Combination gene therapy targeting on interleukin-1β and RANKL for wear debris-induced aseptic loosening

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INTRODUCTION
Aseptic loosening (AL) due to periprosthetic osteolysis remains an important cause of the long-term failure of total joint arthroplasty, accounting for approximately 75% of the failure cases. With increased life spans and numbers of joint prostheses in younger, more active patients, osteolytic aseptic loosening has become the dominant reason for revision surgery. Understanding the precise mechanism of AL and developing potential therapeutic means to halt the loosening process is therefore critical and urgent.

It has been reported that wear particles from the bearing surface have a critical role in periprosthetic osteolysis. Wear particles exert their biological activity via macrophages, foreign body giant cells and fibroblasts within the periprosthetic membrane. Activated cells provoke the periprosthetic inflammation by means of proinflammatory mediators, including IL-1β, TNF-α, IL-6 and PGE2, which in turn contribute to the osteoclast differentiation and survival through the receptor activator of nuclear factor NF-kappa B ligand (RANKL) and its receptor RANK pathway. Extensive research efforts have focused on osteoprotegerin (OPG), a native soluble protein that competes for the binding of RANKL to its signal-transmitting receptor RANK. Animal experiments have established the pivotal role of RANKL/RANK pathway as the central regulator of osteolastogenesis, and demonstrated that the introduction of exogenous OPG to alter the RANKL/OPG ratio may negatively regulate the osteolastogenesis and bone remodeling. Our previous studies have suggested that OPG effectively blocked orthopaedic biomaterial particle-induced bone resorption in animal models. One of the most common pathological findings at the site of osteolytic prosthetic loosening is a layer of periprosthetic tissue with numerous macrophages and osteoclasts. Neale and Athanasou detected strong IL-1, IL-2 and TNF receptors present on macrophages and osteoclasts associated with revision arthroplasty, suggesting that these cytokines have an important role in periprosthetic bone resorption. IL-1, TNF or IL-6 may directly activate osteoclast activity and promote the production and activation of other potent mediators of bone loss. We hypothesize that debris-associated local inflammation and bone resorption are associated, yet separated, pathological processes. Therefore, therapies targeting inflammation and osteolysis might have synergetic effects in controlling wear debris-associated aseptic joint prosthesis loosening. The current study extends our previous findings and aims to evaluate the efficacy and potential molecular mechanisms of a combination gene modification against both IL-1 and RANKL on wear debris-induced aseptic implant loosening in mice.

RESULTS
The anti-inflammation effects of the combination gene therapy The pre-osteoclastic mouse RAW 264.7 cells (2 x 10⁶) were activated in vitro in the presence of titanium alloy particles (1 x 10⁶ m−1), followed by virus-mediated gene transduction of IL-1 receptor antagonist (DFG-IL-1Ra-neo) and osteoprotegerin (rAAV-GFP-OPG), individually or in combination. The combination group received one-half dosage of each individual viral vector (0.4 x 10⁴ pfu) to ensure transduction equivalence. RAW cells
transduced with $10^5$ particles ml$^{-1}$ of AAV-LacZ were used as non-therapeutic control. Enzyme-linked immunosorbent assay (ELISA) for IL-1 in the culture media indicated significant inhibition of IL-1 release in the IL-1Ra-treated group and the combination group at the first week following gene modification in comparison to LacZ controls, and the inhibition effect persisted through the 3-week culture (Figure 1a). During the later time period, all the gene therapy groups resulted in diminished IL-1 expression levels, including OPG gene modification (with less efficiency). At the transcriptional level, real-time PCR on the RAW cell preparations at 4 weeks of culture post treatment revealed a significant decrease of IL-1 mRNA expression in all the therapeutic treatment groups, with best effect in the combination group (Figure 1b, $P<0.05$).

Effect of combination therapy on osteoclast differentiation

RAW cells were harvested at 4 weeks after gene modifications. Tartrate-resistant acid phosphatase (TRAP) staining was performed to quantify mature osteoclasts (Figures 2a–d). Significantly fewer TRAP-positive cells were present following OPG and OPG + IL-1Ra gene modifications, while IL-1Ra modification alone suggested less effectiveness (Figure 2e). Although there were relatively few multinucleated cells in this type of cell line, acquisition of TRAP$^+$ characteristics suggests an indication of mature osteoclastic cells. Real-time PCR data further indicated that the double gene modification resulted in the most marked reduction of RANK messenger RNA expression levels within all treatment groups, thus revealing the influence of synergetic inhibition over the individual exogenous IL-1Ra or OPG gene transduction (Figure 2f, $P<0.01$).

In vivo combination gene therapeutic effects on bone remodeling

The mouse knee pin-implantation-failure model$^{14}$ was used to evaluate the in vivo therapeutic efficacy of the gene modification therapies. Three weeks after titanium pin implantation into the proximal tibia and peri-implant titanium particles challenge, media containing DFG-IL-1Ra and AAV-GFP-OPG (individually or in combination) were injected intra-articularly into the implanted knee joint. Mice with AAV-LacZ viral vector injection were included as controls. Histological assessment of the proximal tibiae harvested 8 weeks following gene modifications exhibited ubiquitous peri-implant pseudo-membranes in the LacZ gene-treated group, while dramatically thinner or disappearing peri-implant soft tissue was exhibited in most of the harvested specimens following therapy (Figure 2f).

Micro-computed tomography (CT) assessment of bone mineral density (BMD) changes and osteolysis

A Scanco in vivo μCT system (Scanco Medical, Basserdorf, Switzerland) was used to quantify peri-implant bone volume and BMD changes following gene modification treatments. Panels (a)–(d) of Figure 4 illustrate representative μCT 3-D reconstruction images of pin-implanted tibiae at 8 weeks post gene modifications. Focal bone osteolysis at the interface was remarkably diminished following the double gene therapy (Figure 4d). Quantification of the bone volume/total volume was done, and the BMDs of the peri-implant bone specimens were also compared and are summarized in Figure 4e. Therapeutic modifications significantly protected against the bone fraction volume and BMD loss in comparison with the LacZ controls (Figure 4d, $P<0.05$).

Biomechanical testing of the knee-implant tibia after combination gene therapy

As peri-implant osteolysis weakens the stability of the pin implant, a pin pull-out test was performed to examine the implant’s biomechanical stability after 8 weeks of gene modification treatments, with a custom-made fixture on a BOSE actuator (Model 3220-AT, Bose ElectroForce System, Eden Prairie, MN, USA); the force curve to dissociate the pin from the surrounding bone was recorded and analyzed (Figure 5, inset). Although there were variations among the individual specimen’s pullout force in the OPG gene modification group, all the pins in the double gene therapy group exhibited well-fixed mechanical stability that required significantly more force to dissociate the pin implants compared with the LacZ control group (Figure 5, $P<0.05$). However, the pullout test on the IL-1Ra transduction group did not show statistically significant difference from the LacZ controls ($P=0.21$).

The molecular exploration of the exogenous gene modifications on osteoclastogenesis

Real-time PCR was performed to examine specific gene expression profiles on cells following the therapeutic gene modifications. Using gene expression data from the LacZ group as the baseline,
c-Fos was diminished 54%, 67% and 88% in IL-1Ra, OPG, and (d) IL-1Ra + OPG; while (e) summaries the quantitative analysis of TRAP+ cells by ImagePro+ software among groups (\( ^* P < 0.05 \) compared with LacZ control; \( ^# P < 0.05 \) compared with the double gene therapy). (f) exhibits the real-time PCR result of the RANK mRNA expressions (\( ^* P < 0.05 \) and \( ^{**} P < 0.01 \) compared with LacZ controls; \( ^# P < 0.05 \) to the double gene group). mRNA, messenger RNA.

DISCUSSION

Wear debris-associated aseptic loosening is the major long-term complication of total joint replacement. Studies suggested that the implant wear particles from the implant-bearing surface attract inflammatory cellular infiltration and initiate the formation of the periprosthetic tissue, which often results in the elevated local expression of pro-inflammatory cytokines, including IL-1, TNF, IL-6, prostaglandin E2, matrix metalloproteinases and other factors.\(^{15,16}\) Those cytokines in turn promote more inflammatory cell activation and osteoclastogenesis, leading to periprosthetic bone resorption and aseptic loosening.\(^7\) In addition, recent research efforts have identified signaling crosstalk between immune and skeletal system in osteoimmunology.\(^7\) As early as 1990, \textit{in vitro} experiments had suggested that the maturation of osteoclasts required the presence of bone marrow stromal cells or their osteoblast progeny.\(^{19}\) Since the identification of two essential molecules’ response for osteoclast differentiation (RANKL and M-CSF) and the naturally existing decoy RANKL
receptor (OPG), the imbalance of RANK, RANKL and OPG expression levels has been theorized as the major cause of bone remodeling disorders including osteolytic aseptic prosthetic loosening.8,19 We have hypothesized that debris-associated local inflammation and bone resorption are associated yet separated pathological processes; thus, therapies targeting inflammation and osteolysis may have synergetic effects in controlling wear debris-associated aseptic joint prosthetic loosening. The current study was designed to test our hypothesis to evaluate a double gene modification targeting both IL-1 and OPG on titanium particle-induced knee pin-implant loosening. We have previously shown the protective effects of the retrovirus-mediated IL-1 receptor antagonist (IL-1Ra) gene modification to wear debris-induced local inflammation and osteolysis on small animal models.19-21 Previous data from our group and others also suggested the protective and therapeutic influence of directly blockade of RANK/RANKL interactions using OPG or RANK:Fc for the wear debris-associated osteolysis in vivo.7,13,22-24 In vitro data in this study confirmed the previous findings that IL-1Ra and OPG gene modification effectively blocked local inflammation and osteoclastogenesis, respectively. More importantly, this study suggested that the combination of OPG and IL-Ra gene transfers with equivalent viral vector load exhibited a strong synergistic therapeutic effect in the blocking of proinflammatory cytokine expression and osteoclast maturation, with a significantly better efficacy over either single gene transduction. The data also

**Figure 3.** Histological appearance of the peri-implant tibiae (hematoxylin and eosin stained) and the measurement of the thickness of the pseudo-membrane. (a) LacZ control; (b) IL-1Ra treatment; (c) OPG gene modification; (d) double gene transduction (all × 40 magnification, except the insets (× 200)); and (e) quantification of the pseudo-membrane thickness at 2 months following gene modifications (*P < 0.05).
supported a close relationship between the immune and skeletal systems in osteoimmunology.25

The knee-implantation-failure mouse model14 was also indicative of the potential long-term therapeutic influence of the combination gene therapy. Peri-implant pseudo-membrane typically develops at the bone-pin interface within 2 weeks of the particulate debris challenge.14 In the current study, therapeutic or control viral vectors were transduced in vivo at 3 weeks post particle challenge to evaluate and compare the therapeutic influence. The double gene-modified mice exhibited a significant decrease in the thickness of pseudo-membrane and a significant increase in the bone fraction volume and bone density, while single gene modification exhibited a slightly less effectiveness. In addition, the double gene therapy reached the maximum implant stability confirmed by the biomechanical pullout test.

Real-time PCR illustrated signal transduction modifications following gene therapy. Takayanagi25 summarized the molecular pathways that RANKL/RANK associated in the osteoclastogenesis. Although the role of the calcium-NFAT pathway25,26 is currently controversial, the RANKL–RANK–c-Fos and RANKL–RANK–TRAF6 pathways have been widely accepted as relevant to osteoclastogenesis.27–30 In this study, significantly decreased expression of TRAF6, c-Fos and JNK1 genes was observed in the cells receiving combination therapy of IL-1Ra and OPG. Although IL-1 is also considered to have an important role through TRAF6, similar to RANKL, to interact with TAK1 and promote osteoclastogenesis,31,32 the data presented here show that combined blockage of both IL-1 and RANKL dramatically enhanced osteoclast depletion and osteolysis, suggesting some synergetic influence. Real-time PCR data further suggested that overexpression of IL-1Ra gene may alter JNK1 expression, while RANKL/RANK inhibition through elevated OPG expression directly blocked the initiation of the osteoclastogenesis. Besides the anti-osteoclastogenesis, the gene modifications also exhibited significantly ameliorated inflammation.

Figure 4. The analysis of bone remodeling. Representative μCT 3D construction images of the mouse prosthetic tibiae after 8 weeks of (a) LacZ control; (b) IL-1Ra treatment; (c) OPG gene modification; (d) double gene modifications. Arrows point out the focal bone erosions. The plot (e) summarizes the bone volume over total volume calculations by the μCT software among groups (*P < 0.05), whereas (f) illustrates the quantitative analysis of bone mineral density changes following treatments (*P < 0.05).
Overall, the combination gene therapy targeted towards anti-inflammation and anti-bone resorption may reduce the individual exogenous gene introduction and provide strong therapeutic influences in controlling wear debris-associated periprosthetic inflammation and osteolysis. The synergistic effect of double gene therapy may be due to inhibition of c-Fos and JNK1 pathways in osteoclastogenesis, but further studies are warranted to explore the long-term safety issues of multiple gene modifications and proper dosages of exogenous genes.

**MATERIALS AND METHODS**

Cell culture and in vitro gene transfer

The RAW 264.7 cell line was purchased from the American Type Culture Collection. The titanium alloy particles (Ti-6Al-4V) used in the experiments was kindly provided by Zimmer, Inc. (Warsaw, IN, USA) and the size of the particles (2.3 ± 0.168 μm) was similar to the wear debris got from the periprosthetic tissue of patients revised for aseptic loosening. The recombinant adeno-associated virus transfer vector coding for OPG (pAAV-CMV-OPG-ires-EGFP) was generated through multiple sub-cloning steps as detailed previously and packaged in the Gene Core Facility, University of North Carolina (UNC) at Chapel Hill (NC, USA), who prepared the purified rAAV-OPG-ires-EGFP using type 2 rAAV. The rAAV-LacZ control vectors were also obtained from the Gene Core Facility, UNC. Retroviral vectors encoding human IL-1 receptor antagonist (DFG-IL1-Ra-neo) and viral β-galactosidase (MFG-LacZ) were constructed in and kindly provided by the laboratories of Dr Robbins in Pittsburgh, PA, USA.

RAW cells at 2 × 10⁴ ml⁻¹ were cultured in the presence of titanium particles (1 × 10⁶ ml⁻¹) for 72 h before dividing into five groups: Group 1 was transduced with DFG-IL-1Ra (retroviral vectors code for IL-1 receptor antagonist gene) by adding 800 μl of 1 × 10⁷ pfu viral medium; Group 2 cells were transduced by AAV-GFP-OPG (Adeno-associated viral vectors encoding osteoprotegerin) at 10⁵ particles ml⁻¹; RAW cells in Group 3 were co-transduced sequentially with a half dose of AAV-OPG (0.4 × 10⁴ particles ml⁻¹) and a half dose of DFG-IL1-Ra (0.4 × 10⁴ pfu), while the Group 4 RAW cells were transduced with 10⁴ particles ml⁻¹ of AAV-LacZ as controls. Group 5 was control cells without viral infections. To achieve the highest concentration of the double gene expressions in the combination therapy group, the infection of AAV-OPG was introduced 3 days later after the retrovirus-IL-1Ra transduction. The culture media were changed every 2 days, pooled weekly and stored in −20 °C for ELISA examination. All the cells were harvested periodically during the 4-week cultivation.

Enzyme-linked immunosorbent assay

Release of proinflammatory cytokines IL-1β and TNFα in the cell culture media following gene transduction was assessed using ELISA kits (R&D Systems, Minneapolis, MN, USA) described previously. The optical density was determined by an ELISA reader (Molecular Devices, Menlo Park, CA, USA) at 405 nm wavelength, and levels of cytokines were determined by regression analysis against a standard curve.

TRAP stain for osteoclasts

The gene-modified RAW 264.7 cells were harvested for TRAP (EC3.1.3.2) staining using a commercial kit (Sigma, Saint Louis, MO, USA). The cells in 200 μl suspension (1 × 10⁵ cells ml⁻¹) were cytopsint onto a histological slide followed by fixation for 30 s in buffered acetic in cold. The slide was incubated at 37 °C for 1 h in 0.1 M acetate buffer (pH 5.2), containing 0.5 mM naphthol AS-BI phosphoric acid, 2.2 mM FastGarnet GBC, and 10 mM sodium tartrate. The reaction was stopped by washing in several changes of distilled water. The presence of dark purple staining granules in the cytoplasm was considered as the specific criterion for identifying TRAP-positive cells.

Real-time PCR for gene expression profile

Real-time reverse transcriptase PCR was performed to assess the efficacy of anti-inflammation and anti-bone resorption after gene modifications. Total RNA from RAW cells after gene modifications was extracted in TRIzol...
The primer pairs for real-time PCR

| Genes | Left primer sequence (5’-3’) | Right primer sequence (5’-3’) |
|-------|-----------------------------|-------------------------------|
| IL-1β | GGGCCTCAAAAGGAAAGTAC      | TACGGTGGGAGGAACCT             |
| RANK  | GGGTGGGGGCGACATTCCAG      | ATGCCAGGGCTGCTGGA             |
| c-Fos | TCTCCGGTCTAGCTTCTGACT      | TTGGCTTTCTGATGGTCCTCTGCA      |
| TRAF6 | GCAGTCGTTGCCGTGTCCTGAG    | GGCAGCAGCAGCAGTGAGAGA         |
| JNK1  | AGAAGGTTCAGGCCTTCCGAG    | TGATGTTGATGGTCCTGGAGA         |
| CPK   | CTCGACAGCAGAAGCAGA        | TAGTCGCTTCTGCCTGCAGA          |

Abbreviations: CPK, cathepsin K; IL, interleukin; RANK, receptor activator of nuclear factor kappa B; TRAF6, TNF receptor-associated factor 6.

 Establishment of the mouse pin-implantation model and in vivo gene transfer(s)

The animal procedures were approved by the Institutional Animal Care and Use Committees (Wichita State University). BALB/c mice (Jackson Labs, Bar Harbor, ME, USA) aged 10–12 weeks were quarantined in our local animal facility for 2 weeks before experimentation. Titanium alloy pins of 0.8 mm diameter and 5 mm length with a flat large top of 1.4 mm diameter were specially manufactured by Stryker Orthopaedic, Inc. (Mahwah, NJ, USA). The mouse model was established as described previously. Briefly, the tibial plateau of the mouse right knee was surgically exposed under strict sterile procedure, and a proximal 6 mm of the tibial intra-medullary canal through the center of the tibial plateau was reamed with a 0.8 mm dental drill. A titanium-alloy pin was then press-fit into the canal in a manner that the surface of the pin head became flush with the cartilaginous surface of the tibial plateau. The limb with tibia pin implantation was surgically harvested at the time of killing. A final scan was performed 8 weeks later at the time of killing the mice, for bone density measurements. The scans were performed with an isotropic voxel size of 30 μm, an energy level of 70 kVp, a current of 114 μA, and an integration time of 200 ms. Reconstruction and analysis were carried out using the manufacturer’s supplied software. A region of interest was defined around the pin, and BMD, bone volume and total volume of the tibia were calculated.

Biomechanical test of pin implantation in tibia

The limb with tibia pin implantation was surgically harvested at the time of killing. All soft tissue around the prosthetic joint was carefully removed to expose the implanted pin surface and proximal tibia. Approximately 10 mm of the distal tibia was cemented into a custom-designed jig with dental cement (Garreco; Heber Springs, AR, USA). Proper vertical alignment of the tibia titanium implant to the loading axis of the BOSE ElectroForce testing system was achieved with a customer-designed fixture, and the actuator positions and loading data during the pulling test were recorded.

Statistical analysis

Statistical analysis among groups was performed by one-way analysis of variance test with the Schafer formula for post hoc multiple comparisons (SPSS v12, Chicago, IL, USA). A P-value of <0.05 was considered as significant difference. Data are expressed as mean ± s.e.m.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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