Osteoprotegerin (OPG) Promotes Recruitment of Endothelial Progenitor Cells (EPCs) via CXCR4 Signaling Pathway to Improve Bone Defect Repair

Background: The aim of this study was to investigate the effect of using osteoprotegerin (OPG) to treat bone defects mediated by endothelial progenitor cell (EPC) recruitment and migration through the CXCR4 signaling pathway.

Material/Methods: The EPCs extracted from human peripheral blood were cultured in vitro and the expression of CXCR4 and its downstream p-AKT was monitored by the Western blot analysis after OPG treatment. Using the scratch wound healing test and Transwell assay, we assessed the variables influencing the effect of OPG on EPCs after pretreatment with CXCR4 blocker (AMD3100) and PI3K blocker (Ly294002). After 4 weeks, the bone defect repair condition was estimated via micro-CT and staining with HE and Masson trichrome. Then, immunofluorescence staining was performed to assess angiogenesis in bone defects, while the expression of EPC marker and vascular endothelial growth factor receptor 2 (VEGFR2) was detected by immunohistochemical staining.

Results: The EPCs treated with OPG had increased levels of CXCR4 and p-AKT. Moreover, the difference in EPC levels among groups in the scratch wound healing experiment and migration experiment indicated that the OPG treatment promoted cell migration and AMD3100 and LY294002 inhibited the function of OPG. In addition, OPG promoted angiogenesis and repair of bone defects, while the expression of EPC marker and vascular endothelial growth factor receptor 2 (VEGFR2) was detected by immunohistochemical staining.

Conclusions: OPG enhanced the proliferation and migration of EPCs through the CXCR4 pathway and promoted angiogenesis and bone formation at bone defect sites.

MeSH Keywords: Angiogenesis Inducing Agents • Osteoprotegerin • Receptors, CXCR4

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Background

Bone defect is the damaged bone structure caused by destructive trauma or osteonecrosis due to inflammatory ischemia; it is one of the most severe diseases treated in orthopedics departments [1]. Bone defects increase the risk of delayed union or nonunion of bone tissues and dyskinesia, as well as increasing the risk of disability. At present, bone grafting is the main treatment used [2], but bone resorption and nonunion often occur in the lesion area due to limited revascularization. Bone is superiorly vascularized tissue; therefore, the rate and range of angiogenesis determine the efficiency of osteoanagenesis and defect repair [3,4]. Rapid vascularization of tissue-engineered artificial bone implanted in the body is needed for its survival and function [5]. Endothelial progenitor cells (EPCs) are a group of immature endothelial cells which can further proliferate and differentiate [6] but they lack the characteristic phenotype of mature endothelial cells and cannot form lumen structure [7]. EPCs function mainly in the angiogenesis and repair of ischemic tissue after birth. EPCs have been reported to play an important role in the treatment of cardiovascular and cerebrovascular diseases, peripheral vascular diseases, and wound healing, which provide new ideas and approaches for research and treatment of ischemic diseases [8]. With recognition of the necessity of vascularization in tissue-engineered bone, construction of osteoblasts combined with EPCs has become one of the common methods used to promote osteogenesis [9]. Recruitment of EPCs promotes angiogenesis at the bone defect site and improves the survival rate of artificial bone grafts [10]. Osteoprotegerin (OPG) is a soluble secretory glycoprotein of the TNFR superfamily, which is mainly produced by osteoblasts and vascular endothelial cells [11]. Other tissues, such as heart, kidney, liver, and spleen, can also secrete it. It is an inductive receptor of the receptor activator of NF-κB ligand (RANKL), which reduces the production of osteoclasts (OCs) by binding to RANKL [12]. In addition, OPG also inhibits endothelial cell apoptosis and promotes vascular endothelial cell maturation [13]. However, the effects of OPG on EPCs in the bone defect model are still unknown. In this study, we explored the effect of OPG on EPCs, and found that OPG promoted recruitment and migration of EPCs through the CXCR4 pathway after bone defects were created, and enhanced angiogenesis to achieve structural remodeling of bone.

Material and Methods

Cell culture and treatment

EPCs were isolated from the blood of healthy persons via flow cytometry (CD133+ and VEGFR2+) and cultured in vitro for experiments. The specific steps were: Purified EPCs inoculated in 25-cm² culture flasks appended to the Dulbecco’s modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin for in vitro amplification. After the cell growth density was satisfactory, EPCs were treated with the same volume of phosphate-buffered saline (PBS), OPG, OPG+AMD3100, and OPG+LY294002 for 24 h following starvation in serum-free DMEM for 24 h. The post-treatment cells were continued to culture for subsequent experiments.

Animals and groups

A total of 60 male Sprague-Dawley (SD) rats, ages 6–8 weeks and weighing 280–320 g, were used in the bone defect model. Rats were house 5 per cage under normal environmental conditions (standard food and drinking water, room temperature of 24°C, 12-h artificial circadian cycle). The experimental animals were randomly divided into 4 groups. In the control group, rats received local injection of 0.9% normal saline after modeling of bone defect. In the OPG group, rats received local injection of OPG solvent into the bone defect site. In the OPG+AMD3100 group, rats were treated with AMD3100 on the basis of OPG solvent. In the OPG+LY294002 group, rats were administered OPG and LY294002 injected into the injury site.

Operative procedure and treatment

The rats were first anesthetized by intraperitoneal injection of 10% chloral hydrate solution, then the fur of the right thigh was shaved off. We made a small incision along the long axis of the femur in parallel direction after disinfection with iodophor and used blunt dissection to separate muscle to expose the femur. The middle femur was taken and a 5×5 mm bone defect was made with a micro-drill. Then, the filler was placed in the defect area, the muscle fascia was closed, and the skin was sutured and sterilized again. Within 1 week of modeling, we injected 0.9% normal saline, OPG, OPG+AMD3100, or OPG+LY294002 locally to the defect site (once every 2 days).

Western blot analysis

The different treated EPCs were transformed into protein using a whole protein extraction kit containing protease inhibitor and phosphatase inhibitor on ice. The protein-containing mixture was centrifuged at high speed (14 000 rpm, 15 min) at low temperature (4°C) to obtain protein supernatant. The obtained protein solution was used to determination the concentration according to the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After protein concentrations were assessed in each group, electrophoretic separation of protein was performed on 10% sodium dodecyl sulfate-polyacrylamide gel. Then, the dispersed protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 4°C for 2 h. The transferred protein membrane was...
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being deparaffinized, we performed hematoxylin-eosin (HE) staining and Masson trichrome staining to assess bone defect healing and formation of bone collagen. In addition, immunohistochemical staining was performed to observe the expression of vascular endothelial growth factor receptor 2 (VEGFR2) and immunofluorescence staining was used to assess vascular growth status.

Microcomputed tomography

At 28 days after injury, the rats were anesthetized and placed on an examination table. Bone defect healing was scanned and imaged by SCANCO vivo CT40 (70 kV 114u A).

Statistical analyses

All the data obtained in this experiment were analyzed and processed by Statistical Product and Service Solutions (SPSS 16.0) (SPSS, Inc., Chicago, IL, USA). The results are expressed as mean ± standard deviation (x±s). The difference between 2 groups assessed by t test. Comparison between groups was done using one-way ANOVA followed by post hoc test (least significant difference). P values <0.05 were considered statistically significant.

Results

OPG expression in EPCs occurred via the CXCR4 signaling pathway in vitro

We first utilized the extracted protein from EPCs to detect the action pathway of OPG. In the group treated with OPG, the CXCR4 protein level was increased and its downstream activation site phosphorylated AKT (p-AKT) was improved correspondingly, yet the total AKT (t-AKT) had no apparent change (Figure 1A, 1B). The protein tendency of the 2 different groups demonstrated that the influence of OPG in the EPCs was mainly mediated by the CXCR4 signaling pathway.

OPG promoted proliferation and migration of EPCs, but the effect was weakened by inhibition of CXCR4 and PI3K

To better understand the role of OPG in EPCs, we investigated the effects of OPG on cell proliferation and migration and administrated 2 different signaling pathway inhibitors (AMD3100 and LY294002). They made rivalry for CXCR4 and PI3K respectively. We observed images from the 4 groups after the scratch wound healing test after OPG treatment, showing a smaller scratch wound compared with the control group, but the proportions were not significantly reduced (Figure 2A, 2B). The Transwell assay showed that OPG treatment enhanced cell migration ability, and the AMD3100 and LY294002 reduced the migration ability of EPCs (Figure 2C, 2D). Our results show that...
Figure 1. The expression of osteoprotegerin (OPG) in endothelial progenitor cells (EPCs) was through the CXCR4 signaling pathway \textit{in vitro}. (A) Results of protein bands of CXCR4, p-AKT, t-AKT, and GAPDH in the control group and OPG treatment groups. (B) There were significant differences in CXCR4 and p-AKT in the 2 groups, as shown by gray value analysis (* means there was a significant difference vs. the control group).

Figure 2. OPG promoted the proliferation and migration of EPCs, but the effect was weakened by inhibition of CXCR4 and PI3K. (A) Scratch area of EPCs in different intervention groups at 0 h and 24 h after the scratch was made. (B) Compared with the control group, there was a significant difference in the ratio of scratch area in the OPG group after 24 h. There were significant differences in the proportion of scratch area between the inhibitor treatment groups and the OPG group (# means there is a significant difference from the OPG group). (C) Number of EPCs in the lower chambers in different intervention groups at 48 h after treatment. (D) Cell count analysis showed that cell numbers in the OPG group were significantly different from that of the control group, and the number of cells in the inhibitors groups were also significantly different from that of the control group and the OPG group.
OPG increased the proliferation and migration abilities of cells via the CXCR4 signaling pathway, and the effectiveness of OPG was suppressed via the pathway inhibitors.

The administration of OPG promoted vascular regeneration in bone defect via enhancing the expression of EPCs

One week after the establishment of the bone defect rat model, we utilized immunohistochemical staining to observe the expression of EPCs at the injured site, and immunofluorescence staining was used to visualize vascular endothelial regeneration. The rats treated with OPG exhibited prominent VEGFR2 expression characteristics, and the images from the OPG group showed the strong expression of EPCs at the defect site. However, the CXCR4 and the PI3K inhibitors reduced the VEGFR2 level, and the fluorescent staining of the 2 inhibitor groups indicated that inhibition of the CXCR4 signaling pathway weakened endothelial cell regeneration, as mediated by OPG (Figure 3A, 3B). These results proved that the OPG treatment in bone defect induced the accumulation and activation of the EPCs and improved the endothelial cell regeneration and angiogenesis, but suppression occurred following inhibition of the CXCR4 signaling pathway.

The treatment of OPG strengthened recovery of the bone defect and collagen formation

Histological staining was used to assess recovery of the bone defect injured site, and we also performed micro-CT and histologic analysis of all samples at 28 days. The images of the OPG-injected group displayed predominant bone growth and high bone density, yet the injection of mixed inhibitors impeded the osteogenic function of OPG (Figure 4A–4C). In addition, the collagen content at the defect site injected with OPG was increased compared with the control group. However, the intervention with the 2 different inhibitors reduced formation of collagen, which impeded defect recovery (Figure 4D). All of the above outcomes revealed that the osteogenic effect and the function of collagen formation of OPG promoted healing of the defect, and blocking of the CXCR4 signaling pathway reversed the effect that OPG exerted.

Discussion

Bone defect is an important and difficult orthopedic disease. Because of the huge defect at its broken end, it leads to serious dysfunction of the affected limb, which seriously affects the lives of patients [14,15]. Effectively promoting osteogenesis and survival of implanted bone is key to the treatment of bone defects [16]. Therefore, angiogenesis at site of the bone defect is critical.
Figure 4. Treatment with OPG strengthened recovery of bone defect and collagen formation. (A) Micro-CT imaging of femur of affected limbs of rats in each group 28 days after modeling. (B) Evaluation of bone mineral density of injury site in each group at 28 days. (C) Evaluation of bone volume at 28 days after injury among groups. (D) HE and Masson staining of bone collagen at defect after injury in each group.
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OPG improves bone defect repair
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OPG is an OC inhibitor that blocks binding of RANKL and the receptor activator of NF-κB (RANK), as well as inhibiting differentiation and maturation of OCs and inducing OCs apoptosis [18–20]. However, there has been no specific research on the role of OPG in vascular regeneration during osteogenesis. OPG has a functional effect on EPCs, and EPCs are specific hematopoietic progenitor cells derived from bone marrow [21,22]. These cells are immature endothelial cells that can be mobilized into the peripheral circulating blood by bone marrow mobilization [23]. Differentiation into endothelialocytes is an important functional characteristic of EPCs, and they can also migrate, adhere, and form tubules [24,25]. EPCs have an important physiological role in maintaining vascular homeostasis and regulating angiogenesis [26,27]. OPG has long been considered as a critical factor in regulating osteoclast function and bone density alterations [28,29]. Previous studies demonstrated that OPG improved osteosarcoma and osteoporosis through modulating osteoclast activity [30,31]. Moreover, some studies have found that OPG may play a potential role in endothelial cell phenotype in angiogenesis [32]. However, the specific therapeutic effect and mechanism of OPG in bone defect repair and angiogenesis have rarely been reported. Based on the unpredictability of OPG in the treatment of bone defects, we performed the above experiments and confirmed that OPG promoted the migration and proliferation of human EPCs, and accelerated angiogenesis and collagenation in a rat bone defect model by independently upregulating the CXCR4 pathway. Therefore, OPG ameliorated healing at the defect site in rats (Figure 5).

The present results suggest that OPG improves the survival rate of artificial bone after bone defect surgery, and may play an important role in accelerating bone growth and plasticity. We did not assess the optimal treatment dose and timing of OPG in treatment of bone defects, and this needs to be explored in further research, as well as the effect of OPG administration on human osteogenesis. We found that OPG is a promising treatment to promote angiogenesis after bone grafting in bone defects.

Conclusions

Treatment with OPG accelerated the migration and proliferation of EPCs through the CXCR4 pathway and promoted angiogenesis and collagenation at the bone defect lesion site. Thus, OPG effectively accelerated bone repair in rats.

Conflict of interest

None.

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