The Inflammatory Milieu Associated with Conjunctivalized Cornea and Its Alteration with IL-1 RA Gene Therapy

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Purpose. This study was designed to gain an insight into the inflammatory milieu into which a donor limbal graft is routinely introduced. The objective of this study was to modulate this environment by gene therapy with the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1 RA).

Methods. In a mouse model, the ocular surface cytokine environment associated with a conjunctivalized cornea was assessed 4 weeks after injury. Total corneal epithelial and limbal debridement was performed with a combination of alkali and scrape injury. The cytokines and adhesion molecules measured included IL-1α, IL-1β, IL-6, VEGF, intercellular adhesion molecule (ICAM)-1, and vascular adhesion molecule (VCAM)-1, by real-time PCR or ELISA. Injured corneas were transfected with IL-1 RA by injection of naked plasmid vector pIRES-EGFP-IL-1 RA immediately after injury. Corneas transfected with pIRES-EGFP served as the control. Expression of corneal IL-1 RA after transfection with pIRES-EGFP-IL1-RA was assessed over a 2-week period by real-time PCR and Western blot analysis. In addition, limbal stem cell grafts transfected with IL-1 RA were assessed for leukocyte influx.

Results. Conjunctivalized corneas showed increased expression of IL-1α, IL-1β, IL-1 RA, IL-6, VEGF, ICAM-1, and VCAM-1, compared with normal cornea. Transfection-efficiency experiments indicated that corneal expression of IL-1 RA peaked between 12 and 24 hours and lasted up to 2 weeks after the initial transfection. IL-1 RA corneal gene therapy resulted in a downregulation of IL-1β and VCAM-1 expression at 4 weeks after injury, whereas downregulation of IL-6 was evident only at 1 week after injury. Corneal neovascularization was also reduced. In addition, corneal limbal stem cell grafts transfected with IL-1 RA showed a decreased leukocyte influx compared with control grafts.

Conclusions. Transfection of a cornea with IL-1 RA immediately after epithelial injury selectively altered the cytokine profile of the resultant conjunctivalized cornea and suppressed corneal neovascularization. Transfection of corneal limbal donor tissue with IL-1 RA before engraftment can reduce leukocyte influx into the graft. The findings demonstrate the feasibility of using transient cytokine gene expression, either in donor or recipient corneal tissue, to alter the ocular surface environment beneficially. (*Invest Ophthalmol Vis Sci.* 2002;43:2905–2915)

Extensive ocular surface injury may result in an ingrowth of conjunctival epithelium to produce a "conjunctivalized" ocular surface.[1] This epithelium is opaque and exhibits morphologic and biochemical characteristics common to conjunctiva rather than cornea.[2] After a period, this surface often alters to become more cornea-like but still does not have major differentiated features common to corneal epithelium.[3] Chronic inflammation is a common secondary feature, as is recurrent epithelial breakdown and degenerative epithelial calcification. Management of these conditions is complex, often requiring lid, conjunctival, and epithelial surgery along with special attention to dry eye states. One of the definitive procedures required is the keratoepithelioplasty,[4] designed to reintroduce corneal epithelial stem cells to rehabilitate the surface. This form of graft surgery has been shown to result in restoration of the normal corneal phenotype, with regression of epithelial vascularization and inflammation. There are a variety of different forms of these grafts including auto-, allo-, large, small, and cadaveric, all of which are designed to reintroduce the corneal limbal stem cell[5] but all of which have limited long-term success. The causes of failure have never been properly elucidated but may include poor levels of stem cells resident within the graft before transplant to the recipient, death of these stem cells when first placed on the inflamed ocular surface, or a gradual ongoing death of stem cells due to an inhospitable environment.

All adult stem cells live within a specialized, protected, homeostatic environment; corneal stem cells are no exception.[2,5,6] The inflamed conjunctivalized surface onto which these transplants are placed may not be a favorable environment to support continued existence of these unique cells. This study was designed to gain further insight into the inflammatory and cytokine milieu into which a donor limbal graft is routinely placed and to attempt to assess the effect of specific anti-inflammatory cytokines on this environment. We chose a mouse model to follow the change in ocular surface cytokine profile from acute injury to 4 weeks after epithelial injury,

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when the acute inflammatory response had passed and a conjunctivalized surface had developed (the mouse cornea fully reepithelialized 3 days after injury). Our hypothesis was that the abnormal epithelial surface on the vascularized conjunctivalized cornea might secrete substances that are proinflammatory and therefore prime the cornea to recruit an inordinate number of inflammatory cells on engraftment of limbal corneal epithelial transplants. Such an exorbitant leukocyte response to the surgical procedure would result in damage to the delicate stem cells residing within the graft.

We directed our attention to inflammatory cytokines and adhesion factors including IL-1α, IL-1β, IL-6, VEGF, intercellular adhesion molecule (ICAM)-1, and vascular adhesion molecule (VCAM)-1. We also looked at the involvement and expression levels of the naturally occurring anti-inflammatory cytokine IL-1 RA. Dana et al.[7] have previously demonstrated the value of IL-1 RA in downregulating corneal inflammation by topical application of IL-1 RA in the form of a recombinant protein. Gene transfer of this anti-inflammatory cytokine has also been successfully used to ameliorate chronic inflammation in rheumatoid arthritis. Previously published work by Stechschulte et al.[8] has also illustrated the potential use of naked gene transfer to the cornea. We therefore attempted to express IL-1 RA in the cornea by transfection of the full coding sequence, within a bicistronic plasmid vector, to alter corneal gene expression after acute injury and to promote survival of the limbal grafts by the inhibition of inflammation.

We demonstrate that by transfecting the cornea shortly after an epithelial injury with IL-1 RA, we can selectively alter the cytokine profile of the resultant conjunctivalized corneal surface. We also show that transfection of corneal limbal donor tissue with IL-1 RA before engraftment can reduce leukocyte influx into the graft.

METHODS

Animals and Anesthesia

Twenty-gram male C57/BL6 mice were obtained from Charles River Laboratories (Wilmington, MA) and transgenic mice expressing green fluorescent protein (GFP) in all nucleated cells (TgN (GFPU) 5Nagy) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Massachusetts Eye and Ear Infirmary Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Before all experimental manipulations, mice were anesthetized with an intramuscular injection of xylazine hydrochloride (4 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO) and ketamine hydrochloride (25 mg/kg; Parke-Davis, Morris Plains, NJ) in endotoxin-free saline.

Corneal Epithelial Injury

Mice were anesthetized, and 2 μL of 0.15 mM NaOH was applied topically. The corneal epithelium was removed with a blunted number 11.0 scalpel blade (Fine Surgical Tools, Belmont, CA).

Quantification of Corneal VEGF, IL1-β, IL1-α, and IL-6 Protein by ELISA

Each cornea (control or injured) was placed in 100 μL lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 1% Triton, 10 mM NaF, 1 mM Na molybdate, and 1 mM EDTA, [pH 6.8]) supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) followed by homogenization with a plastic pestle (Geno Technology Inc., Maplewood, MO) attached to a handheld drill. Corneas were homogenized in three 15-second bursts, and the suspension was incubated on ice for 10 minutes to allow lysis. The lysate was cleared of debris by centrifugation at 18,000*g* for 15 minutes at 4°C and the supernatant assayed. Total protein content was determined by commercial assay (BCA kit; Bio-Rad, Hercules, CA). Supernatant cytokine levels were determined with a sandwich enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) and normalized to total protein.

To enable quantification of mouse corneal IL-6, 1 week after injury, three corneas were pooled into 125 μL lysis buffer and 100 μL lysate per well in the ELISA. To allow accurate quantification, 100 μL assay buffer and 100 μL standards per well were also added.

Quantitative RT-PCR of Corneal IL-1 RA, VCAM-1, and ICAM-1 mRNA

Mouse corneas were treated as described, removed, and stored in preservative (RNA Later; Ambion, Inc., Austin, TX) at 4°C. Total RNA was extracted from RNA (TRIzol Extraction Reagent; Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Briefly, three corneas were pooled and 1 mL extraction reagent added at room temperature. Corneas were homogenized in three 15-second bursts with a plastic pestle (GenoTechnology Inc.) attached to a handheld drill, and the lysate was allowed to sit at room temperature for 10 minutes to allow nucleoprotein dissociation. The lysate was loaded into a shredder (Qiashredder; Qiagen, Inc., Valencia, CA) to aid homogenization, followed by centrifugation at 12,000*g* for 5 minutes. The supernatant was removed to an RNase-free Eppendorf tube, 200 μL of chloroform added, and the mixture vortexed. After incubation at room temperature for 10 minutes, phase separation was performed by centrifugation at 12,000*g* for 15 minutes at 4°C. The upper aqueous phase containing the RNA was carefully removed to an RNase-free tube and RNA precipitated by the addition of 500 μL isopropanol for 15 minutes at room temperature, followed by centrifugation at 12,000*g* at 4°C. The RNA pellet was washed with 75% ethanol and resuspended in 25 μL of diethyl pyrocarbonate (DEPC) water.

Reverse transcription was performed on 2 μg total corneal RNA with a kit (Superscript II Reverse Transcriptase cDNA Synthesis; Gibco BRL), according to the manufacturer's instructions, on a commercial PCR system (GeneAmp PCR System 9700; PE-Applied Biosystems, Foster City, CA). The cDNA was diluted fivefold before PCR amplification.

Real-time PCR analysis was performed with the fluorogenic probe-based 5′ exonuclease assay (*Taq*man; PE-Applied Biosystems) on an automated sequence detection system (model 7700; PD-Applied Biosystems), according to the manufacturer's instructions. Reactions were performed in a 50-μL volume of master mix from the kit (*Taq*man Universal PCR Master mix; PE-Applied Biosystems). The manufacturer's quantitative (q)RT-PCR methodology was used to measure corneal cytokine or the adhesion molecule gene copy number, which was normalized to the ribosomal protein L32 (RPL 32) mRNA copy number.[9] Linear standard curves were created for each amplification, with 10^2 to 10^8 copies of IL-1 RA, VCAM-1, ICAM-1, or RPL32 plasmids (provided by one of the authors [IC]). Control amplification (no probe and no template) samples were included in each reaction to set baselines. All quantitation of corneal gene expression was within the linear range of amplification.

Murine IL-1 RA-pIRES2-EGFP Plasmid Construction

Murine IL-1 RA full-coding-sequence cDNA was obtained in a pGEMEX plasmid (GF IL-1 RA, provided by IC). Murine IL-1 RA cDNA was subcloned into the bicistronic expression vector pIRES2-EGFP upstream of the internal ribosome entry site and enhanced green fluorescent protein (EGFP) to make murine IL-1R A-pIRES2-EGFP. The IL-1 RA CDS was obtained from the pGEMEX plasmid by *Not*I restriction enzyme digestion (New England Biolabs, Beverly, MA). To ensure correct orientation of this cDNA insert into pIRES2-EGFP, PCR amplification primers were designed with 5′ *Nhe*I and 3′ *Bam*HI restriction enzyme overhangs: 5′-TATGCTAGCCCTCGGGATGGAAATCTG-3′ and 5′-TATGGATCCGCAGTACTATTGGTCTTCCT-3′.

After PCR amplification (PCR Super Mix High Fidelity and Platinum *Taq* Antibody; Gibco BRL) the size of the PCR product (murine IL-1 RA coding sequences) was confirmed by gel electrophoresis on agarose gel (Sea Plaque GTG agarose; BioWhittaker Molecular Applications, Walkersville, MD), and the product was purified with an extraction kit (Qiaex II gel extraction kit; Qiagen, Inc.). PCR products were subsequently analyzed by direct sequencing (Prism 377 DNA sequencer with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit; PE-Applied Biosystems), according to the manufacturer's instructions, to confirm full CDS for murine IL-1 RA. The pIRES2-EGFP vector was purchased from Clontech Laboratories, Inc. (Santa Clara, CA). Restriction enzyme digestion of 1 μg pIRES2-EGFP was performed in a commercial buffer (NE Buffer BAMH1; New England Biolabs) with 20 U/μL *Bam*HI and 5 U/μL *Nbe*I for 2 hours at 37°C. Digestion was confirmed by gel electrophoresis, and linearized plasmid was extracted from the gel and purified, as described earlier. Ligation was performed with a 1:1 molar ratio of plasmid to insert for 2 hours at room temperature. JM109 bacteria were transformed with the mIL-1 RA-pIRES2-EGFP ligation mix and grown on kanamycin (50 μg/mL) Luria broth agar (Fermentas Inc., Hanover, MD). The murine IL-1 RA-pIRES2-EGFP plasmid was purified with a kit (Endofree Plasmid Maxi Prep Kit; Qiagen, Inc.) and plasmid was resuspended at 500 ng/μL in endotoxin-free water and stored in aliquots at –20°C.

Corneal Transfection with Murine IL-1 RA-pIRES2-EGFP Plasmid

Under direct microscopic visualization, a small track through the epithelium and into the anterior stroma of the mouse corneal periphery was initiated with a 27-gauge subretinal needle (Visitec; BD Ophthalmic Systems, Anaheim, CA). A 1.2-cm 33-gauge needle with a 45° bevel on a 10-μL gas tight syringe (Hamilton, Reno, NV) was introduced into this track and advanced gently to the corneal center. Two microliters of plasmid solution was injected under pressure into the corneal stroma (control or injured cornea).

Expression of Enhanced GFP

While under general anesthesia, mice injected with the bicistronic murine IL1-RA-pIRES2-EGFP were examined for expression of GFP and photographed with a microscope (Leica, Deerfield, IL) equipped with a digital camera (Dage CCD; Dage-MTI, Michigan City, IN; with Openlab software; ImproVision Coventry, UK).

Analysis of In Vivo IL-1 RA mRNA Production after Transfection with Murine IL-1 RA-pIRES2-EGFP Plasmid

Cornea of mice were transfected as described earlier, and, at various times after transfection, the corneas were removed and stored in preservative (RNA Later; Ambion, Inc.) at 4°C. Total RNA was extracted and quantitative real-time PCR analysis of IL-1 RA mRNA performed as described earlier.

Analysis of In Vivo IL-1 RA Protein Expression by Western Blot after Transfection with Murine IL-1 RA-pIRES2-EGFP

Total protein was extracted from corneas as described earlier and IL-1 RA levels were determined by electrophoresis (NuPAGE Electrophoresis; Invitrogen, San Diego, Ca) and immunoblot (ImmunoBlot; Invitrogen) methodology. In brief, 20 μg total protein was separated under reducing conditions in a 4% to 12% bis-Tris gel with 2-(*N*-morpholino) ethane sulfonic acid (MES) running buffer (NuPAGE; Invitrogen) at a constant voltage of 200 V for 35 minutes. Equal loading of protein samples was confirmed using a Coomassie blue stain. Protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories), by means of a wet transfer system with a constant current of 200 A for 2 hours at 4°C. The membranes were blocked overnight with 20% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS plus 0.1% Tween 20. The membranes were incubated on an orbital shaker for 1 hour at 4°C with goat anti-mouse IL-1 RA primary antibody (1:2000, R&D Systems) followed by three 15-minute washes with PBS+0.1% Tween 20. Secondary antibody donkey anti-goat horseradish peroxidase (1:7000, Jackson ImmunoResearch Laboratories, Inc.) was added for the same incubation period and the wash was repeated. Enhanced chemiluminescence (ECL) was performed with a Western blot analysis detection system (ECL+PLUS; Amersham Pharmacia Biotech, Amersham, UK) and realized on a phosphorescence imager with accompanying software (Storm 840 PhosphorImager and Image Quant Analysis Software; Molecular Dynamics, Sunnyvale, CA).

Visualization and Quantification of Corneal Neovascularization after Corneal Epithelial Injury

Clinical Slit Lamp Examination. Corneal opacification and vascularization were assessed by slit lamp examination of mouse cornea at various times after injury. A simple scoring method that was a modification of the previously published scoring system described by Dana et al.[7] was used to quantify the degree of both opacification and vascularization in each cornea after injury. Opacification was graded from 0 to 4, depending on whether epithelium alone or minimal, moderate, or severe stromal involvement had occurred. The differences in stromal involvement were indicated by decreased visualization of intraocular structures. Vessels were scored according to three parameters: the number of quadrants involved (grade 0–4), the position between the limbus and central cornea (grade 0–4), and the diameter of the vessels (grade 0–3). All scoring was performed by a masked observer.

In Vivo Lectin Vasculature Stain. Mice were perfused with fluorescein isothiocyanate (FITC)–coupled lectin to label the vasculature. With the animal under deep anesthesia, the chest cavity was carefully opened and a 20-gauge perfusion cannula was introduced into the left ventricle. Drainage was achieved with a 20-gauge needle inserted into the right atrium. After PBS perfusion (3 mL), fixation with 1% paraformaldehyde (3 mL) was achieved at physiologic pressure followed by perfusion with FITC-coupled concanavalin-A lectin (3 mL, 20 μg/mL in PBS [pH 7.4], at 5 mg/kg body weight; Vector Laboratories, Burlingame, CA). In mice under general anesthesia, eyes were viewed and photographed with the microscope equipped with a digital camera (Leica equipped with Dage CCD and Openlab image management software; ImproVision). Selected corneas were flatmounted and assessed for corneal neovascularization with a microscope (Orca; Leica) and the image management software Openlab; Improvision). Selected corneas were used for CD45 immunohistochemistry.

Periodic Acid Schiff Staining for Goblet Cell Detection

At various times after injury, corneas were paraffin-embedded and periodic acid Schiff (PAS; Sigma, St. Louis, MO) staining performed to detect goblet cells in the conjunctivalized cornea.

Limbal Stem Cell Corneal Transplantation

In each case, recipient corneas were prepared first by making a superior conjunctival peritomy extending 4 clock hours. The surface was then dried with a Weck-Cell sponge (Weck-Cell spear; Edward Weeks, Inc., New York, NY), and a careful, limited scraping of the of conjunctivalized epithelium from the whole superior corneal surface was performed with a number 11.0 scalpel blade. At the superior anatomic limbal region, another 4-clock-hour scrape was carefully performed to ensure that all epithelium and superficial stroma were removed. A drop of endotoxin-free PBS was then placed on the surface while the donor graft was prepared.

To prepare the donor limbal tissue, 4 clock hours of full-thickness superior limbal tissue was excised from normal eyes with Vannas scissors, taking care to hold the tissue gently in only one area to prevent widespread crush injury. The tissue was then immediately

placed epithelial side down in serum-free DMEM. The peripheral edge of the tissue was gently scraped with a number 11.0 blade until endothelium and Descemet's membrane were separated from the overlying corneal stroma. This tissue was then grasped with toothed forceps and removed from the remainder of the tissue. Care was taken during this procedure to minimize any crush injury to the underlying epithelium, and the tissue was placed in DMEM. The donor graft was sutured in place with four interrupted 11-0 nylon sutures (Sharpoint; Vanguard, Houston, TX). Erythromycin antibiotic ointment was applied to the ocular surface, and the lids were sutured with three interrupted 8-0 nylon sutures.

Limbal Transplant Transfection with Murine IL-1 RA-pIRES2-EGFP Plasmid

Before harvesting, donor corneas were transfected for 12 hours, by methods similar to those described earlier. Briefly, 1 μL of plasmid solution was injected under pressure into both the superior and inferior regions of the peripheral anterior stroma of each donor mouse cornea. The level of expression of GFP in the transfected tissue was assessed before the limbal grafts were harvested. The transplants were excised from the donor corneas, as described earlier.

Quantification of Corneal Polymorphonuclear Neutrophils after Injury

Polymorphonuclear neutrophils (PMNs) were quantified in corneal grafts 3 days after engraftment onto an injured cornea (4 weeks after injury). Donor corneas were transfected with IL-1 RA+GFP or with GFP alone (control plasmid). Corneas were fixed in 4% paraformaldehyde for 30 minutes at 4°C, washed twice in PBS, paraffin embedded, sectioned (6 μm), and stained with hematoxylin and eosin. A cell was deemed a PMN when a multilobed nucleus was clearly identified in cross section. Five cross-sectional slides were analyzed from each cornea. Five standardized high-powered fields per tissue section were counted with a $\times$100 objective. The central pupillary region was taken to be the center of the cornea, and the number of PMNs in this area and in two high-power fields to the left and two fields to the right of this center field were counted. The sum of PMNs for the five fields was presented as the number of PMNs per corneal section. All counts were performed in a masked manner.

Statistical Analysis

All data collected were displayed in a histogram to determine whether distribution was parametric. Parametric data are presented as the mean $\pm$ SEM. Nonparametric data are expressed as the median (with interquartile range; IR). If data were from more than two groups, then the appropriate parametric or nonparametric ANOVAs were used to compare groups. For parametric data from two groups, an independent or paired *t*- test was used for unpaired or paired data, respectively. Statistical analysis was performed on computer (Excel; Microsoft, Redmond, WA; and SPSS for Windows, ver. 9.0; SPSS Science, Inc., Chicago, IL) Significance was accepted at the 5% level ($P < 0.05$).

Results

Conjunctivalized Cornea

To demonstrate conjunctivalization of the corneal epithelium and its relationship to inflammatory cells, we removed corneas at various times after injury. They were paraffin embedded, and sections of the cornea were monitored for reepithelialization of the denuded area. Total epithelial cell debridement was confirmed immediately after total injury (Fig. 1A). Reepithelialization of the cornea surface was noted as early as 3 days after injury. At 4 weeks after injury, PAS staining of the cornea revealed the presence of goblet cells in the injured corneal surface (Fig. 1B) compared with the control eye. As early as 24 hours after injury, a large number of leukocytes (PMNs) were noted in both the corneal stroma and the ciliary body of injured eyes, whereas none were detected in the control eyes (Fig. 1C). Lectin perfusion demonstrated an avascular cornea in control eyes, whereas a large number of vessels and neovascularization was demonstrated in the injured corneas at 4 weeks after injury. CD45 staining demonstrated the presence of leukocytes both within these vessels and within the cornea stroma (Fig. 1D).

Cytokine Protein Expression in Conjunctivalized Cornea

To assess the inflammatory status of the mouse cornea after injury, a number of different cytokines were selected for measurement at the protein level. Cornea IL-1α, IL1-β, IL-6, and VEGF protein expression was measured in control and injured corneas. Control corneas contained no detectable IL1-β or -6, whereas they contained detectable levels of VEGF and IL1-α protein. The greatest increase in protein expression was noted for VEGF (>200 pg/mL) and IL-1α (>300 pg/mL) 24 and 48 hours after injury (data not shown). At 4 weeks after injury no increase in IL-6 protein was detected in injured corneas and a significant increase in VEGF ($P = 0.007$, $n = 6$), IL-1α ($P = 0.05$, $n = 4$), and IL-1β ($P = 0.03$, $n = 5$) was still noted (Fig. 2A). A significant increase in IL-6 protein expression was noted in injured corneas at the earlier time point of 1 week after injury ($P = 0.0003$, $n = 9$, Fig. 2B), but had decreased to normal levels by 4 weeks after injury (Fig. 2A).

Cytokine and Adhesion Molecule mRNA Expression in Conjunctivalized Cornea

Corneal ICAM-1, VCAM-1, and IL-1 RA mRNA expression was quantified using by real-time PCR (*Taq*man; PE-Applied Biosystems). Normal cornea expressed constitutive/basal levels of ICAM-1 and VCAM-1 mRNA and very low levels of IL-1 RA mRNA. Four weeks after injury a statistically significant increase in ICAM-1 and VCAM-1 mRNA levels was detected (Fig. 3; injured cornea versus normal, ICAM-1: 175,000 copies versus 7,000 copies, $P = 0.0008$, $n = 5$; and VCAM-1: 400,000 copies versus 40,000 copies $P < 0.001$, $n = 5$). These adhesion molecules are directly involved in leukocyte attachment to vessels and their extravasation into tissue, which indicates that the tissue is primed to promote leukocyte infiltration. IL-1 RA expression was also significantly increased (50,000 copies versus 4,000 copies, $P < 0.001$, $n = 5$).

Murine IL-1 RA-pIRES2-EGFP Corneal Transfection

Experiments were performed to determine the efficiency of IL-1 RA transfection of the cornea. Murine IL-1 RA-pIRES2-EGFP plasmid (1 μg) resuspended in water was injected into the stroma of mice. At all times after transfection, corneas were optically clear and grossly free of inflammation (Fig. 4A). Effective transfection was assayed by fluorescence microscopy that showed widespread expression of GFP as early as 4 to 6 hours after transfection, peaking at 24 hours and remaining visible for up to 6 days after transfection. Expression was localized to keratocytes and epithelial cells (Figs. 4B, 4C). Mice transfected with the control plasmid without the IL-1 RA insert displayed a similar pattern of expression (data not shown).

IL-1 RA mRNA Expression after Murine IL-1 RA-pIRES2-EGFP Corneal Transfection

Corneal IL-1 RA mRNA levels were assessed by real-time qRT-PCR. Total RNA was extracted from normal mouse corneas at various time points (from 2 hours to 2 weeks, Fig. 4D) after transfection with murine IL-1 RA-pIRES2-EGFP or empty pIRES2-EGFP. The level of IL-1 RA mRNA was quantified as the

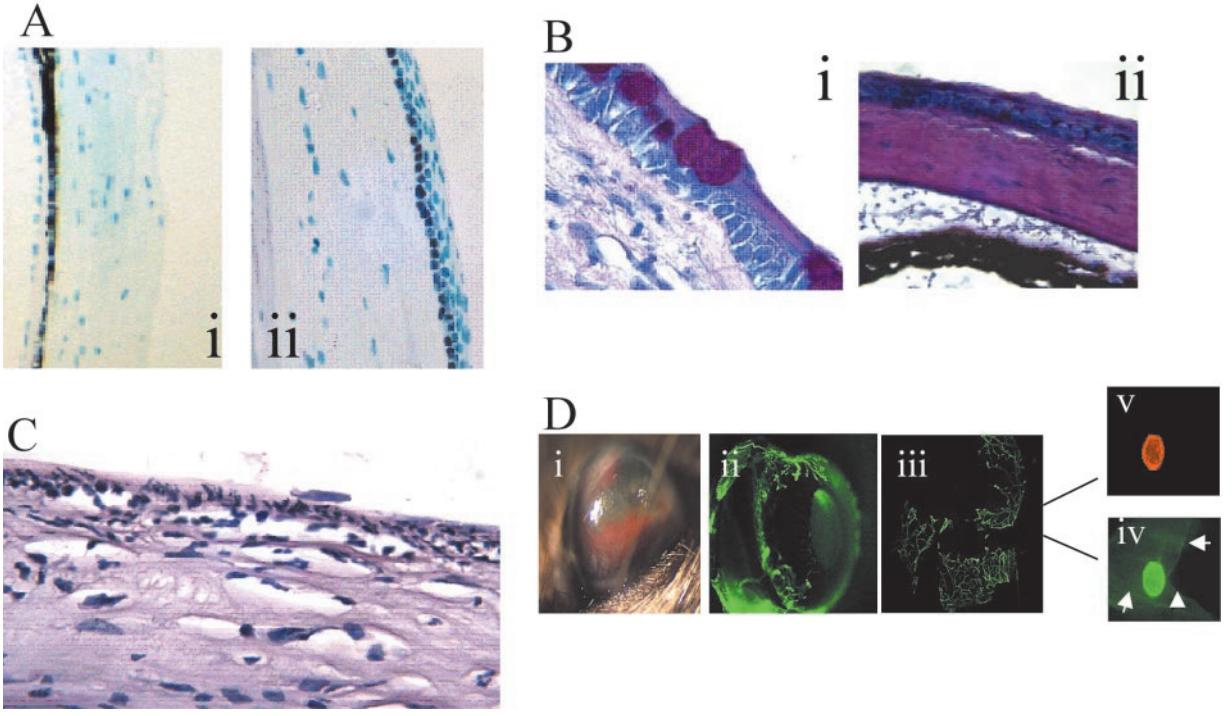

FIGURE 1. (**A**) Total epithelial cell debridement of mouse cornea at time 0 after corneal injury. Normal control eyes or injured eyes were enucleated and paraffin embedded for sections immediately after injury. Total epithelial debridement was noted in injured corneas (**A,** i), whereas the epithelium was intact in all control noninjured corneas (**A,** ii). (**B**, i) PAS staining of a cornea 4 weeks after injury. (**B**, ii) High- power view of PAS staining of an irregular reepithelialized conjunctivalized cornea with interspersed goblet cells 4 weeks after injury. Similar goblet cells were found spread across the epithelial surface of these corneas, with the maximum number found in the periphery, whereas no goblet cells were present in the normal uninjured corneas (**B,** ii). (**C**) Hematoxylin and eosin–stained corneas 24 hours after injury showed a large influx of PMNs into the cornea. In comparison, no PMNs were detected in noninjured control cornea (not shown). (**D**) Corneal neovascularization 4 weeks after injury. (**D**, i) A vascularized cornea. Lectin perfusion (**D**, ii) was also performed to aid in scoring the degree of vascularization. In selected corneas, lectin perfusion 4 weeks after injury (**D**, iii) was followed by CD45 immunohistochemistry to demonstrate the presence of PMNs within the cornea after injury. (**D**, iv) Cells labeled by lectin staining along with vessel wall staining (*white arrows*) were further shown to be PMNs by positive staining with an orange CD45 antibody label (**D**, iv; showing specificity to the PMN with no vessel wall staining).

copy number with real-time PCR and normalized to 5×10^7 copies of ribosomal protein RPL32. No significant difference in IL-1 RA mRNA was noted between normal corneas and those injected with empty plasmid (GFP alone; Fig. 4E). In comparison, a significant increase in IL-1 RA mRNA copy number was noted between the level of IL-1 RA mRNA and the copy number in normal cornea (3,422 ± 709; $n = 6$) when compared with corneas transfected with murine IL-1 RA-pIRES2-EGFP from as early as 4 hours after transfection (24,036 ± 3,370; $P = 0.001$, $n = 6$) remaining up to 1 week after transfection (6,594 ± 763; $n = 6$, $P = 0.0095$; Fig. 4D). No significant increase was noted between normal IL-1 RA mRNA levels and transfection with blank GFP plasmid (3,422 ± 709 vs. 2,279 ± 1,137, control versus GFP transfected; $n = 12$, NS; Fig. 4E). All data are expressed as the mean ± SD.

IL-1 RA Protein Expression after Murine IL-1 RA-pIRES2-EGFP Corneal Transfection

Corneal IL-1 RA protein levels were assayed by Western Blot analysis to confirm the production of IL-1 RA protein after murine IL-1 RA-pIRES2-EGFP corneal transfection (Figs. 4F, 4G). At 24 hours after transfection, an increase in corneal IL-1 RA protein was detected. Data are representative of three pooled corneas from three independent transfection experiments per lane. In contrast, corneas with GFP alone did not show increased production of IL-1 RA protein (Fig. 4E). Additional Western Blot analyses confirmed increased IL-1 RA protein production at 3 days and 1 week after transfection (data not shown).

Effect of Murine IL-1 RA-pIRES2-EGFP Corneal Transfection on Cytokine and Adhesion Molecule Expression Induced by Corneal Epithelial Injury

Experiments were performed to assess whether transfection with IL-1 RA, which resulted in high levels of corneal IL-1 RA, was capable of dampening the chronic inflammatory milieu detected 4 weeks after injury. Levels of IL-1β, VEGF, and IL-6 proteins and of ICAM-1 and VCAM-1 mRNA were measured in corneas that had been injured and transfected with murine IL-1 RA-pIRES2-EGFP. Corneas were transfected immediately after injury, and IL-6 protein levels were measured 1 week after injury, whereas VEGF and IL-1β protein and ICAM-1 and VCAM-1 mRNA levels were measured 4 weeks after injury. During this 4-week period corneas were transfected once more with IL-1 RA.

IL-1 RA transfection resulted in a statistically significant decrease in the amount of corneal IL-1β protein expressed at 4 weeks after injury ($P = 0.006$, Mann-Whitney test [M-W], $n = 6$). IL-6 protein levels 1 week after injury were also reduced by transfection with IL-1 RA ($P = 0.050$, M-W, $n = 9$; Fig. 5). VEGF levels at 4 weeks after injury were not significantly reduced by transfection ($P > 0.05$, $n = 5$, Fig. 5), despite a blinded scoring system showing a significant decrease in formation of corneal vessels up to 1 week after injury (Fig. 6). In addition, we have demonstrated that IL-1 RA corneal transfection decreases VEGF protein expression at the earlier time point of 2.5 days in a mouse model of penetrating keratoplasty (Afshari NA, Moore JE, McMullen CBT, unpublished data, 2001).

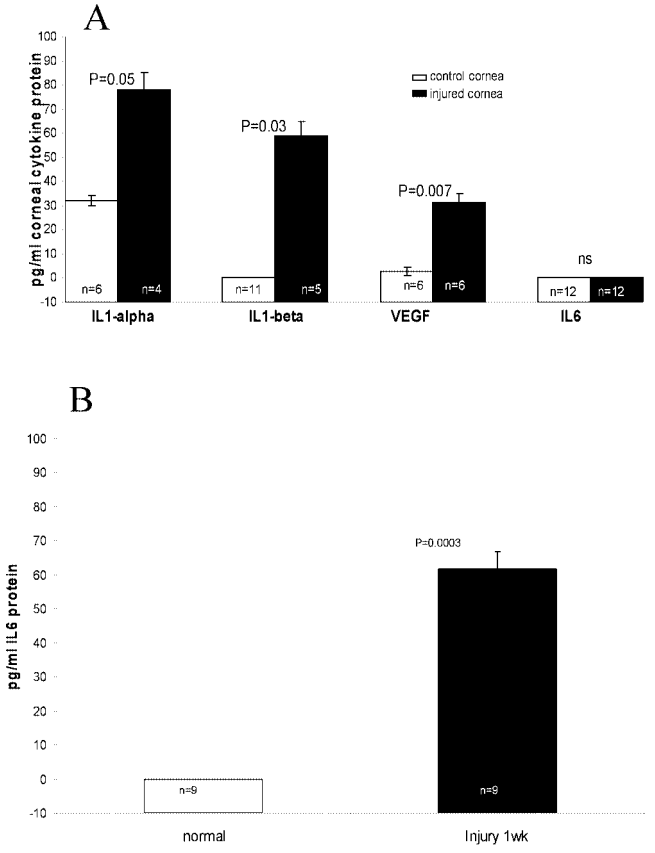

FIGURE 2. (**A**) Increased VEGF, IL-1β, and IL-1α protein expression 4 weeks after injury. Four weeks after total epithelial debridement, VEGF, IL-1β, and IL-1α protein expression was significantly increased above that of normal corneas. In contrast, at this time point after injury, no detectable IL-6 protein was found in injured corneas with no significant difference noted in IL-6 levels between control and injured corneas. Data are expressed at the mean ± SEM. All cytokine levels were normalized to 50 μg of total corneal protein for all samples tested. (**B**) A statistically significant increase in expression of IL-6 protein was detected in injured corneas 1 week after injury. Data are expressed and normalized as in (**A**).

At the mRNA level, no significant difference was noted between mRNA levels of ICAM-1 at 4 weeks after injury followed by transfection with control plasmid pIRES2-EGFP or murine IL-1 RA-pIRES2-EGFP. In contrast, a statistically significant reduction in VCAM-1 mRNA was noted in IL-1 RA–transfected corneas 4 weeks after injury, compared with control injured and transfected corneas ($n = 4$, $P = 0.007$, Mann-Whitney, Fig. 7).

The Effect of Murine IL-1 RA-pIRES2-EGFP Corneal Transfection on PMN Infiltration after Epithelial Injury

In an attempt to determine whether IL-1 RA can alter the inflammatory milieu in injured corneas with limbal transplants, experiments were set up to assess the effect of IL-1 RA transfection on PMN infiltration. Limbal stem cell transplantation was performed on mice corneas 4 weeks after injury. Grafts were transfected with murine IL-1 RA-pIRES2-EGFP (Fig. 8A, ii) or control GFP plasmid (Fig. 8A, i) and, after 3 days, corneas were paraffin embedded and stained with hematoxylin and eosin for PMN counts. A statistically significant decrease was noted in the number of PMNs infiltrating the IL-1 RA transfected grafts, compared with control grafts transfected with GFP alone ($P < 0.001$, $n = 6$, Fig. 8B).

DISCUSSION

Any acute injury to the ocular surface that damages or destroys a significant proportion of the corneal epithelial stem cells results in ingrowth of the conjunctiva. Previous reports have demonstrated that such injuries are associated with a massive influx of PMNs with resultant induction of inflammatory cytokines. Our data agree with data previously published by Sotozono et al.[10] that IL-1α, -1β, and -6 are markedly increased early in a corneal injury but that IL-6 decreases to the level in the uninjured eye before 4 weeks have elapsed after the injury. In our study, IL-1α and -1β remained elevated above levels in control eyes at 4 weeks, indicative of a low-grade chronic inflammatory state. To further elucidate the cytokine and adhesion molecules present 4 weeks after injury, we assessed the mRNA expression of ICAM-1, VCAM, and IL-1 RA. These results showed that, at 4 weeks after an untreated corneal injury, there was increased expression of both inflammatory cytokines and adhesion molecules within the conjunctivalized cornea. Vascularization was a common feature noted in these injured corneas, and we therefore sought to assess VEGF levels. Expression of VEGF protein mirrored IL-1α and -1β levels, showing a large increase early after injury that slowly decreased after the first week to plateau at a level that was still significantly elevated above the level in noninjured control corneas.

Corneal IL-1 expression can lead to an influx of leukocytes that can then release a multitude of enzymes and superoxide radicals, resulting in damage to the surrounding tissue.[11,12] This type of inflammatory reaction sometimes results in a phenomenon known as a "positive feedback loop and chronicity" through activation of IL-6/IL-8 and MMPs by IL-1.[13,14] This causes the influx of more leukocytes, which can themselves both secrete the same cytokines and induce further expression through the surrounding corneal epithelium and stromal keratocytes. Our purpose was to assess whether specific antagonism of the proinflammatory cytokine IL-1, through its naturally occurring antagonist IL-1 RA, would prevent this positive loop and result in an alteration in the final outcome of such an injury. We performed this by gene transfection rather than with topical drops or systemic therapy, thus obviating the need

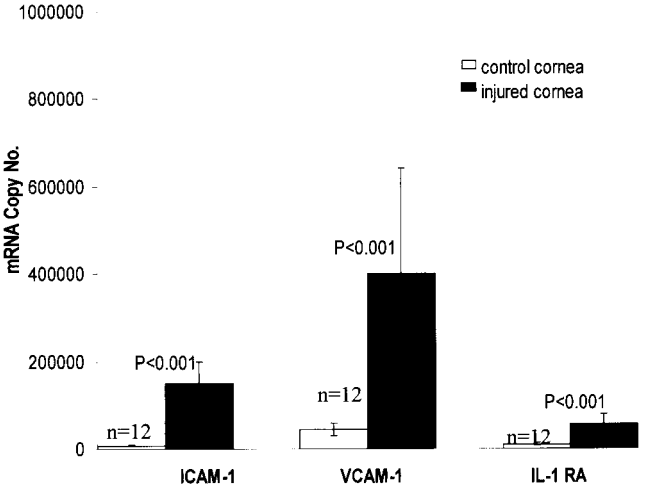

FIGURE 3. Increased expression of ICAM-1, VCAM-1, and IL-1 RA mRNA 4 weeks after injury. At the mRNA level, a statistically significant increase was noted in the expression of adhesion molecules ICAM-1 and VCAM-1 and the anti-inflammatory cytokine IL-1 RA in conjunctivalized cornea. Data are expressed as the mean ± SD. All IL-1 RA results were normalized to a ribosomal protein L32 mRNA copy number of 5×10^7.

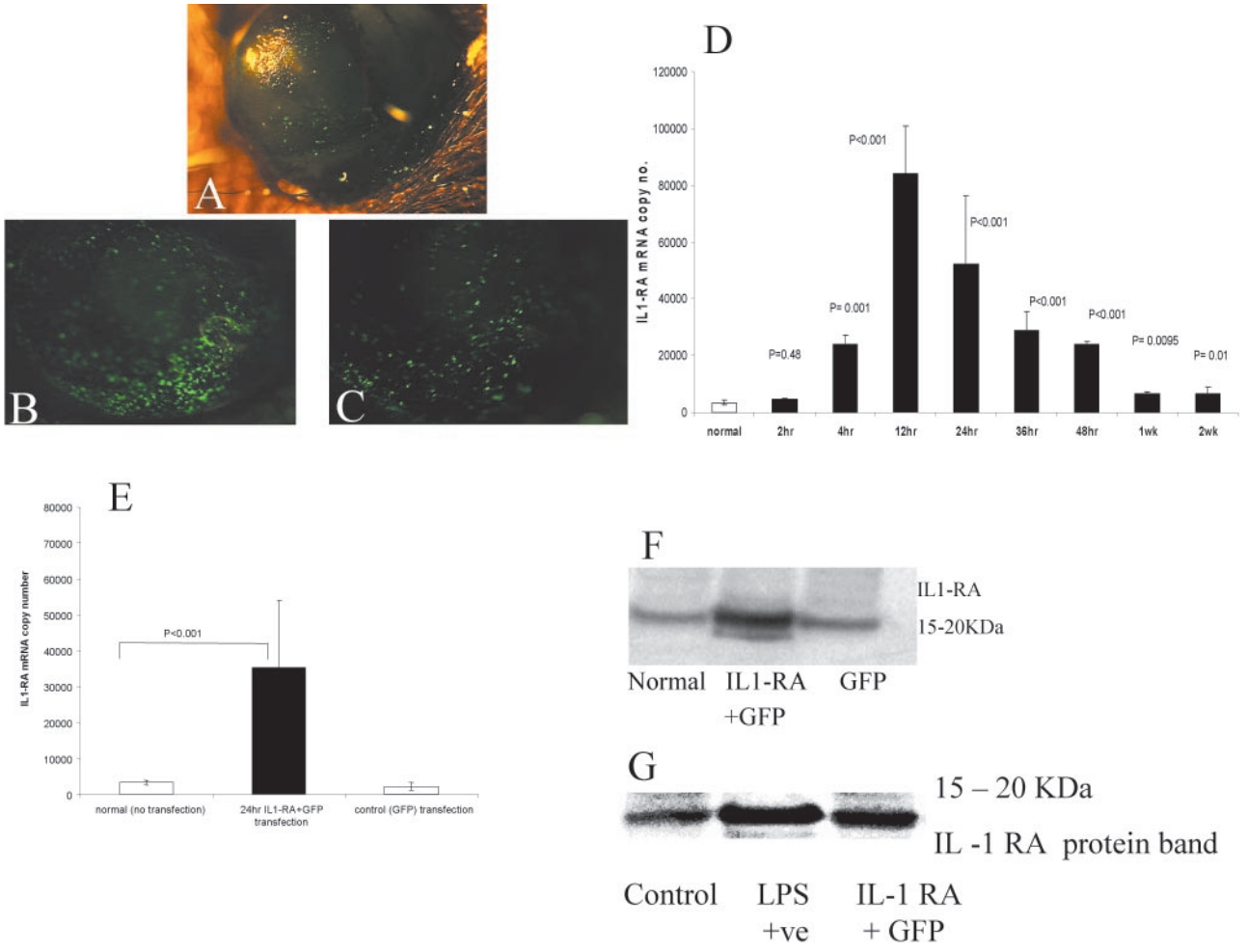

FIGURE 4. (**A**) Appearance of cornea 18 hours after transfection with murine IL-1 RA-pIRES2-EGFP. Eighteen hours after transfection with IL-1 RA-GFP or GFP alone, corneas were optically clear and grossly free of inflammation. (**B**) Expression of GFP in normal (noninjured) mouse cornea 18 hours after transfection with murine IL-1 RA-pIRES2-EGFP. Effective transfection, assayed by fluorescence microscopy, showed widespread expression of GFP as early as 4 hours after transfection that peaked at 24 hours and remained visible for up to 6 days after transfection. Expression of GFP was localized to keratocytes and epithelial cells. IL-1 RA protein was coexpressed with GFP, because of the bicistronic nature of the plasmid used. (**C**) Expression of GFP in injured mouse cornea 18 hours after transfection with murine IL-1 RA-pIRES2-EGFP. In injured corneas after transfection, expression of GFP was located mostly in the keratocytes. (**D**) Real-time qRT-PCR was performed, to demonstrate the corneal expression of IL-1 RA mRNA after transfection with murine IL-1 RA-pIRES2-EGFP. IL-1 RA mRNA increased significantly as early as 4 hours after transfection, peaked at 12 hours after transfection, and remained elevated for up to 2 weeks. Data are expressed as the mean ± SD. All IL-1 RA results were normalized to a ribosomal protein L32 mRNA copy number of 5×10^7. (**E**) Expression of IL-1 RA mRNA was not induced by control injection of the GFP plasmid alone plasmid. The technique of corneal transfection itself did not cause an increase in IL-1 RA mRNA. Data are expressed as the mean ± SD. All IL-1 RA results were normalized to a ribosomal protein L32 mRNA copy number of 5×10^7. (**F**) Western blot analysis of corneal expression of IL-1 RA protein 24 hours after transfection with murine IL-1 RA-pIRES2-EGFP and pIRES2-EGFP. Increased IL-1 RA (15–20 kDa) protein was detected in IL-1 RA–transfected cornea. In contrast, control pIRES2-EGFP–transfected corneas showed no increase in IL-1 RA protein. (**G**) Western blot analysis of corneal expression of IL-1 RA protein 24 hours after LPS stimulation (positive control) or transfection with IL-1 RA-pIRES2-EGFP. At 24 hours after transfection, an increase in IL-1 RA protein was detected. *Lane 1*: the small amount of constitutive IL-1 RA protein present in the mouse cornea; *lane 2*: the amount of IL-1 RA detected in corneas used as the positive control (LPS stimulated); *lane 3*: amount of IL-1 RA expression in corneas 24 hours after transfection with IL-1 RA-pIRES2-EGFP. Each lane is representative of three independent experiments.

for recurrent treatments and opening the possibility of transfection of donor limbal tissue before placement on the recipient cornea.

We produced a corneal epithelial injury through a combination of alkali and scraping to ensure that the limbal region of the mouse cornea was destroyed and hence that vascularization and conjunctivalization of the cornea occurred. PAS staining of the cornea confirmed that mucin-containing cells (goblet cells) were present on the corneal surface, which indicates conjunctival ingrowth. Inflammatory cytokines are known to increase the expression of adhesion factors that promote leukocyte adhesion and transmigration. The upregulated expression of ICAM-1 and VCAM-1 in the conjunctivalized tissue may indicate that these corneas are primed to promote leukocyte adhesion and influx.

This study demonstrated that transient cytokine expression could be induced by naked plasmid transfection, using the bicistronic vector pIRES2-EGFP. The expression of IL-1 RA was at a maximum 18 to 24 hours after transfection and remained elevated throughout the next 10 to 14 days. The results demonstrated the feasibility of inducing prolonged cytokine expression by a single intracorneal stromal injection. Such a technique may be used to modulate various forms of corneal inflammation without use of multiple drops. This same proce-

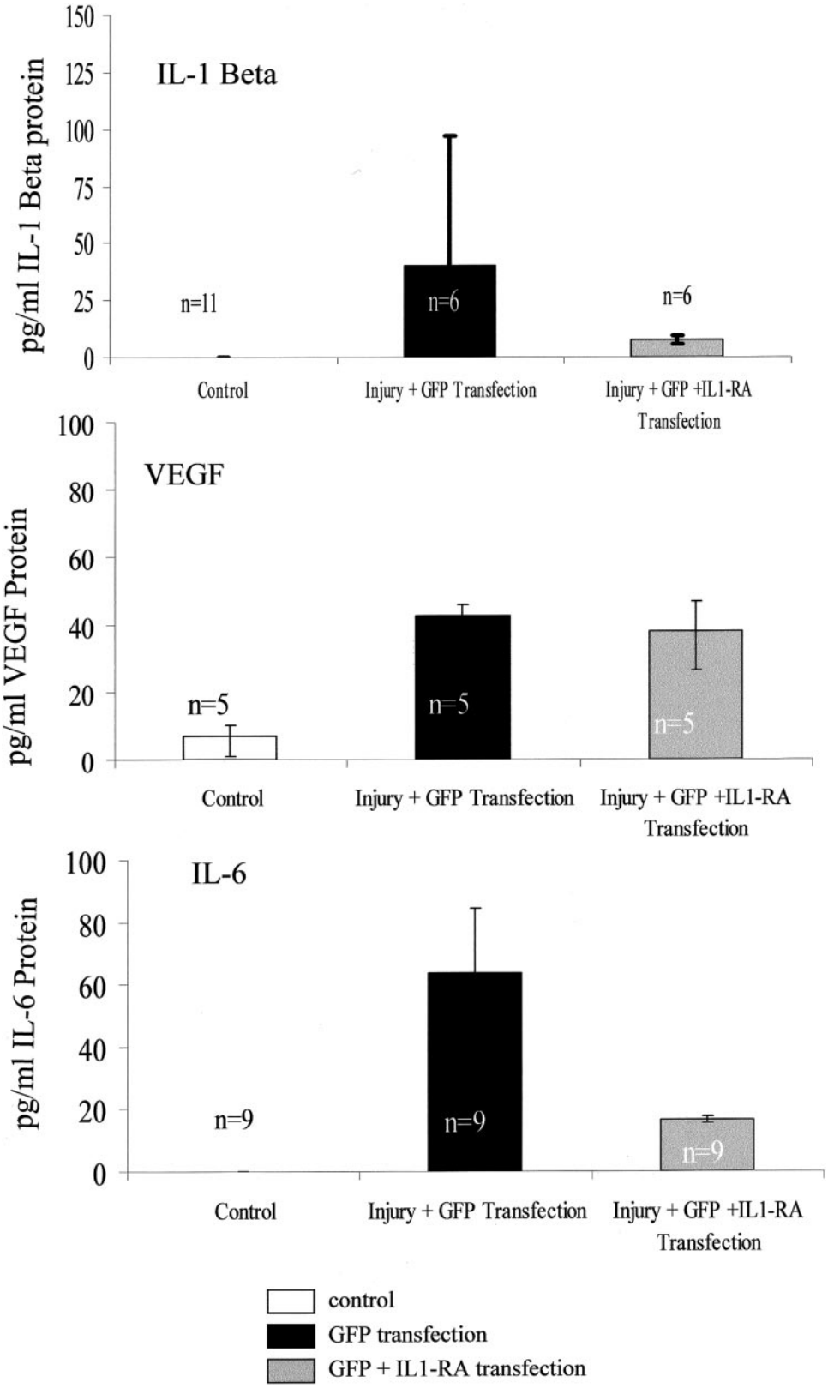

FIGURE 5. Effect of murine IL-1 RA-pIRES2-EGFP transfection on expression of IL-1β (*top*) and VEGF (*middle*) 4 weeks after injury and of IL-6 (*bottom*) 1 week after injury. IL-1β: There was a significant difference in expression between the three groups ($\chi^2 = 20.27$ with $df = 2$, $P < 0.001$; Kruskal-Wallis test). Injury caused a significant increase in expression ($P < 0.001$, M-W), which was attenuated by transfection with IL-1 RA ($P = 0.006$, M-W). VEGF: There was a significant difference in expression between the three groups ($\chi^2 = 13.75$ with $df = 2$, $P < 0.001$ Kruskal-Wallis test). Injury caused a significant increase in expression ($P = 0.002$, M-W). These levels were not attenuated by transfection with IL-1 RA ($P = 0.602$, M-W). IL-6: There was a significant difference in expression between the three groups ($\chi^2 = 13.75$ with $df = 2$, $P = 0.001$ Kruskal-Wallis test). Injury caused a significant increase in expression ($P = 0.001$, (M-W) that was significantly attenuated by transfection with IL-1 RA ($P = 0.050$, M-W). All cytokine levels were normalized to 50 μg total cornea protein for all samples tested. Data represent median +IR.

dure may be useful after routine surgical operations, such as a cataract extraction, in which hydration of the wound edge is commonly used.

Intrastromal injection of plasmid after corneal epithelial injury resulted in decreased expression of inflammatory cytokines 4 weeks later, which illustrates the potential for this type of treatment in clinical practice, whereby specific cytokines may be targeted early in an injury to alter the subsequent cytokine and adhesion molecule expression of the reepithelialized cornea. This surface might then be more suitable to sustain any subsequent corneal limbal graft, whether auto- or allograft or cultured epithelial sheet.

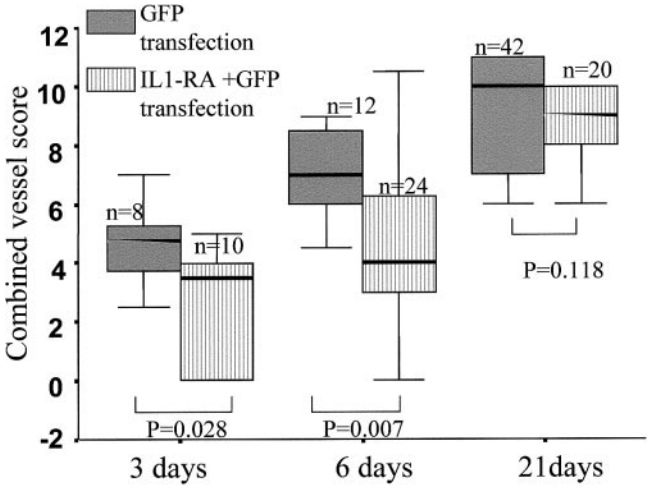

FIGURE 6. Effect of IL-1 RA transfection on vessel formation in injured corneas. Vascularization scores: Transfection with IL-1 RA resulted in clinically significant reduction in the degree of corneal vascularization at days 3 and 6 after injury as demonstrated by a combined clinical scoring system. Data are expressed as the median +IR. Probabilities were calculated by M-W test.

Limbal epithelial grafts appear to contain the putative adult stem cell necessary to replenish the constant XYZ movement of corneal epithelium on the ocular surface. Similar to all stem cells, they require a tightly controlled environment or "niche" to ensure their health and survival.[15] During a transplantation procedure, despite intensive efforts to ensure the health of the transplanted tissue, once grafts are placed on the recipient cornea, they are subject to whatever local cytokine and inflammatory milieu is present. At this moment, very little is known about which factors are critical to the survival of this unique cell type, but inflammation has been associated with both the induction of a stem cell–deficient cornea and the failure of stem cell grafts.[16]

This study has demonstrated the potential use of naked plasmid gene transfection with IL-1 RA immediately after an acute injury to alter the eventual cytokine profile of the conjunctivalized ocular surface. This effect appears to be specific, because IL-1 and VCAM-1 levels at 4 weeks were decreased by transfection with IL-1 RA, whereas VEGF and ICAM-1 levels remained elevated. The findings of increased ICAM-1 in injured cornea are in agreement with previously published data showing VEGF-induced ICAM-1.[17] It has been reported that topical IL-1 RA displays corneal anti-inflammatory effects, largely by

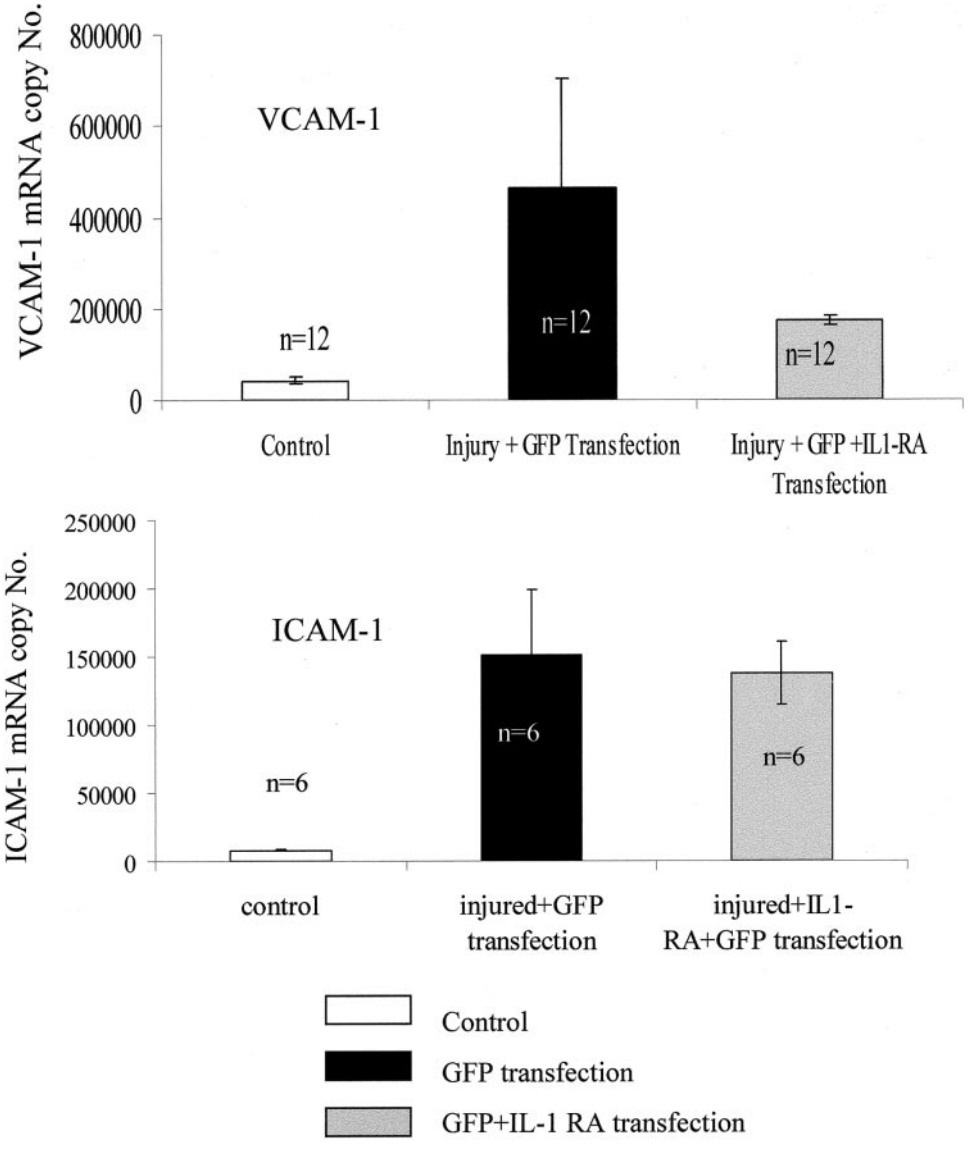

FIGURE 7. Effect of murine IL-1 RA-pIRES2-EGFP transfection on corneal VCAM-1 (*top*) and ICAM-1 (*bottom*) mRNA expression 4 weeks after injury. IL-1 RA transfection specifically reduced the expression of VCAM-1 mRNA in corneas 4 weeks after injury, whereas ICAM-1 levels were not altered by IL-1 RA transfection. ICAM: There was a significant difference in expression between the three groups ($\chi^2 = 14.70$ with $df = 2$, $P < 0.001$, Kruskal-Wallis test). Injury caused a significant increase in ICAM-1 mRNA expression ($P = 0.001$; M-W). These levels were not attenuated by transfection with IL-1 RA ($P = 0.608$, M-W). VCAM: There was a significant difference between the three groups ($\chi^2 = 42.25$ with $df = 2$, $P < 0.001$ Kruskal-Wallis test). Injury caused a significant increase in expression of VCAM-1 mRNA ($P < 0.001$, M-W) that was significantly attenuated by transfection with IL-1 RA ($P < 0.001$, M-W). Data represent mean ± SEM. Adhesion molecule mRNA levels were normalized to a ribosomal protein L32 mRNA copy number of 5×10^7 in all samples.

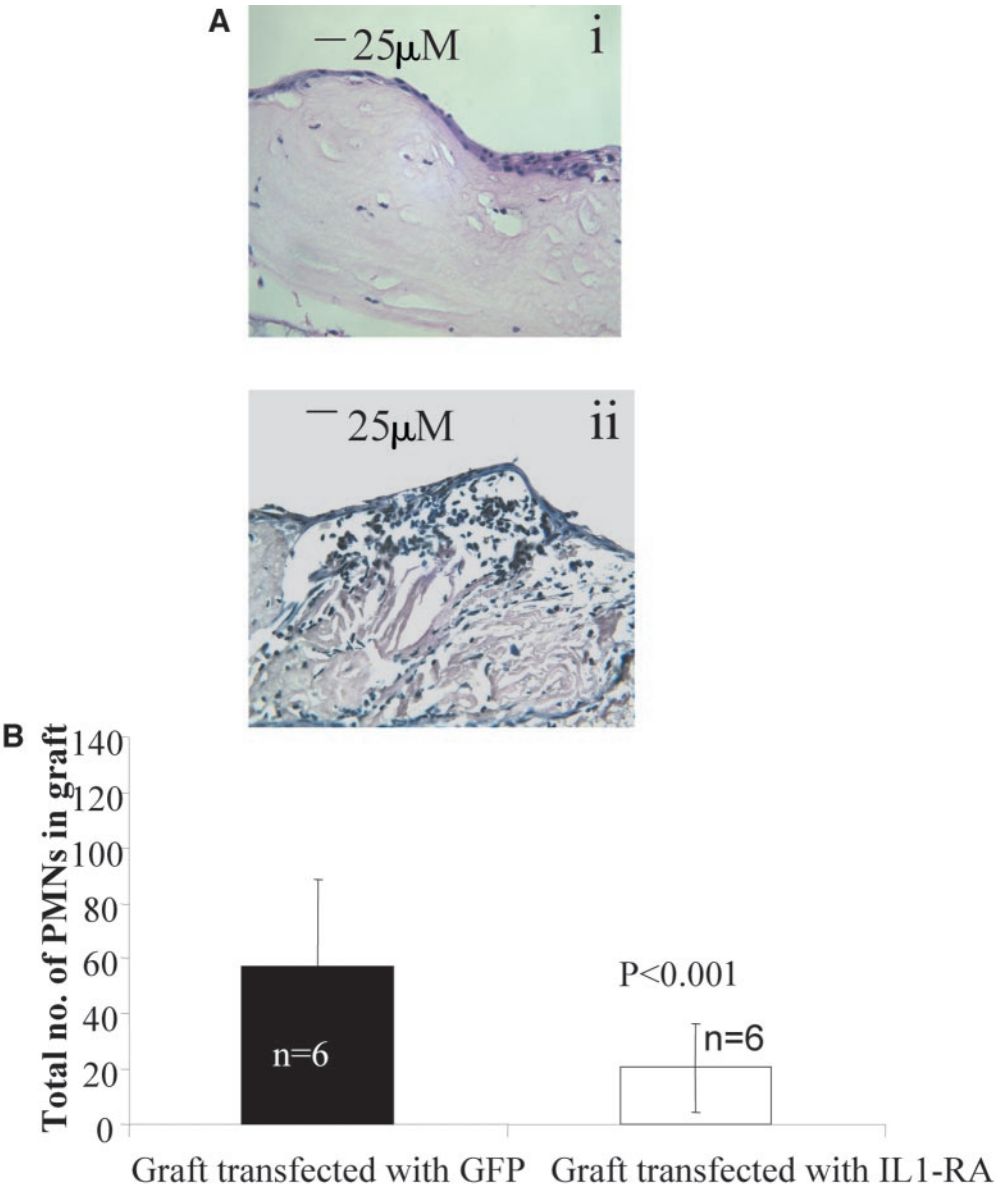

FIGURE 8. PMN infiltrated the grafts (3 days after engraftment onto corneas that had been injured 4 weeks prior) transfected with IL-1 RA+GFP or control GFP plasmid. A significant decrease was noted in the number of PMNs infiltrating into limbal grafts transfected with IL-1 RA (**A**, ii) compared with those grafts transfected with (**A**, i) GFP alone control plasmid (**B**; $P < 0.001$, $n = 6$). Data represent mean ± SD.

suppressing ICAM-1 expression on vascular endothelial cells,[18] whereas IL-1 does not increase expression of ICAM-1 in corneal epithelium.[19] The reduction noted in white blood cell infiltrate may be due to the IL-1 RA–induced reduction in VCAM-1, both on endothelial cells and on leukocytes, in that VCAM-1 has been noted on inflammatory cells of the macrophage-monocyte lineage in inflamed corneas.[20] IL-1 RA–transfected eyes show less neovascularization (Fig. 6) at 3 and 6 days but have similar neovascularization at 21 days. The effect of IL-1 RA treatment on neovascularization may be due to decreased leukocyte influx into the cornea. Downregulation of IL-1–induced leukocyte chemotaxis could indirectly result in less leukocyte expression of VEGF, which may, at least in part, modify the neovascular response.

The ability to target specific cytokines by gene expression not only allows specific targeting of the cytokine of interest but also obviates the requirement for multiple drops or injections of a readily degraded protein. This technique may be used in the future for transfection of beneficial growth factors or other as yet undetermined factors that may protect or expand the stem cell pool.

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