Research Paper

Effects of Recombinant Human Bone Morphogenetic Protein-2 Mediated Wingless-Related Integration Site Beta-Catenin Pathway on Bone Defect Repair and Angiogenesis in Rabbits

J. ZHENG*, A. CHEN AND JING LI

Department of Traumatic Orthopedics, Shanghai Fourth People's Hospital Affiliated to Tongji University, Hongkou, Shanghai 200434, China

The effect of recombinant human bone morphogenetic protein-2 mediated wingless-related integration site beta-catenin pathway on bone defect repair and angiogenesis of rabbit radius was explored. 18 New Zealand rabbits were divided into 3 groups, control group (group A), low dose recombinant human bone morphogenetic protein-2 group (group B), and high dose recombinant human bone morphogenetic protein-2 group (group C). The bone mineral density of each group was compared. Rabbit bone marrow mesenchymal stem cell line cells were resuscitated and the proliferation ability, protein expression, messenger ribonucleic acid expression and cell migration ability of the three groups were detected by cell counting kit-8, Western blot, quantitative polymerase chain reaction and Transwell methods. The radial bone mineral density of rabbits in the group B was higher than that in group A at 4 w and 8 w, while in group C was higher than that group B. The cell proliferation and migration activity in the group B increased than group A, while those in the group C were raised than the group B. The protein levels of angiopoietin-1 and vascular endothelial growth factor in group B increased than those in the group A and these levels in group C raised comparatively than in group B. The relative expression of wingless-related integration site beta and catenin in group B reduced than group A, while this expression in group C was lower than group B. Recombinant human bone morphogenetic protein-2 can promote the repair of radial bone defect and angiogenesis by mediating wingless-related integration beta-catenin pathway in rabbits.

Key words: Recombinant human osteogenic protein 2, wingless-related integration beta-catenin, bone defects, angiogenesis

Bone defect caused by bone tumor, trauma, infection and other factors is a difficult problem in the field of orthopaedics, especially fracture, segmental bone defect, nonunion, pseudarthrosis, persistent pain, decline or loss of motor ability and other symptoms, seriously affect the quality of life and health of patients[1]. There are many methods for clinical application[2]. However, each treatment has its limitations. For example, although autogenous bone transplantation is recognized as the gold standard for repairing bone defects, its source is limited. Although the artificial bone tissue engineering materials developed in recent years have certain ability of bone conduction and bone induction, the effect of bone induction is poor, the degradation rate and plasticity are not ideal[3]. Therefore, it cannot fully meet the clinical needs. The above reasons limit the clinical application of various bone grafts and biological substitutes. The development and utilization of growth factors in the treatment of bone defects has become a research hotspot in the field of orthopaedics. Scholars began to use biological factors to accelerate fracture healing, providing new treatment methods and ideas for solving this kind of clinical orthopaedic problems. Bone Morphogenetic Protein (BMP) is

***Address for correspondence**
E-mail: yiugqtja973@163.com

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a growth factor that induces and promotes bone tissue formation. It can also induce bone marrow Mesenchymal Stem Cells (MSCs) to differentiate into chondroblasts[4]. BMP is mainly involved in the early response to bone tissue injury or trauma. So far, more than 40 members of BMP family have been isolated and cloned by gene recombination technology, among which BMP-2 and BMP-7 are widely used[5]. In particular, BMP-2 is currently recognized as the strongest osteogenic growth factor in the BMP family, with strong osteogenic ability and significant ability to promote bone marrow MSCs to differentiate into osteoblasts[6]. This study was aimed to investigate the effect of recombinant human BMP-2 (rhBMP-2)-mediated Wingless-related integration site (Wnt) Beta (β)-catenin pathway on the repair of radial bone defect and angiogenesis in rabbits, in order to provide reference for the choice of clinical treatment.

MATERIALS AND METHODS

Materials and reagents:

Eighteen 3 mo old New Zealand rabbits were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. rabbit bone MSCs were purchased from American Type Culture Collection (ATCC) cell bank. Fetal Bovine Serum (FBS) and Roswell Park Memorial Institute (RPMI)-1640 medium were purchased from Thermo Fisher Scientific Company of the United States. RhBMP-2 injection was purchased from Hangzhou Jiuyuan Gene Engineering Co., Ltd.; Wnt-β, catenin messenger Ribonucleic Acid (mRNA) primers and β-actin primers (Sigma Co., Ltd.); quantitative Polymerase Chain Reaction (qPCR) detection kit, Cell Counting Kit-8 (CCK-8) detection kit (Shanghai Biyuntian Co., Ltd.); Transwell chamber (Corning Co., Ltd.); Angiopoietin-1 (Ang-1), Vascular Endothelial Growth Factor (VEGF), Wnt-β, catenin antibodies (British Abcam Biotechnology Co., Ltd.).

Methods:

Specimen rabbit, cell culture and treatment: 18 New Zealand rabbits were randomly divided into control group (n=6) (group A), low dose rhBMP-2 intervention group (n=6) (group B) and high dose rhBMP-2 intervention group (n=6) (group C). The rats were anesthetized by intraperitoneal injection of 1 % pentobarbital sodium of 30 mg/kg and fixed on the operation board. The skin and subcutaneous tissue in the middle part of the radius was cut, the muscle was bluntly separated. Once the radius is exposed, 15 mm of middle radius is cut and scraped off the periosteum with bone by peeling, and the model of unilateral critical bone defect of radius was made. Group A received no other intervention after osteotomy. Group B and C were implanted with rhBMP-2 loaded with absorbable gelatin sponge 0.5 and 2.5 mg respectively. Separate cages were used after operation.

After resuscitation of rabbit bone marrow MSC line, RPMI-1640 medium containing 10 % FBS was inoculated in T25 culture bottle and was cultured at 37° using 5 % Carbon dioxide (CO_2). RhBMP-2 at final concentrations of 2.5 and 0.5 mg/ml was configured. Rabbit bone marrow MSCs were inoculated into a 6-well plate, and when the cell density reached about 60 %-80 %, 2.5 and 0.5 mg/ml rhBMP-2 injection was added. Set up group A, B, C. The cells in the group A were rabbit bone marrow MSCs without any treatment and were cultured routinely, while the group B was treated with 0.5 mg/ml rhBMP-2; and group C was treated with 2.5 mg/ml rhBMP-2. The experiment was repeated 6 times.

Bone mineral density measurement: At 4th w and 8th w after operation, the experimental animals were killed, and the bone mineral density of newborn bone in the bone defect area was measured by micro-Computed Tomography (CT) advanced bone analysis software.

Western blot: After 48 h of culture in the incubator, each group of cells were homogenized at 4° to make 10 % homogenate, then centrifuged to determine the protein concentration followed by gel preparation, 90 min of electrophoresis, gel cutting. After 90 min, the membrane was blocked by milk sealing technique. Then the membrane was washed and incubated with primary and secondary antibodies. Finally, the results were analyzed by Bio-Rad image laboratory software.

QPCR: After being cultured in the incubator for 48 h, each group of cells used Ribonucleic Acid (RNA) extraction kit and PrimeScript One Step RT-PCR kit to extract RNA, Micro (mi) RNA and complementary Deoxyribonucleic Acid (cDNA) using cDNA synthesis kit in order to reverse transcription miRNA into cDNA. MiRNA fluorescence qPCR detection kit was used for quantitative real-time PCR, and the cycle was

completed according to the kit instructions. Finally, the relative expression of mRNA was calculated in the software.

CCK-8: Cells in 96-well plates were inoculated and were cultured in 37°, 5 % CO_2 for 24 h, and 10 µl CCK-8 solution was added to each well. The proliferation of cells was detected by enzyme labeling (Optical Density (OD) 450 nm) in a constant temperature incubator.

Transwell assay: The cell density of each group was adjusted to 5×10^5 cells/well, and the complete medium of 10 % FBS was added in the lower chamber. The group B and C were treated with *Astragalus* polysaccharides for 48 h, while the group A was given the same amount of Dulbecco's Modified Eagle's Medium (DMEM) culture medium. Cell migration ability was calculated by counting the purple-stained transmembrane cells under the microscope.

Statistical method:

Statistical analysis was carried out using the software Statistical Package for the Social Sciences (SPSS) 22.0 was used for data processing. Measurement of the data was expressed by ($\bar{x}\pm s$). For multi-group comparisons, F-variance analysis was used while Least Significant Difference (LSD)-t test was used for pairwise comparisons.

RESULTS AND DISCUSSION

The radial bone mineral density of rabbits in the group B was high than that in the group A at 4 w and 8 w, and this in the group C was increased than the group B at 4 w and 8 w (Table 1). The cell proliferation and migration activity in the group B were higher than the group A, while those in the group C were increased than the group B (Table 2). The Ang-1 and VEGF in group B increased than the group A, while these in group C were high than the group B (Table 3). The Wnt-β and catenin mRNA in the group B was reduced than the group A, and these in the group C was decreased than the group B (Table 4). The Wnt-β and catenin protein in the group B was reduced than the group A and these in the group C was reduced than the group B (Table 5).

TABLE 1: EFFECT OF rhBMP-2 ON BONE MINERAL DENSITY OF RABBIT RADIUS (mg/cm³)

Group	n	4 w	8 w
A	6	263.36±37.06	294.38±26.02
B	6	830.23±40.04[a]	1054.24±82.04[a]
C	6	1204.13±170.03[ab]	1535.11±135.04[ab]
F		845.413	747.276
p		0.000	0.000

Note: [a]p<0.05 and [b]p<0.05, compared with group B and C respectively

TABLE 2: EFFECT OF rhBMP-2 ON PROLIFERATION AND MIGRATION

Group	n	Cell proliferation activity	Cell migration
A	6	0.10±0.01	73.25±15.70
B	6	0.18±0.03[a]	106.48±17.44[a]
C	6	0.22±0.04[ab]	142.74±24.34[ab]
F		25.846	19.022
p		0.000	0.000

Note: [a]p<0.05 and [b]p<0.05, compared with group B and C respectively

TABLE 3: EFFECT OF rhBMP-2 ON ANGIOGENIC RELATED PROTEINS IN BONE MARROW MSCS

Group	n	Ang-1	VEGF
A	6	0.41±0.13	0.30±0.11
B	6	0.63±0.14[a]	0.59±0.13[a]
C	6	0.94±0.25[ab]	0.71±0.12[ab]
F		97.823	53.632
p		0.000	0.00

Note: [a]p<0.05 and [b]p<0.05, compared with group B and C respectively

TABLE 4: EFFECT OF rhBMP-2 ON WNT-β AND CATENIN mRNA IN BONE MARROW MSCS

Group	n	Wnt-β	Catenin
A	6	2.47±0.54	2.05±0.46
B	6	1.65±0.40[a]	1.32±0.35[a]
C	6	1.24±0.32[ab]	0.77±0.09[ab]
F		212.744	321.687
p		0.000	0.000

Note: [a]p<0.05 and [b]p<0.05, compared with group B and C respectively

TABLE 5: EFFECT OF rhBMP-2 ON WNT-β AND CATENIN PROTEIN IN BONE MARROW MSCS

Group	n	Wnt-β	Catenin
A	6	0.86±0.28	0.78±0.12
B	6	0.57±0.08[a]	0.44±0.14[a]
C	6	0.28±0.04[b]	0.23±0.14[b]
F		115.521	218.481
p		0.000	0.000

Note: [a]p<0.05 and [b]p<0.05, compared with group B and C respectively

Successful reconstruction of bone defect is a major medical challenge. Although autogenous bone is the gold standard for bone defect reconstruction, the incidence of donor site and limited bone mass are obstacles to its use. Therefore, it is very important to develop suitable bone substitutes, and many candidate materials have been studied. An attractive and potentially useful method for repairing large defects is bone tissue engineering, which can be used to repair tumors, trauma, surgical resections, and congenital malformations[7]. Artificial bone stent implantation has attracted more and more attention because of the limitations and shortcomings of autograft bone substitute, such as shortage of donor, incidence of disease in donor area. This scaffold must have ideal mechanical properties, bone conductivity, biocompatibility, biodegradability, and non-cytotoxicity for bone tissue engineering[8]. As bone tissue engineering technology has developed, the development and utilization of growth factors in the treatment of bone defects has become a research hotspot in orthopaedics.

As a member of Transforming Growth Factor (TGF) β growth factor superfamily, BMP exists widely in bone matrix and can promote MSCs to differentiate into osteoblasts[9]. Osteonecrosis and resorption of the fracture site can release endogenous BMP. The amount of bone formation is proportional to the concentration of BMP and the diffusion distance[10]. Bone regeneration always occurs between the two ends of the bone. When the distance between bone ends exceeds the critical value, the number and distribution of endogenous BMP cannot induce chemotaxis[11] well. Therefore, the application of exogenous BMP can make up for the deficiency of endogenous BMP. However, extracting BMP from different animal or human bone tissues is not only complex, but is also inefficient. Therefore, the production of recombinant human BMP by genetic engineering is the key to the wide application of BMP in clinic[12]. Recombinant BMP plays a similar role in inducing osteogenesis as BMP extracted from bone tissue. Both of them can induce locally undifferentiated mesenchymal cells to differentiate into chondrocytes and form cartilage. With the invasion of blood vessels, endochondral ossification occurred in the cartilage area, and the new bone gradually matured. The clinical efficacy of rhBMP-2 has been widely recognized for more than 20 y. RhBMP-2 has been approved by the Food and Drug Administration (FDA) for the clinical treatment and is considered to be a promising bone induction material[13]. Similar products have been launched in China. Bone Youdao (Hangzhou Jiuyuan Gene Engineering Co., Ltd.) is a kind of bone repair material, with BMP-2 as the main active ingredient, each sample contains 250 μg BMP-2 protein. The bioactivity of this bone material has been verified by cell experiments[14].

The results showed that the radius bone mineral density of rabbits in the group B was high than group A at 4 w and 8 w, while this in group C was increased than the group B. The cell proliferation

and migration activity in the group B increased than the group A, while those in the group C were higher than group B. The protein levels of Ang-1 and VEGF in the group B increased than group A, while these in the group C were raised than the group B. It is suggested that rhBMP-2 can promote the repair of radial bone defect and angiogenesis in rabbits.

Signal transduction is closely related to the progress of osteogenesis. Wnt β-catenin is a critical signal transduction pathway that regulates a wide range of downstream effector molecules, including osteogenesis[15,16]. As a member of the secretory protein family, Wnt-β can activate catenin downstream and bind to Qitong transmembrane crimp receptor[17]. Wnt β-catenin signaling pathway is considered to play critical role in cell proliferation, differentiation, growth, regeneration, self-renewal and determining cell fate[18]. The results showed that the expression of Wnt β and catenin mRNA in group B reduced than that in group A, while this in group C was low than that in group B. The expression of Wnt-β and catenin protein in the group B decreased than that in the group A, while this expression in group C was lower than that in the group B. It is suggested that rhBMP-2 can promote the repair of radial bone defect and angiogenesis by mediating Wnt β-catenin pathway in rabbits. It has been found that the typical Wnt signal through catenin is mainly induced during fracture healing, so it is considered to be bone-induced. Wnt signaling pathway is involved in promoting the role of osteoblasts and osteogenesis, resulting in the improvement of bone mass in adults[19,20].

To sum up, rhBMP-2 can promote the repair of radial bone defects and angiogenesis by mediating Wnt β-catenin pathway in rabbits.

Conflict of interests:

The authors declared no conflict of interests.

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