Original article

Adeno-associated virus-mediated osteoprotegerin gene transfer protects against joint destruction in a collagen-induced arthritis rat model

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A B S T R A C T

Objective: To evaluate the in vivo joint protection effect of recombinant adeno-associated virus-mediated gene transfer of human osteoprotegerin (rAAV-hOPG).

Methods: Collagen-induced arthritis (CIA) rat model was established. CIA rats were randomly divided into three groups: CIA control group (PBS), rAAV-EGFP (enhanced green fluorescent protein) group and rAAV-hOPG (100 μl/d) group, which received corresponding intra-articular injection treatment. The thickness of the palps and soles, arthritis index, radiological score, pathological score, bone damage factor and protein expression of inflammatory factors were measured and compared with normal control group rats.

Results: Positive fluorescence of frozen section confirmed that rAAV-hOPG was efficiently transduced into the synovial tissues of test rats. In rAAV-hOPG group compared with CIA control group, the radiological score was 30.18% lower (P<0.05); the expression of OPG protein was 93.41% higher (P<0.05); the expression of matrix metalloproteinase-3 (MMP-3) protein was 35.38% lower (P<0.05); however, the expression of IL-1β was not significant; the scores of pannus and inflammation in rAAV-hOPG group have no significant difference.

Conclusion: These results suggest that adeno-associated virus-mediated transfer of human osteoprotegerin is effectively transduced into the synovial tissues of CIA model, and protects against articular cartilage and bone destruction, but has no obvious efficiency on inflammation. The results also demonstrate that gene transfer using rAAV-hOPG may be a feasible and effective therapeutic candidate to treat or prevent joint destruction in inflammatory arthritis.

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1. Introduction

In some chronic arthritis, such as rheumatoid arthritis (RA), synovial inflammation is followed by cartilage and bone destruction. RA is a systemic autoimmune disease with progressive articular cartilage and bone destruction as the main character. Osteoclasts are terminally differentiated cells of the monocyte/macrophage lineage that resorb bone matrix. Bone and joint destruction in RA is mainly due to the abnormal activation of osteoclasts (OC) [1,2]. In RA synovitis, synovial hyperplasia can be observed and the hypertrophied synovium (also called pannus) invades and erodes contiguous bone and cartilage. A great number of tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor-positive multinucleated cells (osteoclasts) are located in the synovium at the cartilage-synovial interface producing MMP-2 and MMP-9 [3]. The receptor activator of nuclear factor kappa B ligand (RANKL) is another factor playing a key role in bone erosions in RA. Levels of RANKL are significantly elevated in the inflamed synovium of RA. RANKL, a member of the TNF family, is expressed by osteoblast lineage cells and can bind with two different receptors, the first of which is Osteoprotegerin (OPG), and the second is the receptor activator of NF-κB (RANK) expressed on the surface of osteoclast lineage cells. The binding of RANKL and RANK on OC or OC precursor cell can activate transcription factor NF-κB and protein kinase JNK, which can promote OC proliferation, differentiation, maturation and bone resorption activity [4].

Osteoprotegerin (OPG), belonging to TNF receptor superfamily member, is the natural antagonist of RANKL receptor and can block the linkage of RANKL and RANK, thereby inhibit the biological effects of RANKL such as bone resorption, osteoclast differentiation, activation and induction of apoptosis. Therefore, RANKL promotes osteoclastogenesis, while OPG prevents bone erosion. The RANKL/OPG ratio in the bone marrow microenvironment may regulate osteoclast formation, and any increase of this ratio may be followed by bone loss. Also, RANKL/OPG ratio controls the normal bone remodeling and pathological osteopathy by regulating all of the upstream elements such as 1,25(OH)2D3, PTH, PG, TNFα,
IL-1β and IL-6 [5]. Some previous studies found that increasing levels of sRANKL and decreasing levels of OPG could mediate juxta-articular osteoarthritis and erosions in patients with ERA, poly JIA and RA [5]. Fernanda et al. [6] showed that coupling of bone resorption and bone formation was lower in the diabetic rats than normoglycemic littermates after bone fracture, which might be caused by the decreased expression of RANK, RANKL and OPG. Other research has shown that no expression of OPG was found in synovial membrane of RA or phlogocyte in synovial fluid, which demonstrated that the ratio of RANKL/OPG was unbalance at the joint in RA [7]. Recently, Geusens reported that the ratio of RANKL/OPG in serum or synovia in joints was an independent index to predict the radiology progression of early RA patients [8]. Current studies confirmed [9,10] that OC and RANKL/RANK system were involved in bone and joint destruction of RA, and also demonstrated the potential therapeutic value of exogenous increased OPG to the RA joint damage. Jin et al. [11] reported that OPG-Fc fusion protein had obvious therapeutic effect to the joint destruction of RA. However, it has so many disadvantages such as short half-life, multiple infections required, high cost, and high immunogenicity with OPG antibodies produced. Gene therapy is an excellent alternative method.

Gene therapy, by inducing gene expression and thus produce therapeutic proteins in vivo, can enhance the effectiveness of treatment and long-term expression, and avoid the above-mentioned defects. Adeno-associated virus (AAV) is a small DNA virus of the Parvoviridae family and is safe to humans with no pathogenicity. AAV vectors can infect a broad spectrum of host cells, at any phase of division cycle, with no immune response in vivo, long-term expression of transgenic genes [12,13] and high stability of virus particles. Adeno-associated carrier had been successfully used in IL-4 (interleukin 4) [14], IL-10 [15], IL-1 antagonist [16], TNF-α antagonist [17], soluble TNF receptor, IgFc (TNF-Fc) [18] and angiotatin [19] gene transduction in joint cavity, showed us a new method of OPG treatment for RA joint damage. Also, AAV vector was used for OPG gene transfer in the treatment of bone and joint disorders [20], however few studies was on OPG gene transfer for treating joint destruction of RA. The purpose of this study is to observe the protective effect of OPG to joints destruction by knee intra-articular injection of rAAV-hOPG to collagen-induced arthritis (CIA) rats and to dig out the further mechanism.

2. Methods

Sixty skeletally mature, male, 8–10-week-old Sprague-Dawley rats, weighing 239 ± 13 g, were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. pSNAV2.0-LacZ-α plasmid, HSV-rc/ΔUL2 and pOTB7-hOPG (hOPG gene number: NM_002546) were obtained from Vector Gene Technology Company Ltd. (VGTC, China); BHK-21 cell was purchased from Cell Bank of Chinese Academy of Sciences; EcoRI and Sall were purchased from Sangon (Sangon, China); Human OPG ELISA kit was obtained from Bender Medsystems (Bender, Austrian); rat interleukin (IL)-1β, matrix metalloproteinase (MMP)-3 ELISA kit were purchased from R&D companies (R&D companies, US). Bovine type II collagen (bCII) and incomplete Freund’s adjuvant (IFA) were supplied by Sigma Company (Sigma, US).

2.1. Construction of rAAV-hOPG plasmid and preparation of rAAV-hOPG virus

Firstly, pOTB7-hOPG was amplified by an upstream primer with EcoRI site and a downstream primer with Sall site. Upstream primer (hOPG-EcoRI-1226-F): 5′-CCAGAATTCTGAGAAAGTTCTGTCTGC-3′; downstream primer (hOPG-Sall-1226-R): 5′-TGCGTCGACTTATAACGCAGTTTTTACTG-3′. Secondly, the PCR product of hOPG was digested by EcoRI and Sall, and ligated into the EcoRI and Sall sites of pSNAV2.0-LacZ-ΔUL2. The insertion of hOPG into pSNAV2.0 was identified by PCR, digestion and sequencing. Thirdly, BHK cells were transfected by pSNAV2.0-hOPG and selected by G418 in order to establish stable cell line expressing hOPG. Large-scale preparation of the stable cell was applied. rAAV-hOPG virus was produced by infection with a helper virus HSV-rc/ΔUL2, then collected and purified by chloroform, and its hOPG insertion was identified by PCR. The titer of rAAV2-hOPG, measured by a kit according to the manufacturer’s instruction (Boehringer Mannheim, Netherlands), was 10^5 v.g/mL. rAAV-EGFP was set as control, and its titer of rAAV-EGFP was adjusted to10^5 v.g/mL.

2.2. Collagen-induced arthritis model (CIA model)

The rats were fed for 2 weeks to accommodate the environment. Bovine Type II collagen (bCII) was solubilized (2 mg/mL) in 0.1 M acetic acid at 4°C overnight. The solution was emulsified with the same volume of incomplete Freund’s adjuvant (IFA). The final concentration of bCII emulsified solution was 1 mg/mL and stored at 4°C [21]. Fifty randomly selected rats were injected with 0.2 mL emulsion intradermally into the base of the tail at two or three sites. After 2 weeks, a second immunization booster with another 0.1 mL bCII emulsified solution was administrated. The other 10 rats were kept as normal control group by injecting with PBS at the same dose, frequency and injection position as CIA model animals. The body weight, thickness of palms and soles, and mean arthritis index (MAI) were detected and the infection status was evaluated by comparing with normal control group. MAI was scored as follows [22]: 0, normal; 1, synovial hyperplasia; 2, mild destruction of articular surface; 3, severe destruction of articular surfaces; 4, mild subchondral bone loss; and 5, severe subchondral bone loss.

2.3. Grouping and treatment

According to the arthritis index and palmpoplantar thickness, 30 successfully infected rats were randomly divided into three groups, rAAV-hOPG group, rAAV-EGFP group and CIA control group with 10 rats per group. Including the normal control group (n = 10), the whole experimental groups were four. There was no significant difference with Al and palmpoplantar thickness between three CIA rats groups (P > 0.05). Twenty-five days after the first infection, the four groups were treated respectively with 50 μL of rAAV-hOPG, rAAV-EGFP, PBS, PBS in each side of knee-joint cavity every day for 10 days.

2.4. X-ray examination

At the 40th day after the first infection, the X-ray examination was carried out to the rats. The rats were face lying and fixed. The X-ray conditions were 42.0 kv and 2.00 mAs. The modified Larsen score [23] was applied to the digitized images and the original radiographs. Then the score of 14 joints of rats was estimated, including knees, tarsometatarsus joints and metatarsophalangeal joints. The maximum score was 70 points.

2.5. Histopathological examination and evaluation

At the 42nd day after the first infection, the rats were euthanized. The samples of ankle joints and claws were dissected, cleaned and treated with 10% formaldehyde solution for 48 h, decalcified bone matrix with a mixture solution of hydrochloric acid and
formic acid for 24 h. After decalcification, the samples were washed with lotic water for 2 h and the joints were opened longitudinally. The 4 μm sections were prepared and stained by haematoxylin and eosin (H&E) according to standard histological procedures. The score of samples was evaluated according to the joint inflammation, synovial hyperplasia and bone destruction [24]. Inflammation was divided into five degrees, bone destruction was divided into five degrees, and the pannus was divided into three degrees according to the severity.

2.6. Frozen sections

The samples of knee joints were fixed with 4% paraformaldehyde at 4 °C for 3 days with stirring. The samples were decalcified with fresh 14% EDTA at 4 °C for 5 days with stirring (EDTA changes for every 24 h), washed with PBS for 2 h, and then soaked in PBS with 30% sucrose and stirred for 12 h at 4 °C. A bone spicule was used to examine the decalcification. No obvious resistance force when needling was regarded as well decalcified of the sample. After the samples were coated with OCT and quickly frozen, the 5 μm sections were prepared using Leica RM2235 manual rotary microtome (Leica, Germany) and Feather microtome blade (Feather, Japan), and naturally drying away from light. The sections were detected with green fluorescent under fluorescence microscopy to determine the success of AAV transduction to the joints organizations.

2.7. ELISA analysis of synovial fluid in articular cavity

The ELISA analysis was carried out with 1 mL irrigating solution, which was washed the articular cavity with PBS by self-made trocar. Total protein was determined by the Bicinchoninic Acid (BCA) method. The total protein used for OPG, IL-1β and MMP-3 measurement was about 300–1000 mg/mL. The target proteins (OPG, IL-1β and MMP-3) were detected by ELISA kit according to the manufacturer’s instructions. Then the concentration of target proteins (target protein/total protein) was obtained.

2.8. Statistical analysis

Data were shown as means ± SD and were then analyzed by statistical packages using SPSS software (version 13.0; Chicago, USA). Four groups, 40 rats participated in the experiment; the number of valid data of each group was five or more than five. One-way analysis of variance (ANOVA) analysis and LSD-t test were used to analysis intergroup comparison. P<0.05 was considered statistically significant; P<0.01 was considered highly statistically significant.

3. Results

3.1. CIA model

The rats became sluggish and lost appetite after they were first immunized for 3–5 days. The hair of rats became tarnished; sclerosis or ulcer was found at the intradermal injections site, which was self-cured after 7–10 days. The skin of some rats’ joints became mild swelling and start redness after the rats were first immunized for 16–19 days. The red swelling of the skin became more serious, especially the bilateral ankle joints. The foot pads of rats increased thickness and induced the diffuse redness of fore-claws. The most serious arthritis disease was observed in rats after they were first immunized for 28 days. Some rats had joint swelling at both knees and had walking difficulty. The weight of rats grew slowly or even declined. The joint swelling was regressed and ankylosis can be observed on some rats with significant difficulty in walking. The thickness of the palms and soles of three CIA groups had no obvious difference (P>0.05) (Fig. 1A). Arthritis index of three CIA groups had no obvious difference (P>0.05) (Fig. 1B).

3.2. X-ray examination

Five days after treatment, the X-ray examination was carried out to the rats (Fig. 2A–D). For CIA group and rAAV-EGFP group, the joint space was narrow and the joint structure was broken. It can also be observed that the cystic change under the articular facet and osteoporosis surrounding the joint in these two groups. For rAAV-hOPG group, the destruction level is obviously lighter than the CIA and rAAV-EGFP groups. The radiographs of joints of hind limbs were evaluated using modified Larsen score (Fig. 2E). Score of normal group was 0; score of rAAV-hOPG group was 24.0 ± 3.2 (P<0.01 versus rAAV-EGFP group, P<0.01 versus CIA group); score of rAAV-EGFP group was 35.3 ± 3.4; score of CIA group was 34.4 ± 3.9. The score of rAAV-OPG group was degraded by 30.18% compared with CIA group.

3.3. Histopathological evaluation

HE staining of knee joints of CIA rats exhibited severe synovitis. A great number of inflammatory cells infiltrated and aggregated into the tissue, and a lymph nodule-like structure was formed. Synovial cells proliferated above 10 layers, and chaotically arranged. The number of blood vessels in synovium undertissue increased, and the vessels stretched, cellulose leaked out from them. The pannus was formed, the bone and cartilaginous tissue of joints were destroyed (Fig. 3). The pathological articular damage score of rAAV-hOPG intra-articular injection group is 35.48% lower than the CIA
control group \((P<0.05)\) (Fig. 3B). The inflammation and pannus score had no obvious difference \((P>0.05)\) (Fig. 3B).

3.4. Fluorescence stain sections

Green fluorescence of the frozen sections of synovium taken from the knee joints was observed under inverted microscope (Fig. 4). It showed that rAAV-EGFP was successfully transduced to synovial tissue of knee joints and EGFP was expressed.

3.5. ELISA analysis of synovial fluid

The expression of OPG, IL-1\(\beta\) and MMP-3 in synovial fluid is shown in Fig. 5. In normal group, the expression of OPG and MMP-3 was statistically significant compared with CIA control \((P<0.01)\) and rAAV-EGFP group \((P<0.01)\). In rAAV-OGP treatment group, the expression of OPG in synovial fluid was significantly increased compared with CIA control group \((P<0.05)\) and rAAV-EGFP group \((P<0.05)\), 93.41% higher than CIA control group; the expression of MMP-3 was significantly decreased compared with CIA control group \((P<0.05)\) and rAAV-EGFP group \((P<0.05)\), 35.38% lower than CIA control group; the expression of IL-1\(\beta\) was not significant compared with CIA control or rAAV-EGFP group.

4. Discussion

In the study, we used an in vivo AAV-mediated gene transfer technique to change the ratio of RANKL/OPG in rat’s joint cavity, and evaluated the expression efficiency of rAAV-hOPG in joint cavity and the efficiency on treating joint destruction. EGFP was used for the determination of transfection efficiency and in vivo observation. We found that the expression of soluble OPG protein in joint synovial fluid of rAAV-hOPG intra-articular injection group was significantly increased compared with CIA control group \((P<0.05)\) (Fig. 5), 93.41% higher than CIA control group after 15 days treatment. This result proved the success of rAAV-hOPG transduction into joint cavity effectively. The significant degrade of pathological articular damage score (degraded by 35.48%) (Fig. 3) and the radiology Lasen score (degraded by 30.18%) (Fig. 2) demonstrated that rAAV-hOPG was potent in treating joint destruction. MMP-3 is an effector molecule of destruction of joint and cartilage, which...
can degrade extracellular matrix, promote the angiogenesis, and partly take part in plasminogen activation system [25] to promote the destruction of joint. MMP-3 is partly secreted by osteoclast and macrophages. As shown in Fig. 5, the expression of MMP-3 protein in joint synovial fluid of rAAV-hOPG intra-articular injection group was significantly lower than CIA control group (degraded by 35.38%) (*P < 0.05). We speculated that the lower expression of MMP-3 might be related with the differentiation inhibition or induction apoptosis of osteoclast by OPG. The main inflammatory factor IL-1β was also detected and we found that the protein expression level of IL-1β in CIA groups was obviously higher than the normal group, and have no significant difference between the CIA groups. The result of Pathological Score also proved the scores of pannus and inflammation in rAAV-hOPG group have no significant difference compared with that in CIA control group. These results demonstrated that rAAV-hOPG might have no obvious anti-inflammation effect. This showed the dissociation between destruction and inflammation. Stolina et al. found that RANKL inhibition by OPG could prevent bone loss but did not affect local or systemic inflammation parameters by using rat CIA model and rat adjuvant-induced arthritis (AIA) model [26], which is concordant with our result.

Anti-TNF-α antibodies (anti-TNF-α Ab) can decrease joint inflammation and bone erosions. However, a combination treatment with OPG and anti-TNF-α Ab for CIA mice found no additive effects of OPG and anti-TNF-α Ab [27]. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat RA pain and inflammation. Due to the joint protection effect of OPG, we speculate that remarkable effect will be achieved by combining rAAV-hOPG and NSAIDs for treating chronic arthritis, such as RA. It will be studied in our further research. In conclusion, the elevation of OPG protein expression which mediated by rAAV-hOPG transduction can protect the destruction of cartilage and bone of joint, but has no anti-inflammation effect. The finding provides a new route for treating cartilage and bone destruction of chronic arthritis (include RA).

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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References


Fig. 5. The expression of OPG (A), IL-1β (B) and MMP-3 (C) in synovial fluid. **P < 0.01 versus rAAV-EGFP group, CIA control group. *P < 0.05 versus rAAV-EGFP group, CIA control group.


