesis in response to pro-angiogenic and hypoxic conditions.

Materials and Methods

Cells and cell culture

Human bone marrow was sourced from patients undergoing elective surgery at the orthopaedics department at the Prince Charles Hospital (PCH) in Brisbane, Queensland, Australia. Informed consent was given by all participants and the project had approval from the ethics committees of the PCH and the Queensland University of Technology. Mononuclear cells (MNCs) were isolated from the bone marrow by density gradient centrifugation over Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's protocol and plated out in tissue culture flasks in low glucose Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Australia Pty Ltd., Mt Waverley, VIC, Australia) containing 10% (v/v) fetal calf serum (FCS; InVitro Technology, Noble Park, VIC, Australia) and 1% (v/v) penicillin/streptomycin (Invitrogen). Unattached hematopoietic cells were removed by subsequent media changes. When reaching the 70-80% confluence, the attached mesenchymal cells were subcultured at a seeding density of 3×10^3 / cm^2 after treatment with 0.25% Trypsin/EDTA (Invitrogen). Only early passage cells (P2-P5) were used in this study. Human umbilical vein endothelial cells (HUVECs; Clonetics, San diego, CA, USA) were used as positive controls when required. HUVECs were cultured in a defined endothelial cell growth medium (ECGM) containing VEGF, FGF-2, IGF-1, EGF, ascorbic acid and hydrocortisone (EGM-2; Lonza Australia Pty Ltd., Mt Waverley, VIC, Australia) supplemented with 2% FCS.

The medium was changed for both BMSCs and HUVECs every three days until the cells had reached confluence.

Endothelial cell differentiation of BMSCs under the stimulation of VEGF

BMSCs sourced from five patients (one female and four males ranging from 40 to 78 years of age, average of 61 years) were included in this experiment. At approximately 80% confluence, angiogenic differentiation media, low glucose DMEM supplemented with 5% FCS and 20, 40 or 80 ng VEGF (R&D Systems Inc., Minneapolis, MN, USA) per ml(Oswald et al., 2004; Zhang et al., 2007; Xu et al., 2009), were applied to the cells. The media were replenished halfway through a 6-day culture period, after which the cells were harvested and subjected to real time quantitative PCR (RT-qPCR), western blot, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. The ability of these BMSCs to form *in vitro* vessel-like structures was tested on a Matrigel substrate (Oswald et al., 2004; Xu et al., 2009)(BD biosciences, North Ryde, NSW, Australia).

Endothelial cell differentiation of BMSCs under cobalt chloride-induced hypoxia

BMSCs sourced from six patients (two female and four male patients ranging from 47 to 76 years of age, average 57 years) were used in this experiment. The cells were grown to 80% confluence and then subcultured into the following three groups: (i) BMSCs cultured in normal DMEM containing 5% FCS; (ii) BMSCs cultured in DMEM containing 5% FCS and 100 μM cobalt chloride; and (iii) BMSCs cultured in DMEM containing 5% FCS, 100 μM cobalt chloride and 20 ng VEGF/ml. The media were changed every three days and on day 6, RT-qPCR, western blot, ELISA and immunohistochemistry were conducted. The ability of these BMSCs to form *in vitro* vessel-like structures was tested on a Matrigel substrate.

Real time quantitative polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen) and cDNA synthesized from 1 μg of RNA using SuperScript III reverse transcriptase (Invitrogen).

RT-qPCR was performed with SYBR Green: 12.5 µl 2X SYBR Green QPCR master mix (Roche, Castle Hill, NSW, Australia) was mixed with 5 µl water, 2.5 µl reverse and forward primers (Sigma-Aldrich Pty. Ltd**,** Castle Hill, NSW, Australia) and 2.5 µl cDNA template for a 25 µl final volume in a 96-well PCR plate. The mRNA expressions of PEDF, VEGF, CD31, VEGF receptor 2 (VEGFR2), von Willebrand factor (vWF) and endothelium nitric oxide synthases III (eNOSIII) were assayed and normalized against the 18s house keeping gene. Forward and reverse primers of each assayed gene are detailed in Table 1. Each sample was performed in triplicate and the reactions were run on ABI Prism 7300 sequence detection system (AB Applied Biosystems, Melbourne, Australia). The mean cycle threshold (Ct) value of each target gene was normalized against Ct value of 18s; the relative expression calculated using the following formula: $2^{-(\text{normalized average Cts})} \times 10^4$.

Western blot

Total protein was harvested in a cell lysis buffer supplemented with a proteinase inhibitor cocktail (Roche Products Pty. Ltd., Dee Why, NSW., Australia). The protein concentration was determined with a BCA protein assay kit (Sigma) and 10 µg proteins from each sample were separated on SDS-PAGE gels. The proteins were transferred onto a nitric cellulous membrane (Pall corporation, East Hills, NY, USA) by semi-dry transfer method. The membranes were probed with hypoxia inducible factor 1 alpha (HIF-1α) (1:1000, rabbit anti-human; Santa Cruz Biotechnology Inc., Santa Cruz, CA. U.S.A.), VEGF (1:2,000, rabbit anti-human; Thermo Fisher Scientific, Fremont, CA, USA), PEDF(1:2,000, mouse anti-human; Millipore, North Ryde, NSW, Australia), and α-tubulin (1:5,000, rabbit anti-human; Abcam Inc., Cambridge, MA, USA) primary antibodies. These were bound to HRP-conjugated anti-mouse or rabbit secondary antibodies (1:10,000; Thermo Fisher Scientific, Fremont, CA, USA). The bands were visualized using Super-Signal substrate (Thermo Fisher Scientific, Fremont, CA, USA) and images captured on X-ray films.

Enzyme-linked immunosorbent assay (ELISA)

The protein expression of VEGF and PEDF by BMSCs under different treatment conditions were tested using ELISA assay kits for VEGF (R&D Systems Inc.) and PEDF (CHEMICON, Millipore) . Briefly, BMSCs cultured in flasks were thoroughly washed with phosphate buffer saline (PBS) for three times and cell lysate was produced by adding the lysis buffer supplemented with a proteinase inhibitor cocktail (Roche) and shaking at 4°C for 1h. Then the lysate was transferred into eppendorf tubes and spinned at 10,000 g for 10mins to remove cell debris. The supernatant was collected for the ELISA tests. The test was performed in triplicates and results were expressed as the amount (pg) of VEGF or PEDF in per μg total cell protein, which was measured by the BCA protein assay kit (Sigma).

Immunohistochemistry

BMSCs and HUVECs were cultured in chamber slices and fixed in 4% paraformaldehyde and washed at least three times with PBS. The cells were permeablized with 0.1% Triton X solution for 6 min and endogenous peroxidase activity quenched by incubating the sample slices with 3% H_2O_2 for 15 min, then blocked with 10% swine serum for 1 h. The cells were incubated with the vWF (Rabbit anti-human, 1:300, Millipore) and VEGFR2 (goat anti-human, 1:100, R&D Systems, Inc., Minneapolis, MN, USA), VEGF (rabbit anti-human, 1:50, Thermo Fisher Scientific) and PEDF (mouse anti-human, 1:100, Millipore) primary antibodies overnight at 4°C, followed by incubation with the biotinylated swine-anti-mouse, rabbit, goat universal secondary antibody (DAKO Multilink, CA, USA) for 15 min, and then with horseradish peroxidase-conjugated avidin-biotin complex (DAKO Multilink, CA, USA) for another 15 min. The slides were counter stained with Mayer's haematoxylin (HD Scientific Pty Ltd., Kings Park, NSW, Australia) and the antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 min.

Matrigel Assay

Aliquots (50 µl) of growth factor-reduced phenol red free Matrigel matrix (BD biosciences, North Ryde, NSW, Australia) were added into the wells of 96-well cell culture plates and allowed to polymerize at 37°C for a minimum of 30 min. 3×10^3 BMSCs (randomly sourced from one patient described above), HUVECs or BMSCs cultured under different conditions for 6 days, i.e. with different VEGF concentrations or under cobalt chloride-induced hypoxia, were added onto the matrigel matrix and original culture conditions were maintained. The new vessel-like networks formed on the Matrigel was observed using a microscope at 40× magnification after 6 h incubation at 37°C and 5% CO_2 condition. To study the interactions between the HUVECs and BMSCs, 3×10^3 HUVECs were mixed with either $CoCl_2$-treated BMSCs or untreated BMSCs in serum-free DMEM at the ratio of 1:1, and seeded onto the Matrigel. Unmixed HUVECs and $CoCl_2$-treated BMSCs were used as controls. Vessel networks formed on the Matrigel by different cell combinations were photographed after 24 and 72 h incubation. The total number of vessels in five randomly selected areas from each well was recorded using the Axion software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). The experiment was done in triplicates and the average was taken for statistical analysis.

In vivo vascularization assessment

Three female 6 week-old severe combined immunodeficient (SCID) mice (Animal Resources Centre, Canning Vale, WA, Australia) were used to assess the *in vivo* regulatory role of BMSCs in neovascularization. Ethics approval for this experiment was granted from the QUT Animal Ethics Committee. The animals were anesthetized with 10 µl/g bodyweight of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml), injected intraperitoneally. A total of 5×10^4 cells of one of five conditions: (i) BMSCs, (ii) HUVECs, (iii) BMSCs treated with 20 ng/ml VEGF, (iv) BMSCs treated with 100 µM cobalt chloride or (v) BMSCs treated with both 20 ng/ml VEGF and 100 µM cobalt chloride, were mixed with Matrigel at a ratio of 1:2 to reach a final volume of 300 µl. Gel without cells was used as a negative control.

Each mouse was injected with six gels (one from each group) with three on either side of the dorsal area, approximately 1 cm apart. All animals had recovered by the following day, and were sacrificed after 10 days and the implants were retrieved, photographed and fixed in 4% paraformaldehyde. After paraffin embedding, the implants were sectioned and three serial sagittal slices, close to the centre of each implant, used for immunohistochemical staining. A vWF antibody (rabbit anti-human, 1:300, Millipore) was used to detect the endothelial cells, and all vWF^+ positive cells, capillaries or blood vessels were counted on each slice and normalized to the slice area (mm^2). The average from each group was used for statistical analysis. To determine the relationship of VEGF *vs.* PEDF expression and the degree of neovascularization, the VEGF (1:50) and PEDF (1:100) antibodies were used to stain the slices.

Statistical analysis

Analysis was performed using SPSS software (SPSS Inc., Chicago, Il., USA). All the data was analyzed using Student-*t*, one-way ANOVA or Friedman test. The significance level was set at $p \leq 0.05$.

Results

VEGF and PEDF expressions in BMSCs and HUVECs

RT-qPCR revealed that gene expression of both VEGF and PEDF in BMSCs was significantly higher than that in HUVECs (Student-*t* test, $p<0.01$) (Fig. 1A), and also that the ratio of VEGF to PEDF differed between the two cell types. The PEDF expression in BMSCs was significantly higher compared to VEGF (Student-*t* test, $p<0.05$) (Fig. 1A), whereas in HUVECs the PEDF expression was significantly less than the VEGF expression (Student-*t* test, $p<0.05$) (Fig. 1A). The average VEGF/PEDF expression ratio in HUVECs was around 8.0, which was 16 times greater than that (around 0.5) of BMSCs (Student-*t* test, $p<0.01$) (Fig. 1B). Western blot analysis showed stronger expression of PEDF and VEGF in BMSCs compared with HUVECs (Fig. 1C). Immunohistochemical staining of VEGF and PEDF

confirmed the high expressions in BMSCs and weak VEGF and PEDF expressions were noted in HUVECs (Fig. 1D).

The VEGF/PEDF expression pattern and endothelial cell differentiation of BMSCs under different extracellular VEGF concentrations

When cells were cultured in angiogenic media with increasing VEGF concentrations, the VEGF/PEDF gene expression in BMSCs showed an interesting pattern (Fig 2). The VEGF expression increased to around 1.5 times the original expression at 20 ng/ml extracellular VEGF, returning to its original expression when extracellular VEGF increased from 40 ng/ml to 80 ng/ml (Friedman test, $p<0.05$) (Fig.2 A). When the extracellular VEGF increased from 0 to 40 ng/ml, PEDF expression decreased by nearly half the original expression level. At 80 ng/ml VEGF, however, the PEDF expression returned to approximately 75% of its original expression (Friedman test, $p<0.05$) (Fig.2 B). The VEGF/PEDF gene expression ratio reached a peak average value of 0.8 at 40 ng/ml extracellular VEGF (Friedman test, $p<0.05$) (Fig.2 D). Western blot image showed a similar pattern in VEGF/PEDF expression ratio (Fig.2 C). ELISA results showed the general increasing trend for VEGF and decreasing trend for PEDF despite the absence of statistical differences (Fig.4).

Endothelial differentiation of BMSCs at different extracellular VEGF concentrations was also tested with RT-qPCR of the endothelial cell markers CD31, VEGFR2, vWF and eNOSIII, with HUVECs serving as control. The gene expression of these markers were all significantly higher in HUVECs than in BMSCs cultured in either normal cell culture media or VEGF supplemented media (one-way ANOVA, $p<0.01$) (Fig.2 E). There was no significant difference in the expression of those endothelial cell markers between different BMSCs groups (one-way ANOVA, $p>0.05$) (Fig.2 E). These findings were further confirmed by immunohistochemical staining against two typical endothelial cell markers VEGFR2 and vWF (Fig. 5).

The VEGF/PEDF expression pattern and endothelial cell differentiation of

BMSCs under cobalt chloride induced hypoxia

The hypoxia induced by $CoCl_2$ was firstly confirmed by the accumulated HIF-1α protein within the $CoCl_2$-treated BMSCs revealed by the western blot test (Fig.3 C). When BMSCs were cultured under hypoxic condition induced by 100 μM $CoCl_2$, the VEGF mRNA expression increased, on average, nearly 5 fold (Fig.3 A) and reduced PEDF mRNA expression by nearly 50% (Friedman test, $p<0.05$) (Fig.3 B). The addition of 20 ng/ml VEGF did not significantly influenced the effect of $CoCl_2$ on the VEGF/PEDF gene expression (Friedman test, $p>0.05$) (Fig.3 A&B). The VEGF/PEDF gene expression ratio increased to about 4.0 under $CoCl_2$ culture condition (Fig.3 F). PEDF and VEGF protein expression detected by western blot and ELISA confirmed the gene expression pattern under the $CoCl_2$-induced hypoxia condition (Fig.3 D & Fig.4). Evidence of endothelial cell differentiation of BMSCs under hypoxic conditions was tested with RT-qPCR of CD31, VEGFR2, vWF and eNOSIII endothelial gene expression. There was no significant difference in the expression of these markers between BMSC controls and BMSCs treated with either 100 μM $CoCl_2$ or 100 μM $CoCl_2$ plus 20 ng/ml VEGF (one-way ANOVA, $p>0.05$) (Fig.3 E), and also confirmed by immunohistochemical staining against two typical endothelial cell markers VEGFR2 and vWF (Fig. 5).

Enhanced vessel formation of HUVECs on Matrigel by $CoCl_2$-treated BMSCs

The Matrigel assays showed that HUVECs formed new vessel-like structures more readily within 6 h (Fig.6G), compared to undifferentiated BMSCs (Fig.6A) or endothelial cell-differentiated BMSCs in 20 ng/ml VEGF (Fig.6B), 40 ng/ml VEGF (Fig.6C), and 80 ng/ml VEGF (Fig.6D), as well as in $CoCl_2$ (Fig.6E) or $CoCl_2$ plus 20 ng/ml VEGF (Fig.6F). The number of vessels formed on Matrigel was significantly higher in HUVECs (one-way ANOVA, $p<0.01$) (Fig.6P). There was no difference in the ability to form vessel-like structures between BMSC groups (one-way ANOVA, $p>0.05$) (Fig.6P).

HUVECs, when mixed with $CoCl_2$-treated BMSCs (Fig.6 H&L) in serum-free DMEM, formed more stable vessel-like structures at both 24 (Fig.6 H-K) and 72 h

(Fig.6L-O) incubation on the Matrigel compared to HUVECs with untreated BMSCs (Fig.6 I&M), $CoCl_2$ treated BMSCs alone (Fig.6 J&N) or HUVECs alone (Fig.6 K&O). The number of vessel-like structures formed by HUVECs with $CoCl_2$-treated BMSCs was more than any other group after both 24 and 72 h incubation (one-way ANOVA, $p<0.001$) (Fig.6Q).

In vivo neovascularization of BMSCs

The Matrigel implants were harvested from the SCID mice and images captured with a stereomicroscope (Fig.7 a-f). There was no obvious neovascularization visible in the Matrigel control (Fig. 7a), or in Matrigels containing untreated BMSCs (Fig. 7b) or BMSCs treated with VEGF (Fig. 7c). Matrigels with $CoCl_2$ treated BMSCs (Fig. 7d) or $CoCl_2$+VEGF (Fig.7e), or HUVECs (Fig. 7f) showed an obvious blood vessel formation inside implants, and this was confirmed by vWF staining of the samples. Almost no vWF expression was detected in Matrigel without cells (Fig.7g), with untreated BMSCs (Fig.7h) or with BMSCs treated with 20 ng/ml VEGF (Fig.7i). vWF positive cells were found in Matrigel with BMSCs treated with $CoCl_2$ (Fig.7j), Matrigel with BMSCs treated with $CoCl_2$ plus VEGF (Fig.7k) and Matrigel with HUVECs (Fig.7l). Higher magnification (400x) of vWF staining of the Matrigel with BMSCs treated with $CoCl_2$ showed blood vessels and capillaries (Fig.7m) and a small artery growing into the gel (Fig.7n). Matrigel containg HUVECs also showed positive capillaries in the higher magnification (Fig.7o). The number of vWF positive cells and blood vessels on each slice were counted and normalized against the area of each slice. These results showed that Matrigels containing HUVECs, BMSCs treated with $CoCl_2$ alone or together with VEGF had significantly more vWF^+ cells and blood vessels in comparison with Matrigels containing untreated BMSCs or BMSCs treated with only VEGF (Friedman test, $p<0.01$) (Fig.7p).

When stained with VEGF and PEDF it was revealed in non-vascularized areas of the Matrigels containing untreated BMSCs that stromal cells showed stronger expression of PEDF (Fig.8 A&B) compared to the highly vascularized areas of Matrigels carrying $CoCl_2$-treated BMSCs (Fig.8 C&D). In Matrigels containing $CoCl_2$-treated

BMSCs, robust VEGF expression was seen in both newly formed blood vessel walls (Fig.8 E) and in the surrounding stromal cells (Fig.8 F&G). The positive staining of vWF was also noted in the endothelial cell of newly form blood vessel (Fig.8 H).

Discussion

BMSCs are cells isolated from the bone marrow mononuclear cell and which attach to cell culture plastic surfaces. It has been argued that *in vitro* cultured BMSCs might be capable of differentiating into endothelial cells lineages, or in other words, that *in vitro* cultured BMSCs contained endothelial progenitor cells (Oswald et al., 2004; Zhang et al., 2007). To obtain BMSCs, the standard method for BMSCs isolation and expansion was used in this study, and only cells from passage 2 to 5 were used in an effort to eliminate HSCs contamination. Following a previously described angiogenic differentiation protocol, which prescribes a 6 day angiogenic induction period (Cipriani et al., 2007; Oswald et al., 2004; Zhang et al., 2007), BMSCs were not capable of trans-differentiation into endothelial cells under either VEGF stimulation or $CoCl_2$-induced hypoxic conditions, based on criteria of endothelial cell surface marker expression and Matrigel tube structure formation, when compared with the endothelial cell line of HUVECs. An extended endothelial differentiation period (2 and 3 weeks) was also investigated, including the application of HUVECs-specific media (EGM-2), with similar negative results (data not shown).

BMSCs are reported to be involved in a number of tissue development and regeneration processes, such as osteogenesis, chondrogenesis and angiogenesis (Xia et al., 2008; Connelly et al., 2008; Lamagna and Bergers, 2006; Bexell et al., 2009; Ozerdem et al., 2005). The role of BMSCs during angiogenesis is far from clear, although some studies have shown that BMSCs may support and stabilize newly formed blood vessels as pericytes (Lamagna and Bergers, 2006; Bexell et al., 2009; Ozerdem et al., 2005). Within the angiogenic environment, VEGF is one of the major

pro-angiogenic growth factor that affect endothelial cell differentiation, migration and blood vessel formation (Shibuya, 2008; Otrock et al., 2007; Yamazaki and Morita, 2006), and VEGF concentration tends to be elevated in the angiogenic areas (Harlozinska et al., 2004). PEDF, on the other hand, is probably the most potent angiogenesis inhibitor capable of preventing vascularisation and inducing apoptosis in endothelial cells (Notari et al., 2006; Ohno-Matsui et al., 2003). The ratio or balance between these two antagonistic factors (VEGF/PEDF) in a local environment therefore has a great effect on angiogenesis and vascularization (Zhang et al., 2006). VEGF expression in BMSCs has been investigated in the past (Sena et al., 2007; Wrobel et al., 2003; Cai et al., 2002), but studies concerning the VEGF feedback loop and interactions between VEGF and PEDF in BMSCs has not been forthcoming. The findings reported here reveal for the first time that PEDF is much more strongly expressed in BMSCs than is VEGF. Interestingly, VEGF expression in HUVECs, although quite low when compared with BMSCs, is much higher than PEDF, the VEGF/PEDF ratio being more or less diametrically opposite that of BMSCs. Together these findings suggest that BMSCs may not be an angiogenesis-promoting cell population, when in a normoxia and neutral environment. This view is supported by a recent report which suggests that MSCs have a negatively regulatory role and are capable of inhibiting capillary growth at high cell numbers (Otsu et al., 2009).

In a pro-angiogenic environment, extracellular VEGF concentration increases during the angiogenic process (Harlozinska et al., 2004). In this study, it was found that depending on the concentration, extracellular VEGF set up a general positive feedback with the endogenous VEGF expression of BMSCs, although a certain high level of extracellular VEGF concentration (80 ng/ml in this study) dropped the endogenous VEGF production of the BMSCs; On the other hand, the increasing extracellular VEGF concentration suppressed the PEDF expression of BMSCs. These changes in the VEGF/PEDF expression pattern indicates that when environmental VEGF is at an appropriate concentration, BMSCs may play a more active role in the angiogenic process.

Cobalt chloride is a hypoxia mimicking agent commonly used to activate hypoxia-related responses in cells (Lee et al., 2007). The biochemical and molecular mechanisms of $CoCl_2$ induced hypoxia have been shown to be similar to those in low oxygen tension (Lee et al., 2007). Changes to gene expression in BMSCs as a result of hypoxia has been reported (Ohnishi et al., 2007), however, little is known about hypoxia effect on the endothelial cell differentiation and VEGF/PEDF expression patterns in BMSCs. In this study we showed that $CoCl_2$-induced hypoxia significantly increases VEGF expression and suppresses PEDF expression, suggesting that hypoxia drives BMSCs to favor angiogenesis and neovascularisation. We also demonstrated that $CoCl_2$-treated BMSCs appeared to enhance and stabilize the vessel-like structure formed by HUVECs in Matrigel, further demonstrating a supporting role for BMSCs in neovascularization when BMSCs were in a hypoxia environment.

Our in vivo study showed that the VEGF/PEDF expression ratio in BMSCs was closely correlated with neovascularization. The VEGF/PEDF ratio in undifferentiated or VEGF-treated BMSCs was low (less then "1" at both gene and protein levels), and no *in vivo* blood vessel formation was found after in vivo transplantation. However, in $CoCl_2$-induced hypoxia culture condition the VEGF/PEDF expression ratio in BMSCs reversed up to around "4" at both gene and protein levels, and once transplanted a higher degree of neovasculariztion was observed. The distribution of VEGF and PEDF in the neovascularized areas further confirmed that more VEGF was expressed in blood vessel forming areas, whereas more PEDF was expressed in areas of relatively low vessel formation.

The findings in this study suggest that BMSCs are important regulators in the neovascularization, rather than as a source of endothelial progenitor cell, under both normoxia and hypoxia conditions. The ratio of VEGF and PEDF may therefore be an indicator of the pro- and anti-angiogenic activity of BMSCs.

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References:

Asahara, T., and Kawamoto, A. (2004) Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 287:C572-579.

Bexell, D., Gunnarsson, S., Tormin, A., Darabi, A., Gisselsson, D., Roybon, L., Scheding, S., and Bengzon, J. (2009) Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas. Mol Ther 17:183-190.

Bianco, P., Kuznetsov, S.A., Riminucci, M., and Gehron Robey, P. (2006) Postnatal skeletal stem cells. Methods Enzymol 419:117-148.

Bianco, P., Riminucci, M., Gronthos, S., and Robey, P.G. (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180-192.

Cai, S., Ma, Q., Yu, X., Dang, G., and Ma, D. (2002) Expression of human VEGF(121) cDNA in mouse bone marrow stromal cells. Chin Med J (Engl) 115:914-918.

Chen, J., Zhang, Z.G., Li, Y., Wang, L., Xu, Y.X., Gautam, S.C., Lu, M., Zhu, Z., and Chopp, M. (2003) Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. Circ Res 92:692-699.

Cipriani, P., Guiducci, S., Miniati, I., Cinelli, M., Urbani, S., Marrelli, A., Dolo, V., Pavan, A., Saccardi, R., Tyndall, A., Giacomelli, R., and Cerinic, M.M. (2007) Impairment of endothelial cell differentiation from bone marrow-derived mesenchymal stem cells: new insight into the pathogenesis of systemic sclerosis. Arthritis Rheum 56:1994-2004.

Connelly, J.T., Garcia, A.J., and Levenston, M.E. (2008) Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. J Cell Physiol 217:145-154.

Farhadi, J., Jaquiery, C., Barbero, A., Jakob, M., Schaeren, S., Pierer, G., Heberer, M., and Martin, I. (2005) Differentiation-dependent up-regulation of BMP-2, TGF-beta1, and VEGF expression by FGF-2 in human bone marrow stromal cells. Plast Reconstr Surg 116:1379-1386.

Harlozinska, A., Sedlaczek, P., Kulpa, J., Grybos, M., Wojcik, E., Van Dalen, A., and Einarsson, R. (2004) Vascular endothelial growth factor (VEGF) concentration in sera and tumor effusions from patients with ovarian carcinoma. Anticancer Res 24:1149-1157.

Koch, S., Yao, C., Grieb, G., Prevel, P., Noah, E.M., and Steffens, G.C. (2006) Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF. J Mater Sci Mater Med 17:735-741.

Lamagna, C., and Bergers, G. (2006) The bone marrow constitutes a reservoir of pericyte progenitors. J Leukoc Biol 80:677-681.

Lee, H., Bien, C.M., Hughes, A.L., Espenshade, P.J., Kwon-Chung, K.J., and Chang, Y.C. (2007) Cobalt chloride, a hypoxia-mimicking agent, targets sterol synthesis in the pathogenic fungus Cryptococcus neoformans. Mol Microbiol 65:1018-1033.

Loomans, C.J., Wan, H., de Crom, R., van Haperen, R., de Boer, H.C., Leenen, P.J., Drexhage, H.A., Rabelink, T.J., van Zonneveld, A.J., and Staal, F.J. (2006) Angiogenic murine endothelial progenitor cells are derived from a myeloid bone marrow fraction and can be identified by endothelial NO synthase expression. Arterioscler Thromb Vasc Biol 26:1760-1767.

Notari, L., Baladron, V., Aroca-Aguilar, J.D., Balko, N., Heredia, R., Meyer, C., Notario, P.M., Saravanamuthu, S., Nueda, M.L., Sanchez-Sanchez, F., Escribano, J., Laborda, J., and Becerra, S.P. (2006) Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. J Biol Chem 281:38022-38037.

Ohnishi, S., Yasuda, T., Kitamura, S., and Nagaya, N. (2007) Effect of hypoxia on gene expression of

bone marrow-derived mesenchymal stem cells and mononuclear cells. Stem Cells 25:1166-1177.

Ohno-Matsui, K., Yoshida, T., Uetama, T., Mochizuki, M., and Morita, I. (2003) Vascular endothelial growth factor upregulates pigment epithelium-derived factor expression via VEGFR-1 in human retinal pigment epithelial cells. Biochem Biophys Res Commun 303:962-967.

Oswald, J., Boxberger, S., Jorgensen, B., Feldmann, S., Ehninger, G., Bornhauser, M., and Werner, C. (2004) Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 22:377-384.

Otrock, Z.K., Makarem, J.A., and Shamseddine, A.I. (2007) Vascular endothelial growth factor family of ligands and receptors: review. Blood Cells Mol Dis 38:258-268.

Otsu, K., Das, S., Houser, S.D., Quadri, S.K., Bhattacharya, S., and Bhattacharya, J. (2009) Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. Blood 113:4197-4205.

Ozerdem, U., Alitalo, K., Salven, P., and Li, A. (2005) Contribution of bone marrow-derived pericyte precursor cells to corneal vasculogenesis. Invest Ophthalmol Vis Sci 46:3502-3506.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L., and Verfaillie, C.M. (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98:2615-2625.

Schatteman, G.C., Dunnwald, M., and Jiao, C. (2007) Biology of bone marrow-derived endothelial cell precursors. Am J Physiol Heart Circ Physiol 292:H1-18.

Sena, K., Sumner, D.R., and Virdi, A.S. (2007) Modulation of VEGF expression in rat bone marrow stromal cells by GDF-5. Connect Tissue Res 48:324-331.

Shibuya, M. (2008) Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis. BMB Rep 41:278-286.

Wrobel, T., Mazur, G., Surowiak, P., Wolowiec, D., Jelen, M., and Kuliczkowsky, K. (2003) Increased expression of vascular endothelial growth factor (VEGF) in bone marrow of patients with myeloproliferative disorders (MPD). Pathol Oncol Res 9:170-173.

Xia, Z., Locklin, R.M., and Triffitt, J.T. (2008) Fates and osteogenic differentiation potential of human mesenchymal stem cells in immunocompromised mice. Eur J Cell Biol 87:353-364.

Xu, J., Liu, X., Chen, J., Zacharek, A., Cui, X., Savant-Bhonsale, S., Liu, Z., and Chopp, M. (2009) Simvastatin enhances bone marrow stromal cell differentiation into endothelial cells via notch signaling pathway. Am J Physiol Cell Physiol 296:C535-543.

Yamazaki, Y., and Morita, T. (2006) Molecular and functional diversity of vascular endothelial growth factors. Mol Divers 10:515-527.

Zhang, S.J., Zhang, H., Hou, M., Zheng, Z., Zhou, J., Su, W., Wei, Y., and Hu, S. (2007) Is it possible to obtain "true endothelial progenitor cells" by in vitro culture of bone marrow mononuclear cells? Stem Cells Dev 16:683-690.

Zhang, S.X., Wang, J.J., Gao, G., Parke, K., and Ma, J.X. (2006) Pigment epithelium-derived factor downregulates vascular endothelial growth factor (VEGF) expression and inhibits VEGF-VEGF receptor 2 binding in diabetic retinopathy. J Mol Endocrinol 37:1-12.