Involvement of the Receptor-Associated Prorenin System in the Pathogenesis of Human Conjunctival Lymphoma

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PURPOSE. Extranodal marginal zone B-cell lymphoma (EMZL) is the most common subtype of conjunctival lymphoma, though its molecular mechanisms of pathogenesis are largely unknown. We attempted to explore the association of the renin-angiotensin system (RAS) and (pro)renin receptor ([P]RR) in the pathogenesis of conjunctival lymphoma.

METHODS. Surgically removed conjunctiva EMZL samples were used for gene expression, and immunohistochemical and immunofluorescence analyses of ([P]RR and RAS components. 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). In addition, immunofluorescence analysis of EMZL samples was used to evaluate the in vivo expression of those components.

RESULTS. Gene expression and immunohistochemical analyses revealed the expression of RAS components, including ([P]RR and angiotensin II type 1 receptor (AT1R), in EMZL tissues. Double-labeling analyses demonstrated that ([P]RR and AT1R were detected in cells positive for CD20, a marker for B-cells, where they colocalized with prorenin and angiotensinogen, respectively. Prorenin stimulation of human B-lymphoblast IM-9 cells increased mRNA expression levels of fibroblast growth factor 2 (FGF2), while angiotensin II treatment upregulated the expression levels of basigin (BSG), matrix metallopeptidase (MMP)2, 9, and 14, which were abolished by ([P]RR and AT1R blockades, respectively. Immunofluorescence analyses of clinical samples showed colocalizations of ([P]RR and AT1R with the products of these upregulated genes.

CONCLUSIONS. The present study suggests that activation of ([P]RR and AT1R is associated with the pathogenesis of conjunctival EMZL by stimulating the production of FGF2 and MMPs.

Keywords: renin-angiotensin system, receptor-associated prorenin system, conjunctival lymphoma, angiotensin II type 1 receptor, (pro)renin receptor

The conjunctiva is an ocular tissue that constitutes the first contact barrier against pathogens from the external environment. This barrier contains an immune system composed of conjunctival lymphoid tissue, which recruits B-cells, T-cells, and dendritic cells when the conjunctiva is exposed to pathogenic agents.1 Conjunctival lymphoma is one of the common malignancies found in the ocular adnexa. Extranodal marginal zone B-cell lymphoma (EMZL), a malignant tumor involving inflammatory cells, constitutes 7% of non-Hodgkin’s B-cell lymphoma cases, and is the most common histological subtype of conjunctival lymphoma.2–4 Several studies reported that conjunctival EMZL develops as a result of orbital inflammation.5,6 Moreover, we recently demonstrated that VEGF, a major angiogenic factor, was expressed in human EMZL tissues.7 However, little is known about the molecular mechanism of its pathogenesis.

The renin-angiotensin system (RAS) traditionally has been regarded as a key regulatory mechanism for systemic blood pressure and water balance (circulatory RAS). Recently, components of RAS also were found to be expressed in various tissues independent of the circulatory RAS, hence called tissue RAS. Tissue RAS has diverse roles in the regulation of growth, inflammation and pathological vascular conditions in several organs.8,9 (Pro)renin receptor ([P]RR) binds with prorenin to exert renin activity through the conformational change of the prorenin molecule (nonproteolytic activation of prorenin causing tissue RAS) instead of the conventional proteolysis of the prorenin prosegment by processing enzymes (proteolytic activation of prorenin causing circulatory RAS). Binding of prorenin to ([P]RR triggers dual activation of RAS and RAS-independent signaling pathways, referred to as the receptor-associated prorenin system (RAPS), involved in the molecular pathogenesis of end-organ damage, such as inflammation and angiogenesis, including ocular disorders (e.g., proliferative diabetic retinopathy).10–12 Blockades of RAS and/or RAPS resulted in beneficial effects on the onset and progression of various diseases.9,10,11,13,14 There also is increasing evidence from experimental models and clinical studies that the inhibitors of RAS reduce tumor growth and metastasis,14–17 suggesting a useful strategy against malignancies independent of their classical cardiovascular actions. In this study, we aimed to analyze the expression of RAS components in EMZL tissues and examined the role of RAS/RAPS in the pathogenesis of EMZL of the conjunctiva.

METHODS

Human Surgical Samples

Conjunctival EMZL samples (Ann Arbor classification, Stage 1E or 2E) were surgically removed from five patients and used for...
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. Written informed consent was obtained from all patients after an explanation of the purpose and procedures of the study.

The presence of small- to medium-sized atypical lymphoid cells was confirmed by hematoxylin and eosin (H&E) staining. Immunohistochemistry with anti-CD3, CD5, CD10, CD20, and B-cell surface antigen (BSG; Invitrogen)–mouse anti-CD20 (Dako), mouse anti-CD31 (Dako), mouse anti-fibroblast growth factor 2 (FGF2; Millipore, Temecula, CA, USA), mouse anti-basin (BSG; Millipore), goat anti-matrix metalloproteinase 2 (MMP2; Santa Cruz Biotechnology), goat anti-MMP9 (Santa Cruz Biotechnology), and mouse anti-REN (Millipore) antibodies for fluorescent detection were AlexaFluor 488 and 546 (Life Technologies). Sections were visualized using a BIOREVO microscope (Keyence, Osaka, Japan).

Immunofluorescence Microscopy

Immunofluorescence analyses were performed as described previously. Sections were incubated with the following primary antibodies: rabbit anti-(PRR), rabbit anti-AT1R, mouse anti-prorenin (Abcam, Cambridge, MA, USA), goat anti-angiotensinogen (AGT; Santa Cruz Biotechnology), mouse anti-CD20 (DAKO), mouse anti-CD31 (DAKO), mouse anti-fibroblast growth factor 2 (FGF2; Millipore, Temecula, CA, USA), mouse anti-basin (BSG; Millipore), goat anti-matrix metalloproteinase 2 (MMP2; Santa Cruz Biotechnology), goat anti-MMP9 (Santa Cruz Biotechnology), and mouse anti-REN (Millipore) antibodies. Secondary antibodies for fluorescent detection were AlexaFluor 488 and 546 (Life Technologies). Sections were visualized under a BIOREVO microscope (Keyence).

Statistical Analysis

All results were expressed as mean ± SD with n-numbers as indicated. Two-tailed Student’s t-test was used for determining significant differences between the groups. Differences between the means were considered statistically significant when P < 0.05.

RESULTS

Expression and Localization of RAS Components in EMZL Tissues and IM-9 Cells

In previous reports, we showed 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. To understand the pathological role of RAS/RAFs in conjunctival EMZL, we investigated the gene expression of RAS components in EMZL tissues and human B lymphoma cell line IM-9. Transcripts of RAS components were detected in all clinical EMZL samples and IM-9 cells (Fig. 1A). To validate gene expression results, we performed immunohistochemical analyses on conjunctival EMZL tissues. Signals of (P)RR and AT1R were observed widely in
conjunctival EMZL tissues, including atypical lymphoid cells and vascular endothelial cells (Figs. 1B, 1C).

Localization of Prorenin and (P)RR in Endothelial and Lymphoid Cells of EMZL Tissues

To further study the localization of (P)RR in conjunctival EMZL tissues, we performed immunofluorescence analysis. Double-staining experiments demonstrated colocalization of (P)RR signal with CD20, a B-cell marker (Figs. 2A–C), and with CD31, a vascular endothelial cell marker (Figs. 2D–F). Moreover, expression of (P)RR in B-cells was abundantly colocalized with prorenin (Figs. 2G–I).

Localization of AGT and AT1R in Endothelial and Lymphoid Cells of EMZL Tissues

To further verify our immunohistochemical analysis results, we performed double-staining of AT1R with CD20 or CD31. The AT1R colocalized with CD20 and CD31, which indicates that AT1R is expressed in the B-lymphoid cells (Figs. 3A–C) and vascular endothelial cells of EMZL tissues (Figs. 3D–F). In addition, AT1R immunoreactivity also colocalized with AGT on lymphoid cells of EMZL (Figs. 3G–I).

Upregulation of FGF2 Expression via Prorenin-(P)RR Interaction

Bindings of prorenin to (P)RR and of Ang II to AT1R have been shown to upregulate various gene expressions in vivo and in vitro, related to the pathogenesis of numerous diseases.10,11,19–23 In retinal vascular endothelial cells, we and other groups have shown that prorenin and Ang II stimulations significantly upregulated mRNA expressions, such as MMP2.10,19,21 However, there are no reports on B-cells. In the present study, to investigate the effect of prorenin-(P)RR interaction on B-lymphoid cells, we examined whether stimulation of prorenin affects mRNA expression levels in conjunctival EMZL tissues, including atypical lymphoid cells and vascular endothelial cells (Figs. 1B, 1C).
Figure 4. (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.

FIGURE 4. (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.

human B-lymphoma cell line IM-9 by real-time qPCR analysis. We found that FGF2 expression levels significantly increased (fold change = 2.24, P < 0.01) in IM-9 cells stimulated with prorenin compared to control, but not with Ang II (fold change = 0.87, P > 0.05). Importantly, increased FGF2 expression was inhibited by pretreatment with PRRB (fold change = 1.41, P < 0.05, Fig. 4A).

Based on these results, we performed immunofluorescence experiments to investigate the expression of FGF2 with (P)RR in conjunctival lymphoma samples. Immunofluorescence analysis revealed the colocalization of FGF2 with (P)RR in conjunctival lymphoma (Figs. 4B–D). These data suggested that activation of RAPS, through the interaction between prorenin and (P)RR, induces an increase in FGF2 expression levels in B-lymphocytes, and possibly leads to angiogenesis in EMZL of the conjunctiva.

Upregulation of MMP Family Members Via Ang II–AT1R Interaction

To study the effects of Ang II–AT1R binding in B-lymphoma cells, we administered Ang II to IM-9 cells and examined the changes in gene expression levels using real-time qPCR analysis. Ang II stimulation to cells significantly increased 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 controls. Those pretreatment with valsartan suppressed Ang II-induced BSG, MMP2, MMP9, and MMP14 (fold change = 1.75, P < 0.05; MMP9, fold change = 0.91, P < 0.05; MMP14, fold change = 1.03, P < 0.05, Figs. 5A-D).

Following these in vitro experiment results, we carried out immunofluorescence analysis to examine the colocalization of BSG, MMP2, MMP9, and MMP14 with AT1R in conjunctival samples. Immunoreactivity for BSG, MMP2, MMP9, and MMP14 was widely distributed (Figs. 5E, 5H, 5K, and 5N) and colocalized with AT1R (Figs. 5F, 5G, 5J, 5L, 5M, 5O, and 5P) in the EMZL sections. The results suggest that the binding of Ang II to AT1R causing tissue RAS stimulation increases expression levels of MMP family genes and leads to the following sequence of events at the molecular level to trigger angiogenesis and invasion in conjunctival EMZL.

DISCUSSION

In this study, we provide several unique mechanistic insights into the roles of RAPS and RAS in the pathogenesis of conjunctival EMZL. First, expression of RAS component genes was confirmed in surgically excised EMZL tissues as well as human B-lymphoma cell lines (Fig. 1). The (P)RR and AT1R proteins were immunopositive in B-lymphocytes in the tissues, and colocalized with prorenin and AGT, respectively (Figs. 1–3). Stimulation of prorenin to B-lymphoma cell culture triggered the upregulation of FGF2 through (P)RR interaction (Fig. 4), and (P)RR colocalized with FGF2 in conjunctiva EMZL tissues. Activation of AT1R with Ang II increased BSG and MMPs gene expression levels, all of which were suppressed by pretreatment with valsartan, and immunofluorescence analyses showed colocalization of AT1R with these MMP-related molecules (Fig. 5).

The pro-angiogenic molecule FGF2 is widely expressed in various tumor tissues and its elevated levels are associated with inflammation leading to tumor growth, progression, and metastasis.24 Expression of FGF2 was found to be significantly elevated in sera or tissue specimens of Hodgkin’s and non-Hodgkin’s lymphomas.25–28 In addition, through its interaction with FGF receptor 1, FGF2 is demonstrated to promote angiogenesis in the tumor microenvironment leading to angiogenesis and lymphatic metastasis.27 Excessive FGF2 expression levels in diabetes and hypertension were suppressed by RAS inhibitors.28–29 Here, we found that colocalization of (P)RR and FGF2 in EMZL tissues and prorenin stimulation to B-lymphocyte cell line significantly increased the expression level of FGF2, which was suppressed by PRRB. Taken together, our results suggested 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.

The MMP2 and MMP9 have important roles in the process of tumor neoangiogenesis, and the extent of neovascularization correlates with MMPs expression during the progression such as in multiple myeloma and skin T-cell lymphoma.30 Our current study showed that Ang II induced an AT1R-mediated increase in gene expression levels of BSG, MMP2, MMP9, and MMP14 in human B-lymphocytes. Supporting in vitro data, BSG, MMP2, MMP9, and MMP14 colocalized with AT1R in EMZL tissues. Alternatively called “extracellular MMP inducer” or CD147, BSG is a member of the immunoglobulin superfamily and abundantly expressed on the surface of tumor cells.31 The BSG-positive tumor cells and their supernatants increase expression levels of MMPs, including MMP2 and MMP9.32–33 The MMP2 and MMP9 also are regarded as tumor biomarkers in monitoring response to cancer treatment.34 Degradation of type IV collagen by MMP2 is a significant hallmark of metastasis and invasion in carcinoma.35 The MMPs also can activate growth factor signaling by increasing the bioavailability of factors, such as FGF2, and initiate tumor progression through stimulation of angiogenesis.36 The release of angiogenic growth factors, cytokines, and proteases, like FGF2, MMP2, MMP9, and MMP14, initi into the surrounding extracellular matrix initiates tumor angiogenesis37–39. Studies on MMPs in clinical samples reported that BSG and MMP9
FIGURE 5. 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 $\mu$m.
expression levels are elevated in non-Hodgkin’s and Hodgkin’s lymphomas, which are associated with clinical stages.40,41 Activation of AT1R by Ang II has been reported to trigger upregulation of BSG and MMPs, including MMP2, MMP9, and MMP14, in various cells.21,22,42 Moreover, activity of serum angiotensin-converting enzyme (ACE) increased in non-Hodgkin’s and other types of lymphoma patients. In the bone marrow, ACE cleaves N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), an inhibitor of hematopoietic stem cell proliferation, and renders it inactive.43 Abnormal increased ACE activity may lead to the acceleration of AcSDKP degradation, resulting in hematopoietic cell proliferation. In accordance with these findings, our data suggested that binding of Ang II-AT1R (i.e., stimulation of tissue RAS) has roles in the extracellular matrix turnover and remodeling in B-lymphomas, induces the subsequent sequence of molecular events in the microenvironment, and leads to formation and development of conjunctival lymphoma.

The treatments of conjunctival EMZL are performed mainly with surgical excision and/or irradiation due to their low-grade and radiosensitive malignancy. However, still there are cases that do not achieve complete remission even after these treatments. Therefore, development of additional therapeutic options are required. Our data from clinical human EMZL samples and B-lymphoid cells show the evidence that activations of RAPS and tissue RAS via (P)RR and AT1R 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 (ERK) 1/2 and nuclear factor-kB (NF-kB) pathways, and induce proliferation and differentiation in various cells.10,11,14 Several reports propose the NF-kB signaling pathway as an attractive therapeutic target in T- and B-cell malignancies including EMZL.25 Blockades of (P)RR and AT1R may be promising to prevent the cascade of events essential in the pathogenesis of EMZL of the conjunctiva and serve as clinical tools in the treatment of conjunctival lymphoma.

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