Vascular endothelial growth factor gene silencing suppresses wear debris-induced inflammation

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Abstract

Purpose Aseptic loosening is the most common complication of total joint replacement, which most likely results from an inflammatory response to wear debris shed from the implant. In this study we aimed to investigate whether the lentivirus-mediated microRNA (miRNA) targeting vascular endothelial growth factor (VEGF) could inhibit wear debris-induced inflammation in a murine model.

Methods Titanium alloy particles were introduced into established air pouches on BALB/c mice, followed by implantation of calvarial bone from a syngeneic mouse. After treatment by locally delivered lentivirus-mediated VEGF miRNA, inflammatory tissues were collected for histology and molecular analysis.

Results We found that (1) locally delivered miRNA inhibited titanium alloy particle-induced tissue inflammation, including the diminished pouch membrane thickness and reduced inflammatory cellular infiltration and that (2) locally delivered miRNA inhibited expressions of the inflammatory cytokines VEGF, tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and receptor activator of nuclear factor kappa B ligand (RANKL).

Conclusions These findings suggest that local VEGF inhibition might be a promising therapeutic candidate to alleviate particle-induced inflammation.

Introduction

Total joint arthroplasty is a common surgical procedure to treat osteoarthritis and rheumatoid arthritis. However, aseptic loosening (AL) as the most common complication still affects many postoperative patients. Periprosthetic osteolysis induced by wear debris plays a critical role in the pathogenesis of AL [1]. During the course, cytokine vascular endothelial growth factor (VEGF) plays a critical role in the proliferation of osteoclasts. VEGF could augment the progress of inflammation and enhancement of osteoclast survival and eventually aggravate the inflammatory osteolysis [2]. Thus, the inhibition of VEGF could be regarded as a potential therapeutic target for AL.

Gene therapy is an attractive option for treatment of osteolysis while the effect of traditional anti-inflammatory therapy, such as bisphosphonates, appears insufficient [3]. RNA interference (RNAi) is a post-transcriptional gene silencing mechanism in mammalian cells. In the process, the cellular complex Dicer cleaves a double-stranded RNA (dsRNA) molecule to generate discrete 21–23 nucleotide (nt) small interfering RNAs (siRNAs) or microRNA (miRNAs), which guide the RNAi-induced silencing complex (RISC) to cleave the target mRNAs [4, 5]. Several studies have already demonstrated that local delivery of siRNA in vivo provides a relatively sustained therapeutic effect [6, 7]. This study constitutes initial research to examine the inhibitory effect of lentivirus-mediated miRNA targeting VEGF on wear debris-induced inflammation.

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Materials and methods

Lentiviral vector construction

Four miRNAs were chosen based on the sequence of the mouse VEGF gene (from mRNA sequence; GenBank accession number NM-009505). A missense (MS) miRNA was used as a control (Table 1). The oligonucleotides were synthesised (Invitrogen), annealed and cloned into the pcDNATM6.2-GW/EmGFPmiR (Invitrogen). B16 cells were infected with the four miRNAs (miRNA1, miRNA2, miRNA3 and miRNA4) using Lipofectamine 2000 (Invitrogen). The relative VEGF mRNA levels were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) at two days after infection. Among four miRNAs examined, miRNA2 was selected as the most efficient miRNA. Then we recombined miRNA2 into pLenti6.3/V5-DEST by a recombination system (Invitrogen).

Lentivirus production

293T cells were cultured in Dulbecco's modified Eagle's media (DMEM, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco). The pLenti6.3/V5-DEST vectors containing the miRNA sequences and pPACK Packaging Plasmid Mix (Invitrogen) were cotransfected into 293T cells using Lipofectamine 2000. Infectious lentiviruses were harvested after 48 hours, centrifuged to eliminate cell debris and filtered through 0.45-µm polyvinylidene difluoride (PVDF) filters (Millipore). The viral titres were detected at 2.1×10^8 TU/ml by infecting 293T cells with serial dilutions of concentrated lentivirus.

Particle preparation

Titanium alloy particles (diameter range 0.1–20 µm) were obtained from a commercial source (Alfa Aesar). The

particles were sterilised in 70% ethanol for 72 hours and then washed three times in sterile phosphate-buffered saline (PBS). The particles were then suspended in sterile PBS at 15 mg/ml and stored at 4°C until used. The absence of endotoxin was confirmed using the Limulus assay.

Mouse osteolysis model

The Institutional Animal Care and Use Committee of Shanghai Jiaotong University approved all animal procedures. Mice were randomly assigned to three experimental groups with eight mice in each group. Female BALB/c mice aged ten weeks were purchased from the Shanghai Laboratory Animal Centre (Chinese Academy of Sciences). All mice weighed 20 g or over at the start of the experiment. The dorsal area of each mouse (2×2 cm) was cleaned and shaved, and 2 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. Six days later, mice with established air pouches were anaesthetised by intraperitoneal injection of pentobarbital (50 mg/kg). A 0.5-cm incision overlying the pouch was made and a section of calvarial bone from a genetically identical donor mouse (approximately 0.8×0.6 cm) was inserted into the pouch; 0.3 ml of particle suspension was injected into each pouch to provoke inflammatory responses. Among them eight mice were injected with 0.4 ml of culture medium containing lentivirus-mediated VEGF miRNA (VEGF group), and eight mice were injected with 0.4 ml lentivirus-mediated MS miRNA (MS group). The pouches of the third group injected with PBS alone were included as controls (CON group). The pouch layers and the skin incision were then closed using 4-0 Prolene sutures. Each experimental group included eight mice. The mice were sacrificed in a carbon dioxide chamber at

Table 1 miRNAs and oligonucleotides for shRNA synthesis

miRNA	Oligonucleotides for shRNA synthesis 5' to 3'
miVEGF-1-F	TGCTGAGTAAAGCCAGGGTCCAGTGCGTTTTGGCCACTGACTGACGCACTGGACTGGCTTTACT
miVEGF-1-R	CCTGAGTAAAGCCAGTCCAGTGCGTCAGTCAGTGGCCAAAACGCACTGGACCCTGGCTTTACTC
miVEGF-2-F	TGCTGTGAAGATGTACTCTATCTCGTGTGTTTTGGCCACTGACTGACACGAGATAGTACATCTTCA
miVEGF-2-R	CCTGTGAAGATGTACTATCTCGTGTGTCAGTCAGTGGCCAAAACACGAGATAGAGTACATCTTCAC
miVEGF-3-F	TGCTGAGGGCTTCATCGTTACAGCAGTTTTGGCCACTGACTGACCTGCTGTAGATGAAGCCCT
miVEGF-3-R	CCTGAGGGCTTCATCTACAGCAGGTCAGTCAGTGGCCAAAACCTGCTGTAAACGATGAAGCCCTC
miVEGF-4-F	TGCTGTGGTGATGTTGCTCTCTGACGGTTTTGGCCACTGACTGACCGTCAGAGCAACATCACCA
miVEGF-4-R	CCTGTGGTGATGTTGCTCTGACGGTCAGTCAGTGGCCAAAACGTCAGAGAGCAACATCACCA
miVEGF-MS-F	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
miVEGF-MS-R	CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTT

Four anti-VEGF candidate miRNAs were designed and a MS miRNA was used as a control. The target sequences are underlined

Table 2 Gene-specific primers for VEGF, TNF- α , IL-1 β , RANKL and β -actin

Genes	Primers
VEGF	F: 5'-AAGATGCCGGTTCCAACCA-3' R: 5'-CTTCTTCCACCACCGTGTCT-3'
TNF- α	F: 5'-GAGTCCGGGCAGGTCTACTTT-3' R: 5'-CAGGTCACTGTCCCAGCATCT-3'
IL-1 β	F: 5'-ACCTGGGCTGTCCTGATGAGAG-3' R: 5'-CCACGGGAAGACACAGGTAGC-3'
RANKL	F: 5'-TGTACTTTCGAGCGCAGATG-3' R: 5'-CCCACAATGTGTGTCAGTTC-3'
β -actin	F: 5'-CCTCTATGCCAACACAGTGC-3' R: 5'-GTACTCCTGCTTGCTGATCC-3'

ten days after bone implantation. The pouch membranes containing implanted bone were harvested for histological evaluation and molecular analysis.

Histological evaluation and image analysis

Tissue samples were fixed in 4% polyoxymethylene (pH=7.4) 24 hours and after decalcification in 10% ethylenediaminetetraacetate acid (EDTA); the specimens were processed for paraffin embedding. Tissue sections (6 mm) were stained with haematoxylin and eosin (H&E) to evaluate pouch membrane inflammation and implant bone erosion. The stained sections were examined under a light microscope (Olympus DP70), and digital photomicrographs were captured and analysed using a computerised image analysis system with Image-Pro Plus software (Media Cybernetics). The pouch membrane thickness and the total numbers of infiltrated cells were measured using digital image analysis to evaluate the particle-induced inflammation in the air pouch. Four separate sections per specimen were analysed in a blinded fashion. Pouch membrane thickness was measured at six points on each section, and six random 100- μ m longitudinal pouch areas were selected to count total cells (cells/mm²) based upon nuclei count.

Fig. 1 The relative VEGF mRNA levels in B16 cells were determined by quantitative RT-PCR. Among the four candidate VEGF miRNAs, the miRNA2 led to a 55% reduction in VEGF expression and was the most efficient. The results were obtained from three individual experiments, and the relative VEGF mRNA levels were expressed as the means $\pm$ SD. * P <0.05

Gene expression of VEGF, tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and receptor activator of nuclear factor kappa B ligand (RANKL)

Total RNA was extracted using TRIzol reagent (Invitrogen) from each pouch, and the cDNA was synthesised from total RNA. Quantitative real-time RT-PCR (QPCR) analysis was performed to quantify the relative expression of VEGF, TNF- α , IL-1 β and RANKL using SYBR Green I in ABI 7500. Gene-specific primers for VEGF, TNF- α , IL-1 β and RANKL were designed using Primer 5.0 (Table 2). To standardise the target gene level with respect to variability in RNA and cDNA quality, a housekeeping gene (β -actin) was coamplified as an internal control.

Western blot analysis

Protein concentration was determined with a protein quantitative analysis kit (Biocolor BioScience & Technology Company), and 60 μ g protein was run on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto nitrocellulose membranes. After blocking (5% nonfat milk and 0.5% Tween 20) at room temperature for one hour, the nitrocellulose membranes were separately incubated overnight with goat anti-mouse VEGF antibody (0.1 μ g/ml, R&D Systems) as the primary antibodies; at the same time anti- β -actin antibody (Abcam) was used as a control. After washing with Tris-buffered saline with Tween 20 (TBST), the nitrocellulose membranes were incubated with suitable secondary antibody at room temperature for one hour, and then the membranes were washed three times in TBST. Immune complexes were detected using the chemiluminescent method, and immunoreactive bands were quantified using an imaging system (LAS 4000 mini, Fujifilm).

Statistical analysis

Statistical analysis among groups was performed by the analysis of variance (ANOVA) test. Data are shown as means $\pm$ standard deviation (SD). The difference was con-

Fig. 2 Typical histological appearance of calvaria-implanted pouch tissues. **a** MS group. **b** Control group. **c** VEGF group. The tissue sections were stained with H&E, $\times 200$. Ti particle is marked by *white arrow*. Bone erosion of implanted calvarial bone in the MS and control groups (**a**, **b**) (*black arrow*), while dramatic ameliorations are noted in the VEGF group (**c**)

sidered to be statistically significant at $P < 0.05$. All statistical analyses were conducted using SPSS 11.0.

Results

The miRNA2 was the most efficient among the miRNAs examined

VEGF is highly expressed in B16 cells, which are also widely used in transfection studies [8]. In order to obtain the most efficient miRNA, four candidate miRNAs for VEGF were transfected to B16 cells. As shown in Fig. 1, quantitative RT-PCR analyses revealed that miRNA1, miRNA2, miRNA3 and miRNA4 all led to reductions in VEGF expression in B16 cells, while the MS miRNA did not alter the expression of VEGF. The miRNA2 led to a 55% reduction in VEGF expression ($P < 0.05$) and was selected as the most efficient miRNA for use in this study.

Lentivirus-mediated VEGF miRNA inhibited particle-induced inflammation and osteolysis

In order to evaluate the particle-induced inflammation, we examined the air pouch membrane thickness and total cell number. Figure 2 shows that pouches in the MS and control groups developed inflammatory changes (Fig. 2a, b).

Fig. 3 Histological assessment of the pouch membrane thickness (**a**) and total cell counts (**b**). Four separate sections per specimen were evaluated in a blinded fashion using the Image-Pro software. The *first column* shows that inhibition of VEGF markedly reduced the pouch membrane thickness ($146.6 \pm 32.9 \mu\text{m}$), as compared to the

Lentivirus-mediated VEGF miRNA treatment significantly ameliorated the inflammation in the pouches of the VEGF group (Fig. 2c). The air pouch membrane thickness and total cell number were measured to evaluate the particle-induced inflammation [8]. As shown in Fig. 3, at ten days after transfection, the transfection of lentivirus-mediated VEGF miRNA reduced the pouch membrane thickness to $146.6 \pm 32.9 \mu\text{m}$ in the VEGF group as compared to $207.2 \pm 35.9 \mu\text{m}$ and $211.1 \pm 33.3 \mu\text{m}$ observed in the MS group and control group ($P < 0.05$), respectively. The total cell number in the VEGF group was significantly less (cell number $1,128 \pm 314$) than that in the MS group (cell number $2,312 \pm 378$, $P < 0.05$) and control group (cell number $1,965 \pm 302$, $P < 0.05$). No difference in these two parameters was found between the MS and control groups ($P > 0.05$). These results substantiated that lentivirus-mediated VEGF miRNA interfered with Ti particle-dependent tissue inflammation.

Lentivirus-mediated VEGF miRNA inhibited expression of cytokines

To evaluate the particle-induced inflammation, we examined the expression of proinflammatory cytokines. QPCR was performed with cytokine-specific primers to examine the mRNA levels of proinflammatory cytokines (VEGF, TNF- α , IL-1 β and RANKL) in this murine model and to determine whether the lentivirus-mediated VEGF miRNA

MS ($207.2 \pm 35.9 \mu\text{m}$) and control groups ($211.1 \pm 33.3 \mu\text{m}$). The total cell counts were significantly less in the VEGF inhibitory group (cell number $1,128 \pm 314$) in comparison to the control group (cell number $1,965 \pm 302$). Data expressed as mean $\pm$ SD for a total of 24 mice (8 mice per group). * $P < 0.05$

Fig. 4 Relative mRNA expressions of VEGF, TNF- α , IL-1 β and RANKL in the pouch of each group after transfection. Expressions of VEGF, TNF- α , IL-1 β and RANKL mRNA in the VEGF group were significantly decreased compared with the control group. β -Actin served as a control. Results are shown as mean $\pm$ SD. * P <0.05

affected their expressions. The results of QPCR are shown in Fig. 4. The transfection of lentivirus-mediated VEGF miRNA in vivo significantly diminished the VEGF mRNA level in the VEGF group compared with the MS and control groups; the suppression rate of VEGF mRNA was 68.6% after transfection (Fig. 4, P <0.05). Meanwhile, no significant difference in VEGF expression was detected between the MS and control groups (P >0.05). Expressions of TNF- α , IL-1 β and RANKL mRNA in the VEGF group were also significantly decreased in a similar pattern. The suppression rate of TNF- α mRNA was 44.8% after transfection (Fig. 4, P <0.05), that of IL-1 β mRNA was 48.7% (Fig. 4, P <0.05) and that of RANKL mRNA was 80.6% (Fig. 4, P <0.05). Similarly, there was no significant difference of TNF- α , IL-1 β and RANKL expression between the MS and control groups (P >0.05).

Protein expression of VEGF in air pouch membranes was detected by Western blot analysis (Fig. 5). Intensified densitometric results of VEGF (Fig. 5b) were found in pouches of MS and control groups, and these densitometric results were weakened in the tissues of the VEGF group.

Discussion

AL and osteolysis remain the major causes of failed total joint arthroplasty leading to revision surgery [9]. Wear

debris-induced persistent inflammation and osteoclastogenesis are two critical factors in the aetiology of AL [10, 11]. VEGF is an important proangiogenic cytokine produced by macrophages, fibroblasts, endothelial cells and other cell types [12]. In recent years, VEGF has been recognised as a key regulator of osteoclastogenesis correlated with bone resorption. Several studies have indicated that inflammatory osteoclastogenesis is an important step in the development of AL [13, 14]. VEGF can directly target osteoclasts and enhance osteoclast survival [15].

To mimic the environment of the replaced joint, we constructed a murine air pouch with a cranial bone implantation model and interestingly identified that the lentivirus-mediated VEGF miRNA could alleviate inflammation in the Ti-stimulated tissue. Infusion of Ti alloy particles caused inflammation in the pouch region. By locally delivered miRNA, the pouch membrane thickness and inflammatory cellular infiltration were all decreased, which effectively indicated that locally delivered lentivirus-mediated VEGF miRNA could inhibit Ti particle-induced tissue inflammation.

Although several therapies based on VEGF blockage are found to be feasible, respective weaknesses are also prominent [15]. The limitations of antagonist and antibody therapies dictate the high dose and frequency of administration to maintain a therapeutic level. These limitations provide an opportunity to develop a better VEGF inhibitor that can act more persistently and safely. RNAi mediated by

Fig. 5 Western blot analysis for VEGF (a) and densitometric results of Western bands (b). In a, VEGF was weakened by miRNA inhibition, and the densitometry results shown in b were consistent with the bands. β -Actin served as a control to determine equal protein loading. * P <0.05

miRNA may represent this more potent, longer lasting and safe therapeutic agent.

As a promising vector of miRNA, lentivirus compares favourably with other transgenic methods for transducing genes in vivo [16]. Transfection with lentivirus is more efficient than other methods due to stable expression of miRNA in mammalian cells [17]. The results of QPCR and Western blot analysis demonstrated that the lentivirus-mediated VEGF miRNA could inhibit VEGF expression efficiently both at mRNA and protein levels. Furthermore, we also found that the transfection of lentivirus-mediated VEGF miRNA resulted in a significant down-regulation of the expression of other cytokines such as TNF- α , IL-1 β and RANKL. TNF- α and IL-1 β are both important proinflammatory cytokines and have been proved to play critical roles in response to particles and in causing AL [18]. Besides, TNF- α is also involved in osteoclastogenesis and bone resorption during periprosthetic osteolysis. RANKL is known to be present in the local bone environment and can stimulate the recruitment of osteoclasts. The down-regulation of the expressions of these cytokines also manifests efficacy of this anti-VEGF strategy in inhibiting Ti particle-induced tissue inflammation.

Conclusion

In this murine model, we have demonstrated that the local inhibition of VEGF effectively and safely alleviates Ti particle-induced inflammation. This study provides a novel and promising strategy that will serve as an alternative to the prevention of AL of joint replacement and is of potential clinical importance.

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Conflict of interest The authors declare that they have no conflict of interest.

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