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Electro-transfer of small interfering RNA ameliorated arthritis in rats

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Abstract

RNA interference provides the powerful means of sequence-specific gene silencing. Particularly, small interfering RNA (siRNA) duplexes may be potentially useful for therapeutic molecular targeting of human diseases, although novel delivery systems should be devised to achieve efficient and organ-specific transduction of siRNA. In the present study, we demonstrated that electro-transfer of a siRNA–polyamine complex made efficient and specific gene knockdown possible in the articular synovium. Targeted suppression of the tumor necrosis factor-α gene through this procedure significantly ameliorated collagen-induced arthritis in rats. Our results suggest the potential feasibility of therapeutic intervention with RNA medicines for treatment of rheumatoid and other locomotor diseases.
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RNA interference (RNAi) was first reported in 1998 by Fire et al. [1] who demonstrated that double-strand RNA (dsRNA) induced sequence-specific silencing of gene expression in nematode cells. Molecular mechanisms of RNAi have been intensively investigated in a variety of organisms including fungi, hydra, plants, and amphibians, which indicates that RNAi provides the quite useful means of functional analyses of genes as well as epigenetic engineering of cells of various species [2,3]. Although it was initially difficult to induce this system into the mammalian somatic cells, in which dsRNA induces interferon response leading to non-specific protein synthesis, Elbashir et al. [4] demonstrated that RNAi can be achieved in the mammalian cells by using oligoribonucleotide duplex 21 or 22 bases in length (small interfering RNA; siRNA). The siRNA-mediated genetic knockdown was also demonstrated in vivo in animal organs including the liver [5,6] and skeletal muscle [7]. More recently, several studies applied RNAi to the treatment of various disorders in animal models [8–20] and showed that RNAi may provide promising strategies to treat human diseases by suppressing disease-responsible genes in vivo.

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by inflammation of synovial membranes as well as destruction of the cartilage and bone.

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Upon progression, RA patients suffer from functional impairment of the joints which results in ankylosis or deformation of the joints. Although the etiology of RA still remains unclear, the disease is associated with immunological abnormalities, and a variety of inflammatory cytokines are critically involved in the pathogenesis. In particular, Tumor necrosis factor-α (TNF-α) produced by the synovial tissue is located upstream of the cytokine cascade and is playing responsible roles in the induction of the synovitis, as revealed in previous in vivo [21] and in vitro [22] studies. TNF-α-neutralizing agents have been attracting attention as novel antirheumatic drugs. A TNF-α receptor-IgG Fc fusion protein (Etanercept: Enbrel) [23] and a chimeric monoclonal anti-TNFα antibody (Infliximab: Remicade) [24] have already been applied clinically, demonstrating considerable efficacies in controlling RA. TNF-α-neutralizing agents also demonstrate considerable inhibitory effects on the bone destruction that develops along with the progression of RA, which cannot be achieved by conventional antirheumatics. The anti-TNF-α therapies, however, are associated with high incidences of serious adverse events such as tuberculosis and *Pneumocystis carinii* pneumonia due to immunosuppression. In addition, these drugs should be systemically administered to patients. The treatment is expensive and imposes enormous economic burden on patients. If TNF-α can be locally inhibited at the diseased joints, it would alleviate the burden on such patients who suffer from inflammation at relatively small number of joints. So far previously reported, however, intra-articular administration of TNF-α agonists failed to show successful therapeutic outcomes against the local arthritis lesions [25].

We previously established an in vivo electroporation technique, in which synoviocytes were successfully transfected by transcutaneously applying a voltage load to rat knee joint after an injection with plasmid DNA [26]. We also reported that RNAi was achieved in murine skeletal muscle by transducing siRNA duplexes in vivo through transcutaneous electroporation [7]. In this study, we attempted to deliver siRNA–polyamine complex into a joint by electroporation and investigated therapeutic potential of RNAi against experimental arthritis by silencing TNF-α in a sequence-specific and organ-specific fashion.

Materials and methods

siRNA duplexes. siRNA duplexes targeting rat TNF-α gene were synthesized (A: 5′-GCCCGUAGCCCACGUCGUAd(TT)-3′ and 5′-UACGACGUGGGCUACGGGCd(TT)-3′; B: 5′-UGGGCUCCCUCUCAUCAGUd(TT)-3′ and 5′-ACUGAUGAGAGGGAGCCCAd(TT)-3′; C: 5′-GGAGGAGAAGUUCCCAAAUd(TT)-3′, and 5′-AUUUGGGAACUUCUCCUCCd(TT)-3′; and D: 5′-AGACAACCAACUGGUGGUAd(TT), and 5′-UACCACCAGUUGGUUGUCUd(TT)-3′). Two nucleotide mismatches were introduced into the TNF-α-siRNA-A to generate the mismatched TNF-α-siRNA (5′-GCCCGUAGAACACGUCGUAd(TT)-3′ and 5′-UACGACGUGUUCUACGGGCd(TT)-3′). GAPDH gene-specific and negative control siRNAs were purchased from Ambion (Austin, Texas, USA), while GFP-specific siRNA was purchased from Dharmacon (Lafayette, CO, USA). FAM labeling of GAPDH-specific siRNA was performed using the Silencer siRNA Labeling Kit (Ambion).

Animals. Dark Agouti (DA) rats were purchased from Shimizu Laboratory Suppliers (Kyoto, Japan). The SD-Tg (Act-EGFP) Cz-004sb rats were kindly provided by Professor Masaru Okabe (Genome Information Research Center, Osaka University, Osaka, Japan) [27]. Animal experiments were conducted according to the Guidelines regarding Animal Research of the Kyoto Prefectural University of Medicine.

Electroporation-assisted siRNA transduction in vivo. Rats were anesthetized by an intraperitoneal injection of 1 μl/g of sodium pentobarbital, and 50 μl of the siRNA/siPORT Amine (Ambion) complex containing 800 pmol siRNA and 10 μl siPORT Amine was administered into the left knee joint using a 27-gauge needle. Immediately after the injection, keratin cream (Fukuda Denshi, Tokyo, Japan) was coated around the knee joint, onto which a pair of electrode pads (1.0 cm in diameter) was placed. Using a CUY21 electric pulse generator (NeppaGene, Tokyo, Japan), three square-wave pulses with a pulse length of 100 ms were loaded at 150 V/cm at a frequency of 1 s^{-1} followed by two other pulses with the opposite polarity [26].

Observation of GFP expression. EGFP rats were sacrificed 1 ($n = 3$) and 3 ($n = 3$) days after delivery of GFP siRNA (800 pmol siRNA) into the left knee joint. Synovial membrane was removed from the region surrounding the patella of left knee joint of EGFP rats. After 72 h of fixation in 3.7% formaldehyde, the synovium was dehydrated in 25% sucrose solution for 24 h. The specimens were cryosectioned into 14 μm slices, which were stained with H&E and observed under fluorescence microscope (SZX12).

Induction and evaluation of Collagen-induced arthritis (CIA rats). To induce CIA, collagen type II (Collagen Research Center, Tokyo, Japan) was dissolved in 0.01 M acetic acid (2 mg/ml) and emulsified 1:1 in Freund's incomplete adjuvant (Sigma) on ice (CII/FIA). DA rats weighing 200–250 g were intradermally injected with 200 μl of the CII/FIA solution at the base of the tail [28]. After electro-transduction of siRNA, foot volume was measured using a water replacement plethysmometer (Unicom Japan, Tokyo, Japan) [29]. The left knee and ankle joints were excised 28 days after immunization and fixed in 3.7% formaldehyde. Following decalcification with formic acid, sagittal sections 6 μm in thickness were prepared from the center of the lateral condyle of the femur, as well as from the center of the foot joint. The sections were then stained with H&E or Safranin O. Arthritic changes, such as infiltration of inflammatory cells, synovial proliferation, destruction of articular cartilage, and bone erosion, were evaluated 28 days after immunization using the histological scores as described [30].

RT-PCR. To analyze TNF-α mRNA expression in vivo, the synovium was removed from the region surrounding the left patella of CIA rats 16 days after immunization. Total RNA was extracted and subjected to RT-PCR using a pair of primers for GAPDH (sense: 5′-TACAGCAACCAGGGTGGTGGA and antisense: 5′-ACCACAGTCCATGCCATCAC), or TNF-α (sense: 5′-CGCTCTTCTGTCTACTGAAC-3′ and antisense 5′-TTCTCCAGCTGGAAGACTCC-3′) genes. PCR amplification of GAPDH sequence was performed using the following conditions: denaturing at 94 °C for 60 s, annealing at 57 °C for 60 s, and extension at 72 °C for 120 s, for a total of 30 cycles. PCR amplification of TNF-α sequence was performed as follows: denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C, for a total of 36 cycles. The PCR products were electrophoresed through a 2% agarose gel, and DNA fragments were visualized by ethidium bromide staining.

Statistical analysis. Fisher's exact test and non-parametric Mann–Whitney *U* test were used to evaluate the statistical significance of differences in the incidence of paw swelling, and in paw volume and histological score, respectively.

Results

Electro-transfer of siRNA into the synovium of the rat knee joint

GAPDH-specific siRNA duplex was labeled with 6-carboxyfluorescein (FAM) and injected into the left knee joint

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