BMP2 and VEGF promote angiogenesis but retard terminal differentiation of osteoblasts in bone regeneration by up-regulating Id1

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Inadequate vascularization limits the repair of bone defects. In order to improve angiogenesis and accelerate osteogenesis, the synergism of co-cultured cells with genetic modification in bone regeneration was investigated in this study. Endothelial progenitor cells (EPCs) and bone marrow stem cells (BMSCs) were transfected with the genes of vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP2), respectively. The co-cultured cells, designated as four groups including BMSC + EPC, Ad-BMP2–BMSC + EPC, BMSC + Ad-VEGF–EPC, and Ad-BMP2–BMSC + Ad-VEGF–EPC groups, were seeded on an alginate gel and then implanted into rat intramuscularly to evaluate the effects on angiogenesis and osteogenesis. Both VEGF and BMP2 could induce the overexpression of inhibitor of DNA-binding 1 (Id1) gene which significantly promoted tube formation in vitro and increase the amount of blood vessels in the Ad-BMP2–BMSC + Ad-VEGF–EPC group after implantation. Nevertheless, overexpression of Id1 retarded the terminal differentiation of osteoblasts and the bone formation. Later, osteogenic gene expression at transcriptional level, calcium nodules, and alkaline phosphatase (ALP) activity showed a gradual decrease and the amount of newly formed osteogenesis area exhibited a small increase in the Ad-BMP2–BMSC + Ad-VEGF–EPC group. This finding suggests that a balanced regulation of Id1 expression in VEGF–EPCs and BMP2–BMSCs may be critical to cell-based and gene-based approaches for bone regeneration.

Keywords bone tissue engineering; BMP2; VEGF; angiogenesis; Id1

Received: March 25, 2011 Accepted: June 12, 2011
strategy to overcome the problem of insufficient amount of EPCs [12,13]. BMP2 and VEGF are the primary factors in the cellular communication during angiogenesis and osteogenesis [14]. In vitro studies have revealed a BMP/VEGF-regulated coupling in bone regeneration [15]. A synergistic effect between BMP2 and VEGF may play important roles in enhancing the efficiency of cell-based and gene-based approaches for bone regeneration, while its potential negative effects should not be neglected.

In this study, we combined scaffolds with Ad-BMP2–BMSCs and Ad-VEGF–EPCs, trying to improve the vascularization of the graft and to accelerate ectopic bone formation. We also observed the negative effect of this approach on final bone regeneration and explored the possible reasons.

Materials and Methods

Isolation and culture of EPCs and BMSCs from rat bone marrow

Wistar male rats (3–4 weeks) with weight of 130–140 g were used for isolating BMSCs and EPCs, as described previously [16]. Mononuclear cells (MNCs) from femurs and tibias bone marrow aspirate were isolated by using a Percoll (Sigma, St Louis, USA) density gradient separation method (1200 g for 20 min) at room temperature. After centrifugation, the cells were washed twice with phosphate-buffered saline (PBS), and then suspended in different cell culture medium. For BMSCs differentiation, MNCs were cultured in low-glucose Dulbecco’s modified Eagle’s medium (L-DMEM) (Gibco Brl, Eggenstein, Germany) containing 10% fetal bovine serum (FBS), 10 IU/ml penicillin, and 10 μg/ml streptomycin. For EPCs differentiation, MNCs were cultured in endothelial growth medium (EGM-2) supplemented with the additives of rhEGF, VEGF, rhFGF-B, R3-IGF-1, ascorbic acid, heparin from growth factor bullet kit (Lonza, Cologgne, Germany), 2% FBS, 10 IU/ml penicillin, and 10 mg/ml streptomycin. MNCs were plated into 75 cm² tissue culture flask at a density of 1 × 10^6 cells/ml. After 24 h incubation at 37°C in a humidified atmosphere with 5% CO₂, nonadherent cells were removed and fresh medium was added to the flask. The medium was changed every 3 days. After 5–7 days, the adhered cells with 90% confluence were split for passage at a ratio of 1:3.

Immunophenotype and function of rat EPCs

EPCs from passage 3 were seeded on human fibronectin-coated six-well plates at a density of 2 × 10^5 cells/well. On the next day, the expression of surface markers of cultured cells was determined by using flow cytometry. The cultured cells were washed with PBS and incubated with rat monoclonal antibodies against VEGF receptor 2 (VEGFR2; BioLegend, San Diego, USA), CD133 (Abcam, Cambridge, UK) and phycoerythrin (PE)-conjugated CD34 (Santa Cruz Biotechnology, Santa Cruz, USA) at room temperature for 30 min. In order to detect VEGFR2 and CD133, the cells were further incubated with an isotype-specific PE-Cy 5.5-conjugated (Invitrogen Life Technologies, Carlsbad, USA) and fluoresceinisothiocyanate (FITC)-conjugated (eBioscience, San Diego, USA) goat anti-rat antibodies. Quantitative analysis was performed using CellQuest Pro Software (BD Biosciences, San Jose, USA) on a flow cytometer.

Capillary morphogenesis assay and uptake of Dil-acetylated low-density lipoprotein (Dil-ac-LDL) assay were used to observe the function of EPCs. One hundred microliters of Matrigel (BD Biosciences) at a concentration of 10–12 mg/ml was added into a 96-well plate and polymerized at 37°C for 30–60 min. The EPCs were plated onto the Matrigel (5 × 10^3 cells/ml) in EGM-2 medium and incubated at 37°C for 2 h. Early passage (1–2) EPCs (2 × 10^5 cells/well) were seeded on human fibronectin-coated six-well plates. On the next day, the attached cells were incubated with 10 μg/ml Dil-ac-LDL (Biomedical Technologies Inc., Stoughton, USA) in EGM-2 complete medium at 37°C for 4 h. The cells were then washed three times with PBS and stained with FITC-Letin (Vector Laboratories, Burlingame, USA). The cells were visualized by using fluorescence confocal microscope.

Gene transfer

The adenoviral vectors were designated Ad-BMP2, Ad-VEGF, and Ad-GFP, which were E1-deficient recombinant adenovirus propagated in 293 cells and purified by cesium chloride gradient ultracentrifugation as described earlier [17]. Optimal conditions for adenovirus gene transfer serum concentration, virus incubation time and virus concentration in EPCs and BMSCs were evaluated by preliminary experiments. The cells were transduced with 40 multiplicity of infection of Ad-BMP2, Ad-VEGF, and Ad-GFP for 48 h in the culture media containing 1% FBS. After transduction, the cells were washed with PBS and incubated in complete media.

ELISA for BMP2 and VEGF expression

The levels of BMP2 and VEGF secreted from transfected BMSCs and EPCs were determined by BMP2 ELISA kit and VEGF ELISA kit (R&D system, Minneapolis, USA), respectively. The supernatant of the transfected EPCs and BMSCs was harvested at the 2, 7, 14, 21, and 28 days after transduction. Ad-GFP-transfected and non-transfected EPCs and BMSCs were used as the controls. The assay was performed according to the supplier’s instructions. The absorbance was measured by a multi-label counter (Synergy HT; BioTek, Winooski, USA) at 450 nm.
Co-culture system in vitro
The co-cultured cells were divided into four groups that were designated as BMSCs + EPC group, BMSC + Ad-VEGF–EPC group, Ad-BMP2–BMSC + EPC group, and Ad-BMP2–BMSC + Ad-VEGF–EPC group.

The mixed cells with a ratio of 1:1 were re-suspended in 4.5 ml of osteogenic differentiation medium (L-DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 10 μg/ml streptomycin, 10⁻² μM dexamethasone, and 0.01 M β-glycerophosphate). The cells were cultured in 12-well plates, and incubated at 37°C in an incubator supplied with 5% CO₂ and humid atmosphere. The medium was changed every 4 days.

Alkaline phosphatase (ALP) activity assay
ALP activity was assessed by the time-dependent 4-nitrophenylphosphatehexahydrate (PNPP) method [18]. The cells (5 × 10⁴ cells/ml) were washed with PBS, scraped and lysed with 200 μl 0.1% Triton X-100 (Invitrogen Life Technologies). After centrifugation at 13,000 g for 10 min at 4°C, 40 μl of supernatant extracted from each sample was added to 100 μl PNPP in each well of another 96-well plate and then incubated at 37°C for 30 min. The reaction was terminated by adding 100 μl of 0.4 M NaOH and the absorbance at 405 nm was measured by using the multi-label counter.

Alizarin red staining for mineralized matrix
The cells were fixed with 70% ice-cold ethanol at −20°C for 1 h, and then stained with 40 mM pH 4.2 alizarin red S (AR-S; Sigma) for 10 min at room temperature. Mineralized matrix staining was counted under a light microscope with ×100 magnification and three areas were selected for each sample.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)
RNA was extracted from the cells and transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen Life Technologies). The real-time PCR reactions were run in triplicate in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands). Thermal cycling parameters were as follows: an initial incubation of 95°C for 30 s, and then 40 cycles of 95°C for 5 s and 55°C for 20 s, and 72°C for 15 s. PCR amplification of cDNA was performed using the rat-specific primer sets listed in Table 1. Id1 gene and the osteoblast-related genes including collagen type I (Col I), osteocalcin (OC), and ALP were amplified. A 2⁻^ΔΔCt method was used to evaluate the relative expression level of mRNA for each target gene.

Co-culture cells/alginate gel mixture
The co-culture cells were pooled to a total of 1 × 10⁶ cells/ml, resuspended in 1.2% low-viscosity alginate (Invitrogen Life Technologies) with 0.15 M NaCl at a density of 2 × 10⁶ cells/ml. The 1 ml mixture was transferred to 200 μl 100 mM CaCl₂ solution at room temperature for 15 min of polymerization, and then the alginate was solidified. Alginate gel mixed with cells was implanted into lateral femoral muscle of five rats in four groups, respectively. After implantation for 4 and 6 weeks, the constructs in the rats were cut into several pieces, and immediately stored in liquid nitrogen for future histological analysis.

Histology and immunohistochemistry
The samples were decalcified in 10% formic acid, dehydrated in a gradient ethanol series, embedded in paraffin, sectioned in 4 mm thickness, and stained with hematoxylin and eosin (H&E) and sirius red. Bone and Col I areas were quantified by Image Pro Plus software (Media Cybernetics, Silver Spring, USA). The average bone areas of these sections were used for statistical analysis.

For the growth of blood vessels, 4- and 6-week tissue sections were immunohistochemically stained with rabbit anti-rat CD31 monoclonal antibody (BD Biosciences) to detect endothelial cells. Sections were incubated with anti-CD31 primary antibody (1:50 dilution) at 4°C overnight, and then incubated with a secondary antibody kit (Invitrogen Life Technologies). DAB (3,3'-diaminobenzidin tetrahydrochloride) was used as chromogen. Finally, the slides were counterstained with hematoxylin (Sigma). Blood vessels with CD31 staining were counted under a light microscope with ×100 magnification and three areas were selected for each sample. The average areas of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>Id1</td>
<td>3'-GTTCAGCCGACGACGCAT-5’</td>
<td>3'-CGGTTTGCTTGGGGAACCG-5’</td>
</tr>
<tr>
<td>ALP</td>
<td>3'-GACAAGAGCCCTCTCACACG-5’</td>
<td>3'-ACTGGGGCTGTAGTGTGGT-5’</td>
</tr>
<tr>
<td>Col I</td>
<td>3'-AATGGTGCTCCTCGTTATGGC-5’</td>
<td>3'-GTTACCACTGTGCCCTTT-5’</td>
</tr>
<tr>
<td>OC</td>
<td>3'-CATGAGGGACCTCTCTCTCGC-5’</td>
<td>3'-ATAGATGCCAGTTGAGCGT-5’</td>
</tr>
<tr>
<td>β-actin</td>
<td>3'-TGACTGTGCCGGGAATTTG-5’</td>
<td>3'-GGTGAAAGTGCGAGTGAAACG-5’</td>
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CD31-positive cell structures were calculated and presented as mean ± SD.

**Statistical analysis**

All data were presented as mean ± SD and statistical significance was evaluated by analysis of variance analysis using SPSS software. The statistical significance was considered at \( P < 0.05 \).

**Results**

**VEGF overexpression promotes tube formation of EPCs and up-regulates the level of Id1 gene**

MNCs isolated from bone marrow were cultured in EGM-2 complete medium in the presence of endothelial specific growth factors on fibronectin-coated plates. After culture for 7–10 days, small EPC colonies were observed. Compared with the BMSCs [Fig. 1(A)] which exhibited a flattened, elongated, and fibroblastic shape, the EPCs [Fig. 1(B)] showed spindle shape and cobblestone appearance. EPCs were characterized by measuring the expression of endothelial cell markers such as CD133 and VEGFR2, and stem cell marker CD34. Cells could be considered as the EPCs if these markers were co-expressed. The fluorescence activated cell sorting (FACS) analysis demonstrated that the population of cells with co-expression of CD34, CD133, and VEGFR2 was \( 22.3\% ± 1.6\% \) [Fig. 1(C)].

Besides the immunophenotype, EPCs were also characterized by functional assays in vitro, such as the uptake of diacetylated low-density lipoprotein and tube formation. The partially adherent cells could uptake Dil-ac-LDL and be co-stained with FITC-lectin [Fig. 1(D)]. Moreover, the cells cultured in Matrigel revealed the formation of tube-like structures [Fig. 1(E)].

**Figure 1 Identification of EPCs and expression of VEGF and Id1**

(A) BMSCs exhibited a flattened, elongated, and fibroblastic shape. (B) EPCs showed spindle shape and cobblestone appearance. Magnification, \( \times 200 \). (C) The FACS analysis showed that the population of the cells with co-expression of CD31, CD133, and VEGFR2 was approximately \( 22.3\% ± 1.6\% \). (D) Bone marrow-derived EPCs incorporated with Dil-ac-LDL were shown in a representative imaging with Dil-ac-LDL uptake (red), staining by FITC-lectin (green), 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining and superposition. (E) Angiogenesis assay exhibited the network formation by isolated EPCs on Matrigel. Bar = 100 \( \mu m \). (F) ELISA for VEGF. The Ad-VEGF–EPC group showed markedly increased VEGF levels. \( *P < 0.05 \) compared with EPC and Ad-GFP–EPC groups. (G) Compared with the other groups, the expression of Id1 gene increases 7-fold in the Ad-VEGF–EPC group. \( *P < 0.05 \) compared with EPC and Ad-GFP–EPC groups. (H) Angiogenesis assay exhibited Ad-VEGF–EPC had a better ability of tube formation than the EPC group. Bar = 100 \( \mu m \).
As shown in Fig. 1(F), the expression of VEGF in EPCs exhibited a significant increase after transfection with Ad-VEGF. Id1 is critical to EPCs population formation and angiogenesis, and the expression of Id1 can be up-regulated by VEGF [19]. Consistent with this result, the qRT-PCR revealed that VEGF induced 7-fold increase in the expression of Id1 gene [Fig. 1(G)]. In the tube formation assay, the Ad-VEGF–EPC group has better ability of tube formation compared with the control group [Fig. 1(H)].

**BMP2 overexpression regulates osteogenic differentiation of BMSCs and up-regulates the level of Id1 gene**

The expression of BMP2 exhibited a significant increase in Ad-BMP2–BMSCs, as shown in Fig. 2(A). The AR-S staining and ALP activity assay results indicated that BMP2 could accelerate osteogenic differentiation of BMSCs. As shown in Fig. 2(B,C), at the 21st day, the positive staining in the Ad-BMP2–BMSC + Ad-VEGF–EPC group and Ad-BMP2–BMSC + EPC group were significantly higher than those in other groups ($P < 0.05$). Similarly, ALP activities in the Ad-BMP2–BMSC + Ad-VEGF–EPC and Ad-BMP2–BMSC + EPC groups were also higher than those of other groups ($P < 0.05$) [Fig. 2(D)]. The expression of Id1 gene was found to be up-regulated more than 5-fold in the Ad-BMP2–BMSC group. *$P < 0.05$ compared with BMSC and Ad-GFP–BMSC groups.

**Expression of osteoblastic gene in vitro and osteogenesis in vivo**

Osteoblastic gene expression of cultured cells in vitro was evaluated after transplantation for 7, 14, 21, and 28 days. ALP gene in Ad-BMP2–BMSC + EPC, BMSC + Ad-VEGF–EPC, and Ad-BMP2–BMSC + Ad-VEGF–EPC groups reached a peak level at the 21st day. However, Ad-BMP2–BMSC + EPC and Ad-BMP2–BMSC + Ad-VEGF–EPC groups showed higher ALP expression than other groups ($P < 0.05$) [Fig. 3(A)]. All groups showed slow increase in the expression of OC [Fig. 3(B)]. Interestingly, the Col I expression in all groups increased time dependently, and the Ad-BMP2–BMSC + Ad-VEGF–EPC group had the highest Col I level at the 21st day ($P < 0.05$) [Fig. 3(C)].

For in vivo study, after implantation for 4 weeks, decalcified sections stained with H&E and sirius red from all groups exhibited ectopic bone formation and Col I formation. The volume of the alginate gel became smaller and...
smaller due to the degradation and absorption by host tissues. Long-round or shuttle-like cells were observed along the alginate gel. Many neo-collagen tissues and irregular premature bone-like matrix were also observed on the interface between alginate gel and tissues. Newly formed bone began to cover the surface of scaffolds in all groups after 4 weeks. At week 6, the alginate scaffold still remained in vivo, as shown in Fig. 3(D), and more collagen tissues were observed [Fig. 3(E)]. The quantitative analysis showed that Ad-BMP2–BMSC + EPC and Ad-BMP2–BMSC + Ad-VEGF–EPC groups had higher bone area than others at week 4 and 6, and there was significant difference between these two groups at week 6 ($P < 0.05$) [Fig. 3(F)]. The amount of Col I formation in Ad-BMP2–BMSC + EPC and Ad-BMP2–BMSC + Ad-VEGF–EPC groups were higher than those in the BMSCs + EPC and BMSC + Ad-VEGF–EPC groups at week 4 and 6, but there was no significant difference between Ad-BMP2–BMSC + EPC and Ad-BMP2–BMSC + Ad-VEGF–EPC groups ($P < 0.05$) [Fig. 3(G)].

**Angiogenesis in vivo**

The occurrence of angiogenesis at the 4th and 6th week was confirmed by the expression of CD31, a special marker of endothelial cells. The BMSC + Ad-VEGF–EPC and Ad-BMP2–BMSC + Ad-VEGF–EPC groups displayed higher density of blood vessels than other groups at week 4 and 6, and there was significant difference between these two groups at week 6 ($P < 0.05$) [Fig. 4(A)], which was also verified by the quantitative analysis for blood vessel density [Fig. 4(B)].

**Discussion**

Bone remodeling is a complex process involving angiogenesis and osteogenesis. The correlation between angiogenesis and osteogenesis may offer an exciting therapeutic opportunity for bone regeneration. The understanding of the cellular and molecular interactions in blood vessels and bone cells will be helpful for successful development of vascularized bone scaffold constructs that will offer the survival and integration of bioengineered bone with the host tissues [20].

Bone marrow-derived EPCs are critical regulators of the angiogenic switch [21] and VEGF is one of the most effective angiogenic growth factors for both physiological and pathological angiogenesis [3,22]. Early and late EPCs are two major types of EPCs. Early EPCs have a short lifespan and reveal a gradual loss of endothelial cell-specific markers such as CD133. Compared with the early EPCs, late EPCs have a similar gene expression profile as the endothelial cells and a long lifespan as well as rapid proliferation [10,23]. Based on characterization of EPCs, the late EPCs have been chosen as the seed cells in our present study.
study. VEGF-modified EPCs constitute one option to address the limited number of EPCs. Iwaguro et al. [13] demonstrated that the dose of EPCs used for the in vivo experiments was 30 times less than that required in previous trials of EPC transplantation to improve ischemic limb salvage. In our study, modified EPCs with over-expression of VEGF revealed an efficient increase of blood vessel density in vivo. Moreover, VEGF can up-regulate the expression of Id1 gene which is a critical positive factor in the EPCs, including recruitment, chemotaxis, and mobilization of EPCs [24]. Loss of Id1 led to a complete loss of the EPC populations in the peripheral blood, which was correlated with a block of neovascularization [25].

Previous studies demonstrated that cultured mesenchyme cells genetically modified with adenovirus-BMP2 could produce orthotopic bone in animal models [8,26]. The defects treated with adenovirus-BMP2-transduced cells had an increased density of trabecular bone and an obvious improvement of bone formation. However, we did not observe increase in the expression level of osteoblastic genes (ALP, OC, and Col I) or ectopic bone formation in the Ad-BMP2–BMSC + Ad-VEGF–EPC group at the late time. To gain insight into the reason of this phenomenon, we detected the level of Id1 in BMSCs and found that BMP2 significantly up-regulated expression of Id1 gene. However, according to a previous study from Nakashima et al. [27], Id1 protein might promote proliferation of early osteoblast progenitor cells and down-regulated the terminal differentiation of committed osteoblasts. In this regard, we proposed the overexpression of Id1 induced by BMP2 maybe the reason for this phenomenon.

The vascularization of the scaffold is one of the promising strategies in regenerative medicine, such as co-cultured the vascular endothelial cells and bone cells or mesenchymal stem cells in the scaffold [28,29]. EPCs and BMSCs were the powerful candidate cell types for bone regeneration [30,31]. Cell-to-cell communication between bone vascular endothelium and osteoblasts could be crucial to the coordinated cell behavior, which was necessary for the development and regeneration of bone [32]. Regarding the mode of cell-to-cell communication, cell-soluble factors have been identified and can be used to explain the biological effect on both cell types. VEGF and BMP2 are the necessary factors for osteo-endothelial communication [33–35]. In our study, EPCs and BMSCs were transduced with adenovirus encoding VEGF and BMP2, respectively, and the expression of BMP2 and VEGF revealed a significant increase. The expression of Id1 may be further up-regulated by this synergistic effect, and result in undesired bone formation in the combinatorial group.

Co-cultured EPCs and BMSCs with genetic modifications could enhance the communication between cells and achieve a synergistic effect between VEGF and BMP2. Therefore, VEGF–EPCs and BMP2–BMSCs were powerful candidate cell types for engineered bone tissues. This cell-based and gene-based approach seems feasible due to the following advantages: (i) BM–EPCs and BMSCs are easy to be harvested and cultivated; (ii) high transduction efficiency of transgene can effectively augment cellular functions. This study has demonstrated that VEGF–EPCs and BMP2–BMSCs could enhance bone formation through efficient angiogenesis, although the desired results are not achieved. According to previous studies and our data, we consider that the elevated expression of Id1 gene may retard the terminal differentiation of osteoblasts, and even the bone formation. This finding suggests that a
balanced regulation of Id1 expression in VEGF–EPCs and BMP2–BMSCs may be critical to cell-based and gene-based approaches for bone regeneration.

In conclusion, VEGF-modified EPCs co-cultured with BMP2-modified BMSCs can accelerate osteogenesis and bone formation through promoting the vascularization of transplant tissues. Although this approach did not exhibit perfect bone formation, the relationship between Id1 gene and osteogenesis has the potential to provide a new vision for the engineered bone tissues.

Acknowledgements

The authors wish to acknowledge the excellent technical work of Drs Hongshi Zhao and Heng Liu of Shandong University.

Funding

This work was supported by a grant from the National Natural Science Foundation of China (30772269).

References

Synergism of BMP2 and VEGF in bone regeneration


