Enhanced Experimental Corneal Neovascularization along with Aberrant Angiogenic Factor Expression in the Absence of IL-1 Receptor Antagonist

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Purpose. To address the roles of the endogenously produced IL-1ra in the course of corneal neovascularization (CNV).

Methods. CNV was induced by alkali injury and compared in wild-type (WT), IL-1 receptor antagonist (ra) knockout (KO) mice and anti–IL-1ra antibody-treated WT mice 2 weeks after injury. Angiogenic factor expression and leukocyte accumulation in the early phase after injury were quantified by RT-PCR and immunohistochemical analysis, respectively.

Results. The mRNA expression of IL-1ra, IL-1α, and IL-1β was augmented, together with infiltration of F4/80$^+$ macrophages and Gr-1$^+$ neutrophils, in corneas after alkali injury. Intracorneally infiltrating macrophages, but not neutrophils, expressed IL-1ra. Compared with WT mice, either IL-1ra KO mice or anti–IL-1ra antibody-treated WT mice exhibited enhanced CNV 2 weeks after injury, as evidenced by enlarged CD31$^+$ areas. Concomitantly, the infiltration of F4/80$^+$ macrophages was more significantly enhanced in IL-1ra KO mice than in WT mice. Intraocular mRNA expression enhancement of vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) was greater in IL-1ra KO mice than in WT mice after injury. Moreover, IL-1α and IL-1β enhanced VEGF and iNOS expression by murine peritoneal macrophages.

Conclusions. IL-1ra KO exhibited enhanced alkali-induced CNV through enhanced intracorneal macrophage infiltration and increased expression of VEGF and iNOS. (*Invest Ophthalmol Vis Sci.* 2009;50:4761–4768) DOI:10.1167/iovs.08-2732

Corneal neovascularization (CNV) has various causes, among them corneal infection, misuse of contact lenses, chemical burns, and inflammation,[1–3] and it frequently leads to impaired vision. It is necessary to develop effective measures to prevent and treat CNV based on understanding of its pathogenesis at the cellular and molecular levels. In the early phase of corneal injury, preceding the occurrence of CNV, neutrophils and, to a lesser degree, macrophages infiltrate the cornea.[4–6] Infiltrating monocytes/macrophages are presumed to be a rich source of angiogenic factors and contribute to the development of neovascularization.[7–10] The recruitment of leukocytes to inflammatory sites is regulated by the coordinated action of chemokines and adhesion molecules.[11]

The proinflammatory factor interleukin (IL)-1 is produced by various kinds of cells, such as neutrophils, macrophages, and fibroblasts.[12] Two members of IL-1—IL-1α and IL-1β—exist[13] and exhibit a wide variety of biological actions, including induction of the expression of various growth factors, chemokines, and adhesion molecules.[14] Given this ability, it is reasonable to assume that IL-1 plays roles in various pathologic processes, including neovascularization and tissue repair. The IL-1 receptor antagonist (IL-1ra) can bind to IL-1 receptors with an affinity similar to that IL-1α and IL-1β but cannot deliver any intracellular signals, thereby acting as a potent antagonist against IL-1.[15]

We previously observed that IL-1α and IL-1β mRNA expression was enhanced in the early phase after alkali injury.[16] Our subsequent study demonstrated that IL-1ra expression was also enhanced in cornea after alkali injury. Biswas et al.[17] reported herpes simplex virus infection–induced CNV was diminished in IL-1ra transgenic mice, which produced a much larger amount of IL-1ra than in WT mice. Moreover, topical application of a pharmacological dose of IL-1ra can reduce alkali injury–induced CNV,[18] inhibit inflammatory cell infiltration into the cornea,[19] and promote corneal transplant survival by reducing allosensitization and inducing tolerance.[20–22] These observations have unraveled the protective effects of IL-1ra when it is given exogenously at a pharmacological dose, but it remains elusive on the roles of IL-1ra, which is endogenously produced in a lower amount than a pharmacologic dose. Mice deficient in a particular gene can frequently provide us with invaluable information on the endogenous role of the target gene. Therefore, to elucidate the role of IL-1ra endogenously expressed after alkali injury, we compared the molecular pathologic changes in a frequently used ocular neovascularization model, alkali injury-induced CNV,[4–6,23] in WT mice and mice deficient in IL-1ra.

Materials and Methods

Reagents and Antibodies

Recombinant murine IL-1α and IL-1β were obtained from R&D Systems (Minneapolis, MN). Rat anti–mouse F4/80 (clone A3–1) monoclonal antibody (mAb) was purchased from Serotec (Oxford, UK), and rat anti–mouse CD31 (MEC13.3) and purified rat anti–mouse-Ly-6G mAb (clone IA8) were purchased from BD PharMingen (San Diego, CA). Rabbit anti–IL-1ra (sc-25444) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Neutralizing anti–mouse IL-1ra antibody was prepared as described previously.[24] A mouse VEGF ELISA kit was supplied by R&D Systems (MMV00; Minneapolis, MN). Alexa Fluor

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Supported by the International Cooperative Program of Kanazawa University (NM); National Natural Science Foundation in China Grants NSFC 30572120 and 30771978; Jiangsu Natural Science Foundation Grant BK2006528; and China Postdoctoral Science Foundation Grant 2005038587 and Quing Lan Project (PL).

Submitted for publication August 15, 2008; revised November 29, 2008, and March 30, 2009; accepted July 27, 2009.

Disclosure: **P. Lu**, None; **L. Li**, None; **G. Liu**, None; **X. Zhang**, None; **N. Mukaida**, None

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(AF) 488 donkey anti–rat IgG (H+L) and AF594 donkey anti–rabbit IgG (H+L) were purchased from Invitrogen (Shanghai, China).

Mice

IL-1ra–deficient (KO) mice were generated and backcrossed to BALB/c mice for more than eight generations.[25] Pathogen-free BALB/c mice were obtained from Clea Japan and were designated as wild-type (WT) mice. Age- and sex-matched KO and WT mice were kept under specific pathogen-free conditions in groups of five and fed regular laboratory chow and water ad libitum. A 12-hour day/12-hour night cycle was maintained during the entire course of the study. All animal experiments were performed at the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center, Japan, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

Alkali-Induced Corneal Injury Model

Mice were anesthetized with intraperitoneal injection of 1.8% (vol/vol) tribromoethanol at a dose of 0.15 mL/10 g body weight. In our preliminary experiments, we observed that the removal of the corneal epithelium alone failed to induce corneal neovascularization, consistent with a previous report.[26] We further observed that alkali-induced cauterization of deepithelialized corneas caused significantly greater corneal neovascularization than deepithelialization or cauterization alone. Thus, we combined alkaline burn and epithelial denudation, as described. A 2-mm disc of filter paper saturated with 1 N NaOH was placed onto the right cornea of each mouse for 40 seconds, followed by extensive rinsing with 15 mL phosphate-buffered saline (PBS). The central area of the corneal epithelium, 2 mm in diameter, was removed using a corneal knife in a rotary motion parallel to the limbus by gently scraping over the corneal surface without injuring the underlying corneal stroma.[6,23] Erythromycin ophthalmic ointment was applied immediately after epithelial denudation. At the indicated time intervals (days 0, 2, 4, 7), mice were killed and the corneas were removed from both eyes. These corneas were immediately placed into RNA stabilization agent (RNALater; Qiagen, Tokyo, Japan) and kept at −86°C until total RNA extraction was performed. In another series of experiments, mice were killed at the indicated times (days 0, 2, 4, 7, 14) after alkali treatment, and both eyes were entirely removed from each animal. The eyes were snap-frozen in OCT compound for histologic analysis. In some experiments, the mice were injected intraperitoneally with 100 μg neutralizing anti–mouse IL-1ra[24] or isotype-matched immunoglobulin G (IgG) in 100 μL PBS twice daily from 1 day before to 7 days after alkali injury.

Biomicroscopic Examination

The eyes were examined under a surgical microsystem (MZ16; Leica, Wetzlar, Germany) 14 days after alkali injury. In brief, under anesthesia, photographs of the corneas were obtained using a digital camera linked to an operating microscope. Microscopic assessment was performed by two independent observers with no prior knowledge of the experimental procedures.

Immunohistochemical Analysis

Eight-micrometer-thick fixed cryosections were subjected to immunohistochemical staining. Endogenous peroxidases were quenched in 0.3% (vol/vol) hydrogen peroxide for 10 minutes. After washing with PBS, slides were incubated with blocking reagent for 20 minutes. For the detection of macrophages and granulocytes, the sections were incubated overnight at 4°C with rat anti–mouse F4/80 antibody (1 μg/mL) and purified rat anti–mouse-Ly-6G mAb (2.5 μg/mL), respectively. The sections were further incubated with biotin-conjugated rabbit anti–rat immunoglobulin antibody as the secondary antibody. To detect IL-1ra expression, the sections were incubated overnight at 4°C with rabbit anti–IL-1ra and were further incubated with biotin-conjugated goat anti–rabbit immunoglobulin antibody as the secondary antibody. The immune complexes were detected using an ABC kit and a DAB substrate kit from Vector Laboratories, Inc. (Burlingame, CA), according to the manufacturer's instructions. Slides were then counterstained with hematoxylin (Dako, Carpinteria, CA) and were mounted. The number of positive cells was counted in five randomly chosen fields of corneal sections from each animal at 200-fold magnification by an examiner with no prior knowledge of the experimental procedures. The number of positive cells per square millimeter was calculated.[6,23]

Enumeration of Corneal Neovascularization

Fixed cryosections (8 μm thick) were stained using anti–CD31 mAb (MEC13.3). The number and size of CNV were determined as described previously[6,23] by an examiner with no knowledge of the experimental procedures. Briefly, images were captured with a digital camera and imported into a graphics editing program (Photoshop, version 7.0; Adobe Systems, Mountain View, CA). Then the number of neovascular tubes per square millimeter and the proportion of CNV in the hot spots were determined using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), version 1.62 (National Institutes of Health, Bethesda, MD). Most sections were taken from the central region of the cornea. The numbers and areas of CNV were evaluated on at least two sections from each eye.

TABLE 1. Specific Sets of Primers and Conditions of PCR

Gene Names	Sequences	Product Size (bp)	Annealing Temperature (°C)	PCR Cycles
IL-1ra	(F) TACTGTGGACACCACCCTCA (R) TCTGCCTCCAAGCAAAAGAT	490	59	37
IL-1α	(F) TGGCCAAAGTTCCTGACTTGTTTG (R) CAGGCTATTTAACCAAGTGGTGCT	488	57	38
IL-1β	(F) CAAGGCCACAGGTATTTTGT (R) GAAATGCCACCTTTTGACAG	504	57	38
VEGF	(F) CTGCTGTACCTCCACCATGCCAAGT (R) CTGCAAGTACGTTCGTTTAACTCA	509	57	37
TGF-β	(F) CGGGGCGACCTGGGCACCATCCATGAC (R) CTGCTCCACCTTGGGCTTGCGACCCAC	405	57	37
TSP-1	(F) ACCAAAGCCTGCAAGAAAGA (R) ATGCCATTTCCACTGTAGCC	311	57	37
iNOS	(F) TGGGAATGGAGACTGTCCCAG (R) GGGATCTGAATGTGATGTTTG	360	58	38
β-actin	(F) TGTGATGGTGGGAATGGGTCAG (R) TTTGATGTCACGCACGATTTCC	514	55	25

F, forward primer; R, reverse primer.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from the corneas or cultured peritoneal macrophages (RNeasy Mini Kit; Qiagen), according to the manufacturer's instructions. RNA preparations were further treated with RNase-free DNase (DNase I; Life Technologies Inc., Gaithersburg, MD) to remove residual genomic DNA. Two micrograms of total RNA were reverse-transcribed at 42°C for 1 hour in 20 μL reaction mixture containing mouse Moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Qiagen). Serially twofold diluted cDNA was amplified for β-actin (Table 1) to evaluate the amount of transcribed cDNA. Then equal amounts of cDNA products were amplified for the target genes using the primers (Table 1) under the condition of denaturation at 94°C for 2 minutes, followed by the indicated cycles of 30 seconds at 94°C, 45 seconds at 55°C to 59°C, 1 minute at 72°C, and a final 10-minute extension step at 72°C (Table 1). Amplified PCR products were fractionated on a 1.0% agarose gel and visualized using ethidium bromide staining. Band intensities were measured with the aid of ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) software, and the ratios to β-actin were determined.

Murine Peritoneal Macrophages Isolation and Culture

Specific pathogen-free 8- to 10-week-old male BALB/c mice were injected intraperitoneally with 2 mL sterile 3% thioglycolate medium (Sigma-Aldrich, St. Louis, MO), and intraperitoneal macrophages were harvested 3 days later, as described previously.[23] The resultant cell preparation consisted of greater than 95% macrophages, as verified by flow cytometric analysis on the cell preparation immunostained with anti–F4/80 antibody. The cells were suspended in antibiotic-free RPMI 1640 medium containing 10% fetal bovine serum (FBS) and were incubated in a humidified incubator at 37°C in 5% CO_2 in 24-well cell culture plates (Nalge Nunc International Corp., Naperville, IL). Two hours later, nonadherent cells were removed, and the medium was replaced. The cells were then stimulated with the indicated concentrations of murine IL-1α or IL-1β for 12 hours. Total RNA was extracted from the cultured cells and subjected to RT-PCR, as described. For ELISA to detect VEGF production after IL-1α or IL-1β stimulation, murine macrophages were seeded onto 12-well plates at 5×10^5 cells/well. After adhesion, the cells were stimulated with the indicated concentrations of murine IL-1α or IL-1β for 24 hours in a 37°C incubator with 5% CO_2. Supernatants were collected with a mouse VEGF ELISA kit (R&D Systems) according to the manufacturer's instructions to determine VEGF concentrations.

Double-Color Immunofluorescence Analysis

Double-color immunofluorescence analysis was performed to determine the cells expressing IL-1ra. Briefly, the fixed cryosections (8-μm thick) were incubated with PBS containing 10% normal donkey serum and 1% BSA to reduce nonspecific reactions. Thereafter, the sections were incubated with a combination of rat anti–F/80 and rabbit anti–IL-1ra or a combination of rat anti–Ly-6G and rabbit anti–IL-1ra overnight at 4°C. After they were rinsed with PBS, the sections were incubated with a combination of Alexa Fluor 488 donkey anti–rat IgG and Alexa Fluor 594 donkey anti–rabbit IgG (1/100) for 40 minutes at room temperature in the dark. Finally, the sections were washed with PBS, and immunofluorescence was visualized in dual-channel mode with a fluorescence microscope (Olympus, Tokyo, Japan). Images

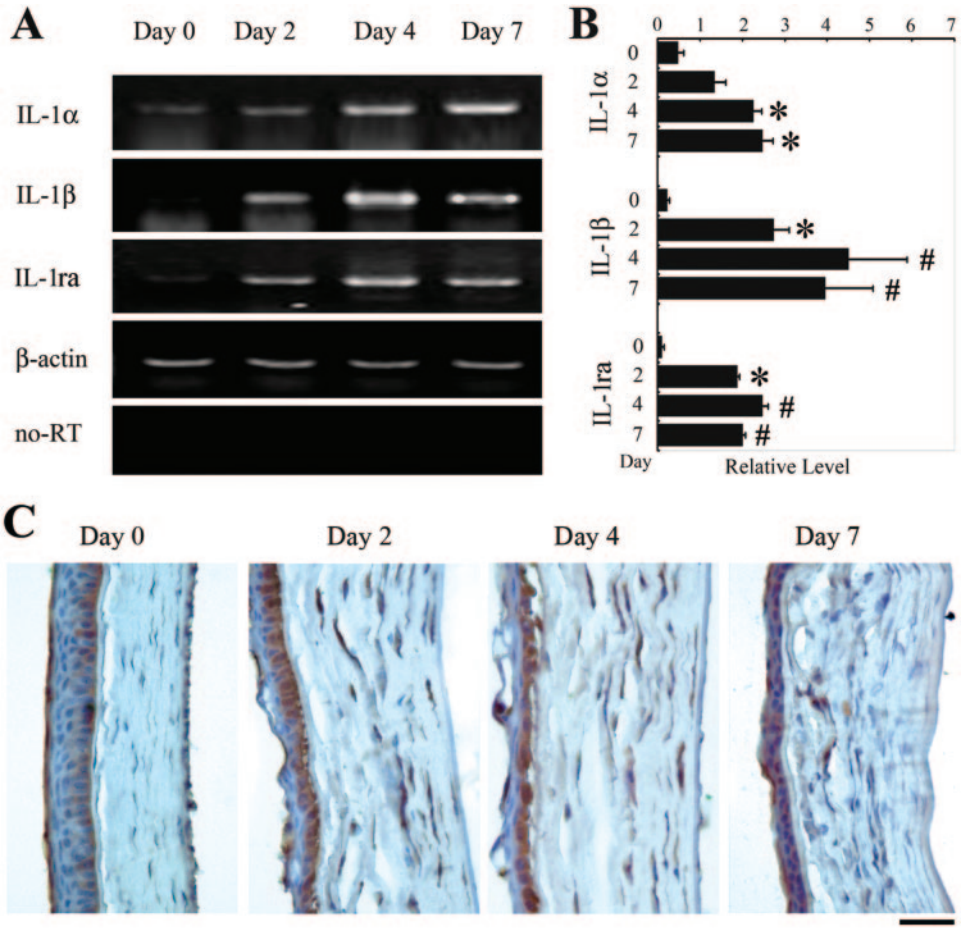

FIGURE 1. Expression of IL-1ra, IL-1α, and IL-1β in corneas after alkali injury. (**A**) Semiquantitative RT-PCR to assess mRNA expression of IL-1ra, IL-1α, and IL-1β. Corneas were harvested at the indicated time points, and five corneas at each time point were pooled to extract total RNAs. RT-PCR was performed using the obtained total RNAs. Analysis for β-actin expression was performed without reverse transcriptase treatment, and the results are shown as no RT-PCR in the lowest. (**B**) The ratios of IL-1ra, IL-1α, and IL-1β to β-actin were determined. All values represent mean ± SEM of three to five independent measurements. $^*P < 0.05$ and $^\#P < 0.01$, compared with untreated. (**C**) Whole eyes were obtained at 0, 2, 4, and 7 days after alkali injury and were processed for immunohistochemical analysis using an anti–IL-1ra antibody. Representative results from five animals are shown. Original magnification, ×200. Scale bar, 50 μm.

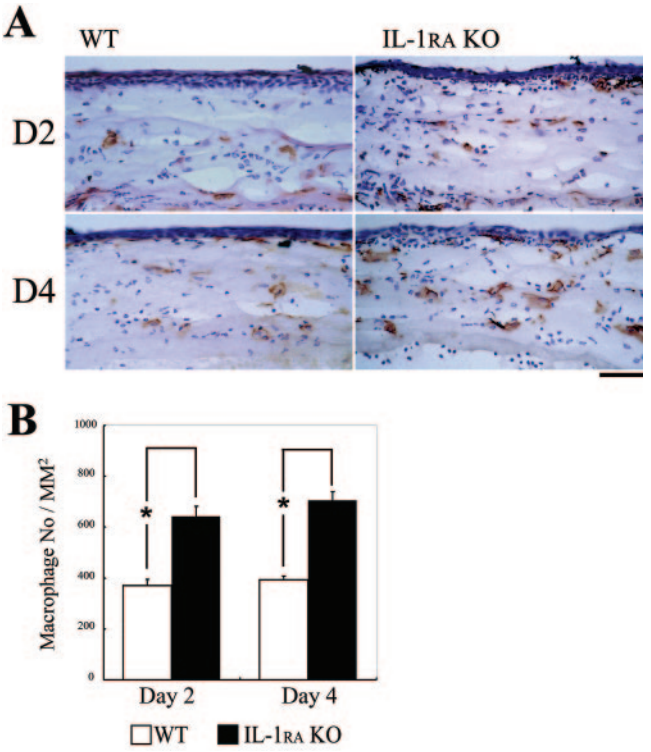

FIGURE 2. Macrophage recruitment in the injured corneas of WT and IL-1ra KO mice. (**A**) Corneal tissues from WT mice (*left*) and IL-1ra KO (*right*) mice were obtained 2 days (*upper*) and 4 days (*lower*) after injury. Tissues were stained with rat anti–F4/80 mAb. (**B**) The numbers of infiltrated F4/80$^+$ macrophages were determined, and the mean ± SEM is shown here ($n = 5$). $^*P < 0.05$.

were processed graphics software (Photoshop, version 7.0; Adobe, Mountain View, CA).

Statistical Analysis

Mean ± SEM was calculated for all parameters determined in the study. Data were analyzed statistically with one-way analysis of variance (ANOVA) or two-tailed Student's *t*-test. $P < 0.05$ was accepted as statistically significant.

RESULTS

IL-1ra, IL-1α, and IL-1β Expression after Alkali-Induced Corneal Injury

We first examined the gene expression of IL-1ra, IL-1α, and IL-1β in the corneas after alkali injury using RT-PCR. mRNA expression of IL-1ra, IL-1α, and IL-1β was detected in untreated eyes, but their expression was increased progressively in eyes starting 2 days after injury and thereafter (Figs. 1A, 1B). IL-1ra protein was detectable in untreated WT mouse corneal epithelial cells (Fig. 1C). After alkali injury, especially at days 2 to 4, IL-1ra immunoreactivities were detected primarily in infiltrating cells and repopulating epithelial cells (Fig. 1C). These observations indicated that alkali injury induced IL-1ra expression by infiltrating cells and repopulating epithelial cells.

Enhanced Macrophage Infiltration at the Wound Sites in IL-1ra KO Mice

We previously observed that Gr-1$^+$ granulocytes and F4/80$^+$ macrophages infiltrated the injured cornea, reaching their peak levels 2 to 4 days after injury in WT mice.[6,23] IL-1 signal can enhance the expression of chemokines, which are vital to macrophage accumulation, and IL-1ra can potently antagonize the various actions of IL-1s by competing the binding of IL-1s to

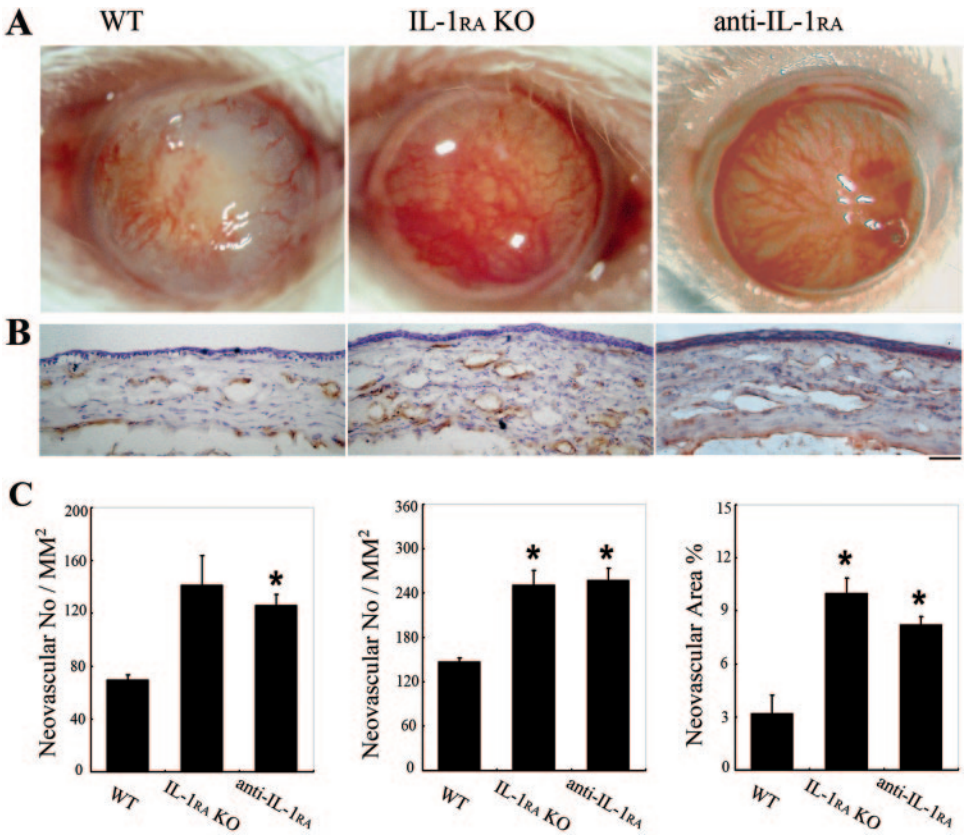

FIGURE 3. Alkali injury–induced CNV. (**A**) Macroscopic appearance of WT, IL-1ra KO, and anti–IL-1ra antibody-treated WT mouse eyes 2 weeks after alkali injury. (**B**) Corneal tissues were obtained 2 weeks after injury from WT, IL-1ra KO, and anti–IL-1ra antibody-treated WT mice. Tissues were immunostained with anti-CD31 antibody, and representative results from five to eight animals are shown here. Original magnifications, ×200. Scale bar, 50 μm. (**C**) CNV numbers per square millimeter in whole section (*left*), CNV numbers per square millimeter in hot spots (*middle*), and percentage CNV areas in hot spots (*right*) were determined from the corneas obtained from WT, KO, and anti–IL-1ra antibody-treated mice 2 weeks after injury. Each value represents mean ± SEM ($n = 5$–8 animals). $^*P < 0.05$ compared with WT mice.

the specific receptors.[13,15] These facts prompted us to assume that the lack of IL-1ra enhanced IL-1 signaling and subsequently augmented leukocyte infiltration into the corneas. To prove this hypothesis, we examined leukocyte infiltration into the injured corneas of IL-1ra KO mice. Neither F4/80$^+$ macrophages nor Gr-1$^+$ granulocytes were present in the untreated corneas of WT and IL-1ra KO mice (data not shown). Gr-1$^+$ granulocytes infiltrated corneas to a similar extent after injury in WT and IL-1ra KO mice (data not shown). On the contrary, F4/80$^+$ macrophage infiltration was markedly augmented in IL-1ra KO mice compared with WT mice (Figs. 2A, 2B). These observations would indicate that IL-1ra deficiency enhanced alkali-induced infiltration of macrophages but not of neutrophils.

Enhanced Alkali Injury–Induced CNV in IL-1ra KO Mice and Anti–IL-1ra Antibody-Treated WT Mice

We next examined the final sequel induced by alkali injury, CNV, in IL-1ra KO and WT mice. CNV was macroscopically evident in WT mice 2 weeks after injury, as previously reported.[6,23] At this time, macroscopic CNV had increased in IL-1ra KO mice compared with WT mice (Fig. 3A). An immunohistochemical analysis using anti–CD31 antibody further demonstrated that vascular areas increased in IL-1ra KO mice to a greater extent than in WT mice (Figs. 3B, 3C). When WT mice were treated with neutralizing anti–IL-1ra antibody, alkali-induced CNV was significantly greater 2 weeks after the injury (Fig. 3). In contrast, isotype-matched control immunoglobulin G (IgG) failed to change the alkali-induced CNV (data not shown). These observations imply that the absence of endogenous IL-1ra enhanced alkali-induced CNV, together with increased macrophage infiltration.

Enhanced Proangiogenic Molecule Expression in IL-1ra KO Mice after Alkali Injury

Monocytes/macrophages can be a rich source of proangiogenic and antiangiogenic factors,[7–10,23,27] and the balance between these two distinct sets of factors can determine the outcome of angiogenesis processes in various situations. Hence, we examined the mRNA expression of proangiogenic and antiangiogenic factors in the corneas after injury. Among the proangiogenic factors we detected, the mRNA expression of VEGF and iNOS was augmented in WT mice in the early phase after injury and further increased in IL-1ra KO mice (Figs. 4A, 4B). The intraocular mRNA expression of the antiangiogenic factor thrombospondin (TSP)-1 increased to a similar extent in WT and IL-1ra KO mice (Figs. 4A, 4B). Similarly, intraocular VEGF mRNA expression was augmented by treatment with anti–IL-1ra antibody compared with control antibody treatment (Fig. 4C). These observations imply that the absence of IL-1ra increased the expression of the proangiogenic factors VEGF and iNOS without affecting antiangiogenic factor expression, thereby tipping the balance to promote angiogenesis.

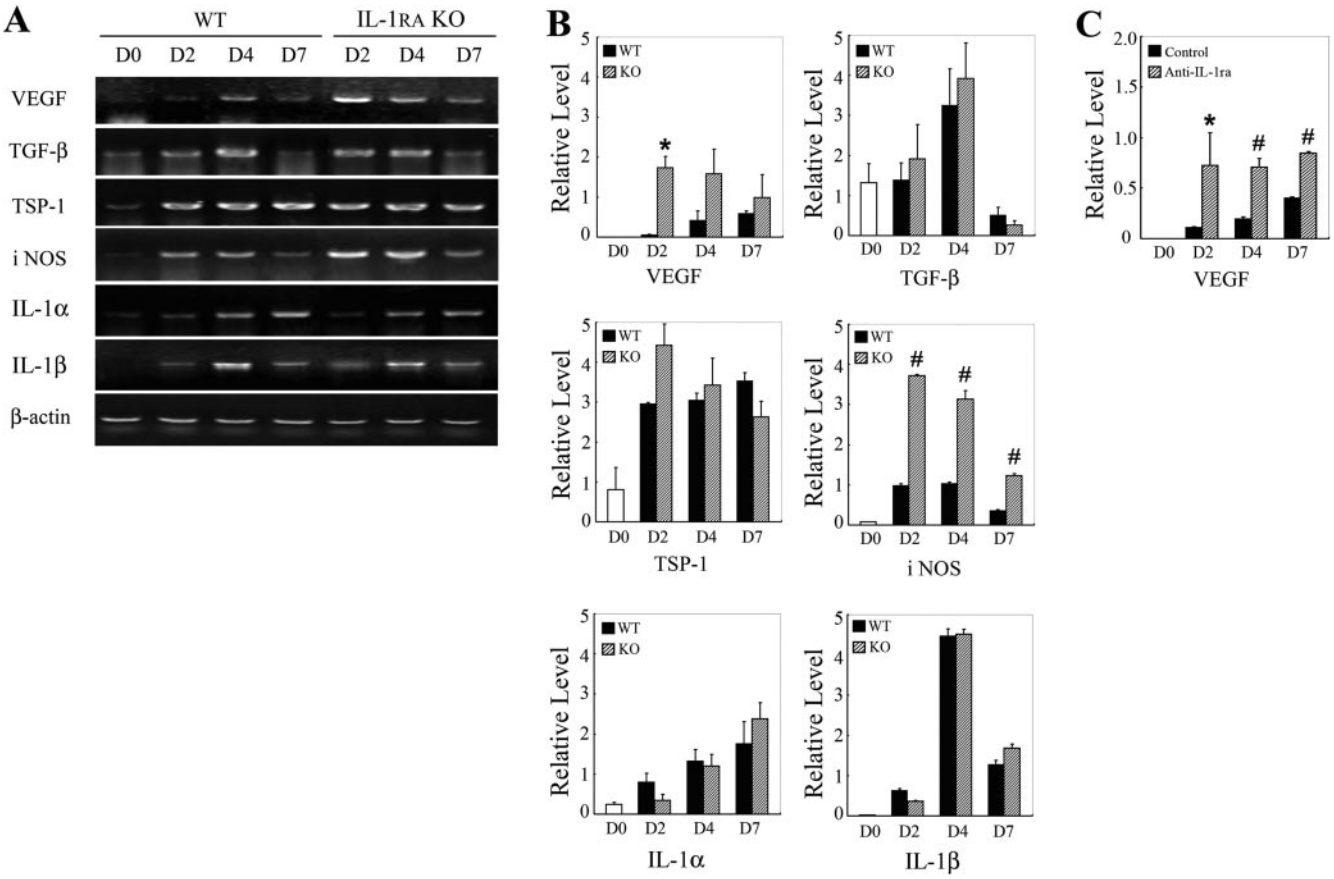

FIGURE 4. RT-PCR analysis of angiogenic and IL-1 gene expression in the injured corneas of WT and IL-1ra KO mice. (**A**) Representative results from three independent experiments of RT-PCR. (**B**) Ratios of VEGF, TGF-β, TSP-1, iNOS, IL-1α, and IL-1β to β-actin of alkali-injured WT (*black bars*) and IL-1ra KO mice (*hatched bars*) and untreated mice (*open bars*) were determined by RT-PCR at the indicated time intervals after alkali injury. (**C**) Ratios of VEGF to β-actin of alkali-injured IgG-treated (*black bars*) and anti–IL-1ra–treated mice (*hatched bars*) and untreated mice (*open bars*) were determined by RT-PCR at the indicated time intervals after alkali injury. All values represent mean ± SEM (n = 3–5 animals). $^*P < 0.05$ and $^\#P < 0.01$; WT versus KO mice or IgG versus anti–IL-1ra treated.

Enhanced VEGF Expression by Murine Peritoneal Macrophages with IL-1 Stimulation

Intraocular IL-1α and IL-1β gene expression was increased to a similar extent in IL-1ra KO and WT mice (Figs. 4A, 4B). Considering that the balance between IL-1 and IL-1ra determines the magnitude of IL-1–mediated signals, these observations indicate that the IL-1 signal pathway was augmented in IL-1ra KO mice. Moreover, because IL-1 can increase VEGF expression in various types of cells,[28–30] we next examined the effects of exogenous IL-1α and IL-1β on VEGF expression by murine peritoneal macrophages. Both IL-1α and IL-1β markedly increased the mRNA expression of VEGF by peritoneal macrophages (Figs. 5A, 5B). IL-1α and IL-1β consistently increased VEGF secretion by macrophages (Fig. 5C). Furthermore, IL-1α stimulation markedly increased the mRNA expression of iNOS by peritoneal macrophages (Figs. 5A, 5B). These observations indicate that the enhanced IL-1 signal pathway in IL-1ra KO mice can augment the expression of the proangiogenic factors VEGF and iNOS by macrophages.

Simultaneous IL-1ra Expression by Intracorneally Infiltrating Macrophages

Our immunohistochemical analysis revealed that the intracorneally infiltrated cells produced IL-1ra (Fig. 1C). We next examined whether intraocularly infiltrating macrophages or neutrophils expressed IL-1ra. A double-color immunofluorescence analysis demonstrated IL-1ra protein expression in a substantial proportion of macrophages, but not in neutrophils (Fig. 6). IL-1ra is a competitive inhibitor of IL-1,[13,15] which can induce the expression of the major proangiogenic molecules VEGF and iNOS. The infiltration of IL-1ra–expressing macrophages can dampen alkali-induced CNV by counteracting a capacity of IL-1 to induce VEGF and iNOS. Thus, the absence of macrophage-derived IL-1ra may result in increased expression of VEGF and iNOS and eventually may accelerate CNV.

Discussion

Tissue injury induces the expression of various growth factors, cytokines, and chemokines, all of which contribute to tissue repair in a coordinated manner. We previously observed that IL-1 expression increased in corneas treated with alkali.[16] IL-1ra is a member of the IL-1 family and can bind to IL-1 receptors with an affinity similar to that of IL-1α and IL-1β, but it cannot induce signal transduction.[13,15] IL-1s and IL-1ra are expressed simultaneously at wound sites.[31,32] Therefore, the balance between IL-1ra and IL-1 levels in local tissues can determine the physiologic or pathophysiologic effects of IL-1 family members as a whole. Because of its potent antagonistic

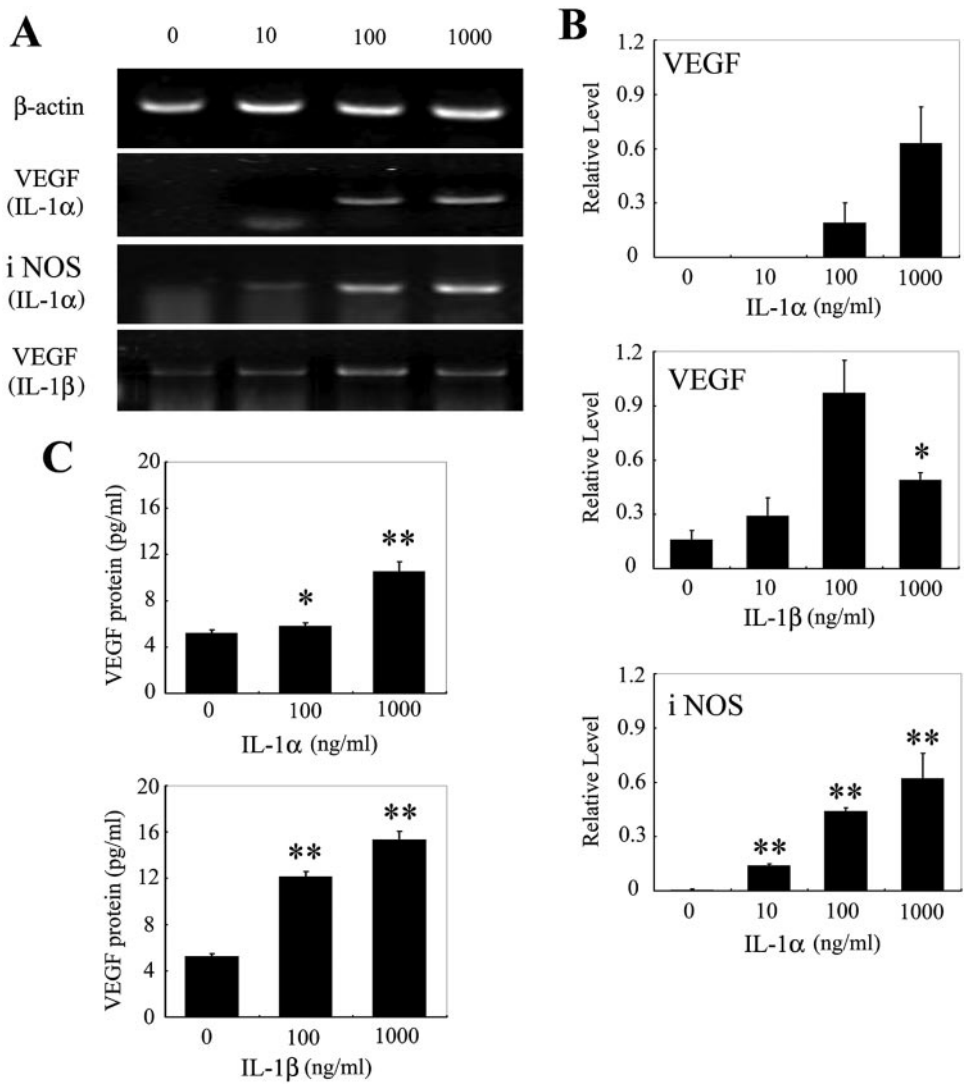

Figure 5. Effects of IL-1α or IL-1β on angiogenic factor expression by murine peritoneal macrophages. Peritoneal macrophages were incubated with the indicated concentration of recombinant IL-1 for 12 hours. RT-PCR was performed on macrophages. Representative results from three independent experiments are shown (**A**). Ratios of anti-angiogenic factors to β-actin were calculated and are shown (**B**). Each value represents the mean ± SEM (n = 3). (**C**) Murine macrophages were stimulated with the indicated concentration of IL-1α or IL-1β for 24 hours. VEGF production in the supernatants was detected with ELISA. Representative results from three independent experiments are shown. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with untreated.

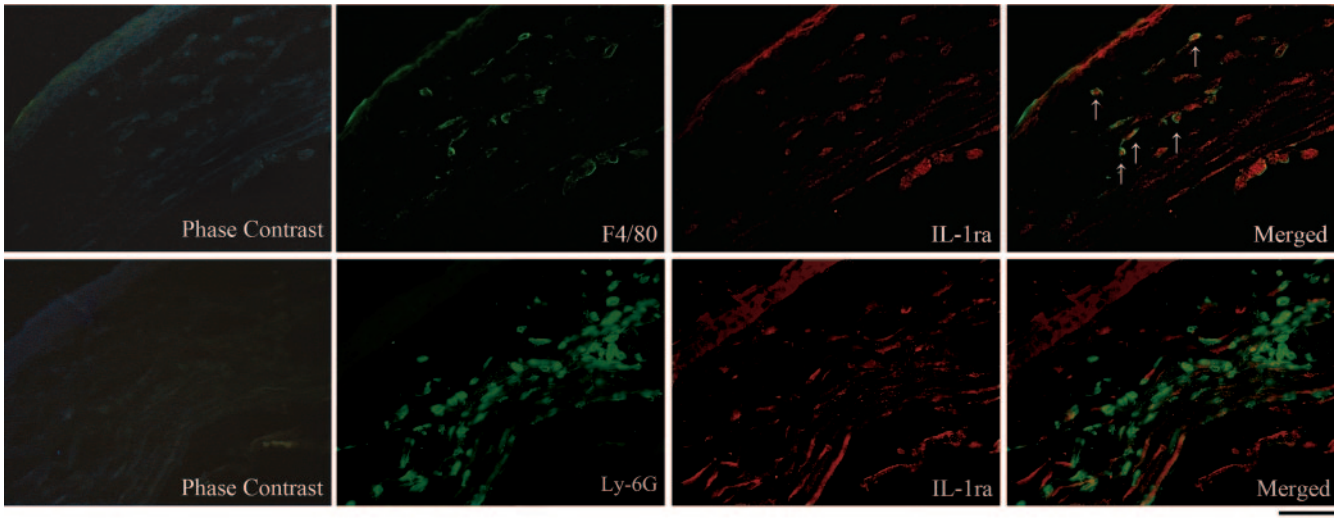

FIGURE 6. Double-color immunofluorescence analysis of IL-1ra–expressing cells. Corneas were obtained from WT mice 2 or 4 days after injury. Samples were immunostained with a combination of anti-F4/80 and anti–IL-1ra antibodies (*upper*) or a combination of anti–Ly-6G and anti–IL-1ra antibodies (*lower*), and were examined using fluorescence microscopy (original magnification, ×400). *Left*: phase-contrast images. *Right*: signals were digitally merged. (*arrows*) Double positively stained cells. Representative results from three independent experiments are shown. Scale bar, 50 μm.

activities against IL-1s, IL-1ra administration is presumed to be effective in preventing tissue damage. Indeed, topical administration of recombinant IL-1ra can inhibit CNV.[18] However, the role of endogenous IL-1ra in CNV remains unclear. We treated wild-type and IL-1ra KO mice with alkali and observed that CNV was more severe in IL-1ra KO mice. To exclude the possibility that IL-1ra gene ablation may cause additional secondary effects, we administered anti–IL-1ra antibody to WT mice during the course of alkali injury. Antibody-treated WT mice exhibited CNV phenotypes similar to those of IL-1ra KO mice, indicating that aggravated CNV in IL-1ra KO mice was caused by the direct effects of IL-1ra deficiency. These observations further implicate IL-1ra as a potent endogenous antiangiogenic factor in alkali-induced CNV.

Normal corneas lack any vasculature, and physiological corneal avascularity is maintained by the net balance between proangiogenic and antiangiogenic factors.[33–36] Alkali treatment increased the expression of VEGF, TGF-β, and iNOS, which play crucial roles in ocular neovascularization.[37,38] Among these proangiogenic molecules, VEGF and iNOS expression further increased in IL-1ra KO mice. The expression of a potent antiangiogenic factor, TSP-1, increased to a similar extent in WT and IL-1ra KO mice. Thus, it is plausible that the absence of IL-1ra augmented the expression of proangiogenic molecules, thereby accelerating neovascularization.

IL-1 profoundly affects a number of functions of endothelial cells, such as the production of proinflammatory cytokines, including IL-6, TNF-α, and IL-8, and the expression of adhesion molecules[39] because these cells expressed a large number of high-affinity IL-1 receptors. IL-1ra is expressed in human endothelial cells and atherosclerotic lesions[40,41] and is presumed to exhibit inhibitory effects on the various functions of endothelial cells.[42] Thus, it is likely that the absence of IL-1ra can further directly intensify IL-1–mediated endothelial cell activation and subsequent CNV development.

IL-1 can induce the migration of leukocytes, including macrophages, by enhancing the production of chemokines and the expression of adhesion molecules by endothelial cells. IL-1ra can also antagonize the activities of IL-1.[13] We recently observed that IL-1ra KO mice exhibited neutrophil and macrophage infiltration at skin wound sites to a greater extent than WT mice.[12] In this CNV model, however, the infiltration of macrophages but not neutrophils was exaggerated in IL-1ra KO mice compared with WT mice. These differences may arise from the differences in the anatomic structures between skin and cornea.

Macrophages can be proangiogenic in ocular neovascularization[7–10] by producing VEGF and iNOS,[43–45] key molecules for ocular neovascularization.[37,38] Moreover, Ambati et al. [4,5] reported that corneal neovascularization and VEGF production were suppressed in mice lacking CCR2 and CCR5, the specific chemokine receptors expressed by macrophages. We observed that CX3CR1-expressing macrophages can dampen alkali-induced CNV by producing antiangiogenic factors such as thrombospondin (TSP).[23] Geissmann et al.[46] proposed the dichotomy of macrophages based on the expression levels of CCR2 and CX3CR1. Supporting this assumption, CX3CL1, a ligand for CX3CR1, can induce TSP but not VEGF expression by macrophages in a CX3CR1-dependent manner, whereas CCL2, a ligand for CCR2, can enhance VEGF but not TSP expression by macrophages in a CCR2-dependent manner.[23] IL-1ra can counteract various activities of IL-1s, including the induction of VEGF and iNOS expression by macrophages, and eventually can exhibit antiangiogenic activities. Consistent with previous reports that IL-1ra can be produced by macrophages,[47,48] some but not all F4/80$^+$ intracorneally infiltrating macrophages expressed IL-1ra. Thus, it is tempting to speculate that CX3CR1-expressing macrophages can also produce IL-1ra, along with TSPs, thereby preventing the development of CNV. If so, the absence of macrophage-derived IL-1ra may augment IL-1–mediated signals and eventually enhance intraocular expression of the major proangiogenic molecules VEGF and iNOS, thereby promoting CNV.

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