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Title	Involvement of (pro)renin receptor in the pathogenesis of inflammatory eye diseases [an abstract of entire text]
Author(s)	,
Citation	. ( ) 12981
Issue Date	2018-03-22
Doc URL	http://hdl.handle.net/2115/70380
Type	theses (doctoral - abstract of entire text)
Note	; 2360
Note(URL)	https://www.lib.hokudai.ac.jp/dissertations/copy-guides/
File Information	Tanerudaru_Ishizuka_summary.pdf

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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）

2018 年 3 月

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博士の専攻分野の名称　　博士（医　学）　　　　氏　名　　石塚　タンエルダル

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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）

【Background and Objectives】

Although advances in science and technology provides longer lives and better welfare, the increasing number of elderly people is accompanied with difficulties in maintaining the welfare and health. In addition, the environment and lifestyles also change bringing various risks that increase the frequency of diseases like diabetes, cancer, cardiovascular diseases, hypertension and others. In ophthalmology field, ocular diseases are dramatically increasing worldwide as the population ages and despite new medical and surgical interventions, they still remain as leading causes of vision loss.

Application of antibodies that inhibit the activity of vascular endothelial growth factor (VEGF), a cytokine responsible for pathological neovascularization leakiness of retinal capillaries, is one of the standard therapies to a number of ocular diseases. This is known as anti-VEGF therapy, where anti-VEGF antibody is injected into the vitreous body of the eye and blocks the activity of VEGF and suppresses pathological angiogenesis and vascular leakage. However, anti-VEGF therapy has limitations due to its application at late stages of the ocular diseases in the state of irreversible degeneration due to inflammation, angiogenesis and fibrosis. Various pro-inflammatory and angiogenic factors other than VEGF play roles in the pathogenesis of the diseases, which are the reasons for resistance to anti-VEGF therapy. Additionally, effective pharmacological therapies need to be established for other inflammatory and neovascular ocular diseases, in which anti-VEGF therapy is not efficient. It is necessary to develop new strategies that target additional and alternative molecular pathways to intercept at the early stages of the diseases.

The renin-angiotensin system (RAS) is proposed as one of the alternative target molecular pathways for the treatment of ocular diseases. RAS is as an important regulatory mechanism for controlling systemic blood pressure and water balance. This type of RAS is called as the systemic or circulatory RAS. Molecular components of RAS were also found to be expressed in various tissues and independent of the circulatory RAS, and therefore is called as the tissue RAS. Tissue RAS plays diverse roles in the regulation of growth, inflammation and pathological vascular conditions in several organs. It was also shown that tissue RAS is present in the ocular tissues and is responsible for ocular pathological conditions as it leads to

increases in the expression of molecules like growth factors and inflammatory cytokines through downstream signaling pathways of tissue RAS.

Prorenin, one of the components of RAS, was known as an inactive precursor of renin. Later, with the identification of (pro)renin receptor [(P)RR], a new RAS activation mechanism was reported, where (P)RR binds to prorenin 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). The binding of prorenin to (P)RR was shown to trigger dual activation of tissue RAS and RAS-independent signaling pathways, which are involved in the molecular pathogenesis of end-organ damage, such as inflammation and angiogenesis, including numerous ocular disorders. 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.

It has been found that blockades of tissue RAS and/or RAPS have 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. Hence, it is probable to hypothesize that RAPS may also play important roles in the molecular mechanism and pathogenesis of various other ocular diseases. Therefore, 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. We also examined the association of RAPS with the molecular pathogenesis of glaucoma in the trabecular meshwork (TM), since glaucoma is a major cause of irreversible blindness in the world. We performed our research on RAPS as it can serve as an alternative target molecular pathway for the treatment of these two inflammatory ocular diseases.

【Methods】

To study the involvement of RAPS in the molecular pathogenesis of conjunctival extranodal marginal zone B-cell lymphoma (EMZL), conjunctival lymphoma tissues were surgically removed. The tissues were used for gene expression studies, immunohistochemical (IHC) and immunofluorescence (IF) analyses of RAS components, including (P)RR. Human B-lymphoblast IM-9 cells were treated with prorenin or angiotensin II (Ang II), and gene expression levels were analyzed using real-time quantitative PCR (qPCR). IF analyses of sections from EMZL samples were performed to evaluate the localization of protein products of the genes with significantly changing expression profiles at prorenin or Ang II stimulations.

To assess the role of RAPS in the molecular pathogenesis of glaucoma in the TM, TM tissues from glaucoma patients were surgically removed. These clinical samples were used for gene expression studies, IHC and IF analyses of RAS components. We stimulated human glaucoma TM (GTM) cells with either prorenin or Ang II, and gene expression levels were analyzed using qPCR. IF analyses of TM samples were used to evaluate the localization of protein products of the genes with significantly changing

expression profiles at prorenin or Ang II stimulations.

【Results】

Expression of RAS components, including (P)RR and AT1R, in EMZL tissues and IM-9 cells were confirmed by reverse transcription PCR. IHC analyses showed that (P)RR and AT1R are localized in EMZL tissues. IF analyses of EMZL tissues demonstrated that (P)RR and AT1R co-localized with CD20, a marker for B-cells, and with CD31, a marker for endothelial cells. In EMZL tissues, (P)RR and AT1R co-localized also with prorenin and angiotensinogen (precursor of Ang II), respectively. Prorenin stimulation of IM-9 cells significantly increased mRNA expression levels of *fibroblast growth factor 2* (*FGF2*), while Ang II treatment increased the expression levels of *basigin (BSG), matrix metallopeptidases (MMP)2, 9,* and *14*, which were abolished by (P)RR and AT1R blockades, respectively. IF analyses of clinical samples showed co-localizations of (P)RR and AT1R with the products of these upregulated genes.

Results of reverse transcription PCR confirmed the expression of RAS components in TM tissues from glaucoma patients and GTM cells. (P)RR and AT1R co-localized with prorenin and angiotensinogen in clinical TM sections from glaucoma patients. Prorenin stimulation of TM cells increased the expression levels of *connexin 43 (CX43)* and *zona occludens-1 (ZO-1)*, while Ang II treatment increased the expression level of *placental growth factor (PlGF)*, which were abolished by (P)RR and AT1R blockades, respectively. IF analyses showed co-localizations of (P)RR and AT1R with the protein products of these regulated genes in TM tissues.

【Discussion】

In our study on the involvement of RAPS in the molecular pathogenesis of conjunctival EMZL, we demonstrated that *FGF2* expression in B-lymphocytes significantly increased through (P)RR-specific signaling. (P)RR and FGF2 co-localized in EMZL tissues. Other reports have shown that inflammation, angiogenesis, lymphatic growth and metastasis are triggered with increased *FGF2* expression levels and RAS inhibitors suppressed increased expression of FGF2 in diabetes and hypertension. We suggest that prorenin-(P)RR interaction (*i.e.*, activation of RAPS) is responsible for the increase in the expression of FGF2, induction of angiogenesis and inflammation in B-cells, and eventually the pathogenesis of conjunctival lymphoma.

Our results also demonstrated that Ang II-AT1R binding increased gene expression levels of *BSG, MMP2, MMP9* and *MMP14* in human B-lymphocytes. BSG, MMP2, MMP9 and MMP14 all co-localized with AT1R in the B-lymphomas of conjunctival EMZL tissues. BSG is known as an extracellular MMP inducer and increases the expression levels of MMPs, including MMP2 and MMP9. The release of angiogenic growth factors, cytokines and proteases like FGF2, MMP2, MMP9 and MMP14 into the surrounding extracellular matrix initiates tumor angiogenesis. It has also been reported that activation of AT1R by the binding of Ang II triggers the upregulation of BSG and MMPs in various cells. In accordance with these findings, our data suggest that Ang II-AT1R binding plays roles in the

extracellular matrix turnover and remodeling in B-lymphomas, leads to uncontrolled B-lymphocyte proliferation and the formation and development of conjunctival EMZL.

In this study, we also investigated the involvement of the RAPS in the molecular pathogenesis of glaucoma in the TM. Gap and tight junction proteins of TM cells are important for the integrity of the TM and abnormality in the TM cell junction elevates the fluid flow resistance to aqueous humor (AH), causing increase in intraocular pressure and subsequently optic nerve damage, which is a risk factor of glaucoma. Previously it was shown that the expression of CX43 and ZO-1 are higher in the TM of glaucoma patients compared to those of normal donors. We have shown that prorenin stimulation increases the expression levels of *CX43* and *ZO-1* genes in GTM cells compared to those in the controls. CX43 and ZO-1 co-localized with (P)RR in the TM tissues. Therefore, it is possible to suggest that the activation of prorenin-(P)RR signaling pathway disturbs the cellular junction in the TM, which subsequently causes a resistance to the AH outflow through the TM in glaucoma.

PlGF, a member of the VEGF family, was shown to be involved in pathological angiogenesis in ocular diseases such as diabetic retinopathy. One of studies also showed that PlGF protein levels are higher in the AH of glaucoma patients compared to those of controls. The findings of our study revealed that Ang II induces an increased *PlGF* gene expression level in GTM cells, and PlGF co-localized with AT1R in TM tissues. These results suggest that activation of Ang II-AT1R axis induces pro-angiogenic cytokine expression in TM cells and contributes to 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 has further 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. 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 a decrease in the number of invasive surgical treatments usually performed after ocular complications develop and reduce the burden on the patients.