

Original Article

Expression of osteoprotegerin, RANK and RANKL genes in femoral head avascular necrosis and related signaling pathway

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Abstract: Femoral head avascular necrosis (AVN) causes the damage of hip joint and related dysfunctions, thus consisting of a clinical challenge. Osteoprotegerin (OPG), receptor activator of nuclear factor κ B (RANK) and its ligand (RANKL) all regulate the formation of bones via gene transcriptional regulation for the balance between osteoblasts and osteoclasts. This study thus investigated the expressional profiles of OPG, RANK and RANKL genes in AVN patients, and explored related molecular mediating pathways. Real-time qPCR was used to measure the gene expression of OPG, RANK and RANKL genes in AVN femoral head tissue samples from 42 patients, along with normal tissues. Western blotting analysis was performed to quantify protein levels of OPG and RANKL. There was a trend but not statistically significant elevation of mRNA levels of OPG in femoral head AVN tissues compared to normal tissues ($P>0.05$). The expression of RANK and RANKL, however, was significantly elevated in necrotic tissues ($P<0.05$). No significant difference in protein levels of OPG or RANKL between groups. The expression of OPG, RANK and RANKL genes exert a crucial role in the progression of AVN, suggesting their roles in mediating bone homeostasis and potential effects on bone destruction.

Keywords: Femoral head avascular necrosis, osteoprotegerin, receptor activator of nuclear factor κ B, ligand for receptor activator of nuclear factor κ B, gene expression regulation

Introduction

Femoral head avascular necrosis (AVN) is a complicated and common disease in orthopedics. Current intervention methods had limited treatment efficacy, thus frequently causing the damage of femoral head and degradation of hip joints [1]. AVN can be further aggravated by the use of steroid, alcoholic intake, systemic lupus erythematosus, sickle cell anemia, metabolic disorders, tumors or trauma [2, 3]. The body's repaired mechanism may function as the vessel proliferation and temporally active osteoclasts at the necrotic lesion and adjacent tissues at the early stage of AVN. Such self-repair, however, had limited effects as the bone formation by osteoblasts will be inhibited, along with the persistent activation of osteoclasts, which finally lead to the degradation of chondrocytes and breakage of joint surface [4-6]. The dynamic balance between osteoblasts and osteoclasts is mediated by various molecular

pathways, which affect the bone formation in a synchronized pattern. The interruption of this balance will break the focal homeostasis of bone metabolism. Osteoprotegerin (OPG), receptor activator of nuclear factor κ B (RANK) and its ligand (RANKL) are all known factors for bone formation and degradation. As the first identified molecule in RANK-RANKL-OPG axis, OPG can inhibit the proliferation of osteoclasts via its competitive binding against RANK. The binding between RANKL and homologous RANK, however, can facilitate the bone reabsorption via a cascade signaling pathway [7, 8]. As the pleiotropic roles of those three genes in bone formation and bone reabsorption, they have been widely used in treating osteoporosis, arthritis, vascular diseases, tumor and bone fracture [9-11]. In this study, the expressional profiles of those three genes were evaluated in a total of 42 AVN patients, in parallel with normal controlled individuals, in an attempt to investigate their roles in focal bone formation.

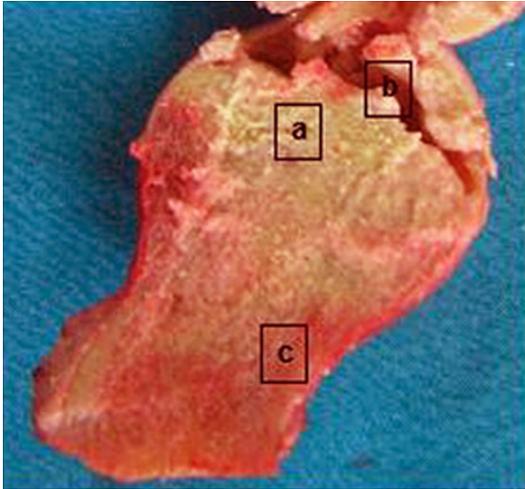


Figure 1. Femoral head tissue sample. a. Necrotic bone tissues; b. Breakage of cartilage; c. Normal tissues.

Materials and methods

Patient information

Tissue samples were collected from a total of 44 femoral head AVN patients, all of which received the total hip arthroplasties (THA) in our hospital. Among all patients, two individuals were excluded due to the lower protein contents in their samples. The condition of femoral head AVN was determined to be grade III or grade IV based on association research circulation osseous (ARCO) standards. This study has been approved by the ethical committee of our hospital. Written consents have been obtained from patients and their families.

Sample collection

The femoral head sample collected from the surgery was longitudinally divided into two parts. Total RNA was extracted from both necrotic and normal tissues at comparable sites. The morphology of sectioning surface was recorded during the surgery for the determination of the borderline between necrotic and normal tissues (**Figure 1**). Necrotic bone tissues were samples from inferior chondrocytes (about 1~3 mm below the cartilage). Normal tissues were collected from the head/neck of the femoral, with more than 1 cm away from the borderline. Collected tissues were rinsed in 0.9% saline for 5 min to clear bone marrow and residual blood. RNA and proteins were then extracted as reported previously [1].

In brief, fresh tissue samples were lysed in Trizol reagents (Invitrogen, UK) for total RNA extraction following manual instructions. RNA was then purified by RNeasy Mini-kit (Qiagen, Germany) and analyzed by 1% agarose gel electrophoresis and UV spectrophotometer. The optical density (OD) ratio at 260/280 nm was between 1.8 and 2.0, suggesting highly purified RNA. We further extracted and cultured cells from seven necrotic tissues along with normal controlled tissues following established protocols [12]. The staining of cultured cells by 85L-2 kit (Sigma-Aldrich, UK) also revealed the existence of living osteoblasts even in necrotic tissues (**Figure 2**).

Real-time qPCR

To quantify the gene expression of OPG, RANK and RANKL genes, we employed fluorescent qPCR technique. The *in vitro* reverse transcription was performed using RNA as the template. PCR system contained 2 μ L cDNA template, 0.5 μ M specific primers, 1 mM $MgCl_2$ and 2 μ L polymerase. The parameters were: initial denature (95°C, 10 min); 45 cycles containing denature (95°C, 15 sec), annealing (54°C, 10 sec) and elongation (72°C, 16 sec). House-keeping gene with stable transcription levels such as h-PBGD gene was used as the reference to quantify the expression level of target genes. A cycle threshold (Ct) value for cDNA copy number was determined from the standard curve of each gene and the reference. The relative expression level was determined by $2^{-\Delta\Delta Ct}$ method.

DNA oligonucleotide primers and hybridization probes were synthesized by TIB Molbiol (Germany). Fluorescent labeling was conjugated on the adjacent sites of hybridization probe. In brief, recipient fluorescent molecule LC Red 640 labeled the 5'-end of the first probe, while fluorescein (FITC, 3FL) was used to label the 3'-end in the second probe. The probe with 5'-label prevents the polymerase elongation during 3'-phosphorylation in PCR. Sequences of primers and hybridization probes were listed in **Table 1**. The lengths of PCR products for OPG, RANKL and RANK genes were 147 bp, 164 bp and 132 bp, respectively.

Western blotting

Both necrotic and normal tissues were firstly rinsed by PBS and were then lysed using NET-

Gene expression in AVN

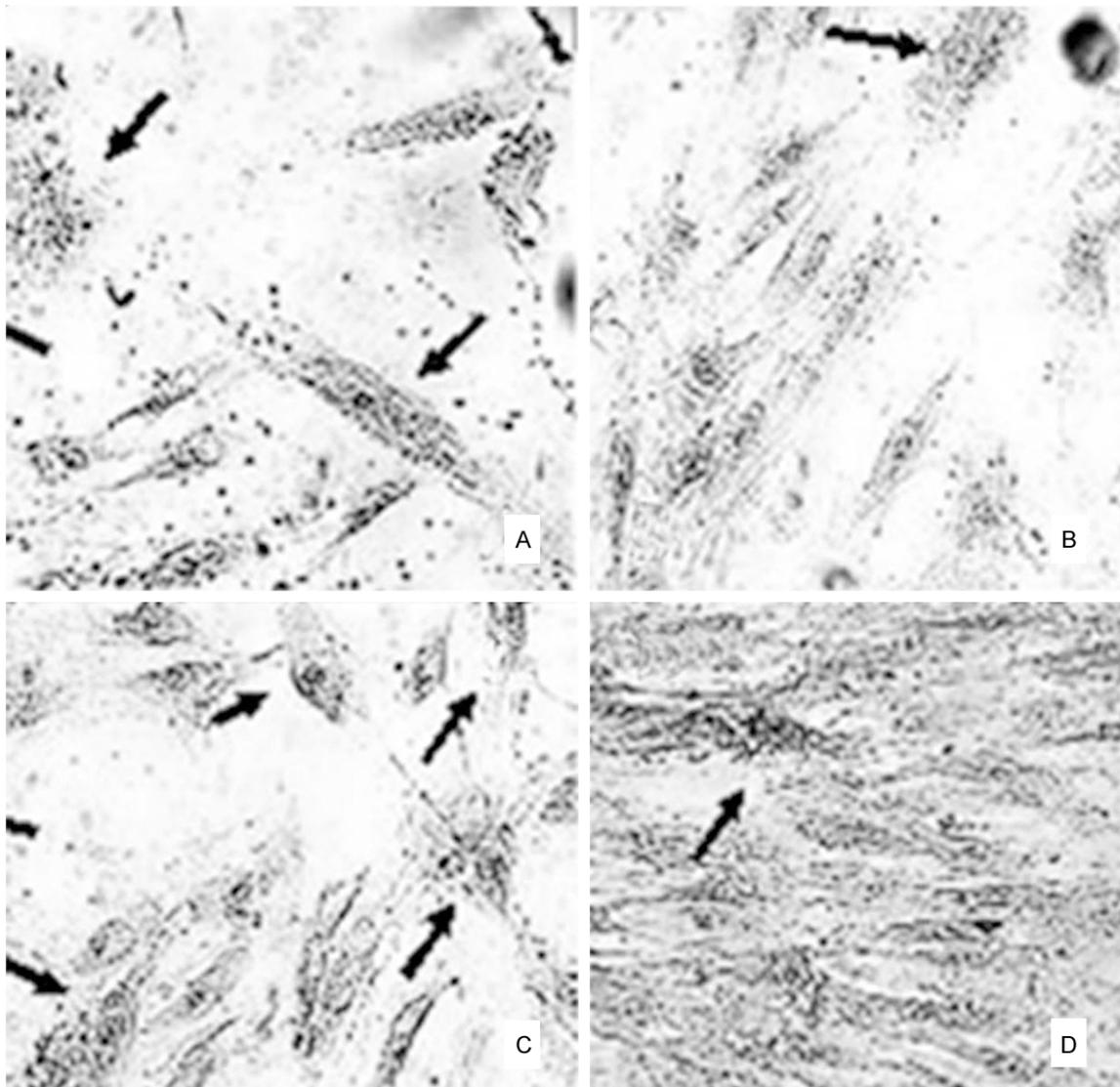


Figure 2. Cultured cells from femoral head samples. A and B. Normal tissues; C and D. Necrotic bone tissues. Arrows, osteoclasts by alkaline phosphatase. Magnification, 40 \times .

Triton lysis buffer (containing 0.01 M Tris-Cl, 0.1 M NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% proteinase inhibitor). Proteins were separated by electrophoresis on Nu-PAGE-Tris acetate gel (Invitrogen, CA) and were transferred to PVDF membrane (BioRad, UK). Non-specific binding sites were blocked by 5% defatted milk powder, mouse anti-RANKL monoclonal antibody (1:100, Santa-Cruz, US) or human anti-OPG monoclonal antibody (1:250, Abcam, UK) was added to detect the protein expression. Human anti-actin monoclonal antibody (1:500, Serotec, UK) was used as an internal reference. After overnight incubation, anti-mouse/human IgG conjugated with

horseradish peroxidase (HRP) (1:500, Abcam, UK) was added to amplify the signal. ELC detection reagent was then used to visualize the positive protein bands. Radioactive autography was performed under the exposure of XAR-5 films (Kodak, Japan). Protein bands were quantified by a laser scanner (Epson, Japan). The relative expression of proteins was calculated as the ratio of OD values of target protein bands against actin.

Statistical analysis

SPSS 16.0 software package was used to analyze all collected data, which were presented as

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Table 1. Primer and probe sequences used in qPCR

Target gene	Primer/probe name	Oligonucleotide sequence (5'→3')
OPG	Sense primer	5'-gaagc tggaa ccca gag-3'
	Antisense primer	5'-gtgtt gcatt tcctt tctga gta-3'
	1 st probe	5'-caatt tgtgt gttt ctaca ggggtg tt-FL
	2 nd probe	5'-LC640-agatg acgtc tcatt tgaga agaac ccat-PH
RANKL	Sense primer	5'-gcaaa aggaa ttaca acata tcggt-3'
	Antisense primer	5'-acttt atggg aacca gatgg g-3'
	1 st probe	5'-ccaga tctaa ccatg agcca tccac c-FL
	2 nd probe	5'-LC640-tcgct ttctc tgctc tgatg tgctg tg-PH
RANK	Sense primer	5'-aggga aagca ctac agcta at-3'
	Antisense primer	5'-acatg ctccc tgctg acc-3'
	1 st probe	5'-agtgg agata aggag tcctc aggtg aca-FL
	2 nd probe	5'-LC640-tgtg tcagt acaca cacgg caaac tt-PH

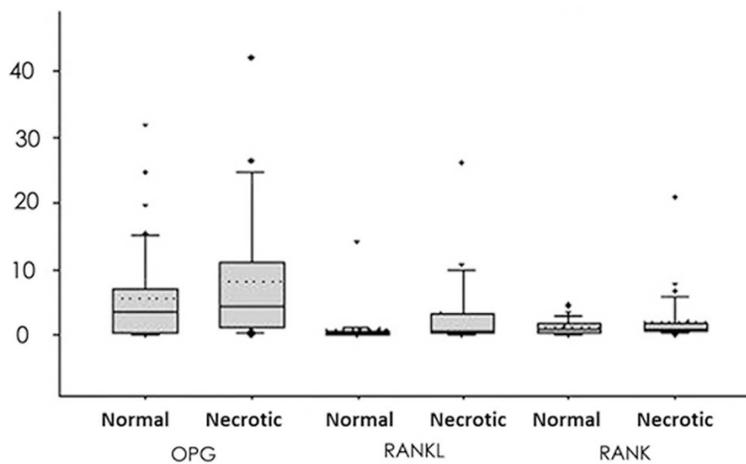


Figure 3. mRNA levels of OPG, RANKL and RANK genes. The box-plot displayed 25% quartile, mean and 75% quartile, in addition to 10% and 90% error bar. Medians were given by dashed lines while outraged data points showed minimal/maximal values. The expression of RANK/RANKL in necrotic tissues was significantly increased compared to controlled ones.

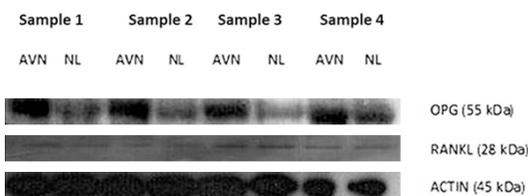


Figure 4. Western blotting for OPG and RANKL proteins. Actin was used as the internal control protein. AVN, necrotic tissues; NL, normal tissues.

mean \pm standard deviation (SD) or medians with minimal/maximal values. Wilcoxon rank-sum test was used in between-comparison, a statistical significance was defined when $P < 0.05$.

Results

Patient information

Of all 42 AVN patients, there were 23 women (10 with menopause) and 19 males. The average age was 43 ± 2.3 years old (20~70 years old). Based on ARCO standard, 4 patients were at stage III while the other 38 patients were at stage IV. A total of 12 patients were smokers. In an epidemiological survey, 15 patients had steroid medicines, one has received kidney transplantation, three of them had sickle cell anemia, three patients were complicated with systemic lupus erythematosus while four patients are alcoholics.

The other four patients had no identified risk factor for AVN.

mRNA levels of OPG, RANKL and RANK

The ratio of OPG/h-PBGD mRNA in necrotic bone tissues was higher than normal tissues but with no statistical significance (**Figure 3**, $P > 0.05$). The expression of RANK and RANKL was significantly higher in necrotic and normal tissues ($P < 0.05$ in both cases).

Western blotting

Total proteins from both necrotic and normal tissues were analyzed for expressions of OPG and RANKL. As shown in **Figures 4** and **5**, OPG

Gene expression in AVN

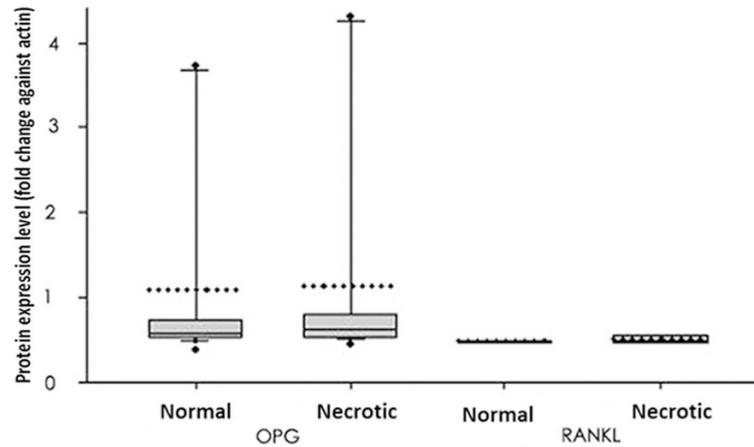


Figure 5. Quantified protein levels of OPG and RANKL. Protein levels were expressed as fold changes against actin in the same tissue and were plotted as the box plot showing 25% quartile, mean and 75% quartile, in addition to 10% and 90% error bar. Medians were given by dashed lines while outraged data points showed minimal/maximal values. No significant has been identified between normal and necrotic tissues in either protein.

protein was expressed in both normal and necrotic tissues with similar levels ($P>0.05$). The RANKL protein also had similar levels in both tissues ($P>0.05$). RANK protein, however, was not identified in either tissue.

Correlation analysis between gene expression and risk factors for AVN

We firstly divided all patients into three age groups: (1) between 20 and 35 years old; (2) between 36 and 50 years old; (5) older than 50 years. The expressional profiles in each group were studied and found no significant correlations between any gene expression level and age ($P>0.05$). We also compared the gene expression level between women before menopause and those after menopause. The RANK level in necrotic tissues was significantly elevated in menopausal women (3.36 vs. 0.95, $P<0.05$). Moreover, RANK gene expression was also significantly potentiated in smokers compared to non-smoking patients (2.23 vs. 1.23, $P<0.05$).

Discussion

The pathogenesis of AVN in femoral heads involves multiple factors, of which the imbalance between osteoblasts and osteoclasts are crucial due to their mediation on bone formation [13]. The focal ischemia in AVN leads to the necrosis of bones and marrows. Although

body's auto-impairment may fix minor lesion (less than 15% of total femoral head), mostly it does not accomplish the fixation [14, 15]. Surgical measures include depressurization, bone dissection and bone transplantation with/without blood vessels. In severe cases, total hip joint replacement is necessary. However, repeated surgeries and postoperative complications often affects the patient's life quality [16].

The growth, development and maintenance of bones are closely regulated by the interaction between osteoclasts, which facilitate bone formation, and osteoclasts for bone reabsorption. OPG, RANKL and RANK have been reported to be important factors in maintaining balances between those two cells [17] as the dysregulation of those factors may lead to the occurrence of various orthopedics diseases [12, 18, 19]. In this study, we investigated the gene expressions of OPG, RANKL and RANK in femoral head samples of AVN patients. Our results showed elevated but not statistically significant difference of OPG mRNA in necrotic tissues. The mRNA of RANKL and RANK, however, were highly expressed in necrotic tissues when compared to control tissues. Protein levels of either OPG or RANKL, were not significantly changed between necrotic and normal tissues. In summary, inconsistent patterns between mRNA and protein expressions existed for those genes, suggesting further post-translational regulation in necrotic tissues.

This study, for the first time, suggested relatively elevated OPG mRNA in necrotic bone tissues, although with no statistical significance. This may be caused by decreased activity of osteoclast, which can disrupt the balance between osteoblasts and osteoclast, further compromising bone reformation. There have been studies reported the elevated bone density and osteopetrosis in mice with OPG-overexpression. In contrast, the gene knockout of OPG may lead to early onset osteoporosis. A recent clinical

survey showed significant correlation between gene expressions of OPG, RANKL and RANK with the risks of bone fracture, suggesting the crucial role of OPG in postnatal bone development. Other studies focused on mutation of RANK or OPG and diseases such as osteolysis, Paget's disease and progressive bone deformity, further supporting the interaction among those three factors in maintain normal bone volumes [20-23]. In our study, the elevation of OPG mRNA in necrotic tissues was consistent with imaging results and mouse model with higher bone density [24]. Whether the elevate OPG is the result of AVN or just one etiological factor for osteopetrosis, however, needs to be elucidated.

In a comparison between RANKL/OPG ratios between necrotic and normal tissues, no significant differences have been identified. However, both gene expression levels were elevated in necrotic tissues, reflecting the requirement of osteoblasts in bone reformation. Genes responsible for osteoblasts maturation and functions, such as bone morphological proteins (BMPs), related transcriptional factors (RUNX2) and other signaling molecules have been demonstrated to be related with bone volume abnormality and bone diseases [25, 26]. For example, recent studies showed the decreasing bone volume by active osteoclasts, which are induced by BMPs via RANKL-OPG pathway, making BMPs as key regulators for osteoclasts [27, 28].

In summary, the expression of OPG, RANK and RANKL genes are closely related with progression of femoral head AVN. Those genes may affect bone homeostasis and may exert certain roles in bone reabsorption and long-term hip joint breakage.

Disclosure of conflict of interest

None.

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