Losartan Attenuates Scar Formation in Filtering Bleb After Trabeculectomy

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Purpose. To examine the effects of losartan on scar formation after trabeculectomy and on fibrotic changes of human Tenon's fibroblasts (HTFs).

Methods. Trabeculectomy was performed on New Zealand rabbits. They were randomized to receive one of the following treatments: 0.9% normal saline, mitomycin-C, or one of the three doses of losartan. Bleb morphology, IOP, and histopathology examination were performed. Primary cultured HTFs were treated with losartan or vehicle, with or without angiotensin II (Ang II). Cell proliferation was assessed by Cell Counting Kit-8 assay, and cell migration was detected by scratch wound and transwell assay. Transdifferentiation was evaluated through the expression of α-smooth muscle actin (α-SMA) by immunofluorescence, real-time PCR, and Western blot. The expression of fibronectin (FN) was evaluated by real-time PCR and Western blot.

Results. An amount of 5 mg/mL of losartan subconjunctival injection significantly decreased IOP postoperatively and attenuated wound healing of the filtering bleb in the rabbit model. Immunostaining results showed less myofibroblast and collagen deposition around the bleb area in the losartan-treated eyes. Losartan (10^{-5} M) in vitro significantly attenuated Ang II's stimulatory effects on proliferation and migration of HTFs. Expressions of α-SMA and FN in these cells were also decreased by losartan pretreatment.

Conclusions. Losartan attenuates scar formation of filtering bleb after trabeculectomy likely via decreasing proliferation, migration, transdifferentiation, and extracellular matrix deposition of Tenon's fibroblasts. These results indicate that losartan may be an effective therapeutic agent in preventing bleb scar formation and in improving surgical outcome after trabeculectomy.

Keywords: losartan, renin-angiotensin system, trabeculectomy, human Tenon's fibroblasts, fibrosis

Scar formation of filtering bleb after trabeculectomy is a multifactorial process including the progressive transdifferentiation of human Tenon's fibroblasts (HTFs) into myofibroblasts.[1] HTFs, physiologically inactive in the human Tenon's capsule, are the main component of scar formation. After surgery or injury, activated HTFs proliferate and migrate toward the wound site within as early as 24 hours.[2] Transdifferentiation from HTFs into myofibroblasts is the key event in bleb scarring. Myofibroblasts are characterized by a high expression level of α–smooth muscle actin (α-SMA) that is insufficient in fibroblasts, and with stress fibers in plasma, the presence of ED-A fibronectin and gap junctions.[3] The subsequent process following transdifferentiation contains the exhibition of an abundant endoplasmic reticulum and Golgi associated with the synthesis and secretion of extracellular matrix (ECM) including collagen type I, type III, and fibronectin (FN).[4,5] The phenotypes of persistent myofibroblasts and ECM synthesis contribute to wound contraction and closure.[3] Different cytokines are involved in the fibrosis process, such as TGF-β, VEGF, and platelet-derived growth factor.[1]

We previously found that angiotensin II (Ang II) treatment in HTFs induced cell proliferation, migration, and transdifferentiated to myofibroblasts with the upregulation of FN expression.[6] Angiotensin II, one of the most important renin-angiotensin-system components, plays a critical role in the regulation of blood pressure. Recent investigations focused on its impact on tissue remodeling and fibrogenesis.[7–9] Angiotensin II mainly exerts its function through two receptor subtypes, the Ang II type 1 receptor (AT1R) and type 2 receptor (AT2R). The expression of AT1R and AT2R has been successfully detected in the eye.[10,11] Our previous immunohistochemical results also showed both AT1R and AT2R are localized to the Tenon's fibroblasts.[6]

There is growing evidence that AT1R blockers (ARBs) play an important role in antifibrosis.[12–15] We are interested in losartan, the selective AT1R orally active antagonist, because it is widely used in the management of blood pressure and heart failure. Losartan binds with high affinity and specificity to the AT1R with a slow dissociation rate, and is 30,000-fold more selective for the AT1R than for AT2R. What is more, losartan was shown to possess an in vivo antifibrotic effect in fibrosis-related diseases, such as Marfan syndrome and colorectal fibrosis.[16–18] These studies indicate that AT1R antagonist may contribute to antifibrotic therapy. We observed the upregulation of AT1R synchronized with Ang II after trabeculectomy,[6] suggesting that Ang II may be involved in the fibrotic process mediated by AT1R. Therefore, we hypothesized that AT1R antagonist has a therapeutic effect on scar formation of filtering

Table. Animal Treatment

Group	Treatment
0.9% NS	0.1 mL, subconjunctival injection after surgery and on POD 1, 2, 3, 5, and 7
MMC	0.4 mg/mL, soaking scleral flaps for 2 min then rinsing with 0.9% NS
Losartan, 1, 5, and 10 mg/mL	0.1 mL, subconjunctival injection after surgery on POD 1, 2, 3, 5, and 7

bleb. The aim of this study was to examine the effects of losartan on scar formation in vitro and in vivo.

Materials and Methods

All animal procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institute Animal Use and Care Committee. The process of obtaining human Tenon's capsule tissue for cell culture experiments was approved by the hospital Ethics Review Board and adhered to the Declaration of Helsinki.

Animal Treatment

New Zealand rabbits, 3 to 5 months old and weighing 1.5 to 2.0 kg, were purchased from the Experimental Animal Center of Shanghai General Hospital (Shanghai, China) and were acclimatized for 1 week before experiments. Thirty rabbits were randomized to one of five treatment groups: the 0.9% normal saline (NS) control (NC) group, the mitomycin-C (MMC) treatment control group, and three different concentrations of losartan-treatment groups (LOS) ($n = 6$). Intraocular pressure of the right eye was measured by Tono-pen (Reichert, Depew, NY, USA) before surgery (baseline IOP). Standard trabeculectomy was performed by one surgeon (X.K.) on the right eyes of all rabbits. As summarized in the Table, rabbits in the NC group were treated with 0.1 mL 0.9% NS by subconjunctival injection immediately after surgery and on postoperative day (POD) 1, 2, 3, 5, and 7. Rabbits in the MMC group were treated with 0.4 mg/mL MMC soaking the scleral flap for 2 minutes during surgery and then washed with 0.9% NS. Rabbits in the LOS groups received subconjunctival injection with 0.1 mL of 1, 5, or 10 mg/mL of losartan immediately after surgery and on POD 1, 2, 3, 5, and 7. Bleb appearance was recorded by photographs and IOP was measured at indicated days after surgery. All rabbits were euthanized on POD 28.

Surgical Procedure

Trabeculectomy was performed under general anesthesia with 846 mistura (0.2 mL/kg; Shengda Co. Ltd., Jilin, China) and local anesthesia with oxybuprocaine eye drops. A peritomy at 5 mm above limbus was performed to form a limbus-based conjunctival flap. A rectangular scleral flap was performed and carefully dissected. An entry into the anterior chamber was made at the surgical limbus and a peripheral iridectomy was performed. No suture was made in the scleral and conjunctival flap to avoid excessive scarring. Subconjunctival drug treatment was performed after surgery.

Histologic Examinations

The eyes were enucleated and fixed with formaldehyde for 48 hours. Samples were dehydrated and embedded in paraffin. Serial sections of 4-μm thick were cut and dehydrated. Sections were stained with hematoxylin-eosin for general histologic examinations, with immunohistochemistry of α-SMA at the Tenon's capsule of surgical site for myofibroblast evaluation and with Masson stain for examination of ECM deposition. Images of each group were taken from five independent fields of the bleb areas in Tenon's capsules and Image J v2.1.4.7 (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used for quantification. The percentage of primary antibody-positive fibroblasts of each image was calculated relative to the number of total fibroblasts.

Cell Culture

Human Tenon's explants were obtained from patients during cataract surgery. These subjects, one male and two females, between 47 and 65 years of age, did not have a prior history of glaucoma or ocular surgery. Primary HTFs were generated as an expansion culture of the human Tenon's explants and were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) with 10% heat-inactivated fetal bovine serum (FBS; Gibco Life Technologies, Karlsruhe, Germany), 100 U/mL penicillin, and 100 mg/mL streptomycin (Biochrom, Berlin, Germany) in 5% CO_2 at 37°C. Cells were maintained in the logarithmic growth phase. Cells from generations 5 to 10 were used for the experiments. Within each experiment, the cells were of the same line and from the same passage. Cells were incubated to a subconfluent status (80% confluence) and starved in serum-free DMEM for 24 hours before experiments.

Cell Proliferation Analysis

To investigate the effect of losartan on HTF proliferation in vitro, we treated cells with Ang II (10^{-7} M) and different concentrations of losartan for 24 hours. Cell proliferation was determined by Cell Counting Kit-8 (CCK-8; Dojindo, Molecular Technologies, Inc., Gaithersburg, MD, USA). Human Tenon's fibroblasts were plated in 96-well plates at a density of 5000 per well (100 μL) and cultured in growth medium with Ang II (10^{-7} M; Sigma-Aldrich Corp., St. Louis, MO, USA) or with different concentrations of losartan (10^{-7} M–10^{-4} M; Selleck Chem, Houston, TX, USA) for 24 hours. Cell proliferation was assessed according to the protocol of the kit. In addition, HTFs were pretreated with 10^{-5} M of losartan for 1 hour and then with Ang II for 24 hours of treatment. Cell proliferation was examined by CCK-8.

Scratch Wound Assay

To evaluate cell mobility, an in vitro scratch wound assay was performed. When HTFs (initial density = 5×10^6 /well) in a six-well plate reached 90% confluence, a single scratch was created in the center of the cell monolayer by gently scraping the attached cells with a sterile 1-mL micropipette tip. Then cells were immediately placed in serum-free media with 10^{-7} M of Ang II with or without losartan (10^{-5} M) pretreatment. Human Tenon's fibroblasts were incubated with serum-free media so that they stopped proliferating and remained in the G1 or G0 stage of the cell cycle.[19] The increasing numbers of cells into the scratch over time were mostly due to cell migration. Migration of cells into the denuded areas was measured by quantifying bright-field images taken at 0, 12, and 24 hours after scratching. Images of each group at each time point were taken from five independent fields of the scratched areas and Image J v2.1.4.7 was used for quantification.

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Transwell Migration Assay

Human Tenon's fibroblasts were trypsinized and resuspended at a concentration of 2.5×10^5/mL in FBS-free DMEM. Media (500 μL) containing Ang II (10^{-7} M) with or without 1 hour of losartan pretreatment was added to a 24-well plate, and an 8-μm pore size insert (BD Falcon, Franklin Lakes, NJ, USA) was added to the wells before the cells (150 μL) were placed inside the insert. After a 24-hour incubation, the under surface was gently rinsed with PBS and stained with 0.25% (wt/vol) cresyl violet (Sigma-Aldrich Corp.) for 15 minutes, rinsed again with sterile water, and allowed to dry. The inserts were viewed under a light microscope and the numbers of cells/field in five randomly chosen fields were counted at ×100 magnification. Images of ×200 magnification were taken for cell phenotype record.

Immunofluorescence

To characterize the effect on transdifferentiation by losartan, we investigated the myofibroblast marker α-SMA, which was absent in fibroblasts. As demonstrated by immunofluorescence, cells cultured in Ang II showed intense staining for α-SMA, indicating a transdifferentiation from fibroblasts to myofibroblasts. Human Tenon's fibroblasts were seeded on coverslips in a 24-well plate with 48-hour incubation of Ang II (10^{-7} M) with or without losartan pretreatment. Cells were fixed in cold 4% paraformaldehyde for 15 minutes, permeabilized in 0.3% Triton X-100 for 5 minutes, blocked in 2% normal goat serum (Jackson-Immuno, Hamburg, Germany) for 1 hour, and conjugated with primary antibody against α-SMA (1:500; Sigma-Aldrich Corp.) overnight at 4°C. Negative controls were incubated with PBS replacing the primary antibody. After incubation with fluorescein isothiocyanate (FITC)-labeled secondary antibody for 1 h at room temperature, coverslips were stained with 4′,6-diamidino-2-phenylindole (DAPI) and observed with a fluorescence microscope (Olympus, Tokyo, Japan).

Real-Time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Afterward, cDNAs were synthesized using the PrimeScript RT Reagent Kit (RR036; Takara, Otsu, Shiga, Japan) and then diluted 10-fold in H_2O before their use in semi-quantitative real-time PCR reactions that contained 5 μL SYBR Premix Ex Taq (Takara), 0.2 μL forward primer, 0.2 μL reverse primer, 0.2 μL ROX Reference Dye II, and 1 μL diluted cDNA. mRNA expression levels were analyzed on the ABI 7500 Detection System (Applied Biosystems, Thermo Fisher, Waltham, MA, USA). The primer sets were as follows: α-SMA, 5′-ATGGTGGGAATGGGACAAAA-3′ (forward), 5′-CGTGAGCAGG GTGGGATG-3′ (reverse); FN, 5′-AATATCTCGGTGCCATTTGC-3′ (forward), 5′-AAAGGCATGAAGCACTCAA-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CAGTGC CAGCCTC- GTCTCAT-3′ (forward), 5′-AGGGGCCATCCA CAGTCTTC-3′ (reverse). The parameters were set at 95°C for 30 seconds for one cycle, then 95°C for 5 seconds, 60°C for 34 seconds for 40 cycles. The fold change in target gene expression was analyzed using the 2^{-ddCt} method.

Western Blot Analysis

Total cell protein was extracted in Radio Immunoprecipitation Assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The protein extracts were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking, the membrane was probed with primary antibody against α-SMA (1:1000), FN (1:1000; Protein-Tech, Chicago, IL, USA), and GAPDH (1:2000; Millipore, Billerica, MA, USA), followed by the appropriate horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Millipore). Specific bands were visualized by a standard enhanced chemiluminescence procedure (Millipore). The signals were analyzed using Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The band density of each sample was normalized to the GAPDH band.

Statistical Analysis

Statistical analyses were performed using 1-way ANOVA followed by the Fisher least significant difference test for comparisons among the study groups using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA), in which $P < 0.05$ was considered significant.

Results

Losartan Improves Surgical Outcome in a Rabbit Trabeculectomy Model

We investigated the effect of losartan on scar formation in a rabbit trabeculectomy model. Among the operated eyes, no intraoperative complication was observed except two eyes (6.7%). One eye of the NS group developed hyphema and the other of the MMC group developed endophthalmitis, both of which were excluded from the study.

We analyzed the bleb scarring through bleb appearances, IOP, and histologic examination. The appearances of filtering blebs were different among the NS control group and the three losartan-treatment (1, 5, and 10 mg/mL) groups. The losartan-treated eyes did not show any adverse effect in the anterior segment. Corneal opacification was observed in one eye of the MMC group. In the control group, the blebs were small and thick; however, in the MMC and losartan-treatment groups, they bulged slightly and were thinner (Fig. 1). The scleral flaps were more visible in the losartan-treatment groups compared with those in the control group. The mean IOP values before and after surgery are presented in Figure 2. Intraocular pressure in the MMC group was lower compared with the NS group throughout the observation period. On POD 28, mean IOP of the LOS 5 (5 mg/mL of losartan) group, which was 10.8 ± 0.9 mm Hg, showed significant reduction compared with that of the NS group and the other two LOS groups ($n = 6$, $P < 0.05$). Intraocular pressure in the LOS 1 (1 mg/mL of losartan) and LOS 10 (10 mg/mL of losartan) groups decreased postoperatively compared with the NS group; however, the difference was not statistically significant ($n = 6$; $P > 0.05$).

Histologic examination was performed to evaluate the effects of losartan on cell infiltration and bleb scarring around the surgery area. Histologic features differed in the NS group and the LOS groups. As shown in hematoxylin-eosin staining, the conjunctiva in the MMC and LOS groups demonstrated localized thinner epithelium in the bleb areas compared with that in the NS control group, with poorly recovered filtering pathway (Fig. 3A). In contrast, massive scarring was observed in the NS control group. To assess the degree of transdifferentiation to myofibroblasts, we performed immunohistochemical staining for α-SMA. Many cells with intensive α-SMA expression were observed in the conjunctival flap in the NS treatment group, which represented severe fibrosis (Fig. 3B). In contrast, the bleb fibrosis was significantly attenuated in the three losartan groups and the MMC group. The degree of fibrosis was presented by the ratio of α-SMA positive number/

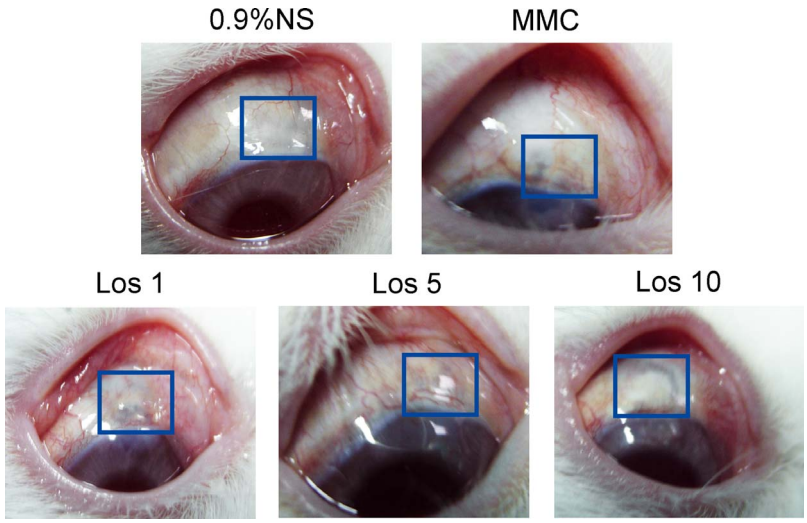

FIGURE 1. The appearance of filtering blebs in the five groups on POD 28. In the control group, the blebs were small and thick; however, in the MMC and losartan treatment groups, they were slightly bulged and thinner and the scleral flap was more clearly visible. *Blue boxes* indicate the bleb.

total cell number. Different concentrations of losartan (1, 5, and 10 mg/mL) and MMC treatment decreased bleb fibrosis by 56.2%, 77.8%, 65.3% and 55.5%, respectively, compared with the NS group (Figs. 3C, 3D).

The degree of collagen deposition was assessed by Masson staining. In the NS group, there was significant collagen deposition (blue stain) in the subconjunctival filtering bleb area, whereas the degree of fibrosis was reduced in the MMC and losartan-treated rabbits (Fig. 3E).

Losartan Inhibits Ang II–Induced HTFs Proliferation In Vitro

Angiotensin II was reported to increase the proliferation of HTFs, which may lead to risk of fibrosis.[6] As shown in Figure 4A, Ang II increased proliferation of HTFs by 45.3% ($P < 0.05$); however, losartan at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M decreased the number of HTFs by 13.5%, 14.7%, 20.4%, and 15.7%, respectively, compared with the control group. Only the cell proliferation with 10^{-5} M of losartan treatment was statistically significantly inhibited ($P < 0.01$). When Ang II was administered after the pretreatment with losartan (10^{-5} M) for 24 hours, Ang II–induced proliferation of HTFs was reduced by 43.9% ($P < 0.01$; Fig. 4A). The proliferation of cells with different treatments at different time points was further assessed. Cell proliferation after 24-hour incubation was significantly reduced with losartan pretreatment by 34.3% ($P < 0.05$; Fig. 4B), compared with the Ang II group. The difference at 12 hours, however, was not statistically significant ($P > 0.05$).

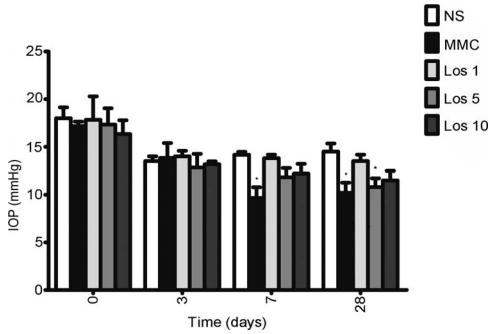

FIGURE 2. Intraocular pressure before and after trabeculectomy. On POD 28, IOP in the losartan groups and MMC group were lower than the control group; however, only the 5 mg/mL of losartan and MMC group showed statistical significance. LOS 1, LOS 5, LOS 10 represented 1 mg/mL, 5 mg/mL, and 10 mg/mL of losartan, respectively. Data represent mean ± SEM from three independent experiments each performed in triplicate. *$P < 0.05$ versus control by 1-way ANOVA followed by Fisher's test.

Losartan Inhibits Ang II–Induced HTF Migration In Vitro

In the scratch wound study, the scratched area was almost completely covered by the migration of HTFs after 24 hours in the Ang II group; however, cells with 10^{-5} M losartan pretreatment had a reduced migratory activity. Quantitative analysis of the denuded area showed that losartan decreased cell migration by 41.3% and 48.3% at 12 and 24 hours compared with the control group ($P < 0.001$). Although compared with the Ang II treatment group, cell migration was significantly decreased by 49.6% and 48.6% with losartan pretreatment ($P < 0.001$) (Figs. 5A, 5B).

Besides the scratch wound assay, we further used the transwell assay to assess HTF migration. Pretreatment with losartan (10^{-5} M) decreased cell migration by 36.6% compared with that without losartan pretreatment ($P < 0.01$; Figs. 5C, 5D). Migration assays revealed that losartan treatment resulted in a marked reduction in Ang II–mediated HTF migration.

Losartan Inhibited Ang II–Induced Transdifferentiation of HTFs

When pretreated with losartan 1 hour before Ang II incubation, cells exhibited little immunoreactivity for α-SMA and showed a fibroblast-like morphology (Fig. 6A). Western blot analysis showed that the amount of α-SMA significantly increased in Ang II–treated cells, which was inhibited by losartan. High concentration (10^{-5} M) of losartan exhibited a stronger inhibitory effect on transdifferentiation of HTFs than low concentration (10^{-8} M; Figs. 6C, 6D). Real-time PCR analysis showed a gene expression pattern consistent with the protein expression. The expression of α-SMA mRNA in HTFs

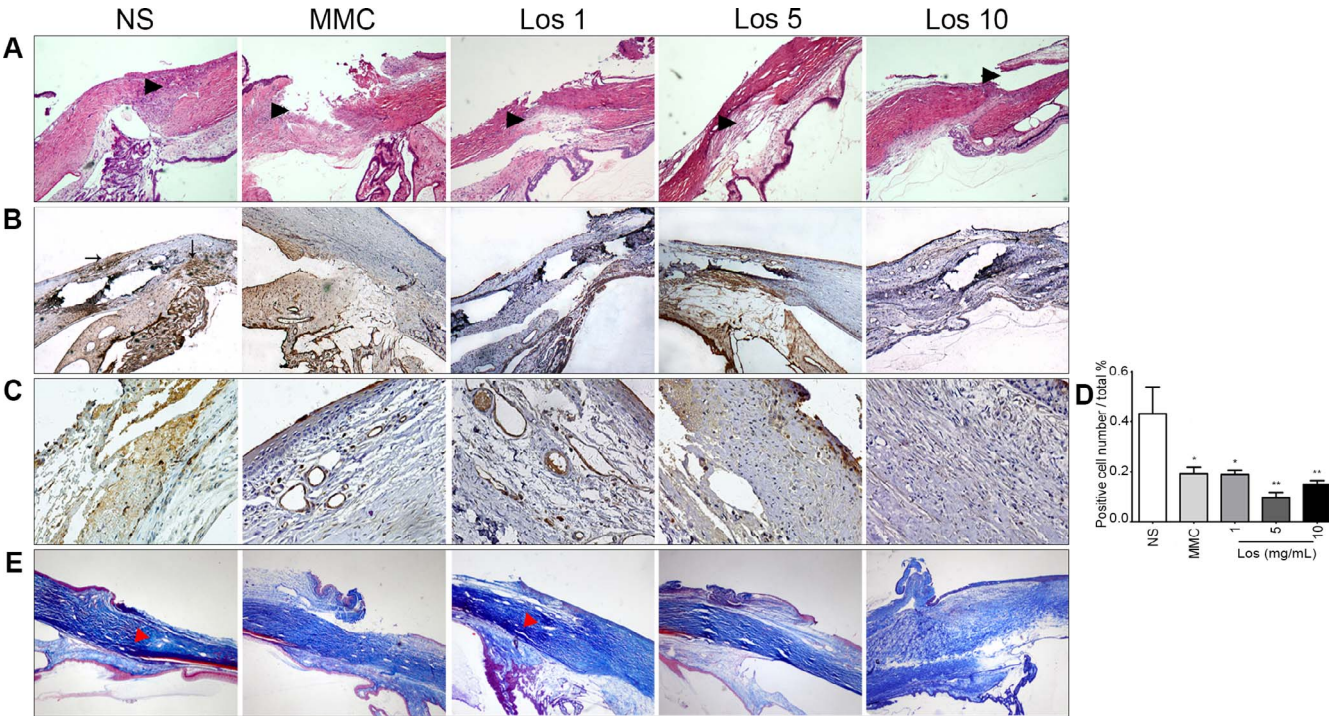

FIGURE 3. Histologic examination of the surrounding tissues of filtering channel on POD 28 after trabeculectomy. (**A**) Hematoxylin-eosin–stained images showed scar formation in the subconjunctival tissue and filtering channel. Scar tissues (*black triangles*) significantly deposited in the subconjunctival area in the NS group; however, were less in the losartan and MMC groups ($n = 6$). (**B**) Immunohistochemical examination of α-SMA of rabbit eyes in the control and experimental groups. Sections were labeled with primary antibody against α-SMA at a dilution of 1:200. Increased expression of α-SMA (*black arrows*) was clearly visible in the subconjunctival tissue in the control group; however, the number of positive-staining cells decreased in the MMC or losartan groups ($n = 6$). (**C**, **D**) At a higher magnification (×400), the ratios of myofibroblasts to total cells in the MMC and losartan treatment groups were significantly decreased from in the control group ($n = 6$). (**E**) Representative Masson staining revealed that with losartan or MMC, the formation of collagen tissue (*red arrowheads*) was attenuated compared with the NS group ($n = 6$). Data represent mean ± SEM from three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control by 1-way ANOVA followed by Fisher's test.

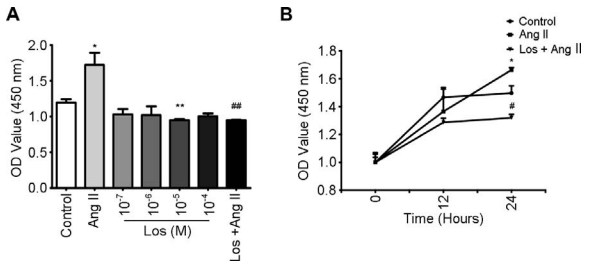

FIGURE 4. Effects of losartan on HTF proliferation. (**A**) Cells treated with Ang II (10^{-7} M) and different concentrations of losartan (10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M). The OD values were measured after 24 hours of treatment. The OD values of groups with 10^{-5} M losartan significantly decreased compared with the control group. When pretreated with 10^{-5} M losartan for 1 hour and then treated with Ang II, cells were largely decreased than those of the Ang II group. Data are mean ± SEM from three independent experiments with triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$ versus Ang II by 1-way ANOVA followed by Fisher's test. (**B**) Cells incubated with Ang II (10^{-7} M) with or without losartan (10^{-5} M) pretreatment. The OD values were measured at 0, 12, and 24 hours after treatment. After 24-hour incubation, losartan significantly inhibited the promotion effects on proliferation of Ang II. Data are mean ± SEM from three independent experiments with triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Ang II by 1-way ANOVA followed by Fisher's test.

was decreased by losartan as compared with the Ang II treatment without losartan (Fig. 6B).

Losartan Inhibited Ang II–Induced Fibronectin Deposition

Pretreatment with losartan before Ang II significantly decreased the expression of FN in both mRNA and protein compared with that for the Ang II treatment group (Fig. 7). Losartan (10^{-5} M) more effectively attenuated the Ang II–promoting expression of FN than losartan (10^{-8} M).

DISCUSSION

Trabeculectomy has been widely used in the treatment of glaucoma; however, scar formation of filtering bleb has reduced the positive outcome of surgery, even leading to surgical failure. Our previous studies showed that the renin-angiotensin-system was localized in Tenon's fibroblasts and upregulated after trabeculectomy.[6] However, there is no report to delineate whether the fibrogenic effect of Ang II on Tenon's fibroblasts is inhibited by ARB. Here we revealed subconjunctival injection of losartan (5 mg/mL, 0.1 mL) attenuated bleb scarring in a rabbit trabeculectomy model. To investigate the possible cellular mechanisms, we treated HTFs with losartan and found that the Ang II–induced fibrosis of HTFs was attenuated by losartan pretreatment. These results suggest that losartan potentially reduces scar formation of filtering bleb by inhibiting fibrosis of Tenon's fibroblasts.

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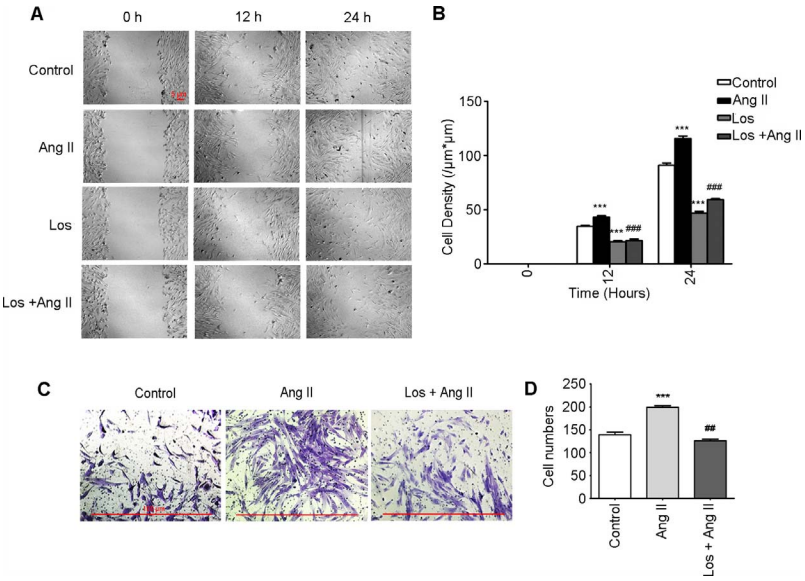

Figure 5. Effects of losartan on HTF migration. (**A**, **B**) Scratch wound assay: A scratch to denude cells was made in the center of confluent HTFs. The cells were incubated with or without losartan (10^{-5} M) pretreatment 1 hour before Ang II (10^{-7} M) treatment for up to 48 hours, or with losartan incubation individually. Then the wound scratches were imaged and quantified. (**A**) Representative images of the different treatment groups at different time points after the scratch. *Scale bar*: 5 μm. (**B**) For quantification, cell numbers in the scratches of each group at each time point were counted and cell densities in the wound areas were calculated. Data from three independent experiments performed in triplicate are shown. $^{***}P <$ 0.001 versus control, $^{###}P < 0.001$ versus Ang II by 2-way ANOVA. (**C**, **D**) Transwell assay: Cells were plated in the upper chamber of filters with or without losartan (10^{-5} M) pretreatment 1 hour before Ang II (10^{-7} M) incubation. Cells migrating to the underside of transwell chambers at 48 hours were imaged and quantified. (**C**) Representative images of the underside of transwell chambers of the different treatment groups. *Scale bar*: 100 μm. (**D**) Quantification for the cell numbers migrating to the underside of the wells. Data are mean ± SEM from three independent experiments with triplicate samples. $^{***}P < 0.001$ versus control, $^{##}P < 0.01$ versus Ang II by 1-way ANOVA followed by Fisher's test.

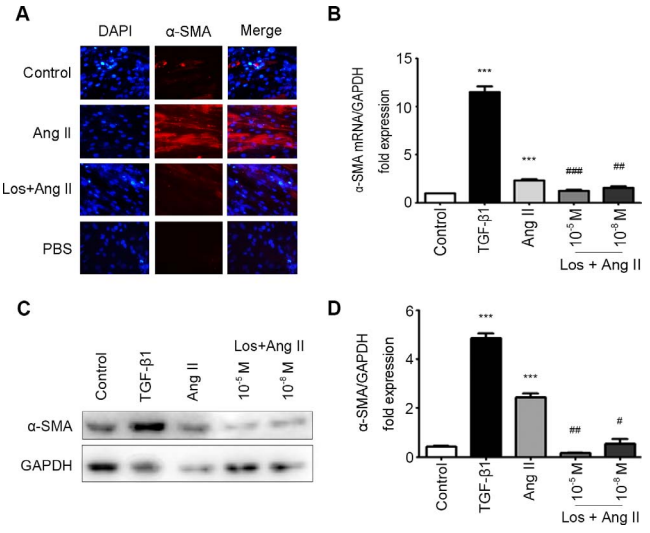

Figure 6. Losartan inhibited the promoting effect of Ang II on phenotype transition from HTFs to myofibroblasts. (**A**) Expression of α-SMA as visualized by immunofluorescence in HTFs treated with or without losartan (10^{-5} M) pretreatment 1 hour before Ang II (10^{-7} M) incubation for 48 hours. (**B**) Inhibition effect of different concentrations of losartan (10^{-8} and 10^{-5} M) on the expression of α-SMA mRNA increased by Ang II. (**C**, **D**) Inhibition effects of different concentrations of losartan (10^{-8} and 10^{-5} M) on the expression of α-SMA protein increased by Ang II. Data represent mean ± SEM from three independent experiments performed in triplicate. $^{***}P < 0.001$ versus control, $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ versus Ang II by 1-way ANOVA followed by Fisher's test.

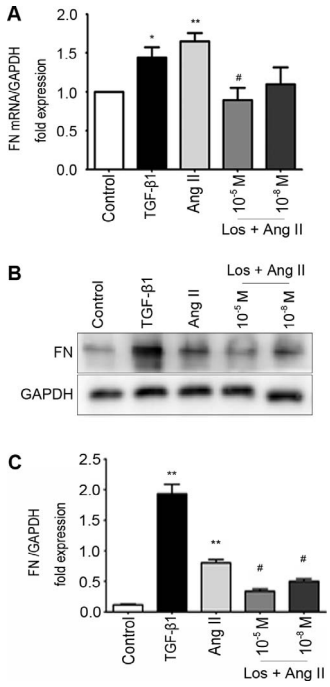

Figure 7. Pretreatment with losartan inhibited the FN expression of HTFs. (**A**) Effects of different concentrations of losartan (10^{-8} and 10^{-5} M) pretreatment before Ang II incubation for 36 hours on FN mRNA expression. (**B**, **C**) Effects of different concentrations of losartan (10^{-8} and 10^{-5} M) pretreatment before Ang II incubation for 48 hours on FN protein expression. With losartan pretreatment, the expression of FN decreased and the effect of 10^{-5} M of losartan was more significant. Data represent mean ± SEM from three independent experiments performed in triplicate. $^{**}P < 0.01$ versus control, $^{#}P < 0.05$ versus Ang II by 1-way ANOVA followed by Fisher's test.

Studies have indicated a strong relationship between AT1R and tissue fibrosis. When AT1R in fibroblasts were depleted, Ang II–induced fibrosis was attenuated, as shown, for example, in medial hyperplasia in the ascending aorta.[20] Conversely, in a mouse model, in which a human AT1R transgene was expressed, progressive cardiac remodeling was stimulated through hypertrophy and death of individual cardiomyocytes, concomitant with infiltration, proliferation, and activation of cardiac fibroblasts.[21] Our in vivo studies showed losartan significantly decreased postoperative IOP and histologically attenuated transdifferentiation of myofibroblasts and ECM deposition after trabeculectomy, thereby improving the surgery outcome. Thinner blebs in the losartan and the MMC groups indicated less fibrosis in the blebs. As an important clinical indicator of surgery success, IOP was shown significantly decreasing in the LOS 5 group. On POD 28, myofibroblasts infiltrated the subepithelial field in the control group; however, there were fewer myofibroblasts and less collagen deposition in the surgical area in the MMC group and the three losartan groups. Thus, we speculate that subconjunctival injection of losartan attenuates bleb scarring by preventing transdifferentiation into myofibroblasts. This hypothesis is confirmed by the cell studies.

Compared with MMC, the most popular antifibrotic therapy after trabeculectomy,[22] losartan did not demonstrate a better inhibitory effect in our study. However, losartan administration did not show adverse clinical complications and drug toxicity related to MMC, such as corneal erosion, corneal opacification, endophthalmitis, and cataract.[23] Thus, we expect losartan injection as a substitute therapy for MMC or a combination with MMC may improve the surgery outcome and safety by reducing the dosage and exposure time of MMC. Clinical trials confirmed the potential beneficial effects of ARBs on attenuating fibrotic-associated diseases, such as chronic heart and kidney failure. Losartan conferred significant renal benefits in patients with type 2 diabetes and nephropathy.[24] It reduced the incidence of end-stage renal disease. The rate of first hospitalization and cardiovascular mortality and morbidity in patients with chronic heart failure was also significantly lower with losartan.[25–27] These studies prompted investigation of losartan as a potential approach in antifibrosis therapy after trabeculectomy; however, it is important to keep in mind that success in an animal model with small sample size does not necessarily translate to success in clinical trials. Further animal studies are needed to determine the optimal regimen of administration. Subconjunctival injection conforms with clinical practice, but with a disadvantage of disturbing and expanding the bleb. Therefore, we plan to compare the advantages and disadvantages among subconjunctival, intracameral, and intravitreal injections. Research to define the optimal dosage with minimal toxicity of losartan awaits further larger sample size studies.

Hemostasis, inflammation, proliferation, and remolding are four main phases involved in the wound-healing process. Surgery stimulates proliferation and migration of fibroblasts and inflammatory cells toward the surgery site to repair the wound. AT1R blockers have previously been demonstrated to decrease cell proliferation and migration in various cell lines. Oral losartan treatment protected retinal ganglion cells by reducing the density of fibroblasts in sclera and affecting scleral remolding in a mouse model.[28] Treatment of ARBs significantly decreases the Ang II–induced proliferation of esophageal squamous cell carcinoma (ESCC) in vitro and decreased the incidence of esophageal tumor in an ESCC murine model.[29] Concomitant treatment of alveolar epithelial cells with ARBs was found to reduce TGF-β–induced cell migration.[30] In agreement, we found a significant inhibition in HTF proliferation and in wound closure with losartan in vitro. Losartan significantly inhibited HTF proliferation only with the concentration of 10^{-5} M in the range of concentration studied, indicating a U-shape dose-dependent suppression of cell proliferation; so, we chose the concentration of 10^{-5} M in the following experiments. Subconjunctival injection of losartan in vivo also showed a best concentration effect. We previously showed the promoting effect on proliferation and migration of Ang II, even stronger than TGF-β.[6] Consequently, losartan effectively inhibited cell proliferation and migration. Besides cell proliferation and migration, ARBs also had an anti-inflammatory effect in the process of fibrosis. Inflammatory response peaks within the first week after trabeculectomy,[31,32] occurring in the early stage of fibrosis simultaneously with fibroblast proliferation and migration. AT1R blockers were shown to decrease the expression of inflammatory cytokines, such as interleukin,[33,34] matrix metalloproteinase,[35] C-reactive protein,[36] and TNF-α[37,38] and to inhibit infiltration of inflammatory cells.[39] Whether losartan reduces inflammation at an early stage after trabeculectomy requires further investigation.

Myofibroblast accumulation and excessive deposition of ECM components are common characteristic features in the late stage of bleb scarring. Our findings were in agreement with previous studies in the liver and kidney. Losartan significantly reduces the drug-induced α-SMA production and ECM deposition in the rat liver and improves liver function.[40] Losartan-treated mice were shown to have less renal injury by suppressing transdifferentiation and the resultant ECM synthesis.[41] Notably, Ang II can induce transdifferentiation and ECM deposition, albeit less efficaciously than TGF-β1.[6] Angiotensin II incubation for 24 hours significantly increased the expression of TGF-β1.[42,43] Thus, we propose that Ang II increases fibrosis by upregulating TGF-β1 and may be mediated with TGF-β/Smad signal pathway. AT1R blockers were shown to suppress TGF-β1 expression in several cell types.[44,45] Sui et al.[46] demonstrated that TGF-β/Smad signal pathway contributes to Ang II–mediated collagen accumulation and valsartan, a blocker of AT1R, significantly attenuates the expression of TGF-β/Smad signaling molecules; however, research results are controversial. Another study showed that ARB inhibits collagen synthesis and metabolic imbalance mediated by Ang II, but had no effect on TGF-β1–induced cardiac fibrosis and Smad signaling molecule expression.[47] Our study showed no significant correlation between losartan and TGF-β by ELISA and Western blot assay (data not shown). However, further research is needed to investigate the interaction between losartan and TGF-β as well as its underlining molecular mechanism.

In conclusion, our study suggests that losartan decreases HTF fibrosis, including cell proliferation, migration, transdifferentiation, and ECM synthesis. In addition, losartan is beneficial in inhibiting the bleb scarring in rabbits. AT1R signaling potentially modifies HTF fibrosis, and the therapeutic effect of losartan likely attenuates fibrosis in the filtering bleb after trabeculectomy.

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