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Effects of Treatment With Small Interfering RNA on Joint Inflammation in Mice With Collagen-Induced Arthritis

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Objective. **RNA interference is a process in which genes can be silenced sequence-specifically. In mammals, RNA interference can be invoked by introduction of small (19–21-nucleotide) double-stranded RNA molecules known as small interfering RNA (siRNA) into cells. Thereby, siRNA offers promise as a novel therapeutic modality. However, siRNA is a relatively large, highly charged molecule and does not readily enter cells. This study was undertaken to investigate the use of electroporation for in vivo transfection of siRNA into joint tissue in arthritic mice to achieve local RNA interference.**

Methods. **Proof of principle that siRNA is able to inhibit gene expression in vivo in the mouse joint was studied by local injection and electroporation of siRNA designed to silence reporter genes. In mice with collagen-induced arthritis (CIA), the disease-modulating activity of siRNA designed to silence tumor necrosis factor α (TNFα) was investigated.**

Results. **Luciferase activity could be reduced by >90% with luciferase-specific siRNA as compared with the activity measured after electroporation without siRNA or with irrelevant siRNA. The effect was observed only locally. In mice with CIA, electroporation of siRNA designed to inhibit TNFα strongly inhibited joint inflammation, whereas electroporation of irrelevant siRNA or injection of siRNA against TNFα without electroporation failed to produce therapeutic effects.**

Conclusion. **Local electroporation of siRNA in joint tissue can inhibit CIA in mice. These results offer promise for the use of siRNA as a new strategy for therapeutic intervention in rheumatoid arthritis and may serve as a tool to study arthritis disease pathways through loss-of-function phenotypes.**

RNA interference (RNAi) is a recently discovered process that utilizes either endogenous or exogenous double-stranded RNA species to inhibit expression of genes in a highly sequence-specific manner and is rapidly supplanting antisense methods (1–3). In mammals, RNAi can be invoked by introduction of short (19–21-nucleotide) double-stranded RNA oligonucleotides, called small interfering RNA (siRNA). The siRNA is taken up by an RNA-inducing silencing complex in the cytoplasm and silences expression of messenger RNA with a complementary sequence. Therefore, siRNA offers promise as a novel therapeutic strategy and, in addition, may be used as a tool for functional genomics to elucidate genes controlling disease pathways.

Rheumatoid arthritis (RA) may benefit from a therapeutic strategy based on the use of siRNA. The disease is characterized by involvement of multiple gene products, whose inhibition can strongly reduce joint inflammation (4). In addition, the exact roles of many of the genes that influence the disease are still unknown (5,6), and siRNA could be a valuable tool to study the function of different genes in disease progression (2).

Unfortunately, siRNA is a relatively large, highly charged molecule and does not readily enter cells to reach its intracellular site of action. Therefore, delivery strategies that enhance intracellular uptake need to be devised. Electroporation, originally developed by Muramatsu et al (7) and further developed by Funahashi et al

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(8), has been shown to be a feasible approach to introduce nucleic acids into embryonic cells. Later the technique was put into practice in adult mice (9), and it was shown that electroporated plasmid could produce functional siRNA (10). Electroporation has been shown to be a feasible approach to deliver DNA into joint tissue (11,12). In the present study we tested, in mice, the hypothesis that local electroporation of siRNA in joint tissue can lead to local RNA interference and effectuate therapeutic effects.

MATERIALS AND METHODS

Inhibition of reporter gene expression with siRNA in vitro. To evaluate whether the electroporation technique could be applied for RNAi-mediated knockdown of gene expression in joints, siRNA targeted against luciferase (siRNA-Luc) and siRNA targeted against enhanced green fluorescent protein (siRNA-EGFP) were designed, based on studies by Elbashir et al (13). Luciferase was chosen because its expression and siRNA-mediated inhibition can be determined quantitatively, and EGFP because its expression and silencing can be visualized. Target sequences were AACCGCTGGAGAGCAACTGCA and AAGCTATGAAACGATATGGGC for luciferase and AAGCTGACCCTGAAGTTCATC and AAGCAGCACGACTTCTTCAAG for EGFP. Corresponding siRNA sequences were validated, by BLAST analysis (http://www.ncbi.nih.gov/BLAST/), to lack significant interfering homology to mouse proteins and synthesized and annealed at Dharmacon (Lafayette, CO). The 2 siRNA duplexes per target were combined in a 1:1 molar ratio.

To validate the sequence-specificity of gene knockdown in vitro, 2×10^5 human embryonic kidney 293 cells were electroporated in the presence of 2 μg of luciferase-encoding pCI plasmid (pLuc) or EGFP-encoding pCI plasmid (pEGFP) with or without 1 μg of siRNA-Luc or siRNA-EGFP as described previously (14). Twenty-four hours after electroporation, green fluorescence in cells was assessed with a fluorescence microscope. For study of luciferase expression, cells were washed and lysed, and reporter enzyme activity was measured using a luciferase assay system (Promega, Madison, WI) with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Protein concentration was measured colorimetrically.

Plasmid DNA reporter gene expression in vivo. Female 6–8-week-old nude mice (Taconic, Germantown, NY) were used. Animals were anesthetized by intraperitoneal injection of Avertin (2,2,2-tribromoethanol) and subsequently received a local injection of 2 μg pEGFP or pLuc in saline solution in the knee joint, followed by electroporation at a setting of 200 V/cm using an ECM 830 electroporator (BTX, San Diego, CA) set to deliver 2×2 pulses at perpendicular angles. Twenty-four hours after electroporation, the leg was dissected, skin was removed, and the knee was examined for EGFP expression, with a fluorescence microscope. For study of luciferase expression, the joint was dissected, weighed, and placed in ice-cold reporter lysis buffer (Promega) in magnetic bead–containing tubes (Q Biogene, Carlsbad, CA). Tissues were homogenized with a Fastprep FP120 magnetic homogenizer (Q Biogene), and samples were measured for reporter enzyme activity using a luciferase assay system, as described above.

Inhibition of reporter gene expression with siRNA in vivo. Reporter gene silencing in vivo was investigated by injecting 2 μg of pEGFP or pLuc together with 10 μg siRNA-EGFP or siRNA-Luc into the knee joint. Immediately after injection, electric stimuli were applied as described above. Twenty-four hours after electroporation, the joint tissue was visualized with a fluorescence microscope or luciferase expression was measured.

To investigate whether siRNA injection followed by electroporation may cause silencing effects in other organs, mice were injected intravenously in the tail vein with 40 μg of pLuc complexed to polyethyleneimine (PEI) as a transfecting agent, in a 1:2 negative (pLuc)–to–positive (PEI) charge ratio. Intravenous injection of PEI-pLuc was immediately followed by local injection and electroporation of 2 μg pLuc or 2 μg pLuc and 10 μg siRNA-Luc into the knee joint. Twenty-four hours after electroporation, the tissue level of luciferase expression was measured.

Induction of collagen-induced arthritis (CIA). Lyophilized chicken type II collagen (Chondrex, Redmond, WA) was dissolved overnight at 4°C in 0.05*M* acetic acid under constant stirring. The collagen was emulsified on ice with Freund's complete adjuvant (Chondrex) to a final concentration of 2 mg/ml, using electric homogenization.

Using a glass syringe, 0.1 ml of emulsion was injected subcutaneously into the tail of DBA/1 mice (6 weeks of age). After 3 weeks, the procedure was repeated with a collagen emulsion in Freund's incomplete adjuvant. To synchronize the onset of inflammation, mice received an intraperitoneal injection with 25 μg lipopolysaccharide (LPS; isolated from *Escherichia coli* serotype O55:B5) (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 0.2 ml phosphate buffered saline (pH 7.4) 72 hours after the second collagen emulsion. Development of arthritis was monitored by scoring inflamed joints in each paw, as follows (15): 0 = no inflammation; 1 = mild erythema and swelling of the joint or individual digits; 2 = moderate erythema and swelling of the joint; 3 = severe erythema and swelling of the entire paw (maximum possible score 12).

Treatment was initiated 24 hours after LPS injection, at which time the first animals were starting to show signs of inflammation. Mice were treated with 10 μg siRNA against murine tumor necrosis factor α (TNFα) (target sequences AAGACAACCAACTAGTGGTGC and AAGTGCCTATGTCTCAGCCTC) or 10 μg siRNA-Luc with or without electroporation. Three local injections were given, separated by 1-week intervals.

RESULTS

Silencing of reporter genes in vitro and in vivo. Electroporation of 293 cells with pEGFP induced strong expression of green fluorescence in the cells, which was substantially reduced in the presence of siRNA-EGFP but not in the presence of siRNA-Luc (results not shown), indicating that silencing of EGFP expression in vitro is dependent on siRNA sequence. The fluores-

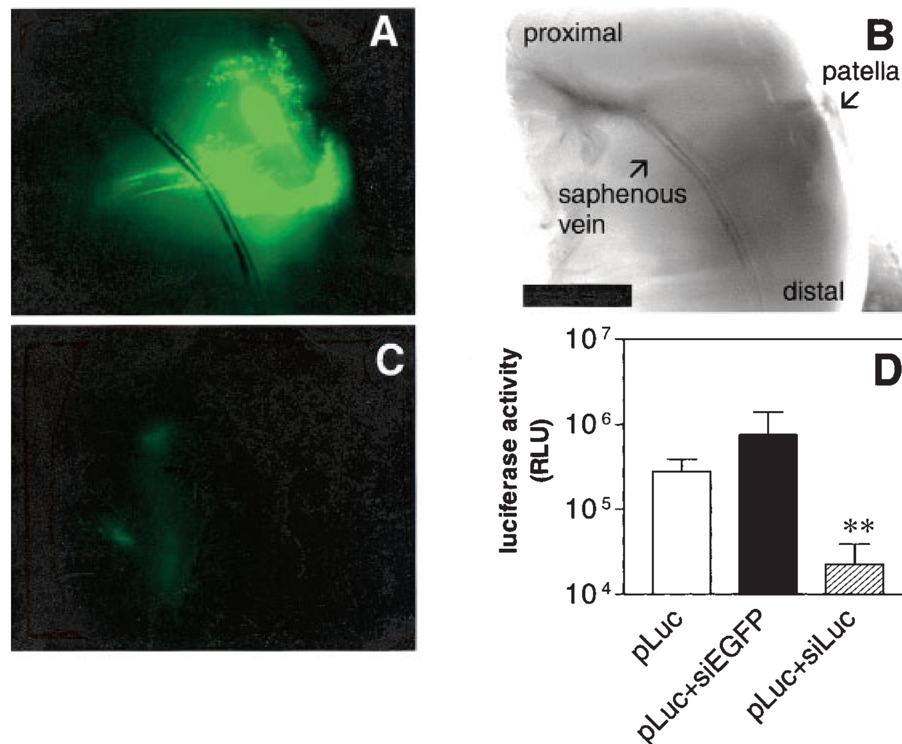

Figure 1. A, Expression of enhanced green fluorescent protein (EGFP) in the joint after electroporation. Two micrograms of EGFP plasmid (pEGFP) was injected locally into the joint, followed by electroporation. EGFP expression was confined to the joint and its immediate vicinity. **B,** Brightfield image of the joint shown in **A**. The positions of the distal and proximal ends of the tissue as well as the patella and saphenous vein are indicated. Bar = 1 mm. **C,** Inhibition of EGFP expression by injection of 10 μg small interfering RNA–EGFP (siRNA-EGFP) into the joint. **D,** Inhibition of luciferase activity by siRNA-Luc in vivo. Two micrograms of pEGFP was injected locally into the joint in the presence or absence of 1 μg siRNA-Luc or siRNA-EGFP, followed by electroporation. Only in the presence of sequence-specific siRNA-Luc was a significant reduction in luciferase activity observed (** = $P < 0.01$ by analysis of variance with Dunnett's post-test) (n = 6). Values are the mean and SD. RLU = relative luciferase units.

cence image of the knee after injection and electroporation of pEGFP showed that the expression of the fluorescent protein was confined to the knee joint and its immediate vicinity (Figure 1A). Figure 1B shows the corresponding brightfield image. Fluorescence was not observed when plasmid injection was not followed by electroporation (results not shown). These findings are consistent with previous observations on plasmid expression after electroporation in the joint (8). Fluorescence was substantially reduced when pEGFP was coinjected with 10 μg of siRNA-EGFP, indicating that siRNA is able to silence EGFP expression in vivo (Figure 1C). Electroporation of siRNA-Luc failed to inhibit EGFP expression (results not shown).

For quantitative measurements we used luciferase as a reporter gene. First we investigated the effects of siRNA on luciferase expression in vitro. One microgram of pLuc was electroporated in the presence or absence of 1 μg of siRNA. In experiments with the sequence-specific siRNA-Luc only 13 ± 9% of luciferase activity remained, as compared with the activity measured after electroporation without siRNA or with irrelevant siRNA-EGFP, for which the activity was 100 ± 21% and 119 ± 15%, respectively (mean ± SD; n = 3) (data not shown).

Luciferase silencing in vivo was investigated by injecting 2 μg of pLuc without siRNA or with 10 μg of siRNA-EGFP or siRNA-Luc into the knee joint (Figure 1D). Luciferase activity was reduced by >90% with the sequence-specific siRNA-Luc as compared with the activity measured after electroporation without siRNA or with irrelevant siRNA-EGFP (Figure 1D).

Effects of siRNA in other tissues. Studies by Filleur et al have indicated that siRNA silencing effects can spread in vivo (16). In their investigations, luciferase expression in tumor tissue could be inhibited by siRNA-Luc injection by several routes of administration (intravenously, subcutaneously, or intraperitoneally). To investigate whether local injection and electroporation of siRNA in the joint would silence luciferase expression in other organs, mice were injected intravenously with PEI-complexed pLuc. These electrostatic complexes of cationic polymer PEI and plasmid are able to transfect several organs. Mice receiving these complexes were subsequently subjected to injection and electroporation of pLuc, with or without siRNA-Luc, in the knee joint. Luciferase expression was similar in the presence and absence of siRNA-Luc in all tissues except for the joint (Figure 2), which was the only site at which a silencing effect of the locally injected and electroporated siRNA was observed.

Therapeutic effects of siRNA. Therapeutic effects of siRNA targeted against murine TNFα were investigated in mice with CIA. TNFα is a well-known proinflammatory cytokine whose inhibition has been demonstrated to affect disease clinically and in mouse CIA models, in which efficacy of nucleic acid–based intervention strategies has been shown (17–19). Electroporation of 10 μg siRNA against TNFα given 3 times (once every 8 days) effectively inhibited paw inflammation in mice as compared with electroporation of siRNA-EGFP at the same concentration, electroporation of saline solution, or injection of siRNA against TNFα but without electroporation (Figure 3).

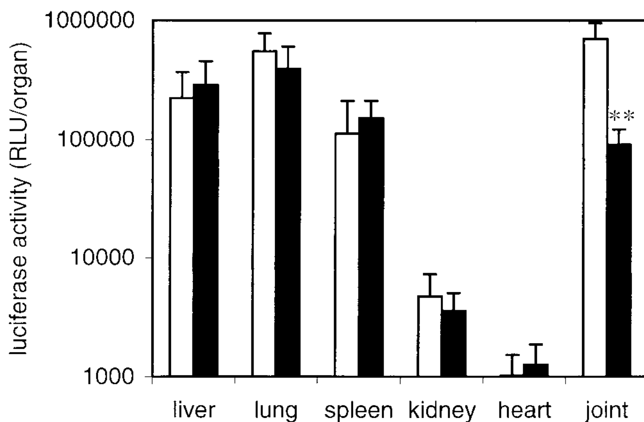

Figure 2. Joint injection and electroporation of siRNA inhibits the target gene locally only. Mice received 40 μg pLuc complexed to polyethyleneimine (PEI) as a transfecting agent, resulting in luciferase expression most notably in the liver, lung, and spleen. Intravenous injection of PEI-pLuc was immediately followed by local injection and electroporation of 2 μg pLuc (open bars) or 2 μg pLuc and 10 μg siRNA-Luc (solid bars) in the knee joint. Local electroporation of siRNA-Luc reduced luciferase expression in the joint only ($** = P < 0.01$). Values are the mean and SD. See Figure 1 for other definitions.

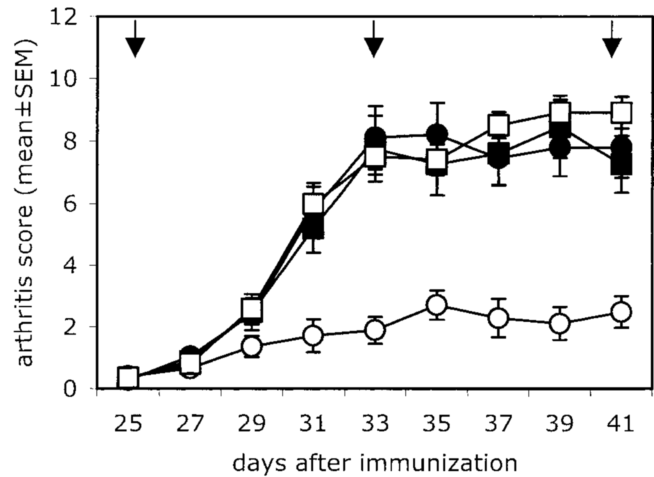

Figure 3. Therapeutic effects of siRNA in collagen-induced arthritis in mice. Injection and electroporation (arrows) of 10 μg siRNA against tumor necrosis factor α (TNFα) (open circles) effectively inhibited paw inflammation as compared with electroporation of siRNA-EGFP at the same concentration (solid circles), electroporation of saline solution (open squares), and injection of siRNA against TNFα but without electroporation (solid squares). See Figure 1 for other definitions.

DISCUSSION

RNA interference has rapidly become the method of choice for gene silencing experiments, and from this standpoint, siRNA can be an important tool to study gene function in RA. In addition, it may have potential as a novel therapeutic strategy. Our ability to silence gene expression in the mouse joint in vivo demonstrates the possibility of invoking RNAi by electroporation of sequence-specific siRNA. Electroporation of siRNA has previously been demonstrated only in muscle tissue (20).

Because of the size and polyanionic nature of siRNA, electroporation is thought to be essential to allow its entry into the cell, similar to findings with single-stranded oligonucleotides and DNA. In contrast, Filleur et al reported silencing of luciferase in tumor tissue in mice after intravenous, subcutaneous, or intraperitoneal injection of a single dose of 3 μg of unmodified siRNA (16). These observations indicate that siRNA can silence gene expression in tissue other than at the site of injection. Our present results indicate that

10 μg siRNA locally injected and electroporated in joint tissue is unable to silence reporter gene expression in other organs transfected using plasmid complexed to PEI. This finding would indicate that the silencing effect of siRNA in the joint is not achieved without electroporation and that joint-injected siRNA is unable to silence gene expression in other organs.

To evaluate whether electroporated siRNA is able to modulate disease progression, siRNA targeted against TNFα was tested in a mouse model of CIA, and this treatment markedly inhibited paw inflammation, whereas irrelevant siRNA or non-electroporated siRNA-TNFα did not. This highlights the importance of sequence specificity and means of delivery.

Taken together, these results offer promise regarding the use of electroporated siRNA as a new strategy for therapeutic intervention in RA. The ability to inhibit various disease pathways based on simple modulation of siRNA sequence may be an interesting approach to obtain more potent therapeutic effects and may even allow personalized treatment based on individual proinflammatory gene expression profiles. In addition, this technique may serve as a tool to study arthritis disease pathways through loss-of-function phenotypes in experimental models.

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