Suppression of ICAM-1 in Retinal and Choroidal Endothelial Cells by Plasmid Small-Interfering RNAs In Vivo

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PURPOSE. Leukocytes play a critical role in ocular diseases such as uveitis, diabetic retinopathy, and choroidal neovascularization. Intercellular adhesion molecule (ICAM)-1 is essential for the migration of leukocytes. Control of ICAM-1 expression may lead to therapies for these diseases. Small-interfering ribonucleic acids (siRNAs) are efficient specific modulators of endogenous gene expression. The authors describe the application of siRNA to suppress ICAM-1 expression on the murine neurosensory retina or retinal pigment epithelial (RPE) cells using a hydrodynamics-based transfection technique (HT) and intravitreal injection (IV) in vivo.

METHODS. ICAM-1-specific plasmid siRNAs designed from the murine gene sequence were transfected into the retina using the hydrodynamics-based transfection technique15–19 and intravitreal injection in vivo. ICAM-1 upregulation was induced by retinal laser photocoagulation and streptozotocin (STZ).

RESULTS. After the administration of GFP expression plasmid with HT and IV, histologic analysis showed GFP fluorescence in every layer of the murine retina. After photocoagulation, ICAM-1 expression was analyzed by enzyme-linked immunosorbent assay and flow cytometry. ICAM-1 upregulation was induced by retinal laser photocoagulation and streptozotocin (STZ).

CONCLUSIONS. SiRNA expression mediated by this plasmid causes efficient and specific downregulation of ICAM-1 expression, suggesting that it can be silenced by plasmid siRNA in mouse retina in vivo. This technology may lead to novel concepts to reduce retinal neovascular disease by inhibiting leukocyte infiltration. (Invest Ophthalmol Vis Sci. 2010;51: 508–515) DOI:10.1167/iovs.09-3457

Leukocytes play a critical role in ocular diseases such as uveitis, diabetic retinopathy, and choroidal neovascularization (CNV). Intercellular adhesion molecule (ICAM)-1 is essential for the migration of leukocytes. Controlling ICAM-1 expression has led to therapies for such diseases.

Specific genes can be manipulated by mechanisms such as neutralized antibody and RNA interference (RNAi). RNAi is a powerful technique for gene silencing. The mechanism by which small-interfering RNAs (siRNAs) recognize and bind to their target RNA in mammalian cells and suppress target gene expression posttranscriptionally is assumed to include base-specific recognition. Double-stranded RNA (dsRNA) induces sequence-specific posttranscriptional gene silencing in many organisms by RNA interference. In several organisms, introduction of dsRNA is a powerful tool for suppressing gene expression through a process known as RNA interference. The siRNA are generated by an RNase III-like processing reaction from long dsRNA.

The first clinical trials of RNAi were directed at the treatment of age-related macular degeneration (AMD) and respiratory syncytial virus infection. The biggest challenge in the systemic use of siRNA-based therapies was delivering them to the cytoplasm. Naked RNA cannot penetrate cellular lipid membranes, and systemic application of unmodified siRNA is unlikely to be successful for resolving these problems and delivering a higher level of siRNA and release for a long period. Various strategies for nonviral and viral delivery of RNAi triggers are effective in disease models, raising the hope that clinical studies of RNAi-based therapies will be applied to more diseases in the near future.

Therefore, this study describes the application of siRNA plasmid vector to suppress ICAM-1 expression on the surface of murine neural retina and retinal pigment epithelial (RPE) cells-choroidal endothelial cells using a hydrodynamics-based transfection technique and intravitreal injection in vivo.

METHODS

Animals

Male wild-type C57BL/6j mice (Japan SLC, Shizuoka, Japan) between 6 and 8 weeks of age were used to minimize variability. For all procedures, anesthesia was induced by intramuscular injection of 50 mg/kg ketamine HCl (Sankyo, Tokyo, Japan) and 10 mg/kg xylazine (Bayer, Tokyo, Japan); the pupils were dilated with topical 1% tropicamide (Santen, Osaka, Japan). All experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Construction of ICAM-1 siRNA-Expressing Plasmid

To explore the function of ICAM-1 by knocking down its expression in mammalian cells, we constructed an siRNA-expressing plasmid. Targeting sequences were chosen according to commercial software (siRNA Target Designer; Promega, Madison, WI). The following sense and antisense sequences were used to target murine ICAM-1: sense,
ICAM-1 Expression Silencing by Plasmid siRNA

**Plasmid Transfection by Intravitreal Injection**

Intravitreal injections of plasmid were performed by inserting a 33-gauge double-caliber needle (Ito Corporation, Shizuoka, Japan) under an operating microscope. Animals received intravitreal injections of 1 μL sterile PBS containing 2 μg plasmid.

**Upregulation of ICAM-1 Expression by Laser Photocoagulation**

Seven days after plasmid, scrambled control siRNA, or naked siRNA transfection, laser photocoagulation (532 nm, 200 mW, 100 ms, 100 μm; Elite [Lumenis, Salt Lake City, UT]) was performed (25 spots/eye). Laser photocoagulation was performed in each animal on day 0 by one clinician masked to the drug group assignment. Laser spots were applied in a standard fashion using a slit-lamp delivery system and a coverslip as a contact lens.

**Quantification of ICAM-1 Expression by ELISA**

To detect ICAM-1 protein levels in the neurosensory retina or RPE/choroid lysates, 1 day after application of the 25 laser spots, the eyes were enucleated and the neurosensory retina or the RPE/choroid complex was sonicated in lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na molybdate, and 1 mM EDTA with protease inhibitor; Sigma-Aldrich, Tokyo, Japan) on ice for 15 seconds. ICAM-1 protein levels in the supernatant were determined by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) that recognizes all splice variants from 450 to 570 nm (Emax; Molecular Devices, Sunnyvale, CA) and were normalized to total protein (Bio-Rad, Hercules, CA). Duplicate measurements were performed in a masked fashion by an operator not involved in photocoagulation.

**Monocyte Detection in the RPE/Choroids by Flow Cytometry**

Single-cell suspensions isolated from mice the neurosensory retina or the RPE/choroids via collagenase D (20 U/mL; Worthington, Lakewood, NJ) treatment were incubated in Fc block (purified rat anti-mouse CD16/CD32 monoclonal antibody, 0.5 mg/mL; BD Biosciences, San Jose, CA) for 15 minutes on ice and stained with Cy5-rat antibody anti-mouse F4/80 (1:30; Serotec, Raleigh, NC). Live cells were detected by gating on forward versus side scatter, followed by analysis of F4/80 fluorescence (FACSCalibur; BD Biosciences). At least 100,000 viable cells were analyzed per condition. Data were analyzed using BD Biosciences software (CellQuest).

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**FIGURE 1.** (A) Schematic depiction of basic vector (psiSTRIKE; Promega). (B) Gene silencing by ICAM-1 siRNA plasmid.

**FIGURE 2.** Schematic depiction of GFP vector.
Upregulation of ICAM-1 Expression by Streptozotocin

Diabetes was induced by one intraperitoneal injection of streptozotocin (STZ) (Sigma Aldrich, Tokyo, Japan) dissolved in 10 mM citrate buffer (pH 4.5; 250 mg/kg body weight). STZ was injected simultaneously into the mice peritoneum, and the ICAM-1-specific plasmid siRNAs or naked plasmid siRNAs were injected into the vitreous space. Diabetes was confirmed 7 days after STZ administration by measuring the glucose concentration in the peripheral blood obtained from the tail vein (Accu-Check Compact Plus; Roche, Tokyo, Japan). Mice with plasma glucose levels exceeding 300 mg/dL were considered diabetic.

Quantification of ICAM-1 Expression by ELISA after STZ Administration

ICAM-1 protein level in the neurosensory retina was determined by ELISA 3 and 7 days after STZ administration.

Statistical Analysis

$P < 0.05$ was considered significant. Results are expressed as the mean ± SD. A post hoc comparison of means was constructed with Bonferroni adjustment for multiple comparisons ($P < 0.01$). ICAM-1 data and the results of flow cytometry were analyzed with a spreadsheet application (Excel; Microsoft, Redmond, WA).

RESULTS

GFP-Expressing Plasmid Distribution and Retinal Uptake by Hydrodynamics-Based Transfection and Intravitreal Injection

Three days after hydrodynamics-based transfection of GFP-expressing plasmid, GFP was observed in a wide retinal area. The GFP expression increased 7 days after transfection of the GFP vectors. Representative results are shown in Figure 3.

Transgene expression after intravitreal injection of GFP plasmid DNA was also observed widely on day 1 in the retina. After transfection of GFP vectors, the GFP expression increased.
Elevated ICAM-1 Expression in the Neurosensory Retina and RPE-Choroid after Laser Photocoagulation

The ICAM-1 protein level increased compared with control mice after laser treatment (Fig. 4). ICAM-1 concentrations in the treated mice increased in the neurosensory retina and RPE-choroid complex; the peak expression of ICAM-1 protein was found on day 1 (2.6 ± 0.41 pg/mL; *P < 0.001) after laser treatment. These levels decreased by day 7 but remained high [†P < 0.01 vs. before laser; n = 6 in all groups].

ICAM-1 Expression after Laser Injury Silenced by Hydrodynamics-Based Transfection Plasmid siRNAs

ICAM-1 expression in the neurosensory retina and RPE-choroid decreased significantly in the group injected with ICAM-1-specific plasmid siRNAs (*P < 0.05 vs. laser only group; n = 12 in all groups; Figs. 5).

Flow Cytometry

The number of neurosensory retina or RPE-choroid infiltrating monocytes decreased markedly in the group injected with ICAM-1-specific plasmid siRNAs compared with the control laser-only group (*P < 0.05 vs. laser only group; n = 6 in all groups; Figs. 6, 7).

ICAM-1 Expression after STZ Administration Silenced by Intravitreal Injection of Plasmid siRNAs

Seven days after STZ administration, glucose concentration increased significantly (*P < 0.05 vs. control mice; n = 6 in all groups; Fig. 8). Seven days after STZ administration, the ICAM-1 expression increased significantly (*P < 0.05 vs. control mice; n = 6 in all groups; Fig. 8). Upregulation of ICAM-1 after STZ administration was silenced in the group injected with ICAM-1-specific plasmid siRNAs (*P < 0.05 vs. day 7 group; n = 6 in all groups). However, ICAM-1 expression was not silenced in the group injected with naked siRNAs (*P < 0.1 vs. day 7 group; n = 6 in all groups; Fig. 8).

Discussion

In the present study, we showed that ICAM-1 expression can be silenced by plasmid siRNA in murine neurosensory retina and RPE-choroid in vivo. We also found that fluorescein-labeled plasmid DNA injected by the hydrodynamics-based transfection...
tion procedure and intravitreal injection was observed in the neurosensory retina and RPE-choroid. We designed siRNA expression plasmid against ICAM-1 and transfected it into the murine retina by intravenous and intravitreal injection. Marked downregulation of ICAM-1 expression occurred in the retinal laser photocoagulation model and in the STZ-induced model.

ICAM-1 is a cell surface glycoprotein in the immunoglobulin superfamily. ICAM-1 is expressed at a low basal level in fibroblasts, leukocytes, keratinocytes, and endothelial and epithelial cells but is upregulated in response to a variety of inflammatory mediators.

Leukocyte adhesion coincides with breakdown of the blood-retinal barrier, capillary nonperfusion, and endothelial cell injury and death. The upregulation of adhesion molecules, such as ICAM-1, on the endothelium and the b2 integrin CD18 on leukocytes is involved in these events. In a murine model, ICAM-1 and CD18 were upregulated by an STZ-induced diabetic model and laser-induced CNV. Antibody-based inhibition of ICAM-1 or CD18 prevents diabetic retinal leukocyte adhesion and blood-retinal barrier breakdown. In addition, targeted disruption of ICAM-1 and the CD18 gene inhibited laser-induced CNV in mice. In human

**Figure 6.** Flow cytometric analysis data with F4/80 staining of neurosensory retina or RPE-choroid 3 days after retinal scatter photocoagulation. Red: no laser control. Blue: laser + plasmid ICAM-1 siRNA. Green: laser only. Three days after laser photocoagulation, the numbers of monocytes significantly increased compared with control mice and were remarkably reduced by intravitreal injection of plasmid ICAM-1 siRNA. $P < 0.05$ vs. laser-only group; $n = 6$ in all groups.

**Figure 7.** The number of neurosensory retina or RPE-choroid infiltrating monocytes decreased markedly in siRNA-treated mice compared with the laser-only group. $^*P < 0.05$ vs. laser only group; $n = 6$ in all groups.
studies, upregulation of ICAM-1 has been reported in several diseases such as uveitis, diabetes, and age-related macular degeneration.

In the normal retina, there was no ICAM-1 expression in the choroid, RPE, or the neurosensory retina. After laser photocoagulation injury in the retina, ICAM-1 expression was upregulated in RPE cells and the choroidal layer at the site of laser injury around 1 to 3 days after laser photocoagulation (Fig. 4). ICAM-1 appeared to be localized to the choroidal vascular endothelial cells and to infiltrating cells. After the upregulation of ICAM-1, the leukocyte (especially, macrophage) appeared in the laser site of the retina by 3 to 5 days after laser photocoagulation, and recruited leukocytes expressed the vascular endothelial growth factor (VEGF). VEGF expressed by intravascular leukocytes may directly induce endothelial cell activation. Macrophage recruitment is an early step in the initiation of the inflammatory and angiogenic process, and ICAM-1 plays an important role in leukocyte recruitment.

Transgene expression in vivo for gene function studies or for therapeutic purposes will require methods that allow for efficient gene transfer into cells. Specific genes can be manipulated by mechanisms such as neutralized antibody and RNA interference (RNAi). RNAi is a powerful technique for gene silencing.

Currently, siRNA is being used in vivo. The most extensively studied method for introducing exogenous genes into animal cells uses a viral carrier. The most commonly used viral carriers are retrovirus, adenovirus, adeno-associated virus, herpes virus, and others. Some viral carriers induce an immune response and cause side effects that are problematic for repeated administration. Therefore, efforts have been made to develop alternative approaches for gene transfer.

Although relatively low efficiency in vivo is the main limiting factor of nonviral gene transfer methods, plasmid DNA has safety advantages compared with viral vectors. Thus, plasmid DNA should be used particularly for long-term and repeated gene therapies requiring improved gene expression efficiency.

Recently, it was reported that a high level of gene expression can be easily obtained by the simple, high-velocity injection of plasmid DNA with a large volume of saline into the tail vein. This is the so-called hydrodynamics-based transfection procedure. This procedure is frequently used as a simple and convenient in vivo transfection method. By rapid injection of a large volume of plasmid DNA into a mouse tail vein, high levels of foreign gene expression in mouse liver, particularly in hepatocytes, were achieved. Under these conditions, high hydrostatic pressure is generated in the inferior vena cava and in the vein linked to that vessel because the volume and speed of injection exceed the cardiac output. The mechanism of gene transfer by this procedure is not clearly understood but must be related to an enhanced uptake and intracellular processing of the injected plasmid DNA in the hepatocytes. To date, the hydrodynamics-based transfection procedure has been used by the gene therapy community for the evaluation of therapeutic activities of various genes. Other reported applications of this technique include studies to define the regulatory functions of DNA sequences, investigations to evaluate gene suppression activity of siRNA, and experiments to establish animal models for viral infection. This procedure is frequently used as a simple and convenient in vivo transfection method. In the present study, we examined the in vivo therapeutic efficiency of ICAM-1 gene downregulation using the hydrodynamics-based procedure.

The hydrodynamics-based procedure seems to be a long way from application in clinical situations because it involves rapid intravenous injection of an extraordinarily large volume of solution. Therefore, all along, one of the major concerns about the hydrodynamics-based procedure has been its safety and invasiveness regarding its feasibility in clinical applications as a practical gene delivery technology for patients is concerned.

Recently, Song et al. reported that frequent hydrodynamic-based injections of synthetic siRNA dramatically reduced mRNA and protein levels of the targeted gene-encoding Fas receptor and protected mice from liver failure and fibrosis in experimental hepatitis. Local administration of synthetic siRNA suppresses endogenous target genes for agouti-related peptide in the brain and for VEGF in the eye.

DsRNA silences homologous gene expression by a mechanism termed RNAi, an evolutionarily conserved phenomenon and a multistep process that involves the generation of active siRNA in vivo through the action of the RNase III endonuclease Dicer. The resultant 21- to 23-nt siRNA mediates degradation of the complementary homologous RNA. RNAi has been used as a reverse genetic tool to study gene function in multiple model organisms, including plants, Caenorhabditis elegans, and Dro sophila, which large dsRNAs efficiently induce gene-specific silencing.

In two ongoing RNAi clinical trials, direct intravitreal injections of siRNAs targeting VEGF or its receptor (VEGFR1) have
been performed to test ocular safety and efficacy. SiRNAs targeting VEGF (Acuity Pharmaceuticals, Philadelphia, PA) and VEGFR1 (Sirna Therapeutics, San Francisco, CA) and delivered into the eye are being evaluated in early-stage clinical trials to treat AMD. Thus far, no adverse events have been reported in patients. This direct injection approach may also be useful for other ocular diseases.13

We showed that siRNA expression mediated by this plasmid causes efficient and specific downregulation of ICAM-1 gene expression, suggesting that ICAM-1 expression can be silenced by plasmid siRNA in murine retina in vivo. This new technology may lead to novel therapeutic concepts to reduce retinal neovascular disease by inhibiting leukocyte infiltration.

References


