学 位 論 文

Involvement of (pro)renin receptor in the pathogenesis of inflammatory eye diseases

(炎症性眼疾患における(プロ)レニン受容体の病態形成への関与)

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Table of Contents

List of Publications and Presentations

A part of this study was published in the following papers.

1. Ishizuka, E.T., Kanda, A., Kase, S., Noda, K., Ishida, S. Involvement of the receptor-associated prorenin system in the pathogenesis of human conjunctival lymphoma. *Invest Ophthalmol Vis Sci*, **56**, 74-80 (2015).

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1. Ishizuka, E.T. Involvement of the receptor-associated prorenin system in the pathogenesis of human conjunctival lymphoma. 11th Japan-Korea International Symposium in Ophthalmology, November 14, 2016, Sapporo, Japan. (Oral presentation)
2. 石塚 タンエルダル. 血管新生緑内障の線維柱帯における受容体結合プロレニン系の病態形成関与 第20回眼科分子生物学研究会、2016年6月25 - 26日、登別 (Poster presentation)
3. 石塚 タンエルダル、神田 敦宏、新明 康弘、田川 義晃、野田 航介、石田 晋. 血管新生緑内障の線維柱帯における受容体結合プロレニン系の病態関与 第120回日本眼科学会総会、2016年4月7 - 10日、仙台 (Oral presentation)
4. 石塚 タンエルダル、神田 敦宏、加瀬 諭、野田 航介、石田 晋. 結膜リンパ腫における受容体結合プロレニン系の病態への関与 第 36 回日本炎症・再生医学会、2015年7月21 - 22日、東京 (Poster presentation)
5. Ishizuka, E.T., Kanda, A., Shinmei, Y., Dong, Y., Inafuku, S., Tagawa, Y., Noda, K., Ishida, S. Role of the receptor associated prorenin system in trabecular meshwork of glaucoma. ARVO (Association for Research in Vision and Ophthalmology) Annual Meeting, May 3 - 7, 2015, Denver, USA. (Poster presentation)

6. 石塚 タンエルダル、神田 敦宏、加瀬 諭、安藤 亮、董 陽子、稲福 沙織、田川 義晃、野田 航介、石田 晋. 結膜節外辺縁帯 B 細胞性リンパ腫におけるレニン・アンジオテンシン系の病態への関与第. 119 回日本眼科学会総会、2015 年 4 月 16 - 19 日、札幌 (Oral presentation)
7. Ishizuka, E.T., Kanda, A., Shinmei, Y., Kinoshita, S., Dong, Y., Inafuku, S., Tagawa, Y., Noda, K., Ishida, S. Role of the renin-angiotensin system in trabecular meshwork of glaucoma. Asia-ARVO (Association for Research in Vision and Ophthalmology), February 16 - 19, 2015, Yokohama, Japan. (Oral presentation)
8. Ishizuka, E.T., Kanda, A., Kase, S., Noda, K., Ishida, S. Renin-angiotensin system is associated with pathological events of human conjunctival lymphoma. 12th International Symposium for Future Drug Discovery and Medical Care, September 4 - 5, 2014, Sapporo, Japan. (Poster presentation)
9. Ishizuka, E.T., Kanda, A., Kase, S., Kinoshita, S., Takashina, S., Dong, Y., Noda, K., Ishida, S. Role of the renin-angiotensin system in human conjunctival lymphoma. ARVO (Association for Research in Vision and Ophthalmology) Annual Meeting, May 4 - 8, 2014, Florida, USA. (Poster presentation)

Introduction

Advances in science and technology provided humankind with longer lives and better welfare. However, in recent years, the number of elderly people is on the rise, which is accompanied with increasing difficulties in maintaining the welfare and health of elderly people. Moreover, the environment and lifestyles have also changed bringing various risks that initiated and dramatically increased the frequency of diseases like diabetes, cancer, cardiovascular diseases, hypertension and others. In ophthalmology field, for instance, the incidences of ocular diseases such as diabetic retinopathy (DR, a complication of diabetes caused by pathological retinal angiogenesis and hemorrhage due to weakening and lesion of the retinal capillaries under hyperglycemia) and wet age-related macular degeneration (AMD, choroidal neovascularization to subretinal macular region through the Bruch's membrane) are dramatically increasing worldwide as the population ages.[1] Despite new medical and surgical interventions, they still remain as leading causes of vision loss in the elderly.

One of the recent common therapies to the above mentioned ocular diseases is the application of antibodies that inhibit the activity of vascular endothelial growth factor (VEGF), a cytokine responsible for pathological neovascularization and leakiness of retinal capillaries.[2-5] This medication is commonly known as anti-VEGF therapy, where anti-VEGF antibody is injected into the vitreous body to block the activity of VEGF and suppresses pathological angiogenesis and vascular leakage. However, anti-VEGF therapy has limitations as it is usually applied at the late stages of the ocular diseases where the retina is in the state of irreversible degeneration due to inflammation, angiogenesis and fibrosis.[6] Furthermore, various pro-inflammatory and angiogenic factors other than VEGF may play roles in the pathogenesis of the diseases, which may be the reasons for resistance to

anti-VEGF therapy.[7-9] In addition, effective pharmacological therapy has not been established even in other ocular diseases. Therefore, it is necessary to develop new strategies that target additional and alternative molecular pathways to intercept at the early stages and achieve a better prognosis before the diseases turn chronic.

For this reason, we propose the renin-angiotensin system (RAS) as an alternative target molecular pathway for the treatment of ocular diseases. RAS has traditionally been regarded as an important regulatory mechanism for controlling systemic blood pressure and water balance.[10] It increases blood pressure by increasing the amount of fluid in them. When the blood pressure decreases, juxtaglomerular cells in the kidneys release renin, which causes the transformation of angiotensinogen to angiotensin I (Ang I). Renin catalyzes the reaction that converts the angiotensinogen, made in the liver, into Ang I, which is also a precursor hormone that is converted to an active hormone called angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) in the lungs. Ang II is a vasoconstrictive hormone that increases systemic blood pressure, renal perfusion pressure and the glomerular filtration rate. This type of RAS is called as the systemic or circulatory RAS. Recently, the above-mentioned components of RAS were also found to be expressed in various tissues and to be independent of the circulatory RAS, and hence to be called as the tissue RAS. Tissue RAS plays diverse roles in the regulation of growth, inflammation and pathological vascular conditions in several organs (Figure 1)[11, 12]. Until recently, it was shown that tissue RAS is also present in the ocular tissues and ocular pathological conditions develop due to the increase in the expression of molecules like growth factors and inflammatory cytokines through signaling pathways downstream of tissue RAS.[11, 13-18]

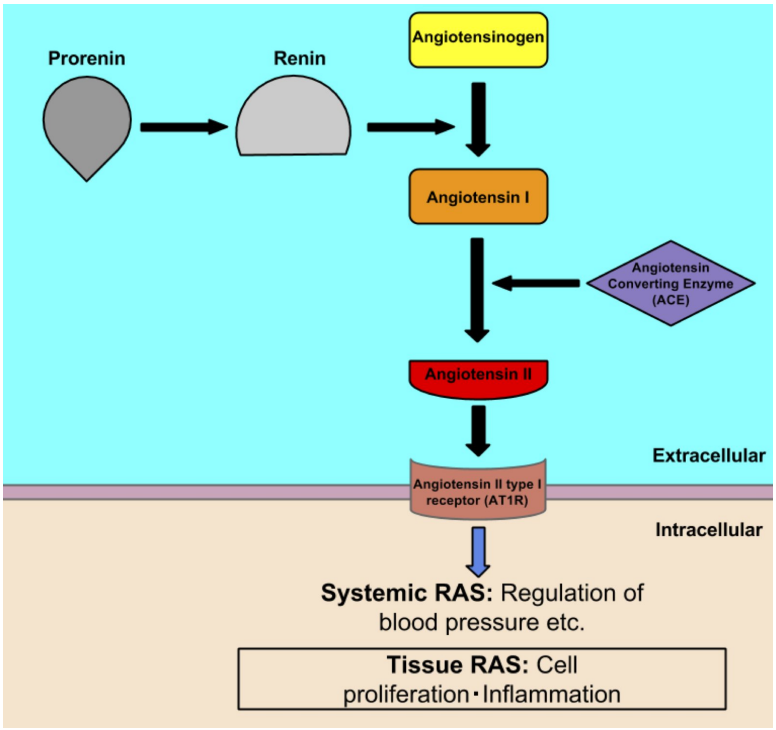

Figure 1. Renin-angiotensin system (RAS)

Previously, prorenin was only known as an inactive precursor of renin, one of the components of RAS. Later on, a receptor for prorenin, called (pro)renin receptor [(P)RR], was identified and a new RAS activation mechanism was reported.[19] (P)RR binds to prorenin, its ligand, to exert renin activity through the conformational change of the prorenin molecule (non-proteolytic activation of prorenin causing tissue RAS) instead of the conventional proteolysis of the prorenin pro-segment by enzymes (proteolytic activation of prorenin in the circulatory RAS). Binding of prorenin to (P)RR triggers dual activation of RAS and RAS-independent signaling pathways, which are involved in the molecular pathogenesis of end-organ damage, such as inflammation and angiogenesis, including ocular disorders [*e.g.,* AMD, proliferative diabetic retinopathy (PDR)].[20-23] We propose to call this system as receptor-associated prorenin system (RAPS) that focuses on (P)RR, which activates both pathways of RAS-independent and RAS-dependent intracellular signals (Figure 2).[22]

Blockades of RAS and/or RAPS have been found to result in beneficial effects on the onset and progression of various ocular diseases some of which were studied extensively by using clinical samples and/or animal models. These ocular diseases and their major pathogenesis are listed on Table 1.

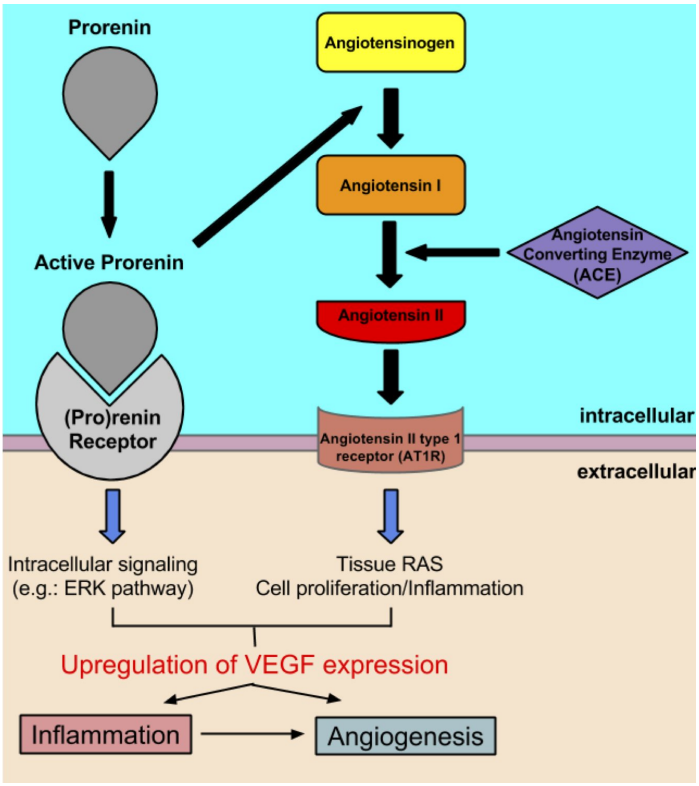

Figure 2. Receptor-associated prorenin system (RAPS)

Table 1. Publications on ocular diseases that RAS and RAPS are involved with RAS

Ocular Disease	Pathogenesis	Experimental samples	Reference
Uveitis	Inflammation	Animal model	11
Ischemic retinopathy	Retinal neovascularization	Animal model	16
AMD	Choroidal neovascularization	Animal model	17
Neuroretinopathy	Inflammation	Animal model	14
DR	Inflammation	Animal model	15
DR	Retinal neuron dysfunction	Animal model	13
Corneal neovascularization	Inflammation	Animal model	18

RAPS

Ocular Disease	Pathogenesis	Experimental samples	Reference
Uveitis	Inflammation	Animal model	23
Ischemic retinopathy	Retinal neovascularization	Animal model	24
AMD	Choroidal neovascularization	Animal model	22
DR	Retinal inflammation	Animal model	25
PDR	Angiogenesis and inflammation	Clinical samples	20
PDR	Angiogenesis and inflammation	Clinical samples	21
Epiretinal membrane	Fibrosis	Clinical samples	26
Uveitis	Inflammation	Clinical samples	27

Therefore, it is plausible to hypothesize that RAPS may also play important roles in the molecular mechanism and pathogenesis of various other ocular diseases. Hence, in the first chapter, we sought to study the involvement of RAPS in the molecular pathogenesis of lymphoma of the conjunctiva, a tissue that covers the surface of the eyeball and serves as the first defense against pathogens from the outside world. In the second chapter, we examined the association of RAPS with the molecular pathogenesis of glaucoma, a major cause of irreversible blindness in the world. We performed our research on RAPS as an alternative target molecular pathway for the treatment of these two ocular diseases.

List of Abbreviations

ACE	angiotensin-converting enzyme
AcSDKP	N-acetyl-seryl-aspartyl-lysyl-proline
AGT	angiotensinogen
AH	aqueous humor
AMD	age-related macular degeneration
Ang I	angiotensin I
Ang II	angiotensin II
AT1R	angiotensin II type 1 receptor
AT2R	angiotensin II type 2 receptor
B2M	beta-2-microglobulin
BSG	basigin
CD	cluster of differentiation
CX43	connexin 43
DAPI	4',6-diamidine-2'-phenylindole dihydrochloride
DR	diabetic retinopathy
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EMZL	extranodal marginal zone B-cell lymphoma
FGF2	fibroblast growth factor 2
GTM	glaucoma trabecular meshwork
H_2O_2	hydrogen peroxide
HPRT1	hypoxanthine phosphoribosyltransferase 1
IOP	intraocular pressure
MMPs	matrix metalloproteinases
NF- κ B	nuclear factor-kappa B
NVG	neovascular glaucoma
PBS	phosphate-buffered saline
PDR	proliferative diabetic retinopathy
PLAT	tissue plasminogen activator
PlGF	placental growth factor
POAG	primary open-angle glaucoma
(P)RR	(pro)renin receptor
PRRB	(pro)renin receptor blocker

RAPS	receptor-associated prorenin system
RAS	renin-angiotensin system
REN	prorenin
SC	Schlemm’s canal
SEM	standard error of the mean
TM	trabecular meshwork
VEGF	vascular endothelial growth factor
ZO-1	zona occludens 1

Chapter 1

Involvement of the receptor-associated prorenin system in the pathogenesis of human conjunctival lymphoma

Introduction

The conjunctiva is a mucous membrane that covers the front of the eye and the inside of the eyelids. It serves an important role in the protection of the eye by forming the first contact barrier against harmful agents and pathogens from external environment. It is not only a physical barrier but also contains an immune system composed of conjunctival lymphoid tissue, which provides immune cells such as B-cells, T-cells, macrophages and other antigen-presenting cells when the ocular surface is exposed to pathogenic agents.[28]

Conjunctival lymphoma is one of the common malignancies and usually appears in the eyelids as a pink and fleshy tumor. A large majority of conjunctival lymphoma cases are divided into four pathological subtypes all of which are classified as non-Hodgkin's B-cell lymphomas.[29, 30] Extranodal marginal zone B-cell lymphoma (EMZL) is the most common subtype of conjunctival lymphoma, followed by follicular lymphoma, mantle cell lymphoma and diffuse large B-cell lymphoma (Figure 3).[29, 31-34] There are studies, which report that conjunctival EMZL develops as a result of inflammation in the ocular orbit.[35, 36] In addition, we recently demonstrated that VEGF, a major angiogenic factor that leads to various pathological conditions, was expressed in human EMZL tissues.[37] However, little is known about the molecular mechanism of EMZL pathogenesis.

Inhibitions of RAS/RAPS resulted in beneficial effects on various diseases[12, 20, 38-40] and evidence from experimental models and clinical studies revealed that the inhibitors of RAS reduce tumor growth and metastasis[38, 41-43], suggesting a useful strategy against

malignancies independent of their classical cardiovascular actions. In this chapter, we analyzed the expression of RAPS components in EMZL tissues, and examined the role of RAS/RAPS in the pathogenesis of EMZL of the conjunctiva with the aim to study the RAPS-related molecules, including (P)RR, as potential targets for novel pharmacologic agents and therefore be able to contribute to future pharmacological research for conjunctival lymphoma.

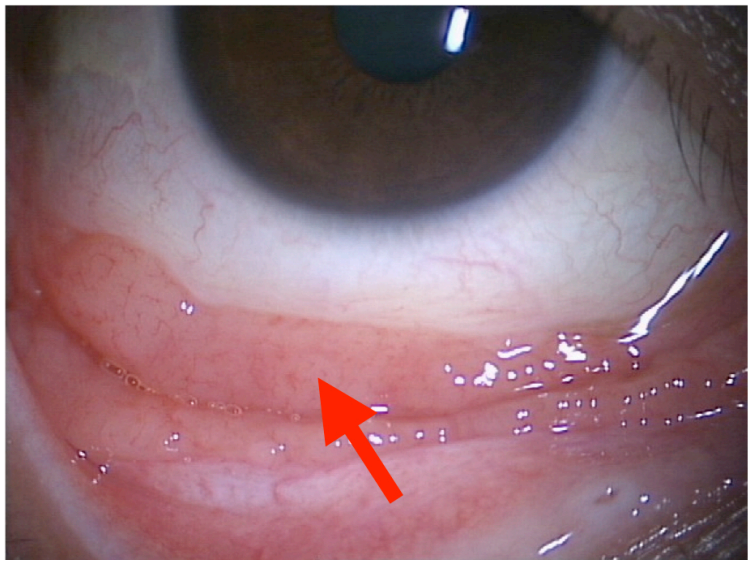

Figure 3. Extranodal marginal zone B-cell lymphoma (EMZL) of the conjunctiva (arrow).

Methods

Human surgical samples

Conjunctival EMZL samples of 5 patients, selected according to Ann Arbor classification Stage 1E or 2E, were removed surgically. The samples were used for further gene expression and immunohistochemical analyses. This study was conducted in accordance with the tenets of the Declaration of Helsinki and after receiving approval from the institutional review board of Hokkaido University Hospital. All patients were informed by explaining the purpose and procedures of the study and written informed consent was obtained.

Hematoxylin and eosin staining was performed to confirm the presence of small- to medium-sized atypical lymphoid cells. Immunohistochemistry using anti-cluster of differentiation (CD)3, CD5, CD10, CD20, and cyclin D1 antibodies (DAKO, Carpinteria, CA) was performed as EMZL cells are $CD3^{-}$, $CD5^{-}$, $CD10^{-}$, and $CD20^{+}$ and are usually cyclin $D1^{-}$. Kappa/lambda deviation was also analyzed. Immunoglobulin heavy chain gene rearrangement was determined by Southern blot analysis or PCR methods, and flow cytometry was applied to clarify the cellular origin and to confirm the B-cell monoclonality in the EMZL tissues.[44] Systemic involvements were evaluated using positron emission tomography–computed tomography, magnetic resonance imaging, and bone marrow puncture.

Cell culture and chemicals

Human B-lymphoblast IM-9 cells were determined as appropriate cells for *in vivo* studies and were purchased from American Type Culture Collection (Manassas, VA). The cells were

cultured under an atmosphere of 5% CO2 at 37°C in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Wako Pure Chemical Industries).

To cover the handle region of the prorenin molecule, which is the binding site of (P)RR [20], decoy peptides NH_2-RIFLKRMPSI-COOH as human (P)RR blocker (PRRB) were synthesized and purified using high-pressure liquid chromatography on a C-18 reverse-phase column by GeneDesign (Osaka, Japan). The purity and retention time of high-performance liquid chromatography was 96.4% and 16.7 min, respectively. The mass of the product was 1261.0 and similar to the theoretical mass value (1260.6). IM-9 cells were seeded in 6-well plate and incubated for 24 hr in serum supplied cell culture medium and then serum-depleted for another 24 hr. After serum deprivation, the cells were pretreated with 1 μM PRRB or 10 μM angiotensin II type 1 receptor (AT1R) blocker valsartan (Sigma-Aldrich, St. Louis, MO) for 1 hr. Prorenin or angiotensin II (Ang II) was then added at a final concentration of 10 nM or 1 μM, respectively. Cells were incubated for another 24 hr and processed for further analyses to detect the mRNA expression levels, as described in the section below.

Reverse transcription-PCR (RT-PCR) and real-time quantitative (q)PCR analyses

Total RNA was isolated from EMZL tissues and cells using TRIzol (Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. Reverse transcription (RT) was performed with GoScript Reverse Transcriptase (Promega, Madison, WI) and oligo dT(20) primers. Briefly, 1 μg of total RNA was mixed with oligo dT(20) primers and random primers in a microcentrifuge tube and the total reaction volume set to 5 μL by adding nuclease-free water. The tube was heated at 70°C for 5 min and then immediately

transferred on ice to facilitate primer annealing. Reverse transcription reaction mix was prepared as described in the manual (total reaction volume 20 μL). The reaction was set at 42°C for 1 hr followed by reverse transcriptase inactivation at 70°C for 15 min. The resulting cDNA was used for conventional PCR and real-time PCR analyses. Conventional PCR was performed using the GoTaq DNA Polymerase (Promega) using the protocol of the manufacturer. Template for the real-time PCR was 1:5 dilution of the first strand reaction mix. Reaction conditions were as follows: one 94°C for 5 min, and 35 cycles of 94°C for 30 s denaturation, 55°C for 30 s annealing and 72°C for 30 s extension. Real-time qPCR was performed using the GoTaq qPCR Master Mix (Promega) and StepOne plus System (Life Technologies) using the protocol of the manufacturers. Template for the real-time PCR was 1:5 dilution of the first strand reaction mix. All the samples to be compared were processed in parallel and 4 independent experiments were performed. Reaction conditions were as follows: one 95°C for 2 min, and 40 cycles of 95°C for 15 s denaturation and 60°C for 1 min annealing and extension. The quantity of mRNA expression was calculated by normalizing the threshold cycle (Ct) of the target genes to the Ct of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene in the same sample, according to the comparative ddCt method. The primers used in this experiment are listed on Table 2.

Table 2. Primer sequences used in RT-PCR and real-time qPCR

Target gene	Sequence
(P)RR	forward 5'-AGG CAG TGT CAT TTC GTA CC-3'
	reverse 5'-GCC TTC CCT ACC ATA TAC ACT C-3'
REN	forward 5'-GTG TCT GTG GGG TCA TCC ACC TTG-3'
	reverse 5'-GGA TTC CTG AAA TAC ATA GTC CGT-3'
AGT	forward 5'-CTG CAA GGA TCT TAT GAC CTG C-3'

	reverse 5'-TAC ACA GCA AAC AGG AAT GGG C-3'
ACE	forward 5'-CCG AAA TAC GTG GAA CTC ATC AA-3'
	reverse 5'-CAC GAG TCC CCT GCA TCT ACA-3'
AT1R	forward 5'-AGG GCA GTA AAG TTT TCG TG-3'
	reverse 5'-CGG GCA TTG TTT TGG CAG TG-3'
AT2R	forward 5'-GGC CTG TTT GTC CTC ATT GC-3'
	reverse 5'-CAC GGG TTA TCC TGT TCT TC-3'
HPRT1	forward 5'-ACC CCA CGA AGT GTT GGA TA-3'
	reverse 5'-AAG CAG ATG GCC ACA GAA CT-3'
FGF2	forward 5'-GCG GCT GTA CTG CAA AAA ACG-3'
	reverse 5'-AAG TTG TAG CTT GAT GTG AGG G-3'
BSG	forward 5'-CCA TGC TGG TCT GCA AGT CAG-3'
	reverse 5'-CCG TTC ATG AGG GCC TTG TC-3'
MMP2	forward 5'-TGA TGG TGT CTG CTG GAA AG-3'
	reverse 5'-GAC ACG TGA AAA GTG CCT TG-3'
MMP9	forward 5'-TTG ACA GCG ACA AGA AGT GG-3'
	reverse 5'-GCC ATT CAC GTC GTC CTT AT-3'
MMP14	forward 5'-GAA GCC TGG CTA CAG CAA TAT G-3'
	reverse 5'-TGC AAG CCG TAA AAC TTC TGC-3'

Immunohistochemistry

EMZL tissue samples, described in the "Human Surgical Samples" section above, were fixed in 4% paraformaldehyde in the operating room soon after excision. Following fixation, the samples were preserved as paraffin-embedded blocks and sectioned on glass slides at 5-μm thickness. Sections were deparaffinised and hydrated by exposing to xylene, series of 100%, 90%, 80% and 70% graded alcohols, water, and rinsed in phosphate-buffered saline (PBS). As a pretreatment, sample antigens were retrieved by inserting the glass slide in a 10 mM citrate buffer (pH 6.0). The glass slide in citrate buffer was then boiled in a microwave oven for 10 min and let to cool down to room temperature. Peroxidase blocking was performed

with REAL Peroxidase Blocking Solution (DAKO) for 30 min. To block unspecific binding of the antibodies, slides were incubated for 30 min with PBS containing 5% goat serum. Afterwards, sections were incubated with rabbit polyclonal antibodies against human (P)RR (1:100, Sigma-Aldrich) and AT1R (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA/0.05% Triton X-100/PBS for overnight. After the washing step, visualization was performed using the Envision HRP kit (DAKO), following the manufacturer instructions. Normal rabbit IgG was used as a negative antibody control. Sections were examined using a BIOREVO microscope (Keyence, Osaka, Japan).

Immunofluorescence microscopy

For immunofluorescence analyses, sections were pretreated with fixation, antigen retrieval and serum blocking as described in the above immunohistochemistry section, except peroxidase blocking. The sections then were incubated with the following primary antibodies: rabbit anti-(P)RR (1:100, Abcam), rabbit anti-AT1R (1:100, Abcam), mouse anti-prorenin (REN; 1:75, Abcam), goat anti-angiotensinogen (AGT; 1:50, Santa Cruz Biotechnology), mouse anti-CD20 (1:100, DAKO), mouse anti-CD31 (1:100, DAKO), mouse anti-fibroblast growth factor (FGF)2 (1:75, Millipore, Temecula, CA), mouse anti-basigin (BSG) (1:50, Millipore), goat anti-matrix metallopeptidase (MMP)2 (1:100, Santa Cruz Biotechnology), goat anti-MMP9 (1:50, Santa Cruz Biotechnology), and mouse anti-MMP14 (1:40, Millipore) antibodies in 1% BSA/0.05% Triton X-100/PBS for overnight. Secondary antibodies for fluorescent detection were AlexaFluor 488 and 546 conjugated antibodies (1:400, Life Technologies). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and sections were visualized under a BIOREVO microscope (Keyence).

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) with their respective n-numbers indicated in the figures sections below. Two-tailed Student's t-test was applied to determine significant differences between the experimental groups. Differences between the means were considered statistically significant when $P < 0.05$.

Results

Expression and localization of RAPS components in EMZL tissues and IM-9 cells

Our previous reports demonstrated that (P)RR and other RAS components were expressed in surgically excised fibrovascular tissues obtained from patients with proliferative diabetic retinopathy, human retinal cell lines and the mouse retina.[11, 20, 45] To study the pathological role of RAPS in conjunctival EMZL, we first needed to confirm whether RAPS components were expressed in EMZL tissues and human B lymphoma cell line IM-9. Gene expressions of RAPS component genes [*(P)RR*, *REN* (prorenin), *AGT*, *ACE*, *AT1R,* and *AT2R*] were confirmed in all of the clinical EMZL samples and IM-9 cells (Figure 4A). To further validate these gene expression study results, we performed immunohistochemical analyses on conjunctival EMZL tissues. (P)RR and AT1R (two major receptors of the RAPS) were observed to be widely expressed throughout the conjunctival EMZL tissues, including atypical lymphoid cells and vascular endothelial cells (Figure 4B, C). Normal IgG served as the negative control (Figure 4D).

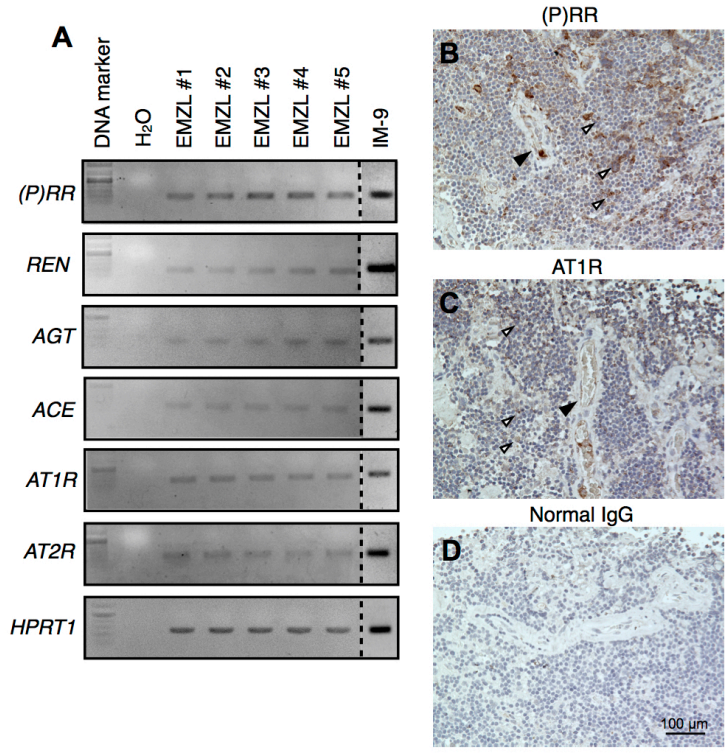

Figure 4. Gene expression of RAPS components and localization of (P)RR and AT1R in human conjunctival EMZL tissues and IM-9 cells. **(A)** Gene expression of RAPS components in EMZL tissues of human conjunctiva and IM-9 cell line. Semi-quantitative reverse transcription PCR analysis was performed to check the expression of RAPS component genes [*(P)RR*, *REN*, *AGT*, *ACE*, *AT1R* and *AT2R*] in five conjunctival EMZL tissues (EMZL #1-5) and IM-9 cells. **(B-D)** Immunohistochemical staining of (P)RR and AT1R in human conjunctival EMZL tissues. Arrowhead: endothelial cells, open arrowhead: B-cells, scale bar: 100 μm.

Localization of prorenin and (P)RR in endothelial and lymphoid cells of EMZL tissues

To further verify the expression and localization of (P)RR in conjunctival EMZL tissues, we performed immunofluorescence analysis. The results of double-staining experiments demonstrated that (P)RR signal co-localized with CD20, a B-cell marker (Figure 5A-C), and with CD31, a vascular endothelial cell marker (Figures 5D-F). These results indicated that (P)RR was expressed in the B-lymphoid and vascular endothelial cells of conjunctival EMZL tissues. Additionally, (P)RR signals abundantly co-localized with prorenin signals in B-cells of conjunctival EMZL samples (Figure 5G-I).

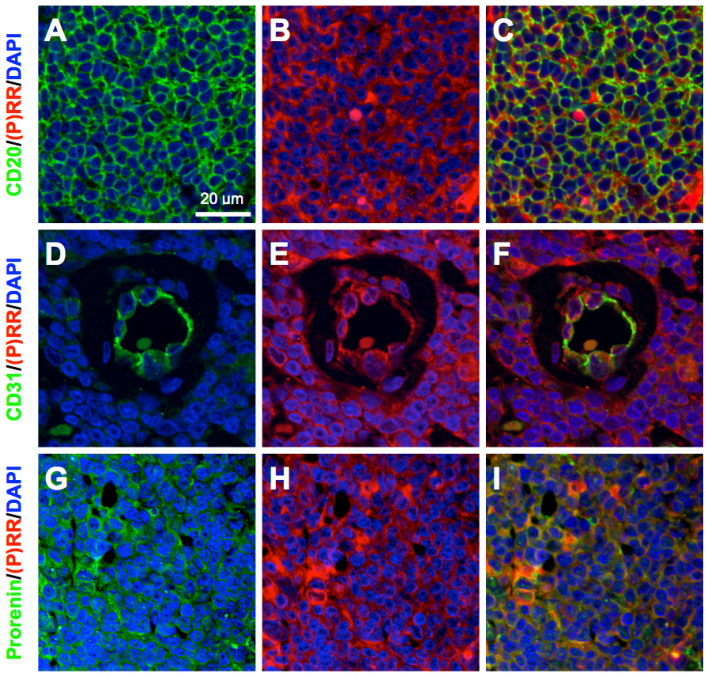

Figure 5. Immunofluorescent analyses of prorenin and (P)RR in human conjunctival EMZL tissues. **(A-C)** Double-labeling of CD20 (*green*), (P)RR (*red*) and DAPI (*blue*). **(D-F)** Double-labeling of CD31 (*green*), (P)RR (*red*) and DAPI (*blue*). **(G-I)** Double-labeling of prorenin (*green*), (P)RR (*red*) and DAPI (*blue*). Scale bar: 20 μm.

Localization of AGT and AT1R in endothelial and lymphoid cells of EMZL tissues

We also performed immunofluorescence double-staining of AT1R (another major receptor of RAPS) with CD20 or CD31. AT1R signal co-localized with CD20 signal, which indicated that AT1R was expressed in the B-lymphoid cells (Figure 6A-C). AT1R signal also co-localized with and CD31, which indicated that AT1R was expressed in vascular endothelial cells of conjunctival EMZL tissues (Figure 6D-F). In addition, AT1R immunoreactivity also co-localized with AGT on lymphoid cells of EMZL (Figure 6G-I).

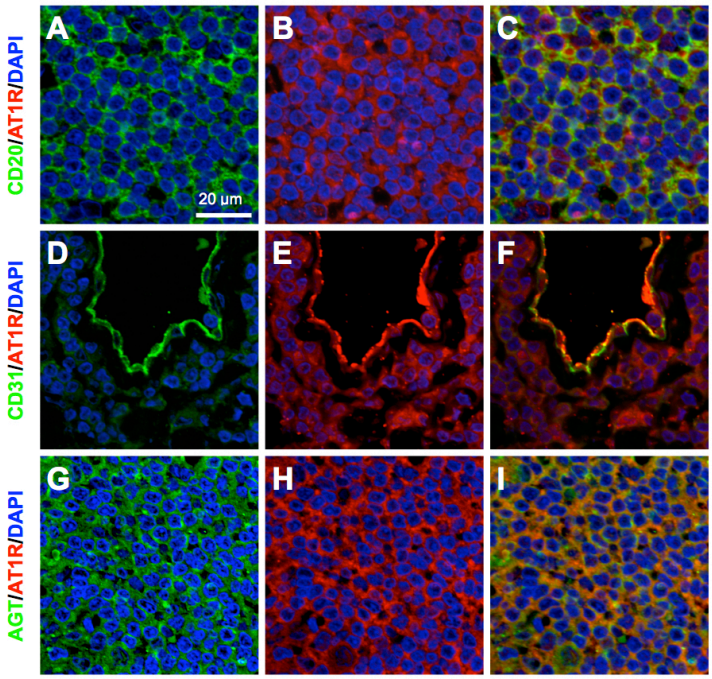

Figure 6. Immunofluorescent analyses of AGT and AT1R in human conjunctival EMZL tissues. **(A-C)** Double-labeling of CD20 (*green*), AT1R (*red*) and DAPI (*blue*). **(D-F)** Double-labeling of CD31 (*green*), AT1R (*red*) and DAPI (*blue*). **(G-I)** Double-labeling of AGT (*green*), AT1R (*red*) and DAPI (*blue*). Scale bar: 20 μm.

Upregulation of *FGF2* expression via prorenin-(P)RR interaction

Previously, it has been shown that when prorenin binds to (P)RR and/or Ang II binds to AT1R, they upregulate the expression of various genes both *in vivo* and *in vitro*, which consequently lead to the pathogenesis of numerous diseases.[20, 46-51] We and other groups have also demonstrated that stimulations of (P)RR and AT1R by their respective ligands, prorenin and Ang II, significantly up-regulate the mRNA expressions of genes such as *MMP2* and *MMP9* in retinal vascular endothelial cells.[20, 46, 48] However, there are no reports showing the effects of prorenin and/or Ang II stimulations on B-cells. Therefore, in this study, we first sought to investigate the effect of prorenin-(P)RR binding on B-lymphoid cells and examined whether stimulation of prorenin affects mRNA expression levels in human B-lymphoma cell line IM-9. The results of our experiments showed that expression level of *FGF2* gene significantly increased (fold change = 2.24, $P < 0.01$) in IM-9 cells stimulated with prorenin compared to the expression level of *FGF2* in control group. On the other hand, AngII-AT1R interaction had no effect on the expression level of *FGF2* (fold change = 0.87, $P > 0.05$). Importantly, the increase in the level of *FGF2* stimulated by prorenin-(P)RR interaction was inhibited by pretreatment of the cells with PRRB (fold change = 1.41, $P < 0.05$) (Figure 7A).

Following these results, we investigated the relevance of (P)RR to FGF2 at conjunctival EMZL tissues. We performed immunofluorescence experiments to investigate the expression of FGF2 with (P)RR in conjunctival EMZL samples. The results of immunofluorescence analysis revealed that FGF2 and (P)RR are expressed and co-localize at conjunctival EMZL tissues (Figure 7B-D). Our data suggest that activation of RAPS through the interaction of prorenin and (P)RR induces an increase in the expression level of

FGF2 in B-lymphocytes, and possibly leads to angiogenesis and pathology of the EMZL of the conjunctiva.

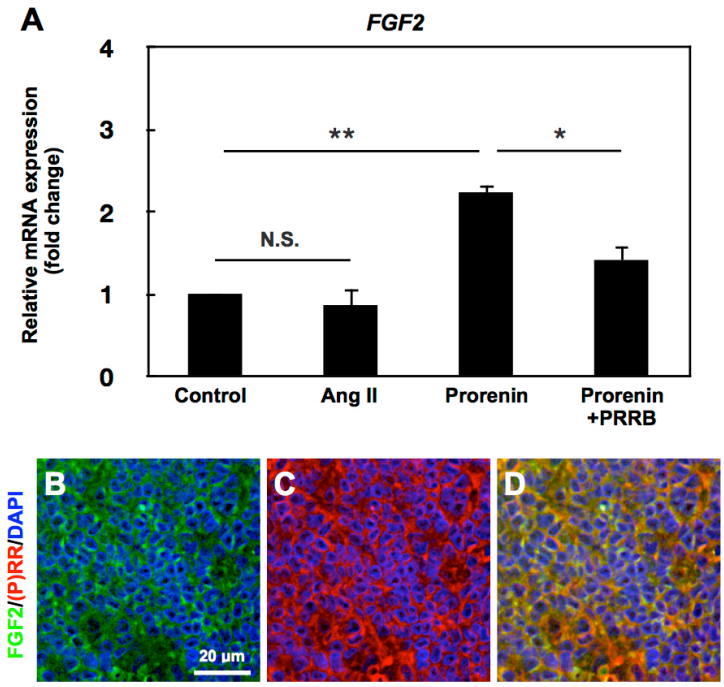

Figure 7. (P)RR-mediated upregulation of *FGF2* in human B-lymphoma cells. **(A)** Relative mRNA expression level of *FGF2* in Ang II or prorenin-stimulated IM-9 cells with or without PRRB compared with control. n = 4, * $P < 0.05$, ** $P < 0.01$, N.S. = nonsignificant. **(B-D)** Double-labeling of FGF2 (*green*), (P)RR (*red*) and DAPI (*blue*) in human conjunctival EMZL tissues. Scale bar: 20 μm.

Upregulation of MMP family members via Ang II–AT1R interaction

To study the effect of Ang II-AT1R interaction to pathogenesis related genes in B-lymphoma cells, we stimulated IM-9 cells with Ang II addition and subsequently examined the changes in gene expression levels using real-time qPCR analysis. Ang II stimulation of IM-9 cells significantly increased the expression levels of *BSG* (fold change = 2.25, $P < 0.05$), *MMP2* (fold change = 2.56, $P < 0.05$), *MMP9* (fold change = 3.64, $P < 0.05$) and *MMP14* (fold change = 1.83, $P < 0.05$) compared to the expression level of the same genes control group. Pretreatment of cells with valsartan before Ang II addition suppressed Ang II-induced increases in *BSG*, *MMP2*, *MMP9* and *MMP14* expression levels (*BSG*, fold change = 0.90, $P < 0.01$; *MMP2*, fold change = 1.75, $P < 0.05$; *MMP9*, fold change = 0.91, $P < 0.05$; *MMP14*, fold change = 1.03, $P < 0.05$) (Figure 8A-D).

In addition to these *in vitro* experiment results, we studied co-localization of BSG, MMP2, MMP9 and MMP14 with AT1R in conjunctival EMZL samples. The immunofluorescence signals of BSG, MMP2, MMP9 and MMP14 were widely distributed in conjunctival EMZL sections (Figure 8E, 8H, 8K, and 8N). BSG, MMP2, MMP9 and MMP14 also co-located with AT1R (Figure 8F, 8G, 8I, 8J, 8L, 8M, 8O, and 8P) in the EMZL of the conjunctiva sections. Our results suggest that when Ang II binds to AT1R in the lymphocytes, this stimulation triggers tissue RAS and causes increase in the expression levels of MMP family genes and leading to the sequence of events at the molecular level to initiate angiogenesis, invasion and therefore the pathogenesis of conjunctival EMZL.

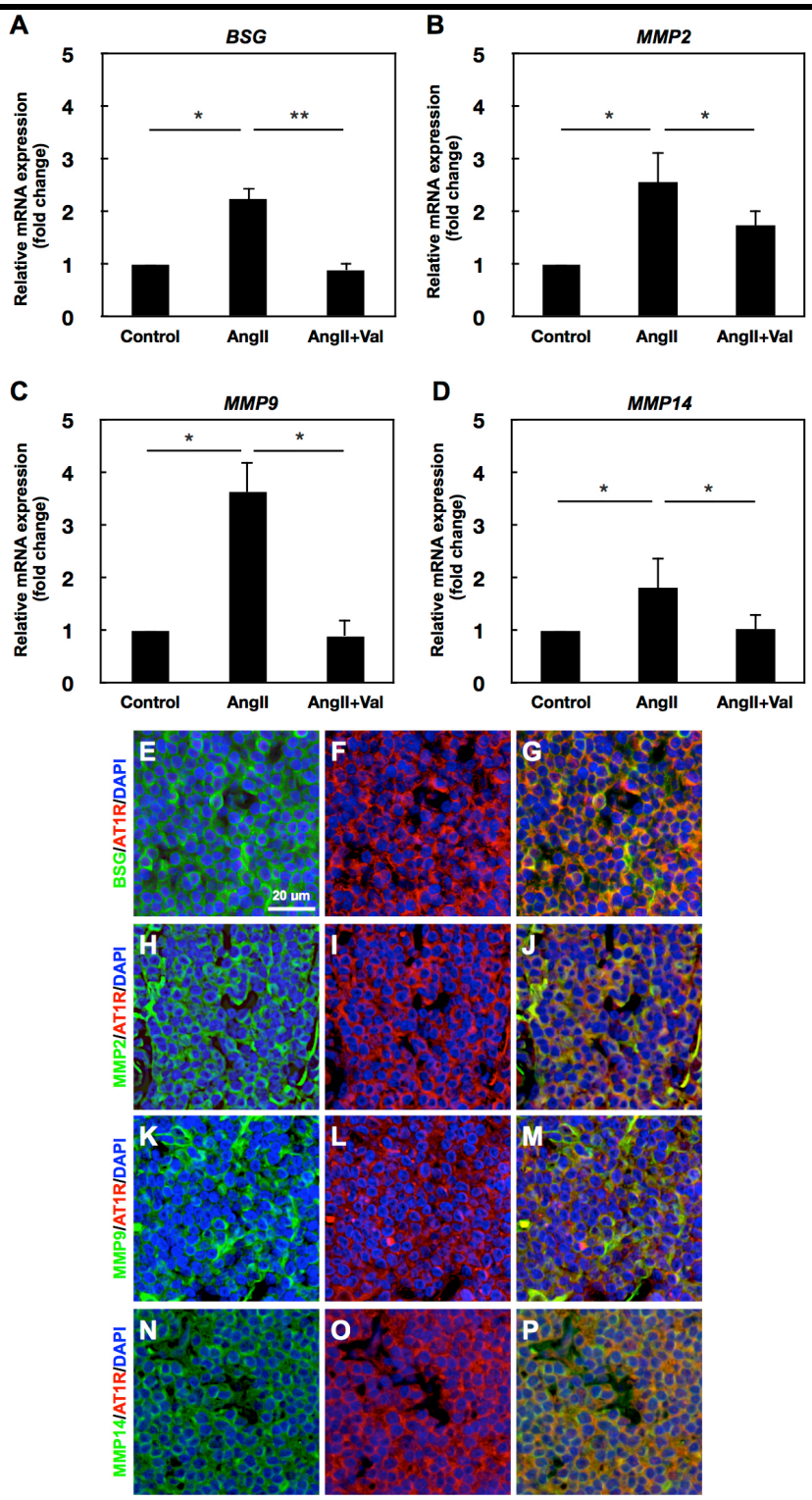

Figure 8. AT1R-mediated upregulation of *BSG*, *MMP2*, *MMP9* and *MMP14* in human B-lymphoma cells. **(A-D)** Relative mRNA expression levels of *BSG*, *MMP2*, *MMP9*, and *MMP14* in Ang II-stimulated IM-9 cells with or without valsartan (Val) compared with control. n = 4. * $P < 0.05$, ** $P < 0.01$. Double labeling of BSG **(E-G)**, MMP2 **(H-J)**, MMP9 **(K-M)** and MMP14 **(N-P)** (*green*), AT1R (*red*) and DAPI (*blue*) in human conjunctival EMZL tissues. Scale bar: 20 μm.

Summary

The results of this study shed a new light in our understanding on the roles of RAPS and RAS in the pathogenesis of conjunctival EMZL. First, we confirmed that RAS component genes were expressed both in surgically excised EMZL tissues and human B-lymphoma cell lines (Figure 4). We could observe the immunofluorescence signals of (P)RR and AT1R in both B-lymphocytes and endothelial cells of the conjunctival EMZL tissues. (P)RR co-localized with prorenin and AT1R with AGT (Ang II precursor) (Figures 4-6). Stimulation of B-lymphoma cell culture with prorenin triggered an increase in the expression level of *FGF2* through (P)RR interaction, and (P)RR co-localized with FGF2 in the B-lymphomas of conjunctival EMZL tissues (Figure 7). Stimulation of IM-9 cell AT1R with Ang II increased *BSG* and *MMP*s gene expression levels. These effects of Ang II were suppressed by pre-treatment with valsartan, and immunofluorescence analyses showed co-localization of AT1R with these MMP-related molecules (Figure 8). Our results indicate that activation of RAPS through the (P)RR/AT1R axis pathways leads to change in the expression profiles of FGF2 and MMPs, and is associated with the pathogenesis of conjunctival EMZL.

Discussion

Here, we investigated the role of RAPS in the molecular pathogenesis of the EMZL of conjunctiva. FGF2 is known for pro-angiogenic properties and is widely expressed in various tumor tissues. When its expression level increases, this effect triggers inflammation, tumor growth, progression, and metastasis.[52] It was found that expression level of FGF2 was significantly elevated in sera or tissue specimens of both Hodgkin's and non-Hodgkin's lymphomas.[53, 54] Additionally, it has been shown that the binding of FGF2 to its receptor, FGF receptor 1, promotes angiogenesis in the tumor microenvironment, which eventually leads to angiogenesis and lymphatic metastasis.[55] Increased expression of FGF2 in diabetes and hypertension were shown to be suppressed by RAS inhibitors.[56, 57] Here, we demonstrated the co-expression of (P)RR and FGF2 in EMZL tissues and also prorenin stimulation of B-lymphocyte cell line significantly increased the expression level of *FGF2*, which was suppressed by PRRB. Taken together, our results suggest that prorenin-(P)RR interaction (*i.e.,* activation of RAPS) is responsible for the increase in the expression of *FGF2*, and the possible induction of angiogenesis and inflammation in B-cells and eventually for the pathogenesis of conjunctival lymphoma.

Previously it was shown that MMP2 and MMP9 are closely involved in the process of tumor neovascularization, and the extent of neovascularization correlates with MMPs expression during its progression gradually towards more advanced states such as in multiple myeloma and skin T-cell lymphoma.[58] The results of our study showed that Ang II - AT1R binding increased gene expression levels of *BSG*, *MMP2*, *MMP9* and *MMP14* in cultured human B-lymphocytes. As further supporting the above-mentioned *in vitro* data, BSG, MMP2, MMP9 and MMP14 all co-localized with AT1R in the B-lymphomas of conjunctival

EMZL tissues. BSG, alternatively called 'extracellular MMP inducer' or CD147, is a member of the immunoglobulin superfamily and it is abundantly expressed on the surface of tumor cells.[59] BSG-positive tumor cells and their supernatants were shown to increase the expression levels of MMPs, including MMP2 and MMP9.[60, 61] MMP2 and MMP9 are also known as tumor biomarkers in monitoring the response of tumors to cancer treatment.[62] Degradation of type IV collagen by MMP2 is a significant hallmark of metastasis and invasion in carcinoma.[63] MMPs can also initiate growth factor signaling by increasing the bioavailability of factors such as FGF2 and facilitate tumor advancement by angiogenesis stimulation.[64] The release of angiogenic growth factors, cytokines and proteases like FGF2, MMP2, MMP9 and MMP14 into the surrounding extracellular matrix initiates tumor angiogenesis.[65-67] Studies on MMPs in clinical samples reported that BSG and MMP9 expression levels are elevated in non-Hodgkin's and Hodgkin's lymphomas, which are associated with clinical stages of the tumors.[68, 69]

It has been reported that activation of AT1R by the binding of Ang II triggers the upregulation of *BSG* and MMPs including *MMP2*, *MMP9* and *MMP14* in various kinds of cells.[46, 50, 70] Moreover, the activity of serum ACE increased in the Non-Hodgkin's and other types of lymphoma patients. In the bone marrow, ACE cleaves N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), which is an inhibitor of hematopoietic stem cell proliferation, and inactivates it.[71] Increased ACE activity may lead to the acceleration of AcSDKP degradation and result in excessive uncontrolled haematopoietic cell proliferation. In accordance with these findings, our data suggest that binding of Ang II to AT1R (*i.e.*, stimulation of tissue RAS) plays roles in the extracellular matrix turnover and remodeling in B-lymphomas, induces the subsequent sequence of molecular events in the microenvironment,

and leads to uncontrolled B-lymphocyte proliferation and finally the formation and development of conjunctival EMZL.

The therapies to cure conjunctival EMZL are performed mainly with surgical excision and/or irradiation, as EMZL is a low-grade tumor and sensitive to radiotherapy. However, there still are cases where complete remission could not be achieved even after these treatments. Therefore, development of additional therapeutic options is required. Our experimental data obtained by using clinical human EMZL samples and B-lymphoid cells show that activation of RAPS and tissue RAS via (P)RR and AT1R axes are associated with the pathogenesis of conjunctival lymphoma. Interactions of prorenin-(P)RR and AngII-AT1R have been reported to activate mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 and nuclear factor-kappa B (NF-κB) pathways, and induce proliferation and differentiation in various cells.[20, 40, 72] There are several reports proposing the NF-κB signaling pathway as an attractive therapeutic target in T- and B-cell malignancies, also including the EMZL.[73] Blockades of (P)RR and AT1R may be promising to prevent the cascade of molecular events essential in the pathogenesis of EMZL of the conjunctiva and serve as clinical tools in the treatment of conjunctival lymphoma.

Chapter 2

The receptor-associated prorenin system is associated with the molecular pathogenesis of glaucoma in the trabecular meshwork

Introduction

Glaucoma, the second leading cause of blindness in the world, is a group of ocular diseases that damage the optic nerve. It is manifested by progressive retinal ganglion cell death and subsequently can severely impair visual function. Primary open-angle glaucoma (POAG) is the most common form of glaucoma; however, the etiology of glaucoma is still unknown and thought to be multifactorial.[74] A fluid called the aqueous humor (AH) flows continuously and leaves the anterior chamber through a tissue called the trabecular meshwork (TM) that serves as a drain, and AH leaves the eye. However, AH drainage through the TM is occluded in pathological conditions and the AH builds up in the eye, therefore increases the intraocular pressure (IOP) to a level that may damage the optic nerve, leading to vision loss.[75] Neovascular glaucoma (NVG) is a secondary glaucoma type (Figure 9), which occurs as a complication of ischemic retinal diseases like PDR. The pathology of NVG is initiated with the formation of new vessels on the anterior surface of the iris and over the TM. This can finally obstruct the AH outflow and thus elevate the IOP.[76, 77] Previous reports showed that angiogenic factors and inflammatory molecules are involved in the pathogenesis of POAG and NVG.[78-81]

It has been reported that extracellular matrix (ECM) plays an important role in the structure of TM cells, which control the AH drainage pathway, and this is consequently involved the pathogenesis of glaucoma.[82] RAS and RAPS are also shown to exist in cornea

and lacrimal gland[18, 83], as well as retina, and are involved in the regulation of expression of MMPs in retinal pigment epithelial cells and human conjunctival EMZL[50, 84, 85] and the production of various inflammatory cytokines.[20, 25-27, 85] Here, using TM tissues of surgical specimen and cultured human TM cells, we aimed to investigate the involvement of RAPS in the molecular pathogenesis of glaucoma in the TM of POAG, most common glaucoma, and NVG, a complication of diabetic retinopathy.

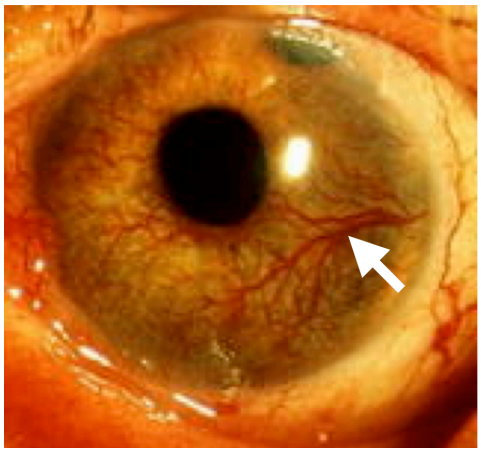

Figure 9. Neovascular glaucoma with pathological neovascularization on the iris (arrow).

Methods

Human surgical samples

TM samples from 4 POAG patients (1 male and 3 females, average age = 71.5 ± 13.2 years) and 5 NVG patients (2 males and 3 females, average age = 60.2 ± 9.3 years) were surgically removed and used for gene expression and immunohistochemical analyses. AH from 18 patients were used for protein expression analysis. Out of these 18 patients, 6 of them (4 males and 2 females, average age = 59.7 ± 8.8 years) were diagnosed with POAG and 6 of them (4 males and 2 females, average age = 62.3 ± 9.2 years) were diagnosed with NVG. AH was collected during surgery. From 6 eyes of 6 age-matched patients (3 males and 3 females, average age = 73.3 ± 6.6 years) undergoing routine surgery for age-related cataract, control AH samples were collected by anterior chamber paracentesis (the removal of AH from the anterior chamber, the area just anterior to the iris and lens, and immediately posterior to the cornea). AH samples were not diluted and frozen rapidly and stored at -80°C until further analyses. The clinical characteristics of the patients are listed on Table 3. The study was conducted in accordance with the tenets of the Declaration of Helsinki and after receiving approval from the institutional review board of Hokkaido University Hospital. All patients were informed by explaining the purpose and procedures of the study and written informed consent was obtained.

Table 3. Clinical characteristics of the patients

Case	Surgical indication	Age	Sex	L/R	Underlying disease (Eye)	Underlying disease (Systemic)	Pre-operative IOP on medication (mmHg)	Surgical specimen	Purpose of use
1	POAG	85	F	L	NA	HT	14	TM tissue	RT-PCR, IF
2	POAG	64	F	R	NA	NA	28	TM tissue	RT-PCR, IF
3	POAG	80	F	R	NA	HT	10	TM tissue	RT-PCR, IF
4	POAG	57	M	R	NA	HT	16	TM tissue	RT-PCR, IF
5	POAG	64	M	L	NA	NA	17	AH	ELISA
6	POAG	75	F	R	NA	NA	21	AH	ELISA
7	POAG	58	M	R	NA	NA	19	AH	ELISA
8	POAG	50	M	L	NA	Dyslipidemia	19	AH	ELISA
9	POAG	57	F	L	NA	NA	21	AH	ELISA
10	POAG	54	M	L	NA	NA	15	AH	ELISA
11	NVG	61	F	R	PDR	DM, DN, HT	29	TM tissue	RT-PCR, IF
12	NVG	44	M	R	PDR	DM, HT, BD	21	TM tissue	RT-PCR, IF
13	NVG	64	F	R	PDR	DM, HT, DN	21	TM tissue	RT-PCR, IF
14	NVG	67	M	R	PDR	DM, HT, DN	18	TM tissue	RT-PCR, IF
15	NVG	65	F	R	PDR	DM, MBA	40	TM tissue	IF
16	NVG	76	M	R	PDR	DM	40	AH	ELISA
17	NVG	55	F	L	PDR	DM, HT, dyslipidemia	46	AH	ELISA
18	NVG	52	M	R	PDR	DM	12	AH	ELISA
19	NVG	66	F	L	PDR	DM	29	AH	ELISA
20	NVG	68	M	L	PDR	DM	34	AH	ELISA
21	NVG	57	M	L	PDR	DM, MS	38	AH	ELISA
22	Cataract	78	F	L	NA	HT	13	AH	ELISA
23	Cataract	70	M	L	NA	Dyslipidemia	12	AH	ELISA
24	Cataract	64	F	R	NA	HT, dyslipidemia	13	AH	ELISA
25	Cataract	81	M	R	NA	AP	10	AH	ELISA
26	Cataract	78	F	L	NA	Dyslipidemia	14	AH	ELISA
27	Cataract	69	M	L	NA	HT	19	AH	ELISA

AH, aqueous humor; AP, angina pectoris; BD, bipolar disorder; DM, diabetes mellitus; DN, diabetic nephropathy; HT, hypertension; IF, immunofluorescence; MBA, megaloblastic anemia; MS, maxillary sinusitis; NA, not applicable; NVG, neovascular glaucoma; PDR, proliferative diabetic retinopathy; POAG, primary open-angle glaucoma; RT-PCR, reverse transcription – PCR; TM, trabecular meshwork.

Cell culture and chemicals

Human glaucoma trabecular meshwork (GTM) cells, isolated from the TM of a glaucoma patient, were a kind gift from Novartis Institutes for BioMedical Research (Cambridge, MA).[86] GTM cells were cultured in high glucose D-MEM medium (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Wako Pure Chemical Industries).

GTM cells were seeded in 6-well plate and incubated for 24 hr in serum supplied cell culture medium and then serum-depleted for another 24 hr. After serum deprivation, the cells were pretreated with 1 μM PRRB for the prorenin stimulation group or 10 μM AT1R blocker valsartan for the Ang II stimulation group (Sigma-Aldrich) for 1 hr. Prorenin or Ang II was then added at a final concentration of 10 nM or 1 μM, respectively. Cells were incubated for an additional 24 hr and then processed mRNA level analysis. GTM cells were also treated with hydrogen peroxide (H_2O_2, Wako Pure Chemical Industries) for 24 hr, and analyzed for gene expression of RAPS components.

Reverse transcription-PCR (RT-PCR) and real-time quantitative PCR (qPCR) analyses

Total RNA from GTM cells was isolated using TRIzol (Life Technologies) according to the protocol of the manufacturer. Total RNA from POAG and NVG TM tissues were extracted using the NucleoSpin totalRNA FFPE XS kit (Macherey-Nagel, Düren, Germany), according to the protocol of the manufacturer. Reverse transcription was performed with GoScript Reverse Transcriptase (Promega) and oligo dT(20) primers. The details of reverse transcription, conventional PCR and real-time qPCR methods are explained in the 'RT-PCR and real-time qPCR analyses' section of Chapter 1 above. All the samples to be compared were processed in parallel and 5 or 6 independent experiments were performed. The

quantity of mRNA expression was calculated by normalizing the Ct of the target genes to the Ct of beta-2-microglobulin (*B2M*) gene in the same sample, according to the comparative ddCt method. The primers used in this study are listed on Table 4.

Table 4. Primer sequences used in RT-PCR and real-time qPCR

Target gene	Sequence
(P)RR	forward 5'-AGG CAG TGT CAT TTC GTA CC-3'
	reverse 5' -GCC TTC CCT ACC ATA TAC ACT C-3'
REN	forward 5'-GTG TCT GTG GGG TCA TCC ACC TTG-3'
	reverse 5'-GGA TTC CTG AAA TAC ATA GTC CGT-3'
AGT	forward 5'-CTG CAA GGA TCT TAT GAC CTG C-3'
	reverse 5' -TAC ACA GCA AAC AGG AAT GGG C-3'
ACE	forward 5'-CCG AAA TAC GTG GAA CTC ATC AA-3'
	reverse 5'-CAC GAG TCC CCT GCA TCT ACA-3'
AT1R	forward 5'-AGG GCA GTA AAG TTT TCG TG-3'
	reverse 5'-CGG GCA TTG TTT TGG CAG TG-3'
AT2R	forward 5'-GGC CTG TTT GTC CTC ATT GC-3'
	reverse 5'-CAC GGG TTA TCC TGT TCT TC-3'
HPRT1	forward 5'-ACC CCA CGA AGT GTT GGA TA-3'
	reverse 5'-AAG CAG ATG GCC ACA GAA CT-3'
CX43	forward 5'-AGG TCT GAG TGC CTG AAC TTG-3'
	reverse 5'-TTG CCT GGG CAC CAC TCT TTT-3'
ZO-1	forward 5'-ACC AGA AAT ACC TGA CGG TGC-3'
	reverse 5'-CGT TAC CCA CAG CTT CCT CTT-3'
PLAT	forward 5'-CAG AAG CAA CCG GGT GGA ATA-3'
	reverse 5'-CGC TGC AAC TTT TGA CAG GC-3'
PlGF	forward 5'-TCA CCA TGC AGC TCC TAA AGA-3'
	reverse 5'-GTG GCA GTC TGT GGG TCT CT-3'
B2M	forward 5'-GAG TAT GCC TGC CGT GTG AA-3'
	reverse 5'-GCG GCA TCT TCA AAC CTC CA-3'

Immunohistochemistry

TM tissue samples were fixed in 4% paraformaldehyde in the operating room soon after excision. Following fixation, the samples were preserved as paraffin-embedded blocks. Dewaxion, rehydration, and antigen retrieval of the samples were performed as explained in the 'immunohistochemistry' section of Chapter 1. The sections were then incubated with rabbit polyclonal antibodies against human (P)RR (1:100, Sigma-Aldrich) and AT1R (1:100, Santa Cruz Biotechnology). Visualization was performed using the Envision HRP kit (DAKO). Normal rabbit IgG was used as a negative control antibody. The sections were examined using a BIOREVO microscope (Keyence).

Immunofluorescence analyses

For immunofluorescence analyses sections were pretreated with fixation, antigen retrieval and serum blocking as described in the 'immunofluorescence analyses' section of Chapter 1. Sections were incubated with the following primary antibodies: rabbit anti-(P)RR (1:100, Abcam), rabbit anti-AT1R (1:100, Abcam), mouse anti-prorenin (REN; 1:75, Abcam), goat anti-angiotensinogen (AGT; 1:50, Santa Cruz Biotechnology), mouse anti-Connexin 43 (CX43; 1:100, Millipore), rat anti-Zona Occludens 1 (ZO-1; 1:50, Millipore), goat anti-tissue plasminogen activator (PLAT; Santa Cruz Biotechnology), goat anti-placental growth factor (PlGF; 1:50, Santa Cruz Biotechnology). Secondary antibodies for fluorescent detection were AlexaFluor 488 and 546 conjugated antibodies (1:400, Life Technologies). Nuclei were counterstained with DAPI, and sections were visualized under a BIOREVO microscope (Keyence).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of human prorenin and Ang II in AH samples were measured using a human prorenin ELISA kit (Abcam) and Ang II ELISA kit (Bertin Pharma, Montigny Le Bretonneux, France) respectively, according to the protocols provided by the manufacturers. The optical density was determined using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland).

Statistical analysis

The results were expressed as mean ± SEM with their respective n-numbers indicated in the figures sections below. Two-tailed Student's t-test was applied to determine significant differences between the experimental groups. Differences between the means were considered statistically significant when $P < 0.05$.

Results

Expression and localization of RAPS components in GTM cells and TM tissues from POAG and NVG patients

Previously, we showed that RAPS components were expressed in surgically excised ocular tissues of patients with diagnosed with PDR and other inflammatory, angiogenic, and fibrotic disorders.[20, 26, 27, 85] At the beginning of our research, we first confirmed the gene expression of RAPS components in GTM cell line and surgical TM tissues from POAG and NVG patients to investigate the pathological role of prorenin-(P)RR and Ang II-AT1R axes in the TM. RT-PCR results showed that RAPS component genes [*(P)RR*, *REN*, *AGT*, *ACE*, *AT1R* and *AT2R*] are expressed in GTM cells, and POAG and NVG TM tissues. *REN* showed some exception as it was expressed in the tissues from only two POAG patients (Figure 10A). We also confirmed the expression of (P)RR (Figure 10B, E) and AT1R (Figure 10C, F), the two main receptors of the RAPS, in the TM tissues from POAG and NVG patients. No signal was observed at the negative control IgG applied tissue sections (Figure 10D, G).

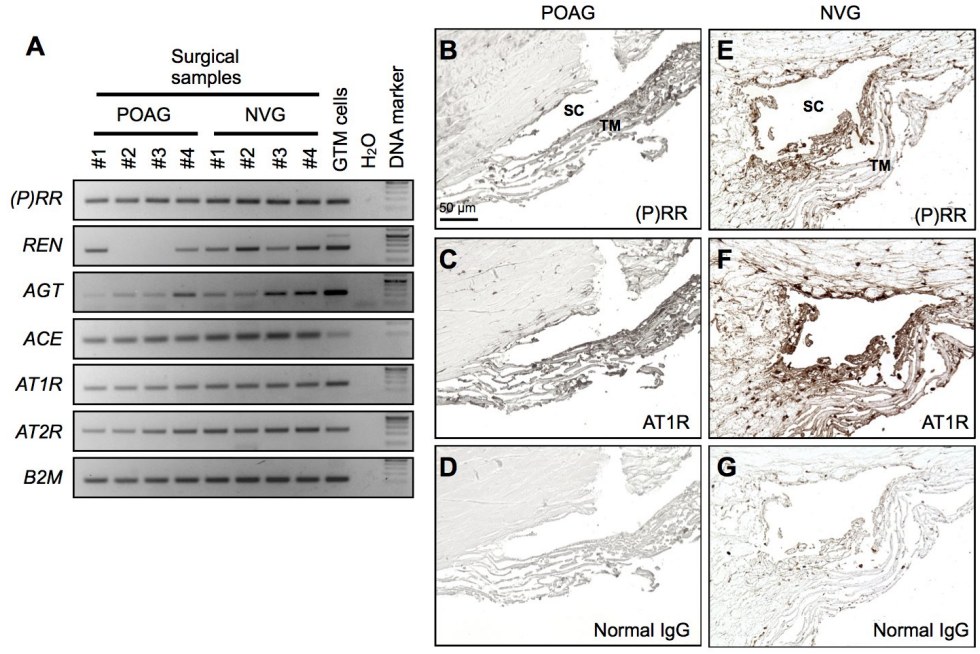

Figure 10. Gene expression of RAPS components in GTM cells and the TM from POAG and NVG patients and localization of (P)RR and AT1R. **(A)** Gene expression of RAPS components [*(P)RR*, *REN*, *AGT*, *ACE*, *AT1R*, *AT2R*] in GTM cells and TM tissues of POAG and NVG patients as tested by RT-PCR analyses. Immunohistochemical staining of (P)RR and AT1R in TM tissues from POAG **(B-D)** and NVG **(E-G)** patients. Scale bar = 50 μm. SC, Schlemm's canal; TM, trabecular meshwork.

Localization of (P)RR and AT1R with their respective ligands in POAG and NVG TM tissues

We performed immunofluorescence analyses to study the localization and hence the possible interaction of (P)RR with prorenin and AT1R with AGT in the TM tissues from POAG and NVG patients. Results of double-staining showed that (P)RR was co-localized with prorenin (Figure 11A–C, G-I) and AT1R was co-localized with AGT (Figure 11D-F, J-L) in TM tissues from both POAG and NVG patients. These results point to the possible involvement of prorenin-(P)RR and Ang II-AT1R axes in the pathogenesis of glaucoma in the TM.

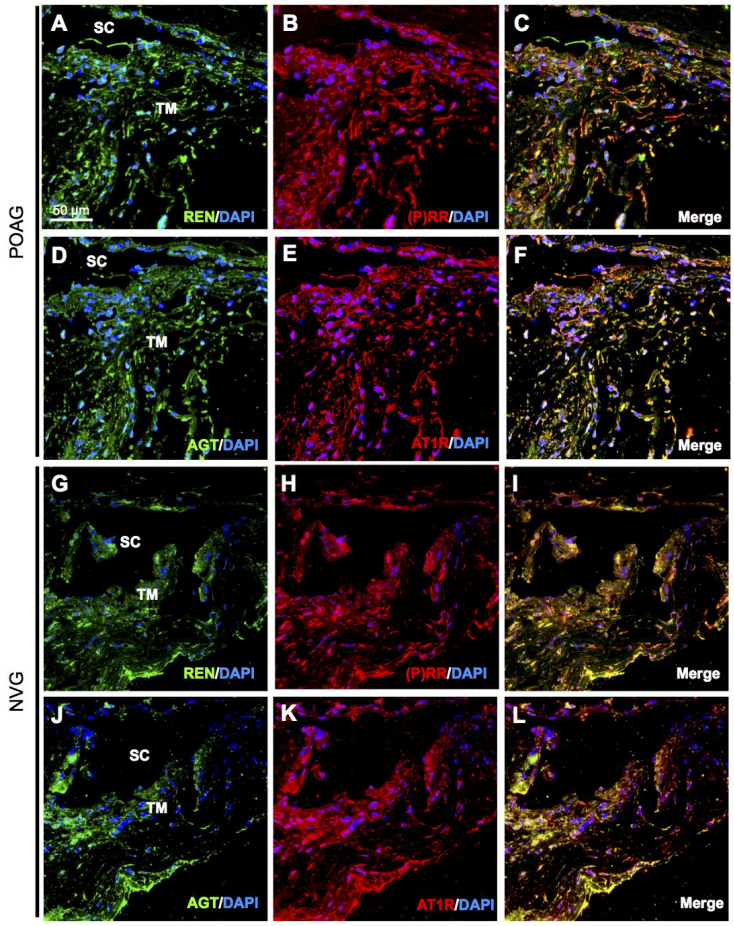

Figure 11. Immunofluorescence analyses of REN, (P)RR, AGT, and AT1R in TM tissues of POAG and NVG patients. Double-staining of REN (*green*) and (P)RR (*red*) with DAPI (*blue*) in TM tissues from POAG **(A–C)** and NVG **(G–I)** patients. Double-staining of AGT (*green*) and AT1R (*red*) with DAPI (*blue*) in TM tissues from POAG **(D–F)** and NVG **(J–L)** patients. Scale bar = 50 μm.

Alteration of prorenin and Ang II protein levels in POAG and NVG aqueous humors

Previous data have shown that intravitreous concentrations of prorenin and Ang II are higher in the eyes of PDR patients compared to that of non-diabetic controls.[20, 87] We performed ELISA experiments to measure and compare the prorenin and Ang II protein concentrations in AH samples collected from the eyes of POAG and NVG patients, and age-matched cataract patients, which served as control group. Prorenin protein levels in the AH of POAG (1.81 ± 0.42 ng/mL, $P < 0.05$) and NVG (2.09 ± 0.42 ng/mL, $P < 0.05$) patients were significantly higher than those of cataract patients (1.12 ± 0.04 ng/mL) (Figure 12A). In addition, Ang II levels were significantly higher in the AH of NVG patients (15.97 ± 4.03 pg/mL, $P < 0.05$) compared with cataract patients (7.62 ± 0.79 pg/mL), while Ang II levels in the AH of POAG patients were lower than those of cataract patients (3.74 ± 1.29 pg/mL, $P < 0.05$) (Figure 12B). The results suggest that the changes in AH prorenin and Ang II protein concentrations may be associated with the pathogenesis of glaucoma.

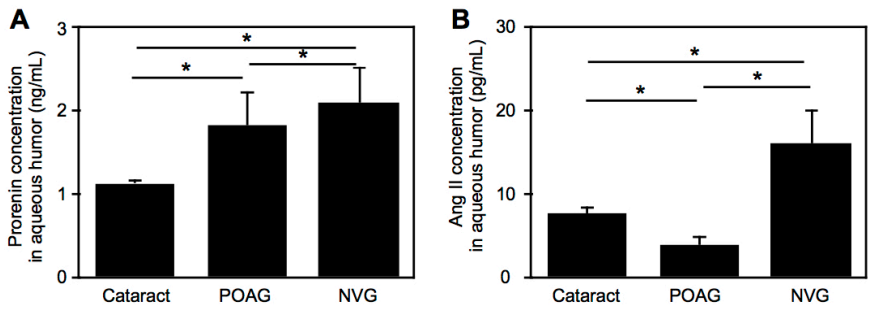

Figure 12. Concentrations of prorenin and Ang II in aqueous humor samples from cataract, POAG, and NVG patients. **(A)** Prorenin protein levels in eyes with cataract, POAG, and NVG. **(B)** Ang II protein levels in eyes with cataract, POAG, and NVG. n = 6 for each group. *$P < 0.05$

Regulation of the mRNA Levels of *CX43* and *ZO-1* genes through prorenin - (P)RR binding

Previously, we have shown that RAPS with the prorenin-(P)RR and the Ang II-AT1R axes regulates the expression of several genes both *in vivo* and *in vitro*, and contributes to the pathogenesis of numerous ocular diseases.[20, 26, 27, 85] Numerous studies have reported that there are various molecules in the TM, which have been shown to be associated with the pathogenesis of glaucoma. Table 5 shows representative proteins from these genes, which we hypothesized to be candidate molecules related to the pathogenesis of glaucoma in the TM such as cell junctions (CX43 and ZO-1)[88, 89], ECM (MMPs and PLAT)[82, 90, 91], angiogenesis (VEGF-A, PlGF, and VEGF receptors)[81, 92, 93], inflammation (intercellular adhesion molecule-1 and monocyte chemotactic protein-1)[79, 80], and others (transforming growth factor-β 2 and uncoupling protein 2).[88, 94]

Table 5. Target genes related to glaucoma pathogenesis

Function	Gene symbol	Gene name
Neovascularization	*EGF*	Epidermal Growth Factor
	PGF	Placental Growth Factor
	NRP1	Neuropilin 1
	VEGFA	Vascular Endothelial Growth Factor A
	VEGFR1	Vascular Endothelial Growth Factor Receptor 1
	VEGFR2	Vascular Endothelial Growth Factor Receptor 2
Inflammation	*ICAM1*	Intercellular Adhesion Molecule 1
	MCP1	Monocyte Chemoattractant Protein 1
ECM turnover	*MMP2*	Matrix Metalloproteinase 2
	MMP3	Matrix Metalloproteinase 3
	MMP9	Matrix Metalloproteinase 9
	MMP14	Matrix Metalloproteinase 14
	BSG	Basigin

	TGM2	Transglutaminase 2
	TIMP1	TIMP Metallopeptidase Inhibitor 1
	PLAT	Plasminogen Activator, Tissue Type
Cell junction	*GJA1*	Connexin-43
	TJP1	ZO-1
Antioxidation	*UCP2*	Uncoupling Protein 2
Fibrosis	*FGF2*	Fibroblast Growth Factor 2
	CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
	TGFB2	Transforming Growth Factor Beta 2

To study the role of RAPS in the molecular pathogenesis of POAG and NVG in the TM, we sought to find whether stimulation of prorenin changes the mRNA levels of those genes in GTM cells and performed real-time qPCR. Among the genes assayed, the analysis results showed that mRNA levels of *CX43* and *ZO-1*, gap and tight junction proteins, significantly increased in GTM cells stimulated with prorenin compared to control group (*CX43*, fold change = 1.78; *ZO-1*, fold change = 2.17, $P < 0.05$). The increases in the mRNA levels of these two genes were inhibited by pretreatment with PRRB (*CX43*, fold change = 1.47; *ZO-1*, fold change = 1.57, $P < 0.05$). No change in the mRNA level of these two genes has been observed in GTM cells stimulated with Ang II (*CX43*, fold change = 1.04; *ZO-1*, fold change = 1.02, $P > 0.05$) (Figure 13A, B). Following these *in vitro* mRNA level results, we also performed immunofluorescence experiments to study whether both CX43 and ZO-1 co-exist with (P)RR in the TM tissues. CX43 and ZO-1 signals co-localized with (P)RR in TM sections from both POAG patients (Figure 13C-H) and NVG patients (Figure 13I-N). Our results suggest that activation of prorenin-(P)RR axis, but not the Ang II-AT1R axis, triggers an increase in CX43 and ZO-1 expression levels in TM cells, which may be responsible for disturbance of cell junctions in the TM.

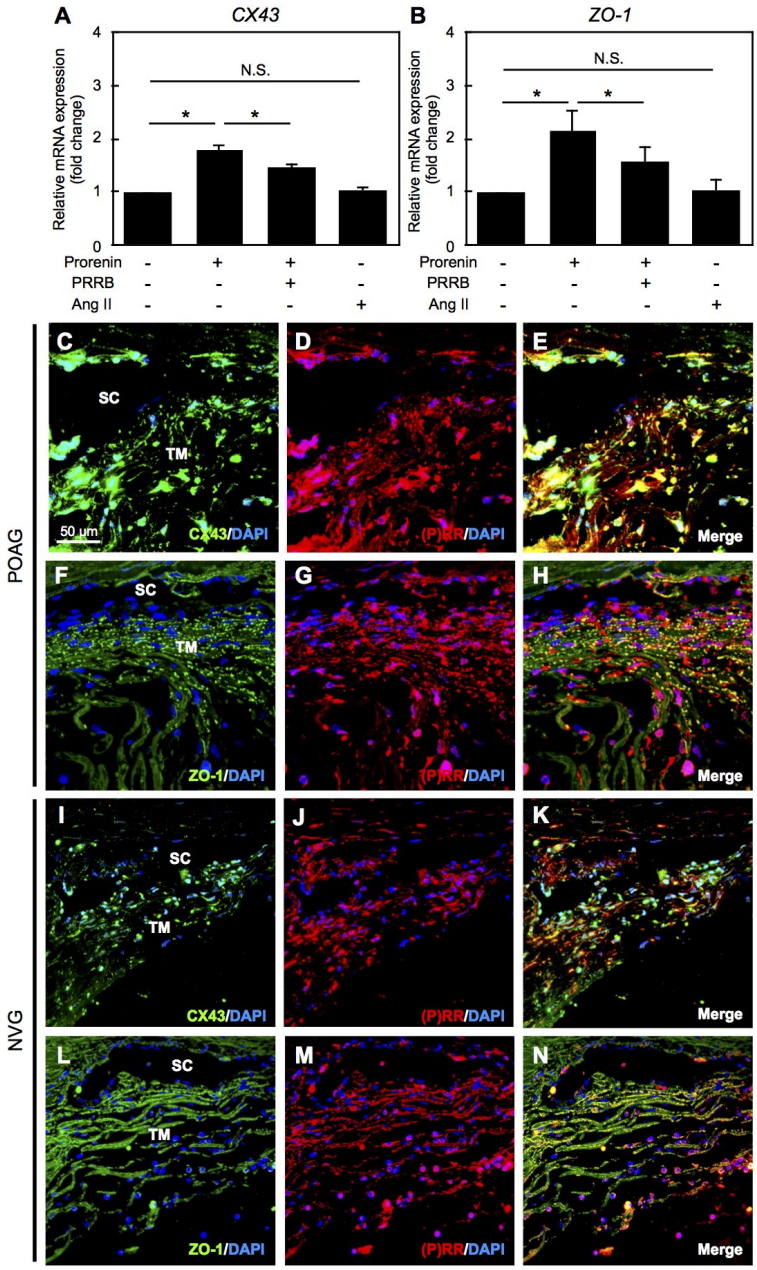

Figure 13. (P)RR-mediated upregulation of *CX43* and *ZO-1* in the human TM. Relative mRNA levels of **(A)** *CX43* and **(B)** *ZO-1* in Ang II or prorenin-stimulated GTM cells with or without PRRB compared with control. n = 5. * $P < 0.05$. N.S., non-significant. Double-immunostaining of CX43 (*green*) and (P)RR (*red*) with DAPI (*blue*) in the TM tissues from POAG **(C-E)** and NVG **(I-K)** patients. Double-immunostaining of ZO-1 (*green*) and (P)RR (*red*) with DAPI (*blue*) in TM tissues from POAG **(F-H)** and NVG **(L-N)** patients. Scale bar = 50 μm.

Downregulation of *PLAT* expression through prorenin-(P)RR binding

On the other hand, mRNA level of *PLAT*, a serine protease (also known as tPA), significantly decreased in GTM cells stimulated with prorenin compared to control group (fold change = 0.56, $P < 0.05$), but not Ang II stimulated group (fold change = 1.17, $P > 0.05$). The change in the mRNA level of prorenin stimulation was reversed close to the control level when the cells were pretreated with PRRB before prorenin stimulation (fold change = 1.41, $P < 0.05$) (Figure 14A). In addition, double-immunostaining results showed that PLAT signals co-localized with (P)RR in the TM sections from POAG patients (Figure 14B-D) and from NVG patients (Figure 14E-G), which altogether suggested that prorenin-(P)RR may contribute to ECM turnover of the TM and pathogenesis of glaucoma.

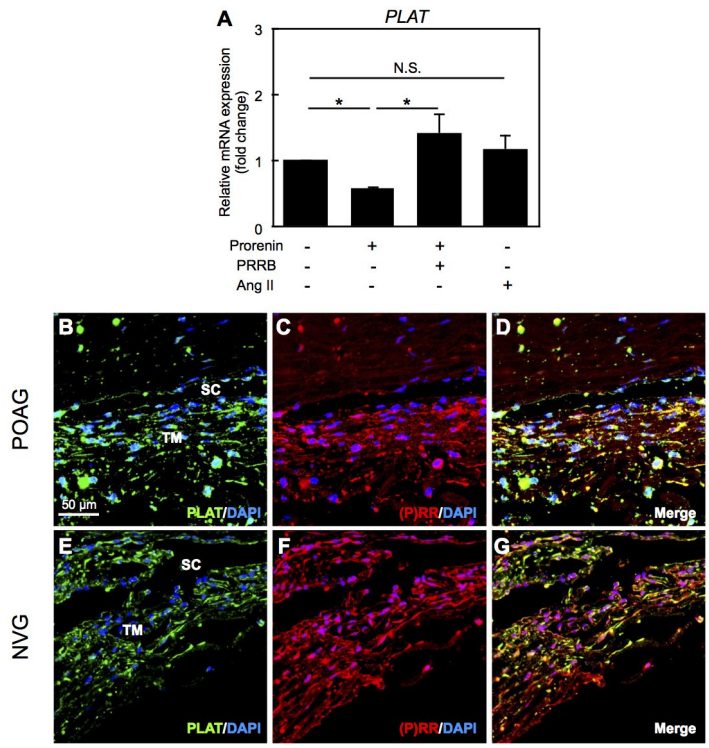

Figure 14. (P)RR-mediated downregulation of *PLAT* in human TM cells. **(A)** Relative mRNA levels of *PLAT* in Ang II or prorenin-stimulated GTM cells with or without PRRB compared with control. n = 6. * $P < 0.05$. N.S., non-significant. Double-labeling of PLAT (*green*) and (P)RR (*red*) with DAPI (*blue*) in TM of POAG **(B-D)** and NVG **(E-G)** patients. Scale bar = 50 μm.

Increase of *PlGF* expression via Ang II - AT1R interaction

We then further examined the changes in the mRNA levels of genes, implied to be involved in the pathogenesis of glaucoma in the TM, by the binding of Ang II to its receptor AT1R. We stimulated the GTM cells with Ang II and then performed real-time qPCR analyses to check the changes in the mRNA levels of the genes. The results showed that Ang II stimulation significantly increased the mRNA level of *PlGF*, a member of the VEGF family, (fold change = 2.93, $P < 0.05$). On the other hand, pretreatment with valsartan inhibited Ang II-induced *PlGF* expression (fold change = 0.82, $P < 0.05$). However, prorenin stimulation had no effect on the mRNA level of *PlGF* (fold change = 1.24, $P > 0.05$) (Figure 15A). We also examined the localization of PlGF in TM tissues obtained from POAG and NVG patients by immunofluorescence microscopy. The results showed that PlGF co-localized with AT1R in TM tissues from POAG patients (Figure 15B-D) and from NVG patients (Figure 15E-G). These results indicate that stimulation of Ang II-AT1R axis in the TM cells may be the cause of angiogenesis in the TM.

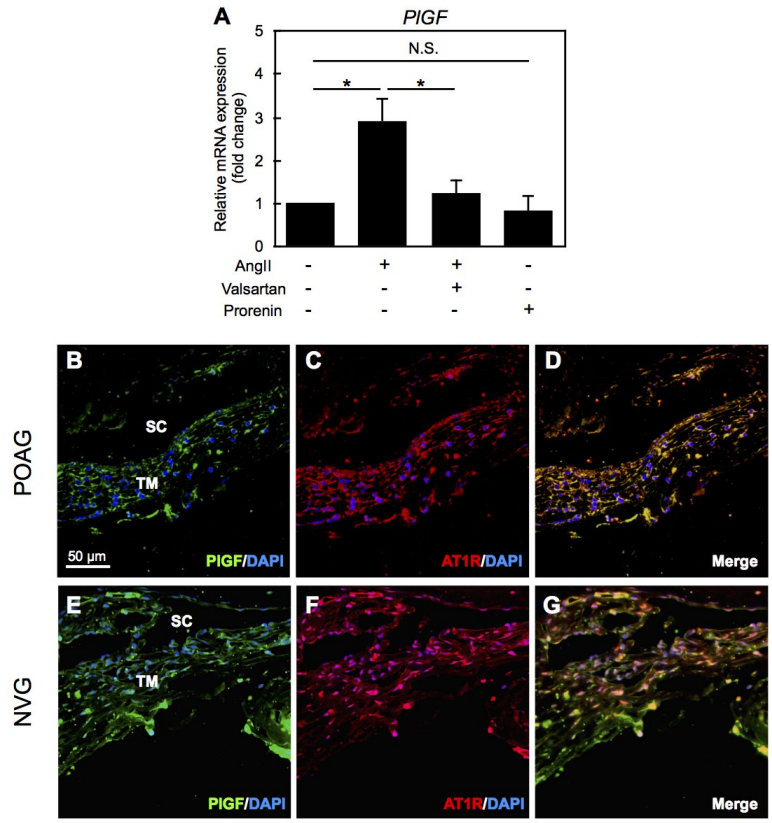

Figure 15. AT1R-mediated upregulation of *PlGF* in human TM cells. **(A)** Relative mRNA level of *PlGF* in prorenin or Ang II-stimulated GTM cells with or without valsartan compared with control. n = 6. * $P < 0.05$. N.S., non-significant. Double-labeling of PlGF (*green*) and AT1R (*red*) with DAPI (*blue*) in TM of POAG **(B-D)** and NVG **(E-G)** patients. Scale bar = 50 μm.

Changes in the mRNA levels of RAPS components in the TM under oxidative stress

Among the various biological pathways suggested to be involved in the pathogenesis of glaucoma, oxidative stress has been shown to be associated with the pathological events in glaucoma.[95-101] We hypothesize that oxidative stress may regulate the mRNA level of RAPS components. In order to study the effect of oxidative stress on mRNA level of RAPS components, we performed qPCR of RAPS components in GTM cells after treatment with H_2O_2. Oxidative stress led to significant upregulation in the mRNA levels of *REN* (60 μM H_2O_2, fold change = 1.75; 90 μM H_2O_2, fold change = 2.56, $P < 0.05$) (Figure 16A) and *AGT* (60 μM H_2O_2, fold change = 2.03; 90 μM H_2O_2, fold change = 1.59, $P < 0.05$) (Figure 16B) in GTM cells. There was no significant change in the mRNA levels of *(P)RR*, *ACE* and *AT1R* (Figure 16C-E). These results imply that by increasing the expression of two main ligands of RAPS (*i.e.,* prorenin and Ang II), oxidative stress may act as an initiator of RAPS activation in TM cells and lead to the pathogenesis glaucoma.

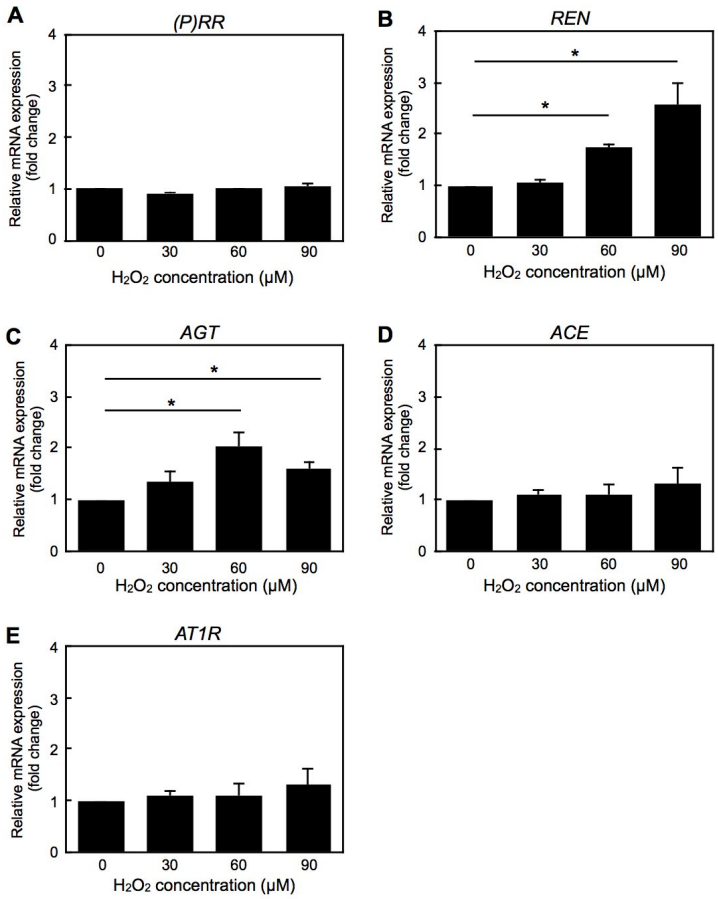

Figure 16. Changes in the mRNA levels of RAPS components in GTM cells under oxidative stress. **(A-E)** mRNA levels of RAPS components [*(P)RR*, *REN*, *AGT*, *ACE*, *AT1R*] were analyzed by real-time qPCR after GTM cells were treated with H_2O_2 (30-90 μM) for 24 hr. n = 6. * $P < 0.05$.

Summary

In this study, we have proven that RAPS has a role in the molecular mechanism of the pathogenesis of glaucoma in the TM. First, we have shown that RAPS component genes were expressed in the TM tissues surgically excised from POAG and NVG patients, and human GTM cell line (Figure 10). Immunohistochemical analyses of (P)RR and AT1R have confirmed that these two receptors are expressed in the TM tissues from POAG and NVG patients (Figure 10), and co-localized with prorenin and AGT, their respective ligands (Figure 11). Concentration of prorenin protein was higher in the AH from POAG and NVG patients compared to that from control cataract patients, while Ang II concentration was significantly higher in the AH from NVG patients than those from POAG and cataract patients (Figure 12). Stimulation of GTM cells with prorenin triggered increase in the expression levels of *CX43* and *ZO-1* (Figure 13) and decrease in the expression level of *PLAT* (Figure 14) through (P)RR interaction, and in addition (P)RR co-localized with CX43, ZO-1 and PLAT in the TM tissues from POAG and NVG patients (Figures 13 and 14). Binding of Ang II to AT1R increased the gene expression level of PlGF, which was suppressed by the addition of valsartan. Immunofluorescence analyses showed the co-localization of AT1R with PlGF in TM tissues from POAG and NVG patients (Figure 15). Oxidative stress in TM cells upregulated the expression levels of *REN* and *AGT*, the main RAPS ligands known to bind (P)RR and AT1R respectively (Figure 16). Our data suggest that oxidative stress increases the expression of prorenin and Ang II, activating RAPS in the TM tissues and changing the expression profiles of cell junction and extracellular matrix related genes, which may have critical roles in the molecular pathogenesis of glaucoma in the TM.

Discussion

In this study, we investigated the involvement of the RAPS in the molecular pathogenesis of glaucoma in the TM. TM dysfunction leads to elevated IOP, which is a major risk factor for pathogenesis and development of glaucoma. Gap and tight junction proteins of TM cells are important for the integrity of the TM, where AH outflow occurs. Abnormality in the TM cell junction elevates the fluid flow resistance to AH, causing increase of IOP.[102] Previous reports showed that the expression of CX43 and ZO-1 are higher in the TM of NVG patients compared to those of normal donors.[88, 89] ECM turnover in the TM is crucial in the maintenance of AH outflow from the TM. Several pro-MMPs are cleaved and converted to their active MMP form by plasmin, which is generated by PLAT. Active MMPs facilitate the turnover and thereby remodeling of the ECM.[103, 104] Previous studies have shown that when steroids are applied to the TM cells, *PLAT* expression is down-regulated, whereas in mice, induced overexpression of PLAT up-regulates the expression of MMPs and reverses the decrease in the AH outflow caused by steroids. [90, 91] These findings suggest that inhibition of ECM turnover leads to a resistance in the AH outflow and an increase in the IOP. In this study, we have shown that prorenin stimulation increases the expression levels of *CX43* and *ZO-1* genes, while it decreases the expression level of *PLAT* in GTM cells compared to those in the controls. CX43, ZO-1 and PLAT co-localized with (P)RR in the TM tissues (Figures 13 and 14). Additionally, prorenin levels were significantly higher in the AH of POAG and NVG patients compared with control patients (Figure 12). From these results, it is possible to suggest that increased level of prorenin in the AH activates prorenin-(P)RR signaling pathway. Activated (P)RR signaling disturbs the cellular junction and ECM turnover in the

TM, which subsequently causes a resistance to the AH outflow through the TM in POAG and NVG.

PlGF is a member of the VEGF family, which is a group of growth factors responsible for pathological angiogenesis in numerous tissues. PlGF was shown to be involved in pathological angiogenesis in ocular diseases such as DR.[105] The results of our studies also recently have shown that PlGF protein levels are higher in the AH of NVG patients compared to those of diabetic macular edema and PDR patients related to the severity of diabetic ocular complication, which all together suggested that the accumulation of proangiogenic factor PlGF in the anterior chamber aggravates iris neovascularization in DR patients.[92] The findings of this present study revealed that increase in the Ang II levels induces an increased *PlGF* gene expression level in GTM cells, and PlGF co-localized with AT1R in TM tissues (Figure 15). These results suggest that activation of Ang II-AT1R axis induces pro-angiogenic cytokine expression in TM cells and contributes to the pathogenesis of NVG.

A growing body of evidence has accumulated to show the association between oxidative stress and glaucoma. Numerous studies reported that levels of oxidative stress markers in serum, AH and TM cells from glaucoma patients were significantly higher than those from controls.[92, 95, 99-101] IOP elevation in experimental glaucoma model rats has been shown to induce oxidative stress, while high systemic oxidative stress is one of the risk factors that increase IOP in glaucoma patients.[96, 98] In addition, hyperglycemia results in increased oxidative stress, and elevated oxidative stress plays a key role in the pathogenesis of PDR.[106] In this study our results showed that *REN* and *AGT* gene levels in TM cells were increased by oxidative stress (Figure 16). When the above mentioned previous reports and

our findings are taken together, it can be suggested that increase in IOP and/or pathological changes owing to increase intraocular oxidative stress prime the activation of prorenin-(P)RR and Ang II-AT1R axes in the TM that consequently induce the pathogenic cascade of glaucoma such as abnormality of cell junctions and ECM via (P)RR, and pathological angiogenesis through AT1R, which eventually result in the elevation of IOP in glaucoma (Figure 17).

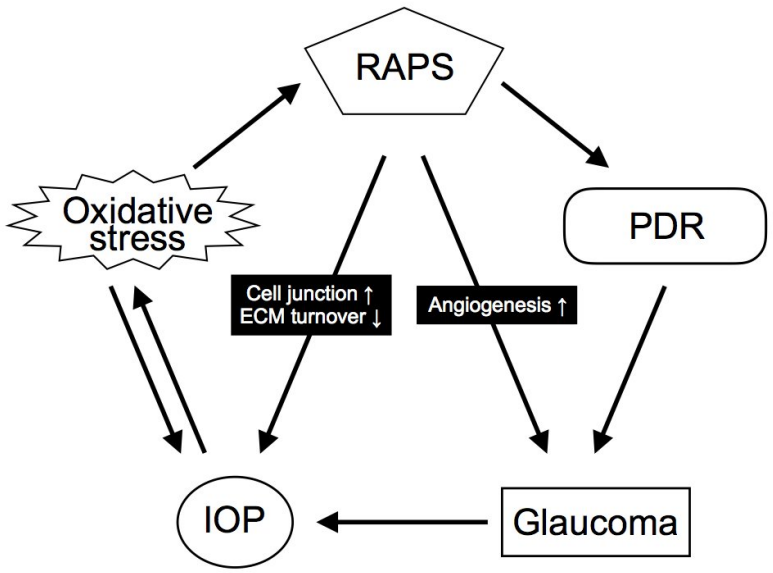

Figure 17. A schema showing the involvement of RAPS and its link to oxidative stress in the pathogenesis of glaucoma. Oxidative stress due to elevated IOP leads to activation of RAPS in TM tissues. Stimulation of prorenin-(P)RR and Ang II-AT1R axes causes abnormality of cell junctions, ECM and angiogenesis-related molecules and associates with the pathogenesis of glaucoma.

Conclusion

Our findings indicate that RAPS plays an essential role in the molecular pathogenesis of ocular diseases, and may lay the foundations for new discoveries and developments of pharmacologic therapies for them.

Vision is the most important of the five senses of human body as around 80% of the information we perceive from the outside world is through the eyes. This underlines the importance of remedies for ocular diseases. Our study newly deepened our insight on the roles of RAPS in the molecular pathogenesis of two crucial ocular diseases, conjunctival EMZL and glaucoma. We propose that (P)RR inhibitors are promising remedies to the ocular diseases by intervening with the molecular pathway of RAPS in the initial stages and before the clinical conditions become chronic. As an alternative and potentially more stable therapeutic agent, we recently developed a new single-strand RNA interference molecule that selectively targets human and mouse (P)RR and is efficient in suppressing acute and chronic ocular inflammation.[27] Future research can be extended to study the inhibitory effects of (P)RR-targeting therapeutic agents on the proliferation and metastasis of conjunctival EMZL by using B lymphocytes and on the pathological changes of cell junction and extracellular matrix in glaucoma mouse models of TM. It can be expected that this will allow decrease the number of invasive surgical treatments usually performed after ocular complications develop and reduce the burden on the patients.

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