Presence and Physiologic Function of the Renin–Angiotensin System in Mouse Lacrimal Gland

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Purpose. To investigate the expression, localization, and physiologic function of renin–angiotensin system (RAS) components in the mouse lacrimal gland.

Methods. Lacrimal glands and cultured lacrimal gland fibroblasts from wild-type (WT) BALB/c (H-2^d) mice were used. Reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry were used to determine the expression and localization of the RAS components, prorenin/renin, angiotensin-converting enzyme (ACE), angiotensin II, angiotensin II type 1 receptor (AT1R), and angiotensin II type 2 receptor (AT2R) in the normal mouse lacrimal gland. To examine the change in tear secretion, mice received ARB (AT1R blocker) or AT2R antagonist. Tear secretion was assessed by cotton thread test before and after drug administration.

Results. The mRNAs coding for angiotensinogen, prorenin, ACE, and both AT1R and AT2R were found in normal lacrimal gland tissue and cultured lacrimal gland fibroblasts. Prorenin/renin and ACE were identified in myoepithelial cells around ducts and acini and in blood vessels. Angiotensin II, AT1R, and AT2R were observed in the ducts and interstitial fibroblasts. AT1R and AT2R were also localized in blood vessels. All the cultured lacrimal gland fibroblasts expressed angiotensin II, AT1R, and AT2R. Tear secretion increased in mice that received ARB.

Conclusions. The results are consistent with the hypothesis that a tissue-specific RAS is present in the lacrimal gland, and suggest that fibroblasts are one of the cell types playing a role in the tissue RAS. Tissue RAS might be involved in tissue function of regulating tear secretion in the lacrimal gland. (*Invest Ophthalmol Vis Sci.* 2012;53:5416–5425) DOI: 10.1167/iovs.12-9891

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The systemic renin–angiotensin system (RAS) plays an important role in the endocrine regulation of blood pressure and of salt and water balance.[1] Angiotensinogen, the precursor of angiotensin, is synthesized primarily in the liver. Angiotensinogen is secreted into the circulation and cleaved by renin to the decapeptide angiotensin I. Angiotensin I is subsequently converted to the octapeptide angiotensin II by angiotensin-converting enzyme (ACE).[2] Renin and ACE are derived from the kidney and lung, respectively. The action of angiotensin II is mediated mainly through its interaction with two pharmacologically defined receptor subtypes, type 1 (AT1R) and type 2 (AT2R), which are distributed in numerous tissues.[3]

Besides systemic RAS, there are reports of many peripheral tissues that are capable of generating RAS components: tissue RAS is recognized in the heart,[4] kidney,[5] vasculature,[6] adipose tissue,[7] liver,[8] nervous tissue/brain,[5] adrenal tissue,[9] gonads/reproductive system,[10] gastrointestinal system,[11] pancreas,[6,12] and breast.[13] Local tissues that possess their own RAS may use it to fine-tune specific functions, frequently via paracrine, autocrine, or intracrine actions.[14,15] The tissue RAS has various roles, including the regulation of cell growth, differentiation, wound healing, tissue reconstruction, embryonic development, apoptosis, and proliferation; metabolism and the generation of reactive oxygen species and free radicals; tissue inflammation and fibrosis; local hemodynamics; and hormone secretion and reproduction.[16–22] Tissue RAS can be subject to fine regulation by several physiologic and pathologic conditions. For example, pancreatic RAS is involved in the physiologic regulation of pancreatic functions such as exocrine acinar and endocrine islet activities, as well as being implicated in the pathogenesis of pancreatic diseases including diabetes, pancreatitis, and pancreatic cancer.[23,24]

In ocular tissue, tissue RAS has been demonstrated by mRNA and protein expression of RAS components in the cornea,[25–29] sclera,[30] ciliary bodies,[25,31] choroid,[26,30] ganglion cells,[25] pigmented epithelial cells,[27,30] and Müller cells[32] of the retina. However, little has been reported about part of RAS components in the lacrimal apparatus, except for a few studies. Vita et al.[33] reported ACE activity in tears, Moss et al.[34] reported that ACE inhibitors lower the risk for dry eye in human patients, and Richards et al.[35] reported androgen exposure increases the expression of AT2R in the mouse lacrimal gland.

In this study, we documented the existence of all of the major RAS components in the wild-type (WT) mice lacrimal gland. The lacrimal gland is the major source of tear fluid. It is well known that the rate of lacrimal secretion is controlled by parasympathetic and sympathetic innervation, where a number of agonists can stimulate tear secretion.[36] Pathologic changes of lacrimal gland, such as inflammation and fibrosis, decrease the quality and quantity of tear fluid leading to the dry eye syndrome.[37] For example, our studies have reported that

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the lacrimal glands of patients with chronic graft-versus-host disease (GVHD) following allogeneic hematopoietic stem cell transplantation (HSCT) show prominent fibrosis and infiltration of inflammatory cells in the glandular interstitium[38] and that dry eye syndrome associated with chronic GVHD is one of the major complications associated with the condition, with 50% of patients developing dry eye or their preexisting dry eye worsened after HSCT.[39]

Recent studies have reported that RAS components contribute to several inflammatory and fibrotic diseases.[40–44] Based on these findings, we hypothesized that tissue RAS is present in the lacrimal gland, and that it is involved in the physiologic functions of the lacrimal gland and possibly in its pathogenic status. Therefore, the goal of this study was to examine the expression and localization of RAS components and the involvement of RAS in physiologic function of tear secretion in the normal mouse lacrimal gland.

Materials and Methods

Mice

Female WT 8-week-old BALB/c (H-2^d) mice, female WT 31-week-old BALB/c (H-2^d) mice, and male WT 8-week-old BALB/c (H-2^d) mice were used in this study. All the animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The ethics committee of our institution approved all surgical interventions and animal care procedures, which were in accordance with the Guidelines and Policies for Animal Surgery provided by the Animal Study Committees of the Central Institute for Experimental Animals of Keio University. The protocol for this study was approved by the Ethics Committee on Animal Research of the Keio University School of Medicine (protocol #09152).

Lacrimal Gland Fibroblast Culture

Lacrimal gland fibroblasts from female WT 8-week-old BALB/c (H-2^d) mice were cultured and used for total RNA extraction and immunohistochemistry. Briefly, lacrimal gland tissue was minced with a sterilized scissors into small fragments, and transferred to plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (200 U/mL), streptomycin (200 U/mL), and 10% fetal bovine serum, for fibroblast growth. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced with fresh medium every 3 days. Confluent cells were detached by incubation with 0.05% trypsin for 1.5 minutes and centrifuged (1500 rpm for 10 minutes at 4°C); the cell pellet was resuspended in DMEM. Fibroblasts were used between passages three and five.

Light and Fluorescence Microscopy

All lacrimal gland specimens were immediately fixed in 10% neutralized buffered formalin, embedded in paraffin wax, and processed according to conventional histologic techniques, including hematoxylin–eosin staining.[45,46] Deparaffinized sections were used for immunohistochemistry as previously described.[47] Briefly, the deparaffinized sections were blocked with 10% goat serum (Invitrogen, Carlsbad, CA) for 30 minutes and then incubated overnight at 4°C with the following primary antibodies: mouse anti-mouse renin (R&D Systems, Inc., Minneapolis, MN), mouse anti-mouse ACE (CD143; Millipore, Billerica, MA), rabbit anti-mouse angiotensin II (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse AT1R (Santa Cruz Biotechnology), rabbit anti-mouse AT2R (Santa Cruz Biotechnology), mouse anti-mouse HSP47 (StressGen Biotechnologies Corp., Victoria, BC, Canada), rat anti-mouse CD45 (BD Biosciences, Franklin Lakes, NJ), and mouse anti-mouse Actin, Smooth Muscle Ad-1 (Thermo Fisher Scientific, Fremont, CA and Cheshire, WA7 1PR, UK).

After washing with PBS, the sections were treated with a peroxidase-conjugated secondary antibody (Nichirei Bioscience Inc., Tokyo, Japan) for 45 minutes, and washed with PBS. The reaction products were developed with a mixture of 3,3′-diaminobenzine-4 HCI (DAB) and H_2O_2 (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Positive staining for renin and ACE was determined by comparison with kidney and lung samples. Isotype-matched antibodies were used as a negative control. To stain paraffin-embedded tissue sections, antigen unmasking was performed, by autoclaving at 120°C for 20 minutes for HSP47 or microwaving for 21 minutes for renin, ACE, angiotensin II, AT1R, AT2R, and α-SMA, in 10 mM sodium citrate buffer.

HSP47 was used as a specific marker for mouse fibroblasts.[48] The coexpression in interstitial fibroblasts of HSP47 and angiotensin II, AT1R, and AT2R was examined by fluorescent double-staining with mouse anti-mouse HSP47 with a dye-conjugated goat anti-mouse IgG secondary antibody (Alexa Fluor 568; Invitrogen/Molecular Probes), and rabbit anti-mouse angiotensin II, rabbit anti-mouse AT1R, or rabbit anti-mouse AT2R with a dye-conjugated goat anti-rabbit IgG secondary antibody (Alexa Fluor 488; Invitrogen/Molecular Probes). Nuclei were counterstained with TO-PRO-3 (Invitrogen/Molecular Probes).

Fibroblasts were cultured on fibronectin-coated chamber slides, to which they were fixed with 10% neutral buffered formalin for DAB and fluorescent staining. For the DAB staining of cultured fibroblasts, the endogenous peroxidase activity was quenched with 0.3% peroxide for 5 minutes. Rabbit anti-mouse angiotensin II, rabbit anti-mouse AT1R, rabbit anti-mouse AT2R, mouse anti-mouse HSP47, and rat anti-mouse CD45 were used in combination with nuclear staining with hematoxylin. HSP47 was used as a positive marker and CD45 as a negative marker for mouse cultured fibroblasts. Isotype-matched mouse antibodies were used as negative controls.

For fluorescent immunostaining, rabbit anti-mouse angiotensin II, rabbit anti-mouse AT1R, or rabbit anti-mouse AT2R followed by a dye-conjugated goat anti-rabbit (Alexa Fluor 488; Invitrogen/Molecular Probes) IgG secondary antibody (Invitrogen/Molecular Probes) was used. Nuclei were counterstained with TO-PRO-3 (Invitrogen/Molecular Probes). The tissue sections and fibroblasts used for fluorescent staining were mounted on glass slides and examined with a confocal microscope (LSM700-ZEN 2009; Carl Zeiss Microlmaging GmbH, Göttingen, Germany).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

A standard RT-PCR protocol was used to measure transcript levels. The gene-specific primers for angiotensinogen, prorenin, ACE, AT1R, AT2R, and β-actin are listed in the Table. Briefly, total RNA was extracted from lacrimal glands and from lacrimal-gland–derived cultured fibroblasts using an RNA isolation kit (Qiagen, Valencia, CA), and the PCR reactions were performed with specific primers. The reaction products were subjected to electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Total RNA was also isolated from the liver, kidney, and lung, and subjected to the same PCR procedure. The liver, kidney, and lung were used as positive controls for angiotensinogen, prorenin, and ACE, respectively. As a negative control, the PCR was performed with sterilized water instead of primer.

Administration of ARB (AT1R blocker) or AT2R Antagonist and Measurement of Tear Secretion

Female WT 8-week-old BALB/c (H-2^d) mice were divided into four groups of 10 mice based on the type of treatment. The vehicle and ARB and AT2R antagonists were administered simultaneously. Tear secretion was measured by cotton thread test prior to administration,

Table. Primer Sequences Used in the RT-PCR Analysis to Amplify Different Targets and the Predicted Size of the PCR Products

Target	Predicted Size (bp)	Sense (5′ - 3′)	Antisense (5′ - 3′)
Angiotensinogen	622	GGGCTGCCCAGGTCGCAATG	GACACCGAGATGCTGTTGTCCACC
Prorenin	332	GAACGGGTCCGACTTCACCATCC	CTTGATAATGCTGCGGGTCGCTAC
ACE	918	CGCTGCTGGTGCTGTTGCTG	TGTGTGTGGCGTTCCAGCCC
AT1R	204	TCACCTGCATCATCATCTGG	AGCTGGTAAGAATGATTAGG
AT2R	679	CCAGCAGCCGTCCTTTTGATAA	GTAATTCTGTTCTTCCCATAGC
β-Actin	837	ATGTGGCACCACACCTTCTACAATGAGCTGCG	CGTCATACTCCTGCTTGCTGATCCACATCTGC

15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 8 hours, and 24 hours after treatment.

Blockage of AT1R and AT2R

Animals were treated with intraperitoneal injections of two different vehicles (dimethylsulfoxide [DMSO] diluted with phosphate-buffered saline [PBS] and PBS), an ARB (valsartan; LKT Laboratories, Inc., St. Paul, MN), and AT2R antagonist (PD123319; Sigma, St. Louis, MO). The groups were designated the DMSO group, PBS group, ARB group, and AT2R antagonist group.

Valsartan was dissolved in DMSO, diluted with PBS, and injected into mice at a dose of 2.6 mg/kg. The dose of 2.6 mg/kg is the same as the maximum tolerated dose in humans. The dilution rate of DMSO in PBS injected to the DMSO group was the same as that of valsartan. PD123319 was dissolved in PBS and injected into mice at a dose of 10 mg/kg. The dose of 10 mg/kg was used in other reports on tissue RAS.[49–51] We prepared two types of vehicle since the solvent of valsartan and PD123319 were different. The effect of valsartan in the ARB group was compared with the DMSO group, and the effect of PD123319 in AT2R group was compared with the PBS group.

Measurement of Tear Secretion by Cotton Thread Test

A phenol red thread was placed on the temporal side of the lower eyelid margin for 15 seconds. The length of the moistened thread from the edge was measured. The tear secretion was measured in both eyes and the average was used for analysis.

Statistical Analyses

All results are expressed as the mean ± SD. The data were processed for statistical analyses (Bonferroni/Dunn test). Differences were considered to be statistically significant at $P < 0.05$.

Results

Lacrimal Gland and Primary Fibroblast Culture

We first confirmed that the extracted tissue was lacrimal gland by hematoxylin–eosin staining. The tissue appearance was consistent with that of the mouse lacrimal gland, which is comprised of many lobules separated from one another by loose connective tissue. Each lobule had many acini, interlobular ducts, and capillaries (Fig. 1A).

Primary culture of fibroblasts was successfully generated from the lacrimal glands of five female WT 8-week-old BALB/c (H-2^d) mice using the explant technique.[52] We performed immunohistochemical staining using HSP47 and CD45 to confirm that the cells were fibroblasts. All the cells in these cultures had a spindle shape and were positive for HSP47, a marker of collagen-secreting cells, consistent with the fibroblast phenotype. No cells were positive for CD45, indicating there were no contaminating hematopoietic cells (Figs. 1B, 1C).

Expression of RAS Component mRNAs in the Mouse Lacrimal Gland

RT-PCR coupled with specific primers for the angiotensinogen, prorenin, ACE, and angiotensin II receptor subtype (AT1R and AT2R) genes was performed to elucidate the expression levels of these RAS components in the mouse lacrimal gland. The lacrimal glands from five female WT 8-week-old BALB/c (H-2^d) mice expressed all the RAS components examined (Fig. 2). To examine whether the expression of RAS component mRNAs differs by age and sex, mRNAs were also examined using female WT 31-week-old BALB/c (H-2^d) mice and male WT 8-week-old BALB/c (H-2^d) mice (see Supplementary Material and Supplementary Figs. S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9891/-/DCSupplemental), and all the RAS components were detected in both mice.

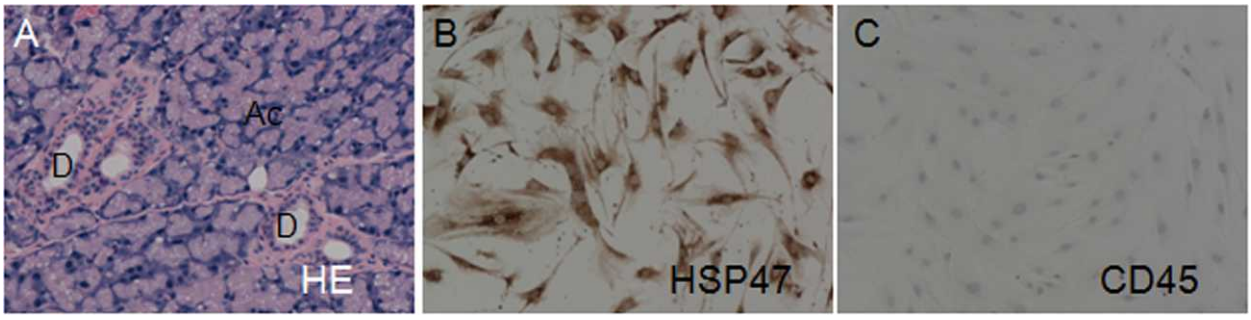

Figure 1. Light microscopic findings of mouse lacrimal gland specimens and phenotype of cultured lacrimal gland fibroblasts. Hematoxylin–eosin (HE) staining (**A**) of a lacrimal gland section shows each lobule had many acini and intralobular ducts. Immunostaining for HSP47 (**B**) and CD45 (**C**) in fibroblasts from mouse lacrimal gland indicate that all the fibroblasts are positive for HSP47 and negative for CD45. D, duct; Ac, acini. Magnification: (**A**) ×200; (**B**, **C**) ×100.

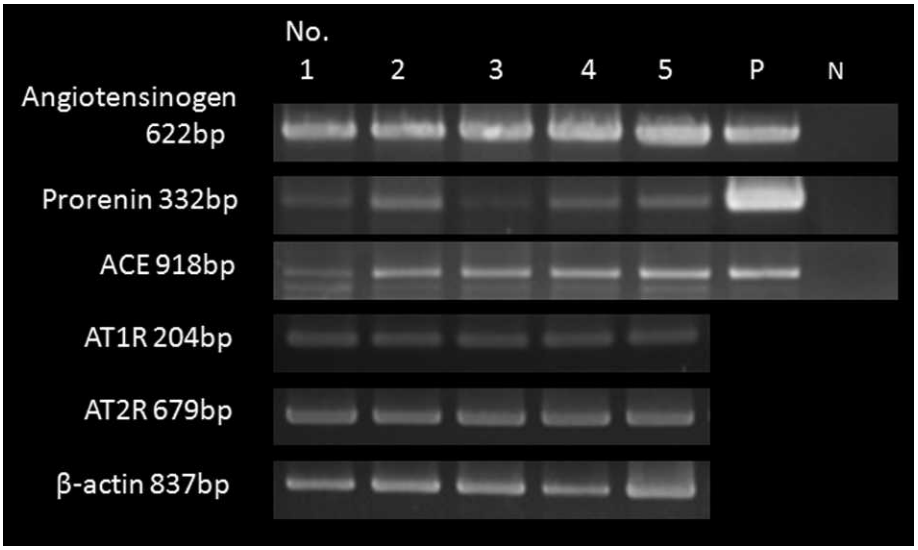

Figure 2. Expression of the mRNAs for RAS components in mouse lacrimal gland. The mRNA expression of RAS components angiotensinogen, prorenin, ACE, AT1R, and AT2R were detected in five different lacrimal gland samples. *Lanes 1–5* (Nos. 1 to 5), mRNA expression in the lacrimal gland from different mice. *Lane 6* (P, positive control), angiotensinogen mRNA expression was examined in the liver, prorenin mRNA in the kidney, and ACE mRNA in the lung. *Lane 7* (N, negative control), the PCR was performed with sterilized water instead of primer.

Localization of RAS Components in the Mouse Lacrimal Gland

The localization of RAS components (prorenin/renin, ACE, angiotensin II, AT1R, and AT2R) was studied by immunohistochemistry using specific antibodies against each component. The mouse anti-mouse renin antibody detected both prorenin and renin. In the mouse lacrimal gland, prorenin/renin and ACE were localized to interstitial cells around the duct and acini (Figs. 3A, 3B) and blood vessels (Figs. 3E, 3F). The colocalization of α-SMA in these cells suggested that they were myoepithelial cells (Fig. 3C).

Angiotensin II, AT1R, and AT2R appeared to be localized exclusively to the ducts and interstitial cells, which had a spindle-shaped morphology with oval nuclei, suggesting that they were fibroblasts (Figs. 3I–K). AT1R and AT2R were also localized to the blood vessels (Figs. 3M, 3N). To observe the localization of angiotensin II, AT1R, and AT2R in the ducts at higher magnification, we performed immunofluorescence staining. The results showed that angiotensin II was localized to the epithelial cells of the ducts (Fig. 3O), AT1R to the basolateral membrane of the ducts (Fig. 3P), and AT2R to the apical membrane of the ducts (Fig. 3Q). To confirm that the cells positive for angiotensin II, AT1R, and AT2R in the mouse lacrimal gland were fibroblasts, we examined the coexpression of HSP47 and angiotensin II, AT1R, or AT2R in tissue sections. We found that the interstitial cells coexpressed HSP47 and angiotensin II, AT1R, or AT2R (Figs. 3R–T), confirming that the interstitial fibroblasts were positive for angiotensin II, AT1R, and AT2R.

mRNA Expression and Immunohistochemical Staining of RAS Components in Cultured Lacrimal Gland Fibroblasts

To confirm that fibroblasts were one of the cell types involved in the local RAS of the mouse lacrimal gland, we cultured the fibroblasts from lacrimal glands and subjected them to the RT-PCR and immunohistochemical analyses. Figure 4 shows the mRNA detection of RAS components in five preparations of lacrimal gland fibroblasts from female WT 8-week-old BALB/c (H-2^d) mice. The lacrimal gland fibroblasts expressed all the RAS components examined. We next performed immunohistochemical staining for angiotensin II, AT1R, and AT2R in the cultured fibroblasts. All the preparations of cultured fibroblasts established from the lacrimal gland of female WT 8-week-old BALB/c (H-2^d) mice were positive for angiotensin II (Figs. 5A, 5E), AT1R (Figs. 5B, 5F), and AT2R (Figs. 5C, 5G).

Effects of Administration of ARB or AT2R Blocker

The mean values of tear secretion are depicted in Figures 6A, 6B. Tear secretion was statistically analyzed using the multiple comparison during the experimental period of 24 hours.

There was a significant difference in the cotton thread test between the DMSO group and the ARB group (Fig. 6A). (Bonferroni/Dunn test; $P < 0.01$).

Discussion

The presence of a tissue-specific RAS is well documented in a number of organs, implying that various tissues have the ability to synthesize angiotensin II independent of the systemic RAS.[53,54] Our present study clearly demonstrated the presence of tissue RAS in the mouse lacrimal gland.

First of all, we examined the expression of RAS component mRNAs using female WT 8-week-old BALB/c (H-2^d) mice, female WT 31-week-old BALB/c (H-2^d) mice, and male WT 8-week-old BALB/c (H-2^d), and demonstrated that all the RAS components were detected irrespective of age and sex.

Recent findings have revealed that locally generated angiotensin II plays a pivotal role in normal physiology as well as pathophysiology in various tissues and organs. Under normal physiologic conditions, RAS is involved in regulating exocrine and endocrine functions. AT1R is abundant in secretory epithelial tissue, and angiotensin II has a widespread role in maintaining epithelial structure and function (e.g., in regulating water and electrolyte transport).[13,55,56] In the pancreas, AT1R may regulate pancreatic ductal bicarbonate and chloride secretion, and local angiotensin II may influence insulin release partly through the regulation of islet blood flow.[24]

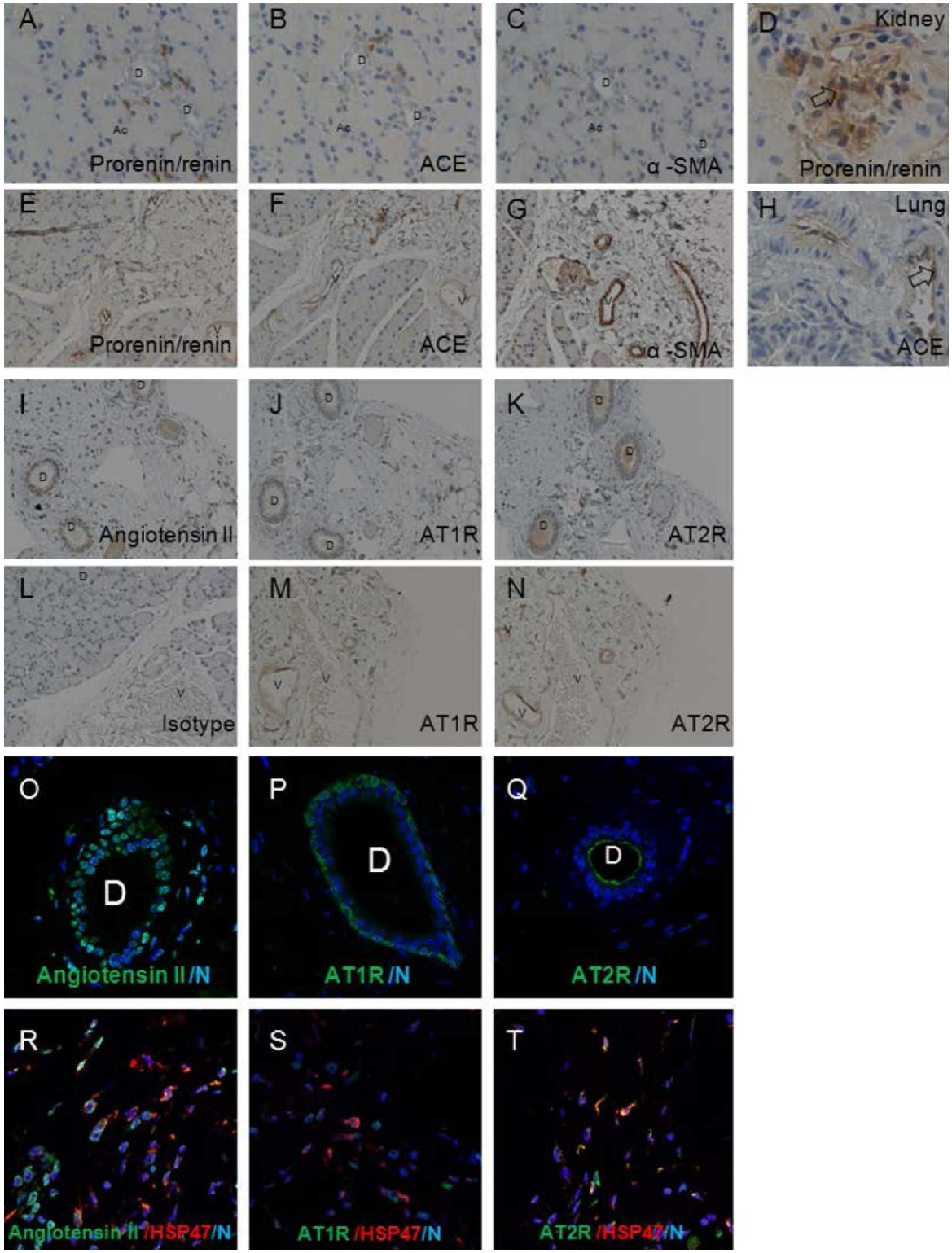

Figure 3. Immunohistochemical analysis of RAS components (prorenin/renin, ACE, angiotensin II, AT1R, and AT2R) in mouse lacrimal gland and prorenin/renin and ACE in kidney and lung specimens. Immunostaining for prorenin/renin and ACE in the lacrimal gland shows intense brown staining on cells around the ducts and acini (**A**, **B**) and on blood vessels (**E**, **F**). Myoepithelial cells (**C**) and blood vessels (**G**) are positive for α-SMA. Kidney (*arrow* shows juxtaglomerular cells) and lung (*arrow* shows vascular endothelium) samples were examined as positive controls for prorenin/renin (**D**) and ACE (**H**), respectively. Angiotensin II, AT1R, and AT2R are localized to the duct and interstitial cells, which were probably fibroblasts (**I**–**K**). AT1R and AT2R were also localized to blood vessels (**M**, **N**). Control sections, in which isotype-matched antibody was used, shows no immunostaining (**L**). Immunofluorescence staining for angiotensin II, AT1R, and AT2R in the ducts reveals that angiotensin II is localized in the epithelial cells (**O**), AT1R in basolateral membrane (**P**), and AT2R in the apical membrane (**Q**). Immunofluorescence double-staining demonstrates that some of the $HSP47^{+}$ fibroblasts also express angiotensin II, AT1R, and AT2R (**R**–**T**). D, duct; Ac, acinus; V, blood vessel; N, nuclei. Magnification: (**A**–**D**, **O**–**T**) ×400; (**E**–**N**) ×200.

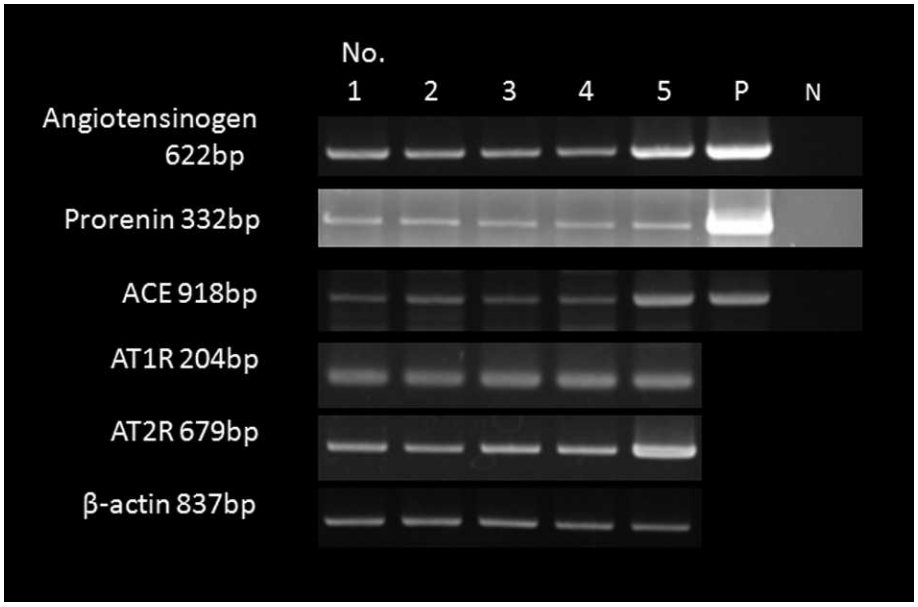

Figure 4. Expression of RAS components mRNAs in cultured fibroblasts from mouse lacrimal glands. The mRNA expression of RAS components angiotensinogen, prorenin, ACE, AT1R, and AT2R was detected in all five samples of lacrimal gland fibroblasts. *Lanes 1–5* (Nos. 1 to 5), mRNA expression in the lacrimal gland fibroblasts prepared from 5 different mice. *Lane 6* (P, positive control), angiotensinogen mRNA expression was examined in the liver, prorenin mRNA in the kidney, and ACE mRNA in the lung. *Lane 7* (N, negative control), the PCR reaction was performed with sterilized water instead of primer.

Concerning the correlation of RAS and a factor involved in secretion, Aquaporins (AQPs) are reported to correlate with RAS. AQPs are a family of water-permeable channel proteins, and they have been shown to account for transcellular permeability in many organisms.[57] Numerous studies evaluated the expression of AQPs in the secretary gland, which indicate that AQPs play a functional role of secretion.[58] Yao et al.[59] reported that ARB (telmisartan; Micardis) attenuates the enhanced expression of aquaporin-2 (AQP2) in renal medulla and concluded that AQP2 and RAS might be involved in the pathogenesis of urinary concentration lesion under a diabetic nephropathy condition. Stegbauer et al.[60] reported that levels of AQP2 protein in renal inner and outer medulla after water deprivation are significantly lower in transgenic mice lacking AT1A receptors only in the collecting duct (CD-KO) mice and concluded that AT1A receptors in epithelial cells of the collecting duct directory modulate AQP2 levels and contribute to the concentration of urine.

In the mouse lacrimal gland, aquaporin-4 (AQP4) labeling was located at the basolateral membrane, whereas aquaporin-5 (AQP5) was situated at the apical membrane of acinar and ductal cells.[61] The localization of AT1R and the AT2R was very similar to that of AQP4 and AQP5; thus, there might be a possibility that RAS correlates with AQPs in the lacrimal gland.

In exocrine organs, myoepithelial cells are observed between the basal lamina and the acinar or ductal epithelial cells. Myoepithelial cells have structural features of both epithelial and smooth muscle cells, and they contract when the gland is stimulated to secrete.[62] In the human lacrimal gland, immunostaining for α-SMA shows that myoepithelial cells form basketlike structures around the acini.[37] In mouse lacrimal glands, renin and ACE were identified in the cells around the ducts and acini. The location of these cells

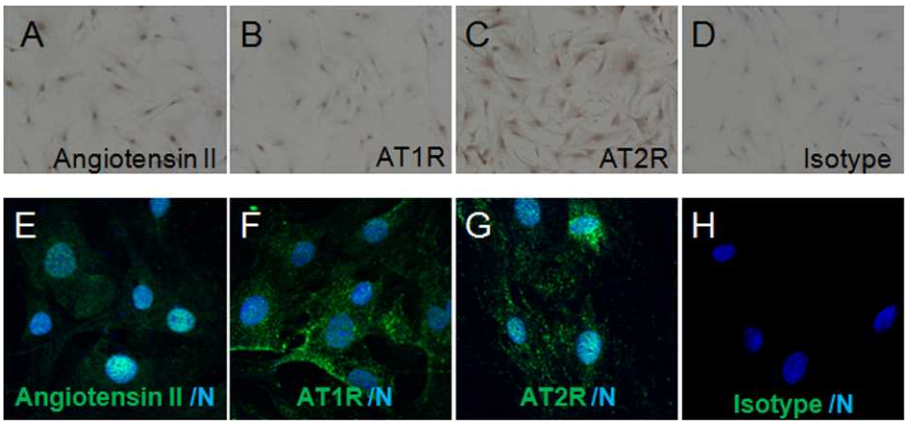

Figure 5. Immunohistochemical analysis of angiotensin II, AT1R, and AT2R in the cultured fibroblasts from mouse lacrimal grand. All the lacrimal gland cultured fibroblasts were positive for angiotensin II (**A**, **E**), AT1R (**B**, **F**), and AT2R (**C**, **G**). Isotype-matched mouse antibodies were used as a negative control (**D**, **H**). N, nuclei. Magnification: (**A–D**) ×100; (**E–H**) ×400.

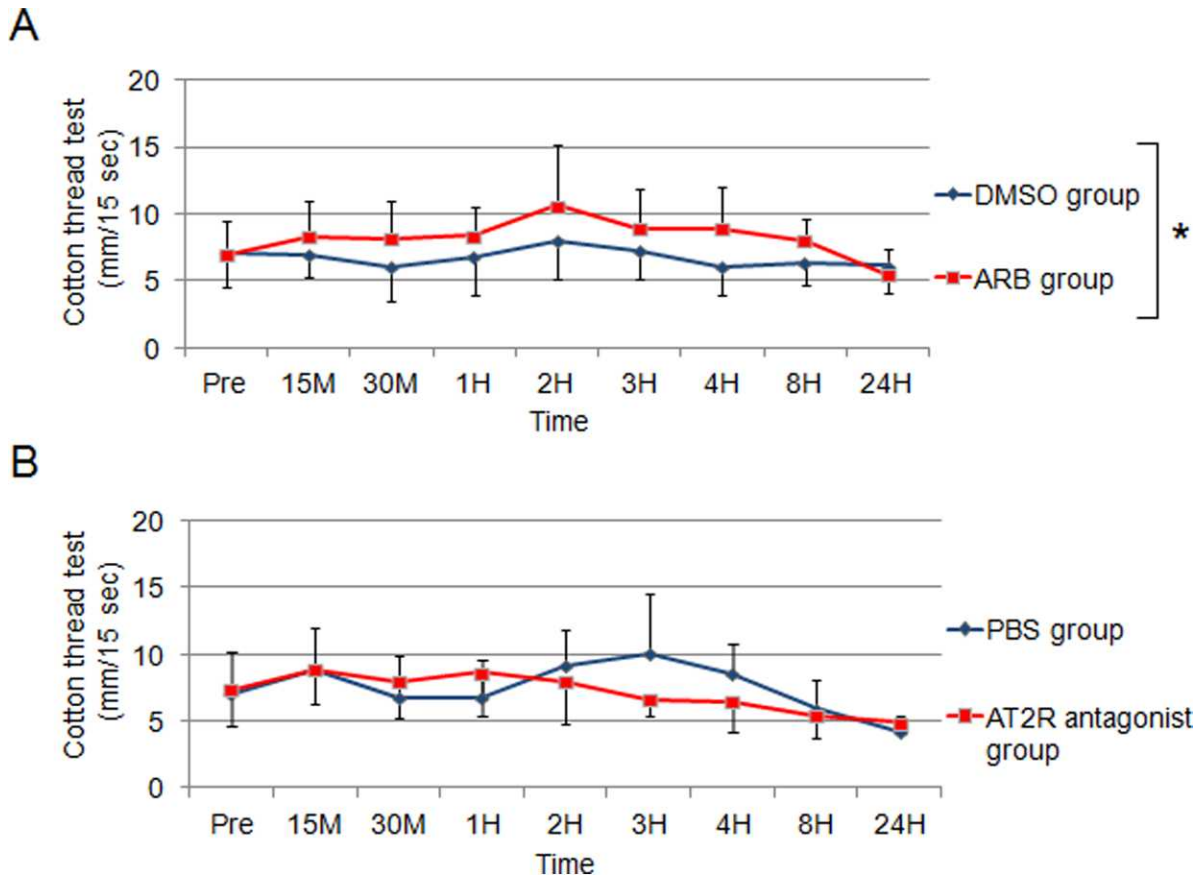

Figure 6. Cotton thread test results before and after drug administration in mice of DMSO, PBS, ARB, and AT2R antagonist groups. (**A**) Effects of ARB on tear secretion in ARB group were compared with DMSO group. (**B**) Effects of AT2R antagonist on tear secretion in AT2R antagonist group was compared with PBS group. There was a significant difference between the DMSO and ARB group during the experimental period of 24 hours. (**A**) $P < 0.01$. The results represents the mean ± SD; $n = 10$ in each group. $^*P < 0.01$ by Bonferroni/Dunn test.

coincided with the α-SMA–immunostained region, suggesting that these were myoepithelial cells. It is suggested that the secretary function of the breast is influenced by nonsecretory cells. Tahmasebi et al.[13] reported that prorenin/renin is identified in fibroblasts and myoepithelial cells in the breast. Veerappan et al.[63] reported that mast-cells release renin, which is a trigger for bronchoconstriction in the airway. These reports indicate a colocation mechanism in the tissue RAS. In the lacrimal gland, myoepithelial cells around the acini may produce angiotensin II and be activated to constrict, to promote secretion.

In this study, administration of ARB increased the mean tear production in the ARB group compared with the DMSO group during the experimental period of 24 hours. There are probably several mechanisms by which valsartan affects tear secretion; we propose three mechanisms. First, tissue RAS may interact with expression of AQP4 and AQP5, and valsartan has effects on the expression of aquaporins as previously reported in the kidney.[59,60] Second, tissue RAS may regulate the blood flow of lacrimal gland through AT1R and AT2R in the blood vessels, and valsartan increased the blood flow. Although it is reported that 200 mg valsartan has no significant effect on blood pressure and heart rate in normotensive subjects on day 1 of administration,[64] the peripheral blood flow may be increased in the lacrimal gland. Third, valsartan may have increased the angiotensin II concentration and renin activity. It is reported that plasma angiotensin II concentration and renin activity are increased after the valsartan administration.[64,65] It is reported that activated airway RAS is triggered by release of mast-cell renin and locally produced angiotensin II induces bronchoconstriction.[63] Based on these reports, we suspected that angiotensin II concentration and renin activity may be locally increased by valsartan, which induced tear secretion.

Taken together, our results suggested that the tissue RAS in the lacrimal gland is involved in the physiologic regulation of tear secretion.

Under pathogenic conditions, tissue RAS has profibrotic activity, which can affect fibroblasts' growth and synthetic properties, and mainly is related to the production of extracellular matrix components.[66–69] Angiotensin II was recently implicated in pathogenic fibrosis in the kidney, heart, lung, and liver.[40–44] Angiotensin II has potential as a profibrotic mediator, and fibroblasts from several organs are reported to be activated by angiotensin II.[70–72]

Furthermore, reactive oxygen species (ROS) have been implicated in the signal transduction of angiotensin II–dependent cellular response via activation of redox-sensitive signaling cascades. One of the main sources of ROS is nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase (NOX), a multiprotein enzyme complex that uses NAD(P)H as a substrate to convert molecular oxygen to ROS.[73] NAD(P)H oxidase activation and increased ROS production are implicated in angiotensin II–induced effects such as vascular smooth muscle hypertrophy, hypertension, pancreatitis, and fibrosis of heart, liver, and kidney.[41,73–75] Our study has reported that the accumulation of oxidative stress has an

influence on the corneal epithelial alternation in blink-suppressed dry eye.[76] If the tissue RAS in the lacrimal gland generates ROS and causes dry eye, medicines that block the RAS can be effective for patients with dry eye. Generation of ROS by RAS might be one of the reasons why the ACE inhibitors lower the risk for dry eye in human patients.[34]

There is a possibility that tissue RAS connects with parasympathetic and sympathetic nerves and that overstimulation or stress mechanisms alert RAS function. It is reported that an angiotensin-sympathetic interaction plays a critical role in long-term blood pressure and that a lack of the normal suppression in angiotensin II and/or sympathetic activity in response to an increase in sodium intake produces salt-sensitive hypertension.[77] In chronic kidney disease, it is reported that parallel activation of the renin and sympathetic system exists.[78] The neural regulation by sensory afferent nerves of the cornea and conjunctiva and the efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland play an important role regulating lacrimal gland secretion.[36] However, these nerve functions are impaired in the lacrimal gland of patients with dry eye. The mechanism is reported that proinflammatory cytokines block the release of neurotransmitters from the afferent sensory nerves and the efferent parasympathetic and sympathetic nerves preventing their stimulation of lacrimal gland secretion, resulting in an inadequate secretion leading to aqueous-deficient dry eye.[36] The angiotensin-sympathetic interaction may present in the lacrimal gland and their imbalance may cause dry eye.

The tissue RAS in the lacrimal gland may play a role in the dry eye disease process. Further evidence is needed to demonstrate that the tissue RAS is relevant to dry eye disease. The main diseases for which the lacrimal gland is one of the target organs are IgG4-related systemic disease (IgG4-RSD), Sjögren's syndrome, and chronic GVHD. The lacrimal gland of the patient with IgG4-RSD shows fibrosis and a prominent infiltration of lymphocytes and IgG4-positive plasma cells.[79] Sjögren's syndrome is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the lacrimal glands.[80] The pathogenesis of chronic GVHD in the ocular surface and lacrimal gland involves excessive fibrosis, in which a subset of fibroblasts plays an important role.[38,48] Periductal fibroblasts may interact with T cells in the lacrimal gland of patients with cGVHD, to elicit fibrogenic and immune processes that result in rapidly progressive dry eye.[81,38]

In this study, we investigated whether the interstitial fibroblasts from mouse lacrimal glands express RAS components, and found that the cultured lacrimal gland fibroblasts expressed angiotensin II, AT1R, and AT2R. Fibroblasts may augment the expression of RAS components in the presence of inflammatory events, and fibrosis may be increased in the lacrimal gland microenvironment. Therefore, the tissue RAS might be involved in the pathogenesis of inflammation and fibrosis in the lacrimal gland. Further study using a model that demonstrates the fibrotic change in the lacrimal gland[82,83] may reveal how inflammatory processes affect the expression of lacrimal gland RAS components.

Nonproteolytic activation of prorenin results from the interaction of prorenin receptor with the handle region of the prorenin segment is hypothesized to play a critical role in the activation of tissue RAS.[84,85] Further studies to investigate the expression of prorenin receptor and the presence of processing enzyme such as cathepsin B, which achieve the conventional proteolysis of the prorenin prosegment, are needed in the near future.

In summary, we have provided evidence that all the major RAS components that are indispensable for the production of angiotensin II are present in the mouse lacrimal gland. Tissue RAS might be involved in certain aspects of the physiology and pathology in the lacrimal gland.

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