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Inhibition of osteolysis and increase of bone formation after local administration of siRNA-targeting *RANK* in a polyethylene particle-induced osteolysis model

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ABSTRACT

Receptor activator of nuclear factor kappa-B (RANK) and RANK-ligand are relevant targets for the treatment of polyethylene particle-induced osteolysis. This study assessed the local administration of siRNA, targeting both human *RANK* and mouse *Rank* transcripts in a mouse model. Four groups of mice were implanted with polyethylene (PE) particles in the calvaria and treated locally with 2.5, 5 and 10 μg of *RANK* siRNA or a control siRNA delivered by the cationic liposome DMAPAP/DOPE. The tissues were harvested at day 9 after surgery and evaluated by micro-computed tomography, tartrate-resistant acid phosphatase (TRAP) immunohistochemistry for macrophages and osteoblasts, and gene relative expression of inflammatory and osteolytic markers. 10 μg of *RANK* siRNA exerted a protective effect against PE particle-induced osteolysis, decreasing the bone loss and the osteoclastogenesis, demonstrated by the significant increase in the bone volume ($P < 0.001$) and by the reduction in both the number of $TRAP^+$ cells and osteoclast activity ($P < 0.01$). A bone anabolic effect demonstrated by the formation of new trabecular bone was confirmed by the increased immunopositive staining for osteoblast-specific proteins. In addition, 5 and 10 μg of *RANK* siRNA downregulated the expression of pro-inflammatory cytokines ($P < 0.01$) without depletion of macrophages. Our findings show that *RANK* siRNA delivered locally by a synthetic vector may be an effective approach for reducing osteolysis and may even stimulate bone formation in aseptic loosening of prosthetic implants.

1. Introduction

During the periprosthetic osteolysis process, wear particles released from bearing surfaces result in a local inflammatory response challenged by proinflammatory macrophages (M1) that produce interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) [1]. The largest particles (>10 μm) coated with proteins may act as damage-associated molecular pattern molecules (DAMPs) and are sensed by macrophagic toll-like-receptors (TLRs) [2–4], leading to the activation of an innate inflammatory immune response such as a "foreign body reaction" [5,6]. Otherwise, the smallest particles (<10 μm) and ions are up taken, activating the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome, leading to an adaptive immune response [3,5–7]. Both immune pathways activate the transcription factors NF-kB and NF-IL), increasing synthesis of the receptor activator of nuclear factor kappa-B ligand (RANKL) and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) [8,9]. These cytokines were recognized as the main molecules responsible for maintaining the periprosthetic inflammatory environment and for increased osteoclastogenesis [8].

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Inhibition of the RANKL–RANK axis is an efficient therapeutic approach for decreasing osteoclast differentiation/activation. Anti-resorptive drug-based therapies, such as bisphosphonates and the anti-RANKL monoclonal antibody (Denosumab™), have evolved from experimental models of osteolytic bone diseases [10–13] to clinical uses [14–16]. In addition, new inhibitors, such as small peptides designed to target a specific region of the RANK membrane, are currently in preclinical development [17]. The prevention of bone loss by blocking the RANK–RANKL axis was reported in an experimental model of particle-induced osteolysis using a recombinant protein of RANK (RANK-Fc) [18] and by inducing the osteoclast apoptosis using zoledronic acid (ZOL) [19]. Despite these encouraging preclinical results, they have still not been transferred to clinical use [20].

Small interference ribonucleic acid (siRNA) regulates the synthesis of proteins by means of a specific gene silencing mechanism [21–23]. The use of siRNA-based therapy is a specific and biocompatible approach that has led to significant advances in cancer, age-related macular degeneration and viral diseases [24]. Two key aspects need to be considered in this strategy: the identification of clinically relevant targets and the use of efficient delivery vectors. Targeting of the key RANKL–RANK axis was first reported in vitro in murine cells by Wang et al. [25] and Ma et al. [26] with effective inhibition of *Rank* expression, osteoclast differentiation and osteolysis using *Rank*-siRNAs and *Rank*-shRNAs, respectively, which target the mouse *Rank* transcript [25–27]. The systemic delivery of therapeutic siRNAs using biological and synthetic vectors was reported in bone disease experimental models, including bone-metastatic cancer (targeting Luciferase (LucF) and delivered by atelocollagen) [28] and rheumatoid arthritis (targeting *TNF-α*, *IL-1ß*, *IL-6* and *IL-18* and delivered by the cationic liposome DMAPAP/DOPE) [29,30]. Similarly, a siRNA targeting of the type I bone morphogenetic protein receptor transcript (*BMPR-IB*) systemically delivered by a recombinant adenoviral vector was reported [31]. However, the nature of aseptic loosening by wear debris seems to be a confined condition that requires a local intervention. The local delivery of siRNAs is then a logical strategy for bypassing the anatomical barriers and optimizing its biotransformation during its transport. In this context, the local delivery of *Rankl*-siRNA by the cationic liposome DMAPAP/DOPE in a murine model of osteosarcoma [32] and a local lentiviral delivery of *β110*-siRNA, targeting a subunit of the PI3K/AKT pathway in a particle-induced osteolysis model, were reported [33]. However, there are no scientific reports using siRNA-based technology targeting the key RANKL–RANK axis by local delivery using a synthetic vector in an in vivo model. We hypothesized that siRNA targeting *RANK*, locally delivered in situ by a cationic liposome might be an effective approach for inhibiting osteoclastogenesis in vivo. The aim of our study was to unveil the therapeutic effect of three doses of siRNA targeting both human *RANK* and mouse *Rank* transcripts (*RANK*-811 siRNA) in a mouse model of polyethylene (PE) particle-induced osteolysis.

2. Material and methods

2.1. SiRNAs

All siRNAs were ordered from Eurogentec (Angers, France) with 3′ overhanging dTdT and with annealed sense and reverse strands. The primer sequence sense strand 5′-GUGGAAAUAAGGAGUCCUC-3′ was designed to target *Homo sapiens RANK* mRNA (NM_003839; Tumour Necrosis Factor Receptor Superfamily 11A, TNFRSF11A) at start positions 811 and was named *RANK*-811. The antisense strand of *RANK*-811 siRNAs presents perfect complementarity with *Mus musculus Rank* mRNA (NM_009399.2) at start positions 804. *RANK*-811 siRNA was selected based on its efficacy for decreasing *RANK* expression in *RANK*-overexpressing human embryonic kidney 293 (HEK 293) cells [17] and *Rank* in murine RAW 264.7 monocytic cells (American Type Culture Collection, Promochem, Molsheim, France) (Supplementary Information Fig. S1).

The siRNA duplex (sense strand 5′-UUCUCCGAACGUGUCACGU-3′) which did not show significant homology with any mouse mRNA sequence according to BLAST database searches, was used as a negative control and designated Ct-siRNA. A previously validated siRNA (*LucF*-siRNA sense strand 5′-CUUACGCUGAGUACUUCGA-3′) was used in vivo as an innocuous siRNA [32].

2.2. Particles of polyethylene

Pure PE particles (Ceridust 3610™) were purchased from Clariant (Gersthofen, Germany). The morphology of particles was assessed by scanning electron microscopy (JEOL, model 6400F). The particle size and distribution were determined by five consecutive measurements obtained with a Coulter Counter™ (Beckman Coulter Inc., USA). The mean size particle was 7.23 μm and with a distribution of d10 = 1.15, d25 = 3.94, d50 = 7.14, d75 = 10.28 and d90 = 13.05. To eliminate endotoxins, the particles were washed in ethanol, dried and then aliquoted until use [34]. Endotoxin levels were measured using a quantitative Limulus Amebocyte Lysate (LAL) assay (Lonza, Belgium). The threshold of positivity was 0.25 EU ml^{-1}.

2.3. Implantation of polyethylene particles in mouse calvaria

The mice (Elevages Janvier, Le Genest Saint Isle, France) were housed in pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes) in accordance with the institutional guidelines of the French Ethical Committee (CEEA PdL 06 ethical committee, authorization no. 1280.01) and under the supervision of the authorized investigators. All surgical procedures were also performed according to international ethical guidelines for animal care (authorization no. 2012-198). Twenty-one C57BL/6 male mice (Janvier, Le Genest-Saint Isle, France) aged 10 weeks were randomly divided in two groups (Table 1). Eighteen of the mice were surgically implanted using the adapted mouse calvaria model [35] with 20 mg of dried PE particles (PE-implanted group). Briefly, under general anaesthesia (2–3% isofluorane in 100% oxygen at flow rate of 1 l min^{-1}), a 0.5 × 0.5 cm^2 area of periosteum was exposed by a midsagittal incision in previously shaved and aseptic head skin (Betadine, France). The dried PE powder was uniformly spread over the periosteum with a sterile surgical spoon. The surgical approach was carefully closed with 5-0 non-absorbable sutures. A subcutaneous injection of buprenorphine (Buprecar 0.1 mg kg^{-1}) was performed after the surgical procedure (Palier 1 protocol). One group of three mice underwent the same surgical procedure but without the PE particle implantation (Sham group).

2.4. Local injections of formulated siRNAs

For in vivo injections, siRNAs were premixed with an equal quantity of a deoxyribonucleic acid (DNA) adjuvant in 150 mM sodium chloride as described in Ref. [36] and mixed with an equivalent volume of cationic liposome DMAPAP/DOPE at a ratio of 6 nmol of cationic lipid per microgram of nucleic acid as previously described [37]. The lipoplexes were formed at room temperature for at least 30 min. Injections of 50 μl of lipoplexes containing 2.5, 5 or 10 μg of siRNAs were used in vivo.

The PE-implanted mice were randomly divided in four groups which received siRNA injections, while the Sham group (non-implanted; $n = 3$) received 50 μl of saline solution (NaCl 0.9%) (Table 1). Three groups of PE-implanted mice received a total of