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Efficient Inhibition of wear debris-induced inflammation by locally delivered siRNA

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ABSTRACT

Aseptic loosening is the most common long-term complication of total joint replacement, which is associated with the generation of wear debris. The purpose of this study was to investigate the inhibitory effect of small interfering RNA (siRNA) targeting tumor necrosis factor-α (TNF-α) on wear debris-induced inflammation. A local delivery of lentivirus-mediated TNF-α siRNA into the modified murine air pouch, which was stimulated by polymethylmethacrylate (PMMA) particles, resulted in significant blockage of TNF-α both in mRNA and protein levels for up to 4 weeks. In addition, significant down-regulation of interleukin-1 (IL-1) and interleukin-6 (IL-6) was observed in TNF-α siRNA-treated pouches. The safety profile of gene therapy was proven by Bioluminescent assay and quantitative fluorescent flux. Histological analysis revealed less inflammatory responses (thinner pouch membrane and decreased cellular infiltration) in TNF-α siRNA-treated pouches. These findings suggest that local delivery of TNF-α siRNA might be an excellent therapeutic candidate to inhibit particle-induced inflammation.

Total joint replacement is a widely successful approach that reduces pain, restores joint function, and allows arthritis patients to return to varied activities of daily living. However, despite the popularity and initial success, aseptic loosening of the prosthesis has become the most common complication of total joint replacement and remains a major problem for the long-term success and survival of prosthesis [1]. Wear debris-associated local inflammation and consequent osteolysis are believed to play critical roles in the pathogenesis of aseptic loosening [2]. PMMA particles are widely used in total joint replacement and generally found in peri-prosthetic tissue. Repeated phagocytosis of the particles results in aseptic inflammation and release of proinflammatory cytokines, which contributes to the activation of osteoclasts and the final prosthetic loosening [3]. Tumor necrosis factor-α (TNF-α), a major proinflammatory cytokine in the osteolytic process, can induce aseptic inflammation and differentiation of osteoclast precursors into mature osteoclasts in direct and indirect manners [4,5]. Therefore, we proposed that the inhibition of TNF-α represents a potential therapy to reduce chronic inflammation provoked by wear debris in the peri-prosthetic tissue.

Current systemic anti-inflammation therapies have several weaknesses including high dose requirements with modest efficiency, systemic side effects, and tendency for poor patient compliance [6,7]. In this regard, gene therapy is a novel approach that avoids most of the weaknesses of conventional therapies. RNA interference (RNAi), mediated by small interfering RNA (siRNA), is a recently discovered process that induces post-transcriptional gene silencing in a highly potent and sequence-specific manner [8,9]. Several studies have demonstrated that local delivery of siRNA in vivo provided a relatively sustained therapeutic effect and reduced side effects caused by systemic delivery [10–12]. The present study is an initial research to examine the inhibitory effect of lentivirus-mediated siRNA targeting TNF-α on particle-induced inflammation without systemic side effects.

Materials and methods

Lentiviral vector construction, virus production, and transfection: The lentivector (System Biosciences) used in this study was designed to co-express green fluorescent protein (GFP) cloned from the copepod. The sequence of the siRNA targeting murine TNF-α, 5′-GACAACCAACTAGTGGTGC-3′, has previously been proven to down-regulate murine TNF-α effectively [13]. A missense siRNA (MS siRNA) whose sequence was 5′-TAATCGTCGTAGACGGTTG-3′ was designed as a control. Pairs of complementary oligonucleotides containing these sequences were synthesized (Invitrogen) and cloned into the pshRNA-H1-GFP Lentivector. 293TN producer cells were co-transfected with pPACK Packaging Plasmid Mix (System Biosciences) and the pshRNA-H1-GFP Lentivector containing the shRNA sequences using Lipofectamine™ 2000 (Invitrogen). Viral supernatants were harvested after 48 h, and the titers were determined with serial dilutions of concentrated lentivirus.

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PMMA particles: Spherical PMMA particles (mean diameter 4.8 μm, range 0.1–16 μm) were obtained from a commercial source (Polysciences). Ninety percent of the particles were <10 μm in diameter as measured by a Coulter Multisizer II (Coulter Electronics). The particles were rinsed in 70% ethanol three times and sterilized in 70% ethanol for 48 h, then washed three times in sterile phosphate buffered saline (PBS). Limulus assay (Endosafe) was performed to confirm the absence of endotoxin. The particles were then suspended in sterile PBS at 5 mg/ml (1×10^8 particles/ml).

Animal model: Female BALB/c mice aged 8 weeks were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences) and quarantined in our local facility for 2 weeks prior to experimentation. All mice weighed 20 g or over at the start of the experiment. All procedures were approved by the Laboratory Animal Care and Use Committee of Shanghai Jiaotong University. The dorsal area of each mouse (2×2 cm) was cleaned and shaved, and 3 ml of sterile air was injected subcutaneously to establish an air pouch. Then 0.5 ml of sterile air was injected every day to maintain the pouch. At 6 days after air pouch formation, 0.5 ml of particle suspension was injected into each pouch to provoke inflammatory responses. Pouches were randomly divided into 3 groups with 12 mice in each group the following day. They were injected with 0.5 ml of culture medium containing lentivirus-mediated TNF-α siRNA (TNF-α group) or lentivirus-mediated MS siRNA (MS group) at 2.0×10^8 infectious units/ml. The same volume of the medium without viral vector was injected into a virus-free control group. The mice were sacrificed at 14 or 28 days after transfection, and the pouch tissue was surgically removed. One half of each pouch was fixed in 4% polyoxymethylene (pH = 7.4) for histological evaluation, while the other half was flash-frozen in liquid nitrogen for molecular and immunological analysis.

Molecular analysis of cytokines expression: Total RNA was extracted using Trizol reagent (Invitrogen) from each pouch, and the cDNA was synthesized from total RNA. Quantitative real-time RT-PCR (QPCR) analysis was performed to quantify the relative expression of proinflammatory cytokines in pouches, using Syber Green I (Molecular Probes) in TP800 (TaKaRa). Gene-specific primers for TNF-α, interleukin-1β (IL-1β), and interleukin-6 (IL-6) were designed using Primer 5.0 (Table 1). To standardize the target gene level with respect to variability in RNA and cDNA quality, a housekeeping gene (β-actin) was co-amplified as an internal control.

Enzyme linked immunosorbent assay (ELISA): The levels of TNF-α protein in pouches were detected at 14 and 28 days after transfection by commercial ELISA kit (R&D system) according to the manufacturer's instructions.

Histological analysis: The fixed pouches were mounted with a consistent orientation to preserve the original shape of the pouch membrane. Sections (6 μm) were cut along a longitudinal axis at approximately midline and stained with hematoxylin & eosin (H&E). The stained sections were examined under a light microscope (Olympus DP70), and digital photomicrographs were captured and analyzed using a computerized image analysis system with Image-Pro Plus software (Media Cybernetics). The pouch membrane thickness and the total number of infiltrated cells were measured using digital image analysis to evaluate the particle-induced inflammation in air pouch. Four separate sections per specimen were analyzed in a blinded fashion. Pouch membrane thickness was measured at six points on each section, and six random 100 μm longitudinal pouch areas were selected to count total cells (cells/mm^2) based upon nucleus count [14].

Imaging and quantification of bioluminescence data: Bioluminescent assay is a high-sensitivity and non-invasive technique used for monitoring specific cellular and genetic activity in a living organism. The in vivo Xenogen IVIS 50 Bioluminescent System was used for data acquisition and analysis. At 14 and 28 days after transfection, the photon emission transmitted from each live mouse was measured using IVIS 50 to detect GFP expression and distribution. A region of interest (ROI) was manually selected to measure the flux (photons/sec) of signal intensity and compare it to the background signal. The area of ROI was kept constant, and the bioluminescent flux was recorded as photons/s/cm^2/sr (steradian).

Statistical analysis: Mice were randomly assigned to 3 experimental groups with 12 mice in each group. Statistical analysis among groups was performed by analysis of variance (one-way ANOVA) with S-N-K post-hoc *t*-test. Data were shown as means ± standard deviation (SD). Difference was considered to be statistically significant at $P < 0.05$. All statistical analyses were conducted using SPSS 11.0.

Table 1
Gene-specific primers for tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and β-actin.

Gene	Primers
TNF-α	5′-GAGTCCGGGCAGGTCTACTTT-3′ (forward) 5′-CAGGTCACTGTCCCAGCATCT-3′ (reverse)
IL-1β	5′-ACCTGGGCTGTCCTGATGAGAG -3′ (forward) 5′-CCACGGGAAAGACACAGGTAGC -3′ (reverse)
IL-6	5′-AACCACGGCCTTCCCTACTTC -3′ (forward) 5′-TCATTTCCACGATTTCCCAGAG -3′ (reverse)
β-actin	5′-CCTCTATGCCAACACAGTGC-3′ (forward) 5′-GTACTCCTGCTTGCTGATCC-3′ (reverse)

Results

Lentivirus-mediated TNF-α siRNA inhibits expression of cytokines

QPCR was performed with cytokine-specific primers to examine the mRNA levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) in this murine model, and determine whether the lentivirus-mediated TNF-α siRNA affected their expression. The result of QPCR is shown in Fig. 1. The transfection of lentivirus-mediated TNF-α siRNA in vivo significantly diminished TNF-α mRNA level in the TNF-α group compared with the MS and control groups at each time point, with the interference rate being 82.78% at 14 days and 79.72% at 28 days after transfection (Fig. 1A, $P < 0.01$). Meanwhile, no significant difference in TNF-α expression was detected between the MS and control groups ($P > 0.05$). Expression of IL-1β and IL-6 mRNA in the TNF-α group was also significantly decreased in a similar pattern. The suppression rate of IL-1β mRNA was 36.72% and 40.27% at 14 and 28 days after transfection respectively (Fig. 1B, $P < 0.01$), and that of IL-6 mRNA was 62.63% and 55.61% (Fig. 1C, $P < 0.01$). Similarly, there was no significant difference of IL-1β or IL-6 expression between the MS and control groups ($P > 0.05$).

At 14 and 28 days after transfection, the ELISA result (Table 2) revealed significant reduction of TNF-α protein in the TNF-α group compared with MS and control groups ($P < 0.01$). No significant difference in TNF-α protein level was found between the MS and control groups ($P > 0.05$). In each group, no significant difference in the above-mentioned data was found between two time points ($P > 0.05$) Table 2.

Lentivirus-mediated TNF-α siRNA inhibits particle-induced inflammation

No infection was found in all of the mice. The histological appearance of pouch membranes harvested at 28 days after transfection is illustrated in Fig. 2. The pouches in the TNF-α group (Fig. 2C) appeared less cellular than those in both the control (Fig. 2A) and MS groups (Fig. 2B). Fig. 3A shows the summary of