Vascular Endothelial Growth Factor Receptor 1 Morpholino Increases Graft Survival in a Murine Penetrating Keratoplasty Model

Yang Kyung Cho,1,2 Xiaobui Zhang,1 Hironori Uehara,1 Jason R. Young,1 Bonnie Archer,1 and Balamurali Ambati1

PURPOSE. This study sought to determine whether a Vascular Endothelial Growth Factor Receptor 1 (VEGFR1)-specific morpholino (MO) could decrease neovascularization, thereby enhancing murine cornea transplant survival, and if this effect is synergistic with steroid therapy.

METHODS. Graft survival, corneal neovascularization, and corneal lymphangiogenesis were compared among the VEGFR1_MO, STD MO, and PBS groups following subconjunctival injection in mice that underwent normal-risk penetrating keratoplasty (NR PK) and high-risk penetrating keratoplasty (HR PK). Graft survival, corneal neovascularization, and corneal lymphangiogenesis in groups treated with both VEGFR1_MO and steroid therapy were also analyzed in HR PK.

RESULTS. In NR PK, the VEGFR1_MO decreased angiogenesis, lymphangiogenesis, and increased graft survival compared with the PBS group (P = 0.055, P = 0.003, P = 0.043, respectively). In HR PK, VEGFR1_MO decreased angiogenesis, lymphangiogenesis, and increased graft survival compared with the STD MO (P = 0.000, P = 0.000, P = 0.029, respectively) and PBS groups (P = 0.004, P = 0.002, P = 0.024). In HR PK, when the VEGFR1_MO was combined with steroid therapy, a significant increase in graft survival was seen compared with steroid treatment alone (P = 0.045). The 2-month graft survival rate for HR PK was 27% in the combination group compared with 0% in the triamcinolone only group.

CONCLUSIONS. VEGFR1_MO decreased angiogenesis and lymphangiogenesis, resulting in increased graft survival in both NR PK and HR PK. This beneficial effect is synergistic with steroid treatment in HR PK. (Invest Ophthalmol Vis Sci. 2012;53:8458–8471) DOI:10.1167/iovs.12-10408

Penetrating keratoplasty (PK) is one of the most common and successful organ transplant procedures worldwide, with first-year survival rates as high as 90% in normal-risk patient populations.1–3 Unfortunately, even with current immunosuppression techniques, rejection rates in high-risk patients can be over 70%.4–7

Risk factors for transplant rejection include corneal vascularization, regrafting, elevated intraocular pressure, trauma, and active inflammation.7–9 Corneal vascularization is a recognized risk factor for transplant rejection10,11 and one of the most important causes of corneal graft failure.5,12 Thus, minimizing corneal angiogenesis has the potential to decrease immunologic rejection and graft failure rates.

Corneal angiogenesis is driven by VEGF and acts in an important pathogenic role in other ocular diseases.12,13 VEGF acts by binding tyrosine kinase receptors on the cell surface, causing dimerization and activation through phosphorylation of the receptors13,14. Angiogenic responses to VEGF-A are mediated by VEGF receptor 1 (R-1, Flt-1) and VEGFR-2 (KDR). While the tyrosine kinase activity of VEGFR-1 is usually weaker than that of VEGFR-2, VEGFR-1 has stronger VEGF-binding affinity than VEGFR-2.12,15 Furthermore, VEGFR-1 is involved in the VEGF-dependent migration and gene expression of monocytes and macrophages.15–17 VEGFR-1 has two isoforms, a full-length membrane-bound form (mFlt-1) and a shorter nonmembrane-bound form (sFlt-1),14,15,18 Soluble VEGFR-1 (sFlt-1) can be generated from alternative splicing or from proteolytic cleavage of the ectodomain from the cell surface.14,19 The molecular role of sFlt-1 is believed to be the sequestration of the VEGF ligands, causing decreased activation of VEGF receptors. Potential biological functions of sFlt-1 include inhibiting angiogenesis by dampening the VEGF-VEGFR2 signaling pathway and by acting as an anti-inflammatory by attenuating VEGF-VEGFR1 signaling, thus decreasing monocyte and macrophage activation and migration.13,14

Morpholinos (MOs) are synthetically produced molecules similar to DNA oligonucleotides which can bind mRNA or premRNA to sterically block translation or alternative splicing.20–24 Although morpholinos have been shown to be very effective for splice modification by blocking splicing events, morpholinos can also be used to block translation of mRNA or inhibit micro-RNA activity, depending on the specific binding site of the RNA.20–24

In a previous study, we reported that a VEGFR-1 specific morpholino that promotes the shift from mFlt-1 to sFlt-1 can inhibit neovascularization and inflammation in a murine corneal suture model.25 This study sought to determine whether a VEGFR1-specific morpholino can inhibit neovascularization and lymphangiogenesis enough to result in an increased graft survival in the normal risk (NR) and high-risk (HR) murine PK models. We also tested for a potential synergistic effect of combining VEGFR1 morpholino with steroid treatment (triamcinolone).
**Materials and Methods**

The experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the IACUC (Institutional Animal Care and Use Committee) of the University of Utah and the Catholic University of Korea, St. Vincent’s Hospital.

**Morpholino Oligomer Construction**

Morpholino constructs were designed to target the exon 13/intron 15 junction of the murine Flt-1 transcript. The Flt morpholino (VEGFR1-MO: 5'TCT TTT GCC GCA GTG CCT ACC TCT A-3') and the nonspecific standard (STD) morpholino (STD MO: 5'CCT TTT ACC TCA GTT ACA ATT TAT A-3') were synthesized by Gene Tools (Philomath, OR). Vivo-morpholino was used to introduce morpholino oligomer into tissues.

**Normal Risk Corneal Transplantation**

Penetrating corneal transplantation was done using male mice (8 to 12 weeks old) of the BALB/c strain as graft recipients and mice of the C57BL/6 strain as graft donors (The Jackson Laboratory, JAX Mice and Services, Bar Harbor, ME). Mice were anesthetized by intramuscular injection with ketamine (100 mg/kg) and xylazine (10 mg/kg). The donor cornea was marked with a 2 mm trephine; the anterior chamber was penetrated with a knife (ClearCut, Alcon, Inc., Fort Worth, TX); and the cornea was cut with intraocular scissors (Vannas; Katena Products Inc., Denville, NJ) and placed in Balanced Salt Solution (BSS Alcon Laboratories, Inc). The recipient mouse was anesthetized as described previously. A 1% tropicamide ophthalmic solution was used to dilate the pupil and 0.5% proparacaine ophthalmic solution was used to anesthetize the cornea. The recipient cornea was marked with 1.5 mm trephine and removed by the same method as the donor cornea. Viscoelastic material (Healon, 1% sodium hyaluronate, Abbott Medical Optics, Inc, IL) was used during recipient cornea dissection. The donor graft was sutured into the recipient bed using 8 to 10 interrupted sutures (11-0 nylon, CS160-6, Ethicon, Inc, Cincinnati, OH). After transplantation, the eye was covered with 0.5% erythromycin ophthalmic ointment and the recipient cornea was marked with 1.5 mm trephine and removed using an operating microscope (OPMI 9-FC; Carl Zeiss, Jena, Germany) and corneal microscopic pictures were taken with a camera (CVMV-K59; Ecwcox, Putian Fujian, China).

**High Risk Corneal Transplantation**

Female mice (8 to 12 weeks old) of the BALB/c strain were graft recipients and mice of the C57BL/6 strain served as graft donors (The Koatech Laboratory, Pyeongtak, Korea). Mice were anesthetized by intramuscular injection with 1:1 mixed solution of tiletamine and zolazepam (30 mg/kg, Zoletil 50, Virbac Korea Co, Ltd) and xylazine (10 mg/kg).

To induce vascularization of the recipient corneal bed prior to PK, three corneal sutures (10-0 nylon, CS140-6, Ethicon, Inc) were placed between the corneal center and the limbus at the 12, 4, and 8 o’clock positions. After 2 weeks, the three corneal sutures were removed and penetrating corneal transplantation was done according to the methods used in the normal risk PK. The recipient mouse was anesthetized as described previously. Viscoelastic material (Hyal 2000, 1% sodium hyaluronate, LG life sciences, Seoul, Korea) was used during recipient cornea dissection. The donor graft was sutured into the recipient bed using 8 to 10 interrupted sutures (10-0 nylon, CS140-6, Ethicon, Inc). After transplantation, the eye was covered with 0.5% ofloxacin ophthalmic ointment and the lid was sutured with 8-0 coated vicryl (BV130-5, Ethicon, Inc). Sutures remained in the recipient’s eye for 1 week after transplantation.

**Subconjunctival Injection of Antiangiogenic Agents**

In the conjunctiva, which is just beneath the limbal vascular arcades, we made a hole using a 30-gauge needle and injected each test plasmid into subconjunctival space using a 33-gauge needle with a 45° bevel on a gas-tight syringe (Hamilton, Reno, NV).

Fifteen microliters of the respective treatment (40 ng/µL of VEGFR1 morpholino, 40 ng/µL of standard morpholino, and PBS) were injected into the subconjunctival space using a gas-tight syringe.

In the normal risk PK, VEGFR1_MO (15 µL, 40 ng/µL); STD morpholino (15 µL, 40 ng/µL); and PBS (15 µL) for each group were injected in the subconjunctiva on the day of transplantation and postoperatively at 1, 2, 3, and 4 weeks.

In the high risk PK, VEGFR1_MO (15 µL, 40 ng/µL); STD morpholino (15 µL, 40 ng/µL); and PBS (15 µL) for each group were injected in the subconjunctiva on the day of transplantation and weekly for postoperative weeks 1 to 7.

To test for a synergistic effect of VEGFR1_MO and triamcinolone acetonide (40 mg/mL, Dong Kwang Pharm, Inc, Pyeongtak, Korea) in high-risk PK, the triamcinolone and triamcinolone plus VEGFR1_MO groups were compared. In the triamcinolone only group, triamcinolone acetonide was injected in the subconjunctiva on the day of transplantation and weekly for postoperative weeks 1 to 7. In the triamcinolone plus VEGFR1_MO group, 10 µL of triamcinolone acetonide (40 µg/µL) and 15 µL of VEGFR1_MO were injected in the subconjunctival space on the day of transplantation and weekly during postoperative weeks 1 to 7.

**Clinical Evaluation of Rejection**

In NR PK, the mice were examined and photographed weekly through postoper week 8 under general anesthesia with intramuscular tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO) using an operating microscope (SZX7; Olympus, Tokyo Japan) with an attached camera (U-TV05XG; Olympus, Tokyo, Japan). In HR PK, the mice were examined twice a week and photographed weekly through postoperative week 8 under general anesthesia with inhalation anesthesia (isoflurane) using an operating microscope (OPMI 9-FC; Carl Zeiss, Jena, Germany) and corneal microscopic pictures were taken with a camera (CVMV-K59; Ecwcox, Putian Fujian, China).

We evaluated clinical graft rejection according to the grading system for orthotopic corneal graft opacity and neovascularization as previously described.26 The opacity grading (0–5) was as follows: 0 - clear; 1 - minimal, superficial (nonstromal) opacity, pupil margin and iris vessel readily visible through the cornea; 2 - minimal, deep (stromal) opacity, pupil margin and iris vessel visible; 3 - moderate stromal opacity, only pupil margin visible; 4 - intense stromal opacity, only a portion of pupil margin visible; 5 - maximum stromal opacity, anterior chamber not visible. Opacity grades 3 and above were considered a graft rejection. Mice with complications such as severe inflammation and hemorrhage were excluded from the study.

**Analysis of Angiogenesis and Lymphangiogenesis**

For both the NR PK and HR PK, after the planned injections and observation periods, mice eyes were harvested and the corneas were trimmed of any remaining limbus and iris. Immunohistochemical staining for vascular and lymphatic endothelial cells were performed on corneal flat mounts. Fresh corneas were dissected; rinsed in PBS for 30 minutes; and fixed in 100% acetone (Sigma-Aldrich) for 20 minutes. After washing in PBST (0.1% Tween20/PBS), nonspecific binding was blocked with 3% BSA (bovine serum albumin)/PBS for 3 nights at 4°C. Incubation with fluorescein isothiocyanate (FITC)–conjugated monoclonal anti-mouse CD31 antibody (558738, BD Pharmingen, San Diego, CA) at a concentration of 1:500 and rabbit anti-LYVE-1 (ab 14917; Abcam Inc, Cambridge, MA) at a concentration of 1:200 in 3% BSA/PBS at 4°C overnight was followed by 1:1000 goat antirabbit antibody-Alexa.
Fluor 546 (A11071, Invitrogen Corporation, Carlsbad, CA) for 1 hour with subsequent washes in PBST at room temperature. Corneas were mounted with an anti-fading agent (GeL Mount; Biomeda, San Francisco, CA).

After immunohistochemical staining for vascular endothelial cells and flat mounting of the cornea, images of the corneal vasculature were captured using a camera attached to a fluorescent microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY in NR PK and OLYMPUS BX51 in HR PK). Neovascularization and lymphangiogenesis were quantified by setting a threshold level of fluorescence above which only vessels were captured and processed using a Java-based image processing program (ImageJ; National Institutes of Health, Bethesda, MD). The total corneal area was outlined using the innermost vessel of the limbal (rim of the cornea) arcade as the border and the graft cornea area was outlined using the star line between recipient and donor graft. Total and graft neovascularization, and total and graft lymphangiogenesis was calculated as follows: total neovascularization (%) = neovascularized area of total cornea / total cornea area × 100; graft neovascularization (%) = neovascularized area in graft / graft area × 100; total lymphangiogenesis (%) = lymphangiogenesis area of total cornea / total cornea area × 100; graft lymphangiogenesis (%) = lymphangiogenesis area in graft / graft area × 100.

Comparison of Graft Survival, Angiogenesis, and Lymphangiogenesis in NR PK and HR PK

The three groups (VEGFR1_MO, STD MO, and PBS) were compared in normal and high-risk PK. In addition, the previously mentioned outcomes for the steroid only group (triamcinolone injection) and the combination group (VEGFR1_MO and triamcinolone) were compared in the high-risk group. Graft survival, neovascularization (NV), and lymphangiogenesis (LY) were compared.

Comparison of the Inflammatory Infiltration in HR PK Model

To test inflammatory infiltration in HR PK model, we tested four groups: VEGFR1_MO (15 μL, 40 ng/μL); STD MO (15 μL, 40 ng/μL); VEGFR1_MO (15 μL, 40 ng/μL) plus triamcinolone (10 μL, 40 mg/mL); and STD MO (15 μL, 40 ng/μL) plus triamcinolone (10 μL, 40 mg/mL) group. High-risk penetrating keratoplasty was done using the same HR PK Model. High-risk penetrating keratoplasty was done using the same

In each stack image, the inflammatory infiltration was quantified by setting a threshold level of fluorescence above which cells were captured and processed using Java-based image processing program (National Institutes of Health). The percentage area of CD11b+ cell infiltration was analyzed in each stack image using pixel area.

Expression of VEGF-A, Membranous VEGFR1 (mbVEGFR1) and Soluble VEGFR1 (sVEGFR1) in HR PK Model

Eighteen Balb/c mice were used for recipient and nine C57/Bl6 mice for donor in HR PK model. Four groups were tested to observe the expression of VEGF-A, mbVEGFR1 and sVEGFR1.

VEGFR1_MO (15 μL, 40 ng/μL); STD MO (15 μL, 40 ng/μL); VEGFR1_MO (15 μL, 40 ng/μL) plus triamcinolone (10 μL, 40 mg/mL); and STD MO (15 μL, 40 ng/μL) plus triamcinolone (10 μL, 40 mg/mL) were injected into the subconjunctival space using a gas-tight syringe (Hamilton) in each group.

The subconjunctival injection was done on the day of PK and on day 4 postoperatively. Corneas were enucleated and fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) for 2 hours at 4°C. For preparing cryosections, the corneas were cryoprotected in 30% sucrose overnight and embedded in a compound (Tissue-Tek OCT Sakura Finetek USA Inc, Torrance, CA). Sections (12 μm) were cut on a cryostat. After permeabilization with 0.25% triton-X 100/PBS for 15 minutes, the sections were blocked in 5% donkey serum/0.02% triton-X 100/PBS (blocking buffer) for 1 hour at room temperature. For staining mbVEGFR1 and VEGF-A, we incubated the sections with rabbit anti-mbVEGFR1 antibody (ab2350, Abcam) and rabbit anti-VEGF-A (sc-507, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 5 μ/mL in blocking buffer for 1 hour. VEGFR1 staining was done using rabbit anti-sVEGFR1 antibody (gift of Dr. Pedro Lacal). After washing with PBS, the sections were stained with Alexa 546 conjugated secondary antibody for 30 minutes at room temperature. After washing three times, the sections were stained with DAPI followed by washing three times, and mounted with anti-fading reagents. Images were taken with an inverted fluorescence microscope (Carl Zeiss Micro-Imaging, Inc).

Statistical Analysis

Statistical analysis was performed using statistical software (SPSS 11.5; SPSS, Chicago, IL). Postoperative survival of the allografts was analyzed using Kaplan-Meier survival curves and the log rank test. Neovascularization and lymphangiogenesis of each group was compared with the corresponding control group using an unpaired two-tailed t-test. A P value < 0.05 was considered statistically significant.

RESULTS

Normal Risk Corneal Transplantation

Normal risk corneal transplantation was done in 16 eyes in the VEGFR1_MO group, 18 eyes in the STD MO group, and 11 eyes in the PBS group.

Graft Survival. Figure 1 shows the survival curve and biomicroscopic pictures at postoperative week 8 in NR PK. VEGFR1_MO increased graft survival compared with the PBS group (P = 0.043). There was no difference in graft survival between the VEGFR1_MO and the STD MO groups (P = 0.14). The 8-week graft survival rate for each group was 9.4% in VEGFR1_MO group, 6.9% in STD MO group, and 0% in PBS group.

Neovascularization. The VEGFR1_MO group (22.67 ± 1.39%) showed less total neovascularized area than PBS group.
Figure 1. Survival graph in NR PK. Survival curve for NR PK and biomicroscopic pictures of each group at 8 weeks postop. VEGFR1_MO increased graft survival compared to the PBS group (*P < 0.05).

**High Risk Corneal Transplantation (Combination Therapy vs. Triamcinolone Alone)**

To test the potential synergistic effect of VEGFR1_MO, high risk corneal transplantation was done in 8 eyes in the triamcinolone only group and 10 eyes in the triamcinolone plus VEGFR1_MO group.

**Graft Survival.** Fig. 5 shows the survival curve and biomicroscopic pictures at postop week 8 in HR PK for combined group. The 8-week graft survival rate of each group was 27% in the triamcinolone plus VEGFR1_MO group and 0% in triamcinolone only group. There was a significant difference between the two groups (*P = 0.045).

**Neovascularization.** The VEGFR1_MO plus triamcinolone group (9.10% ± 1.12%) showed less total neovascularization compared with the triamcinolone only group (22.02% ± 3.11%, *P = 0.005; Fig. 6). For graft-specific neovascularization, there was no difference between VEGFR1_MO plus triamcinolone group (11.75% ± 4.06%) and triamcinolone alone group (23.55% ± 6.44%, *P = 0.160).

**Lymphangiogenesis.** In total lymphangiogenesis, there was no difference between VEGFR1_MO plus triamcinolone group (5.71% ± 1.24%) and triamcinolone only group (8.58 ± 7.13%, *P = 0.081; Fig. 6). For graft-specific lymphangiogenesis, there was no difference between VEGFR1_MO plus triamcinolone group (7.80 ± 3.38%) and triamcinolone alone group (14.17 ± 3.88%, *P = 0.252).

**Comparison of Inflammatory Infiltration in High Risk Corneal Transplantation**

There were differences of CD11b+ cell infiltration between groups. The percentage area of CD11b+ cells were 1.63 ± 0.38% in VEGFR1_MO group, 4.44 ± 1.56% in STD MO group, and 0.50 ± 0.15% in VEGFR1_MO plus triamcinolone group, and
FIGURE 2. Normal-risk penetrating keratoplasty. (A) Comparison of neovascularization in total cornea area and graft area in NR PK. For total neovascularization, the VEGFR1_MO group showed less neovascularized area than the PBS group ($P = 0.055$). In graft neovascularization, the VEGFR1_MO group showed less graft neovascularization compared with STD MO and PBS groups ($P = 0.005$, $P = 0.039$, respectively). (B) Comparison of lymphangiogenesis in total cornea area and graft area in normal risk PK. In total lymphangiogenesis, the VEGFR1_MO group showed less lymphangiogenesis than the PBS group ($P = 0.003$). In graft lymphangiogenesis, VEGFR1_MO group showed less graft lymphangiogenesis compared with the STD morpholino and PBS groups ($P = 0.030$, $P = 0.014$, respectively). (C) Representative pictures of NR PK of flatmounted cornea under fluorescent microscope. Upper: CD31 staining. Lower: LYVE-1 staining. Scale bars, 500 μm.
2.28 ± 0.83% in STD MO plus triamcinolone group. The VEGFR1_Mo plus triamcinolone group showed less inflammatory cell infiltration than the STD MO plus triamcinolone group (P = 0.024). VEGFR1_Mo group showed less inflammatory infiltration than STD MO group, but not significant (P = 0.12; Fig. 7).

**Expression of VEGF-A, mbVEGFR1, and sVEGFR1 in High Risk Corneal Transplantation**

There was no difference in the expression of VEGF-A between groups treated with VEGFR1_MO and STD MO. There was also not a difference in the expression of VEGF-A between the VEGFR1_MO plus triamcinolone group and the STD MO plus triamcinolone group. However, there was less expression of VEGF-A in groups treated with triamcinolone (Fig. 8).

In the expression of mbVEGFR1, there was less staining of mbVEGFR1 in the VEGFR1_MO group compared with the STD MO group and in the VEGFR1_MO plus triamcinolone group relative to the STD MO plus triamcinolone group (Fig. 8). With respect to the expression of sVEGFR1, VEGFR1_MO increased the expression of sVEGFR1 significantly compared to the STD MO. However, there was no difference in the expression of sVEGFR1 between the VEGFR1_MO plus triamcinolone and STD MO plus triamcinolone groups (Fig. 9).

**Discussion**

Corneal transplantation often represents the last option available for restoring sight in patients with blindness due to corneal opacification. Although a low 2-year rejection rate of approximately 10% is seen in transplantations performed in avascular, immune privileged (e.g., low risk) recipients, grafting into pathologically prevascularized corneal beds (e.g., high risk) leads to a greater than 50% immunologic rejection rate.

It has been known for many decades that the presence of preexisting blood and lymphatic vessels in corneal tissue is a strong risk factor for subsequent immune rejection. More recently, researchers have shown that postoperative growth of blood and lymphatic vessels into the preoperative avascular recipient's bed is also a strong promoter of subsequent immune rejection in the mouse model of low-risk corneal transplantation.

Corneal angiogenesis is driven by VEGF due to its pathogenic role in causing ocular surface disease through activation of two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). VEGF is not only expressed by vascular endothelial cells, but also by monocytes and macrophages and plays a major role in several proangiogenic factors. Because sFlt-1 is believed to play a role in the sequestration of VEGF, strategies to increase the levels of sFlt-1 may lead to reduced angiogenesis.

In our previous study, we found that the VEGFR1 morpholino leads to the shifting of mFlt-1 to sFlt-1, resulting in reduced angiogenesis and inflammation in murine corneal suture model. For this study, we hypothesized that the VEGFR1_MO can increase graft survival by reducing angiogenesis through increasing sFlt-1 in the mice corneal keratoplasty model. Because there have been several published studies detailing the differences between NR PK and HR PK, we tested the effect of VEGFR1_MO’s effect on graft survival in these two different conditions. Some reports have shown that inflammatory corneal NV leads to the
Figure 4. HR PK comparing each group therapy. (A) Comparison of neovascularization in total cornea area and graft area. In total neovascularization, the VEGFR1_MO group showed less neovascularization than the STD MO and PBS groups ($P = 0.000$, $P = 0.004$, respectively). (B) Comparison of lymphangiogenesis in total cornea area and graft area. In total lymphangiogenesis, the VEGFR1_MO group showed less lymphangiogenesis than the STD MO and PBS groups ($P = 0.000$, $P = 0.002$, respectively). (C) Representative pictures of flatmounted cornea under fluorescent microscope. Upper: CD31 staining. Lower: LYVE-1 staining. Scale bars, 500 μm.
loss of immune privilege during the first week of the NV induction.

Compared with NR KP, eyes with HR KP exhibited significantly higher levels of chemokines such as macrophage inflammatory protein (MIP)-2 and monocyte chemotactic protein-1 (MCP-1) in the early prerejection postoperative period. The overexpression of MIPs in HR eyes correlated with a significant increase in the number of infiltrating macrophages and neutrophils in HR recipients.

In NR PK, our study found that the VEGFR1_MO showed significantly improved graft survival compared with STD MO and PBS through 5 weeks post-PK. But, after 6 weeks of follow-up, the difference in graft survival was only between the VEGFR1_MO and PBS groups, not between the VEGFR1_MO and the STD MO group. We suspect the reason for this is the difference in the steady state concentration of VEGFR1_MO due to the injection schedule. We assumed that after 6 weeks, the steady-state concentration of VEGFR1_MO cannot be maintained due to the cessation of VEGFR1_MO injection after 5 weeks post-PK. As a result, the injection schedule was changed to prolong the effect of the test plasmid in the HR PK, a condition in which shorter graft survival and increased neovascularization and lymphangiogenesis is expected. We injected test material a total of eight times in HR PK and five times in NR PK due to our hypothesis that longer anti-angiogenesis treatment is required in HR PK compared to NR PK.

Morpholinos are one of the available gene knockdown agents. Morpholinos are not dependent on RNase H and do not have significant interaction with extracellular and cellular structures, which can result in off-target effects. In addition, morpholinos have an exceptionally high targeting success rate (70%–98% knockdown of the expression of their intended target) which is likely a consequence of their high affinity for complementary RNA sequences. Because of this specificity, they could be an attractive potential treatment option to decrease corneal angiogenesis. In this study, we used a morpholino designed to block splicing at the junction of exon13 and intron13 of VEGFR1.

When we compared the VEGFR1_MO’s effect on graft survival between the NR PK and HR PK through 5 weeks of follow-up, there was a significant difference in the graft survival rate between NR PK and HR PK, as expected. However, we could not directly compare the effects of VEGFR1_MO between NR PK and HR PK through 8 weeks of follow-up as the morpholino injection frequency differed between the two groups (i.e., injection was more frequent in the HR PK group).

There are several published reports that insist on early initiation of anti-inflammatory agents after NV induction in HR PK. Treatments that cause angiostasis but have a delayed onset do not have a significant effect on the eye's capacity to promote deviant immunity. Our results showed that the VEGFR1_MO decreased angiogenesis and lymphangiogenesis compared with the two control groups (STD MO and PBS) in both NR PK and HR PK. However, as opposed to the results of NR PK, in high risk PK, the VEGFR1_MO decreased neovascularization and lymphangiogenesis in total NV and total LY, but not in graft NV and graft LY, compared with the STD MO and PBS groups even though we injected it throughout the full 8 weeks of follow-up.

**Figure 5.** Survival graph comparing combined group therapy in HR PK. Survival Graph of combination therapy and biomicroscopic pictures of each group at 8 weeks postop in HR PK. The combination group of triamcinolone plus VEGFR1_MO showed better graft survival than triamcinolone alone group (*P* = 0.045). *P* < 0.05.
FIGURE 6. HR PK comparing combined group therapy. (A) Comparison of neovascularization in total cornea area and graft area. In total neovascularization, VEGFR1_MO plus triamcinolone group showed less neovascularization than triamcinolone alone group ($P = 0.005$). In graft neovascularization, there was no difference between the VEGFR1_MO plus triamcinolone group and the triamcinolone alone group ($P = 0.160$). (B) Comparison of lymphangiogenesis in total cornea area and graft area. In total lymphangiogenesis, there was no difference between the VEGFR1_MO plus triamcinolone group and the triamcinolone alone group ($P = 0.081$). In graft lymphangiogenesis, there was no difference between VEGFR1_MO plus triamcinolone group and triamcinolone alone group ($P = 0.252$). (C) Representative pictures of flat mounted cornea under fluorescent microscope. Upper: CD31 staining. Lower: LYVE-1 staining. Scale bars, 500 µm.
Figure 7. Comparison of inflammatory cell infiltration. (A) The percentage area of CD11b⁺ cell infiltration per corneal area of confocal microscopic field. The VEGFR1_MO plus triamcinolone group showed less inflammatory cell infiltration than STD MO plus triamcinolone group ($P = 0.024$). VEGFR1_MO group showed less inflammatory infiltration than STD MO group, but this was not statistically significant ($P = 0.12$). (B) Representative confocal images of corneas showing CD11b⁺ cells. Scale bar, 100 μm.
When we analyzed the NV and LY of recipient only area and donor only area separately, there was a significant decrease in NV of recipient only area for the VEGFR1_MO group compared with the STD MO and PBS groups \((P = 0.000, P = 0.003, \text{respectively})\). There was also a significant decrease in LY of recipient only area for the VEGFR1_MO group compared with STD MO and PBS groups \((P = 0.004, P = 0.021, \text{respectively})\). Our interpretation is that in HR PK, the significant effect of VEGFR1_MO compared with controls would be more easily detected in a recipient-rim only area than donor area. In a recipient-rim only area, there are already established NV(LY) with inflammation due to sutures prior PK as well as newly developed NV(LY) from the limbus after PK (NV starts from the limbus, migrates across the recipient cornea, and finally reaches the donor graft). The expression of VEGFR1 is increased in inflamed, vascularized human corneas. The VEGFR1 which is a target for VEGFR1_MO would be weakly expressed in graft only area than in the recipient only area. As a result, there could not be significant difference in graft specific NV and LY between groups in HR PK. The other possibility is the diffusion of morpholino from the limbus to corneal center after subconjunctival injection would be limited due to positively charged molecular characteristics of vivo morpholino. Another possibility is that the graft specific NV(LY), which were analyzed at 8 weeks postop, can be affected by other factors, such as growing speed of NV(LY) after rejection time point, as well as anti-VEGF treatment itself. The difference in NV and LY in recipient area was related to the final graft survival in HR PK in our study. This result demonstrates the importance of early treatment of antiangiogenesis in HR PK.

These results differ somewhat from those of Hui et al. who insisted the importance of the degree of lymphangiogenesis crossing the grafting border on the graft rejection in HR PK. In our HR PK, even though there was no difference in NV and LY in donor area between the VEGFR1_MO and control groups, the VEGFR1_MO groups showed better survival with less neovascularization and lymphangiogenesis in the recipient corneal area in HR PK.

There are other reports that insist on the importance of early treatment in HR PK. Whereas grafts placed in high-risk eyes induce rapid donor specific sensitization (within 7 days), presumably because antigens have access to draining lymph nodes through preestablished lymphatics, allografts placed in normal-risk eyes do not generate sensitization until 2 to 4

### Figure 8
Immunofluorescence of mbVEGFR1 and VEGF-A in HR PK cornea. VEGFR1_MO reduced mbVEGFR1 expression with and without triamcinolone compared with STD MO control groups. VEGF-A did not show a decrease with VEGFR1_MO treatment, but triamcinolone reduced VEGF-A (Right panel). Bottom panel is isotype control. Scale bar, 100 μm.
weeks, likely reflecting the time needed for lymphangiogenesis to develop.\textsuperscript{3} As a result, in HR PK, late treatment might not have appreciable effect on the eye’s capacity to promote ACAID.\textsuperscript{2}

There are a variety of reasons that can nullify immune privilege other than allo-immunization, such as a loosened suture, bacterial or viral suture associated infection, vascularization of the graft recipient bed, or inflammation at the time of transplantation.\textsuperscript{1,3,6,37} The main determinant of chemokine expression after PK could be related to the degree of inflammation in the recipient bed (i.e., whether the host is normal or high risk) and not on the presence of allodispersity.\textsuperscript{33} Antiangiogenic intervention using VEGFR1_MO may have possible benefits in both allo-dependent and allo-independent events in a PK model. VEGFR1 is expressed on both vascular endothelial cells and inflammatory cells, such as macrophage and monocyte, which simultaneously function as antigen presenting cells. Thus, VEGFR1_MO can affect both cells by shifting mFlt-1 to sFlt-1, thereby decreasing both angiogenesis and inflammation.

Future studies are needed to define the optimal dosage and frequency of administration of morpholinos considering the importance of early treatment to achieve the best outcomes in HR PK. The relative importance of heme versus lymphangiogenesis after both NR PK and HR PK for subsequent immune rejections remains unknown.\textsuperscript{1,6} In this study, we were not able to determine this because the VEGFR1_MO inhibited both angiogenesis and lymphangiogenesis, presumably because sequestration of VEGF-A blocks both (as VEGF-A can induce lymphangiogenesis through VEGFR-2, independently of VEGFR-3 effect).\textsuperscript{3,10,32,38} Furthermore, VEGF-A activates inflammatory macrophages through VEGFR1, which releases VEGF-C and -D, indirectly leading to lymphangiogenesis.\textsuperscript{3,10,32,38} Our VEGFR-1–promoting morpholino would be expected to inhibit each of this.

In contrast to other reports,\textsuperscript{36,39,40} which indicate that antilymphangiogenesis alone promotes graft survival, we found that only a significant antihemangiogenic effect of VEGFR1_MO was enough to increase graft survival in combination with triamcinolone in HR PK. This likely indicates that vascular invasion of the cornea is also important in compromising cornea transplant survival, consistent with findings from human clinical series.\textsuperscript{3,5,7,32,41,42}

When we compared the VEGFR1_MO and triamcinolone in high-risk PK, there was no statistical difference in graft survival rate ($P > 0.05$) between the two groups and no statistical differences in NV and LY. This is not necessarily surprising as steroid treatment, (triamcinolone acetonide in

\textbf{Figure 9.} Immunofluorescence of sVEGFR1 in high risk PK cornea. VEGFR1_MO increased sVEGFR1 expression compared to the STD MO. However, cotreatment with triamcinolone did not show a difference. \textit{Scale bar}, 100 \textmu m.
this case) is one of the most effective agents that is used as a mainstay of treatment to increase graft survival after PK. However, the synergistic effect of the VEGFR1 MO combined with triamcinolone was remarkable. When combined with triamcinolone, VEGFR1 MO can decrease total neovascularization and total lymphangiogenesis, resulting in increased graft survival compared with triamcinolone alone in HR PK.

In immunohistochemical staining, we did not find difference of VEGF-A staining in VEGFR1 MO groups compared with other groups. We believe this was due to VEGF-A expression which does not depend on VEGF1 level but rather on hypoxia or injury itself. Therefore, even though VEGFR1 was blocked by VEGFR1 morpholino, VEGF-A expression does not decrease.

Compared with our previous study, VEGFR1 MO did have an effect on decreasing the expression of CD11b+ cell infiltration in the HR PK model. We believe that this difference of CD11b+ cells in cornea was due to differences between the suture model and HR PK model. In the HR PK model, the control group (STD MO group or STD MO plus triamcinolone group) has so many CD11b+ cells that the significant effect of VEGFR1 MO can be easily detected compared with suture model.

The combination of VEGFR1 MO plus triamcinolone did not show a significant difference in the expression of sVEGFR1. The triamcinolone reduces CD11b+ cell migration, which corresponds to triamcinolone function (anti-inflammation). In the current HR PK model, when we used VEGFR1 MO plus triamcinolone, CD11b+ inflammatory cells decreased significantly. If the VEGFR1 MO has a preferential action on the CD11b+ inflammatory cells, the CD11b+ cells would be the major cell to produce sVEGFR1. As a result, there would be relatively decreased sVEGFR1 expression in the group with triamcinolone treatment.

In conclusion, the VEGFR1 MO can decrease neovascularization and lead to increased graft survival in both NR PK and HR PK. Furthermore, in HR PK, there was a significant synergistic effect when the VEGFR1 MO was combined with triamcinolone.

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References


