Letter to the Editor

Construction of recombinant adenovirus vector containing hBMP2 and hVEGF165 genes and its expression in rabbit Bone marrow mesenchymal stem cells

**A R T I C L E   I N F O**

**A B S T R A C T**

To construct an adenovirus vector co-expressing human bone morphogenetic protein (hBMP2) and human vascular endothelial growth factor (hVEGF165) as well as green fluorescence protein (GFP) as a marker, with which the intracellular expression of the inserted genes could be identified in Bone marrow mesenchymal stem cells (BM-MSCs). BMP2 and VEGF165 genes were PCR amplified from a cDNA library and inserted to the polycyonal site of adenovirus shuttle plasmid pAd-MCMV-GFP. The virus solution (Ad-BMP2-VEGF165) was generated by co-transfecting HEK293 cells with the constructed recombinant shuttle plasmid pAd-MCMV-BMP2-VEGF165 and adenovirus helper plasmid pBHlOxΔ (delta) E1, 3Cre. The virus solution was further purified and virus titer was determined accordingly. The expression of the target genes was subsequently detected and quantified in rabbit BM-MSCs by using real time PCR, ELISA and Western blotting. The recombinant adenovirus vector containing BMP2 and VEGF165 (Ad-BMP2-VEGF165) was successfully constructed, which was confirmed by Sanger sequencing, colony PCR, as well as visually detection of GFP, and the titer of the adenovirus was 1 × 10^10 PFU/mL, and the proteins level of BMP2 and VEGF165 secreted in the supernatant are significantly higher than the control. Recombinant adenovirus vector containing hBMP2 and hVEGF165 genes was successfully constructed. The transfection rate of BM-MSCs by the adenovirus was high (95% at 100 MOI) and the BMP2 and VEGF165 genes was highly expressed in the cells. The present study provides a method to efficiently express the target genes in BM-MSCs and an vector for further research of bone defect repair using dual genes of BMP2 and VEGF165.

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1. Introduction

The critical bone defect caused by trauma and tumor can be surgically treated with various procedures. In such cases, the use of autologous bone is still regarded as the golden standard (Gamradt and Lieberman, 2003). The transplantation of autologous bone, however, is limited by its quantitative availability, loss of vascularity, invasion of normal tissue, and risk of infection during harvesting. Recent research has been focused on the bone regeneration favored by a rapid development of MSCs-based therapies (Nakamura et al., 2010; Khojasteh et al., 2008). Bone marrow mesenchymal stem cells (BM-MSCs) are characterized by its self-renewal capacity, immunosuppressive property, and the pluripotency to differentiate into several lineages of cells including osteocytic, chondrocytic, musclecytic and adipocytic cells. Accordingly, BM-MSCs are considered as an potential therapeutic target of stem cells in tissue engineering filed. In addition, several growth factors have been proved to increase bone regeneration facilitated by a rapid development of gene therapy technology, especially as the progression of application of vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) in vascularity and bone regeneration.

As a member of the transforming growth factor-β (TGF-β) super family as evidence by the presence of homologous 7 cysteine sequence in the C-terminal of the protein (Khojasteh et al., 2008; Reddi, 1994; Urist et al., 1982), BMPs are the only signal molecules that are individually sufficient for the induction of bone formation at orthotopic and heterotopic sites. The VEGF family is one of the most important angiogenesis-regulating that is characterized by its ability to induce angiopoiesis and promote endothelial proliferation. VEGF plays a vital role in regulating bone formation and repair, by attracting endothelial cells and osteoclasts, and enhancing the osteoblasts differentiation (Dai and Rabie, 2007; Clarkin et al., 2008), especially for large and critical bone defects (Peng et al., 2002; Huang et al., 2005).

As bone defect repair is a highly coordinated and well organized process involving both the BMP and VEGF (Hou et al., 2009; Young et al., 2009), it is postulated that gene therapy of both genes have an improved or synergistical efficacy compared with the individual one. Moreover, numerous aforementioned limitations such as compromised vascularity, limited bone stock, and
abundant fibrous tissue necessitate a combined therapy to achieve an adequate osteogenic signal (Ojima et al., 2006). Adenovirus vector-based transfer system have many favorable features such as high infection efficiency, low toxicity, and its ability to infect both dividing cells and non-dividing cells, which make it outstand other vectors. To improve the effect of vascularization and osteogenesis during bone defect repair, we constructed adenovirus vector co-expressing hBMP2 and hVEGF165 and examined their gene expression in BM–MSCs.

2. Materials and methods

2.1. Reagents

cDNA library (coming from hepar, human) and pAd-MCMV-GFP (Hanbio Technology Co., Shanghai), HEK293 Cells (jika gene, Shanghai), pBHGllox (delta) E1, 3Cre (Microbix Biosystem, USA), Competent Escherichia coli (E. coli) DH5α, Lipofectamianle transfection 2000 Kit (Invitrogen, USA), SYBR premix Ex Tag (Takara, Japan), human BMP2 EIIIA Kit and human VEGF165 EIIIA Kit (Sangon, China), Anti-CD14 APC (AbD serotec, UK), Anti-CD29 PerCP (Biolegend, USA), CD34 FITC (Miltenyi Biotec, Germany), Anti-CD44 PerCP, normal IgG1-PE, IgG2a-APC, IgG2b-PerCP (Santa Cruz, USA).

2.2. Methods

2.2.1. Construction and identification of recombinant shuttle plasmid vectors

Human BMP2, T2A and human VEGF165 were PCR amplified from cDNA library, digested by the corresponding restriction enzymes, and purified using agarose gel electrophoresis (Tables 1 and 2).

Then hBMP2, T2A and hVEGF165 were subcloned into the pHBAd-MCMV-GFP as follows: the plasmid pHBAd-MCMV-GFP was digested with BamHI and EcoRI, which respectively located on the upside and downside of T2A sequence at 4°C by T4 DNA ligase connection overnight. The T2A sequences were incorporated directly into the multiple cloning sites of plasmid to construct a bicistronic vector. The positive recombinant clone was named pHBAd-MCMV-T2A-GFP. Then the hBMP2 and hVEGF165 genes were respectively inserted into the upstream and downstream T2A in pHBAd-MCMV-T2A-GFP after enzyme digestion. The positive clone combining hBMP2 and hVEGF165 was named pHBAd-hBMP2-hVEGF165.

The pHBAd-hBMP2-hVEGF165 were transfected and amplified into DH5α by hot shock method and screened by Amp resistance screening. The bacterial clon PCR was determined and positive clone was sequencing to confirm correct cloning of desire genotype.

2.3. Virus preparation, purification, and titer determination

To generate recombinant adenovirus vectors expressing dual-genes, the pHBAd-hBMP2-hVEGF165 and the adenovirus helper plasmid pBHGllox (delta) E1, 3Cre were co-transformed into HEK293 cells with lipofectamine 2000 Kit according to the manufacture's instructions. 6 h after transformation, the medium was replaced with new DMEM, and cytopathic effects (CPE) was observed every 2 days.

The HEK293 cells were lysed by three consecutive freeze-thawing cycles in ice-ethanol. The viral particles in the supernatant was harvested at 3000 rpm for 5 min. To achieve high titer viral stocks, the infection was repeated three times. The adenovirus were then purified by two-step ultracentrifuge on cesium chloride gradient and blue-white band was distracted by 20G needle. Finally, the viral particles went through 0.4 µM syringe filter to remove inadvertent materials.
The viral titers (Ad-hBMP2-hVEGF165) were subsequently determined on 1 × 10⁴ HEK293 cells monolayers per well in a 96-well plate by means of the TCID₅₀ standard method and calculated using the Reed–Muench protocol (Blake and O’Connell, 1993). The recombinant adenovirus was stored in PBS-EDTA containing 10% glycerol buffer at −80 °C.

2.4. BM-MSCs preparation and identification

Male New Zealand rabbits (2 month old, weight 2–2.5 kg) were used to collect BM-MSCs. All procedures followed the guidelines and were under the supervision of the Animal Care and Experiment Committee of Jining Medical College. The bone marrow was harvested by ilium puncture and diluted with PBS and gently laid on the top of Percoll at a ratio 1:2, which was then centrifuged at 3000 rpm for 25 min at 20 °C. The mononuclear layer was collected and diluted with PBS. This suspension was centrifuged at 1000 rpm for 6 min and washed twice with PBS. The pellet was resuspended with DMEM/F12 containing 10% fetal bovine serum (FBS), 100 μg/mL penicillin and 100 μg/mL streptomycin. Then cells were plated onto 25 cm² culture plates at confluence of 1 × 10⁶ cells/cm² and incubated in a 5% CO₂ incubator at 37 °C, with the medium being replaced every 2 days. Cells were split at ratio of 1:2 when reaching about 80% confluence.

BM-MSCs phenotype characterized by CD29, CD44, and CD14, CD34 were determined by flow cytometers. The third generation cells were detached and washed in PBS containing 1% goat serum albumin. The antibodies against phenotypic antigens were mixed with cells at 1 × 10⁷/mL for 20 min on ice in dark. All analyses were normalized against corresponding negative control.

BM-MSCs were cultured in the osteoblast induced medium containing ascorbic acid, β-glycerophosphate and dexamethasone. The cells mineralization effects were detected by alizarin red staining and observed using light microscope 2 weeks later.

2.5. GFP expression and infectivity test

1, 2, 3, 5, 7, 9, 11, 14, 21 and 28 days following the infection with Ad-hBMP2-hVEGF165 ex vivo, the expression of GFP protein was observed under an inverted fluorescence microscopy. BM-MSCs employed to evaluate the best MOI were seeded onto a 6-well plate at 1 × 10⁵ cells/well and cultured in DMEM/F12 containing 10% FBS at 37 °C/5% CO₂ for 24 h before adenovirus infection. The adenovirus vectors were diluted into 5 gradient at MOI of 40, 60, 80, 100 and 120. The medium was replaced after 2 h and the infectivity was determined by observation of GFP expression after 48 h. It was reckoned that the rules for determining the best MOI is defined as a low CPE with a high efficiency of infection.

2.6. Real time PCR analysis

The BM-MSCs were cultured in 6-well plate and divided into 3 groups: BM-MSCs, Ad-GFP, Ad-hBMP2-hVEGF165 to analyze and determine the gene expression. These cells were harvested 48 h after infection. Gene-specific primers for hBMP2, hVEGF165 and β-actin were designed to detect the relative mRNA expression level (Table 2). Total RNA was extracted using total RNA extraction kit and cDNA was synthesized with the corresponding specific primers. SYBR Green detection kit was used to evaluate the PCR product signal. Reaction cycles included denaturation cycle at 95 °C for 5 s, annealing at 60 °C for 30 s, and extension at 72 °C for 10 s. The resultant SYBR Green I was evaluated after 40 cycles.

2.7. Enzyme linked immunosorbent assay (ELISA)

The cultured supernatant was harvested and measured for levels of hBMP2 and hVEGF at the 1, 3, 5, 7, 9 days following Adenovirus infection, with an ELISA kit according to the manufactures instruction. The medium was replaced every day during the ELISA assay.

2.8. Western blotting analysis

BM-MSCs were grown in 6-well plate and infected with adenovirus when the cells reached 70% confluence, and then cells were incubated for 7 days. Western blotting was performed according to conventional protocol. Antibodies used were: mouse anti-hBMP2 monoclonal antibody, mouse anti-hVEGF165 monoclonal antibody and secondary antibody of goat anti-mouse IgG conjugated horseradish peroxidase.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 13.0 software and data were presented as mean ± standard deviation (SD). Comparison among multiple groups was evaluated using ANOVA with Tukey Post hoc analysis for Pairwise comparisons. P-value of <0.05 was considered statistically significant.

3. Result

3.1. Identification of PCR of specific gene

The BMP2 and VEGF165 gene were amplified from cDNA library by PCR, and the products were subsequently purified 1% agarose gel electrophoresis. As shown in Fig. 1, length of hBMP2 and hVEGF165 is 1191bp and 576bp respectively, which is identical with the consensus sequences of the two genes.
3.2. Colony PCR identification of recombinant shuttle plasmid vector

Single colony of grown DH5α transformed with pAd-BMP2-VEGF165 was picked as a template to test strain, and the results of sequencing confirm a successful construction of the vector (Fig. 2).

3.3. Quantification of recombinant viral particles

The first generation of adenovirus vectors were undergoing several times of transformation by means of TCID50 standard method and then the result was transformed by calculated formula to PFU/mL. The viral titer is $1 \times 10^{10}$ PFU/mL and reach the basis of test in vitro and in vivo (Fig. 2).

3.4. Identification of BM-MSCs

BM-MSCs appeared to be a homogeneous population of fibroblast-shaped cells, and the virus infection did not change the morphology of the cells. After 3 passages, we found that the expression levels of cell surface antigens such as CD29 and CD44 were significantly increased, which the expression levels of the detection markers of hematopoietic cell such as CD14 and CD34 were substantially decreased (Fig. 3). Meanwhile, the mineralization effect of cells cultured in induced medium was detected by alizarin red staining and mineralized nodules were observed (Fig. 4).

3.5. GFP gene expression and infectivity test

GFP expression can be observed as early as 24 h post-infection and increased from the 1 day through 5 day, and still visually detectable 28 day post-infection.

When the MOI selected from 40 to 120, the rate of GFP positive cells increased gradually. However, infection efficiency could be improved when cells were infected at an MOI above 40, and CPE started to appear at 24 h post infection at an MOI of 120 which a certain number of dead cells were simultaneously detected after the infection (Fig. 5).

3.6. Analysis of gene expression

To confirm hBMP2 and hVEGF165 gene expression ex vivo, real-time PCR (Fig. 6), ELISA (Fig. 7) and Western blotting (Fig. 8) were performed. As shown in Fig. 7, the expression of hBMP2 and hVEGF165 were markedly higher than the control (either 3 day or 28 day). Notably, the gene expression levels have been gradually enhancing from 3 to 5 day. The data detected by ELISA for hBMP2 and hVEGF165 expression in the Ad group on the 1, 3, 7, 14, 21 and 28 day post-infection, respectively. There was little hBMP2 or hVEGF165 expression which was determined in Ad-GFP and BM-MSCs, respectively.

4. Discussion

Bone defect repair is a complex biological process that is always complicated with pain and disability. In recent years, implantation of seed cells which can accelerate the process of bone regeneration has provided a bright prospect for bone defect repair. Three elements, seed cells, biomaterial and growth factors are critical in tissue engineering bone (Jiang et al., 2008). MSCs derived from bone marrow appear to be a good candidate of seed cells to repair bone defect, not only because that it is easily available, but also because that they are multipotent cells of mesodermal origin capable of differentiating into bone and cartilage under certain internal environment. Therefore, BM-MSCs are considered as a potential source of cells for bone engineering (Shi and Wang, 2010). The typing of BM-MSCs surface antigens using flow cytometry is thought to be the gold standard for cell type identification. In our study, CD44, CD14 and CD34 were used to identify the cell phenotype. According to the results of flow cytometry typing, we...
concluded that the purity of the BM-MSCs are satisfactorily high, which is sufficient for the following experiments.

The main purpose of gene therapy is focused on bone regeneration and neoangiogenesis, and a method that could combined those aspects together might achieve a better curative effect (Murphy and Mooney, 1999). BMP2 has been tested appricatal in multiple clinical trials, and its superiority to autograft in spinal fusions has been repeatedly verified (Boden et al., 2002, Yasko et al. (1992) and Bostrom and Camacho (1998) described that bone healing of the defects of critical size in animal models had been promoted by application of BMP2. Geiger et al. (2005) have reported the therapeutic effect that VEGF165 significantly enhanced bone formation and vascular density. Zhang et al. (2010) showed that neither BMP

\[ \text{ Fig. 6. Relative mRNA expression of BMP2 and VEGF165 in BM-MSCs for each group (BM-MSCs group, Ad-GFP group and Ad-BMP2-VEGF165 group) at 3 days after transfection. Each assay was performed in triplicate.} \]

nor VEGF165 alone is sufficient to significantly improve bone formation. Peng et al. (2002) demonstrated a synergistic promoting effect of bone formation and healing by applying muscle – derived stem cells expressed BMP4 and VEGF165. In addition, individual

\[ \text{ Fig. 7. BMP2 and VEGF165 concentrations in harvested media samples from BM-MSCs of each group were performed by ELISA at 3, 5, 7, 9, 14, 21 and 28 days. (a) BMP2 concentration was analyzed at different points in time. (b) VEGF165 concentration was analyzed at different points in time. Each assay was performed in triplicate.} \]
application of VEGF165 did not affect bone regeneration, but it could enhance vessels density in combination with BMP4 in their trial. Therefore, the combined application of BMP2 and VEGF165 may be more effective than single use of either for increasing bone regeneration in bone tissue engineering.

The rapid development of transgenic technology and gene therapy has provided efficient methods to solve aforementioned problems. Generally, delivery of the genetically modified materials to the seed cells can be accomplished by both viral and nonviral vectors (Jiang et al., 2008). Nonviral vectors are usually used to overexpress exogenous genes in tissue engineering, which is limited by factors such as the short term expression duration, low transfection rate. The adenovirus-associated virus has the priority to function as a transfer method of target gene due to its ability to infect both dividing and non-dividing cells as well as low immunogenicity. Adeno-associated viral vectors, however, also have some limitations, such as over long-term expression and lower expression in the early period of transfection. The limitation of HIV-based lentivirus include its ability to incorporate the target gene into the host cell genome and prolong the target gene expression duration. Thus, the host genome would be altered, which may potentially affect the function of the genes located in or nearly of the insert site on the chromosome. Adenovirus has many natural features that makes it an attractive candidate vector. It is able to transfect a variety of cells, either dividing or non-dividing cells. The advantages of adenovirus also include its high efficiency of transduction (up to 95%), its ability to achieve high level gene expression in early time after infection in defect bone tissue. Therefore, the sufficient dose of the target gene could be ensured and the side-effects of over-lying gene expression also could be avoided to the greatest extent.

The adenoviral vector in our study was produced by the AdMax packaging system and consisted of a type 5 replication defective adenovirus with double loss of E1 and E3 (Shayakhmetov et al., 2002). The framework plasmids containing most of the adenovirus genome with the Cre/loxP recombinase system and shuttle plasmids inserted the exogenous genes, and they were assembled in HEK293 cells which thereby avoided the recombination in bacteria, as well as the corresponding shuttle plasmid linearization and improved recombination efficiency. Additionally, the T2A-like (Thosaea asina virus 2A-like) sequence was selected as the multicistronic connection in the vector, ensuring separate but coordinated expression of several genes. In our study, the hBMP2 and hVEGF165 genes were inserted into the opposite side of the T2A to construct a bicistronic frame, and secreted in supernatant of BM-MSCs. The result revealed that the T2A sequence may be a superior strategy for multiple genes co-expression with adenovirus vector.

By direct sequencing, visual detection of GFP expression, RT-PCR, ELISA and Western blotting assays, we showed that Ad-BMP2-VEGF165 co-expressing double genes was successfully constructed and has favorable ability of gene expression.

5. Conclusion

In conclusion, we used Adenovirus as a gene delivery system to co-express BMP2 and VEGF165 genes in BM-MSCs, which was verified by direct sequencing, visual detection of GFP expression, RT-PCR and ELISA assays. The synergistic biological effects of BMP2 and VEGF165 facilitated by the present method might be able to provide theory support and experimental basis for gene therapy of critical bone defect.

Author contributions

Conceived and designed the experiments: Chun-ying Meng. Performed the experiments: Cong Zhang, Hong-mei Liu, Guo-wu Chen, Xiao Liang. Analyzed the data: Qing-wei Li. Contributed the reagents/materials/analysis tool: Chun-ying Meng. Wrote the paper: Cong Zhang All authors approved the final manuscript version for submission.

Conflict of interest

No competing interests have been declared.

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Cong Zhang a
Hong-mei Liu b
Qing-wei Li a
Guo-wu Chen a
Xiao Liang a
Chun-yang Meng a,*

a Department of Orthopedics, Affiliated Hospital of Jining Medical University, Guhuai Road, Jining 272000, Shandong Province, China
b Department of Pathological Teaching and Research, Jining Medical University, Guhuai Road, Jining 272000, Shandong Province, China

*Corresponding author. Tel.: +86 0537 2903236; fax: +86 0537 2213030.
E-mail address: blueskymaster@hotmail.com

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