[Frontiers in Bioscience 13, 3884-3895, May 1, 2008]

Pathologic roles of prorenin and (pro)renin receptor in the eye

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1. ABSTRACT

Recent reports indicated that tissue renin-angiotensin system (RAS) was upregulated and angiotensin II type 1 receptor signaling plays crucial roles in ocular inflammation and neovascularization; however, the precise mechanism for activating tissue RAS had not been defined until recently. (Pro)renin receptor, a recently identified molecule existing in the major organs but not in the circulation, has attracted growing attention as an activator of tissue RAS. When the handle region of the prorenin prosegment binds to (pro)renin receptor, prorenin undergoes a conformational change to its enzymatically active state without the conventional proteolysis of the prorenin prosegment. Systemic treatment with a peptide with the structure of the handle region (handle region peptide; HRP), which competitively binds to (pro)renin receptor as a decoy peptide and inhibit the nonproteolytic activation of prorenin, resulted in the suppression of retinal inflammation and neovascularizaion in the rodent models. Retinal expression of RAS-related inflammatory and angiogenic molecules, such as intercellular adhesion molecule-1, monocyte chemotactic protein-1, and vascular endothelial growth factor, was also suppressed with application of HRP. These findings demonstrate that nonproteolytically activated prorenin plays a significant role in the ocular inflammation and neovascularization.

2. INTRODUCTION

Recently, it was revealed that angiotensin II type 1 receptor (AT1-R) blockers, widely and safely used for anti-hypertensive therapy, have an inhibitory effect on retinal inflammation and neovascularization (1, 2). Retinal expression of intercellular adhesion molecule (ICAM)-1, monocyte chemotactic protein (MCP)-1, and vascular encothelial growth factor (VEGF) was upregulated during retinal inflammation and neovascularization, and AT1-R blocker application led to significant suppression of these angiogenic and inflammatory molecules (1, 2). These findings are supported by several recent reports showing that the renin-angiotensin system (RAS), originally regarded as an important controller of systemic blood pressure, plays crucial roles in pathologic vascular conditions including inflammation and neovascularization via interaction of angiotensin II with AT1-R (3-6). The initial step for upregulation of the RAS is classically known as proteolytic activation, whereby prorenin is converted to active (mature) form of renin by the processing enzymes to remove the prorenin prosegment, which folds into an active-site cleft of mature renin (Figure 1A). Renin is well known to be a rate-limiting enzyme in the RAS for the cleavage of angiotensinogen to angiotensin I, which angiotensin-converting enzyme (ACE) processes to angiotensin II, a final effector molecule that interacts with its cognate receptors AT1-R and AT2-R.

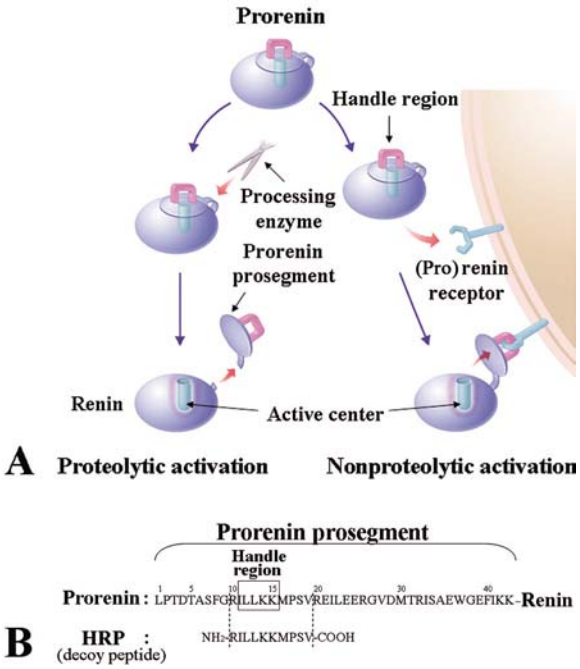

Figure 1. (A) Proteolytic activation of prorenin by processing enzymes versus nonproteolytic activation by (pro)renin receptor binding to the handle region of the prorenin prosegment. (B) Preparation of the decoy peptide corresponding to the HRP. Amino acid sequences of the rat prorenin prosegment and HRP. Reproduced with permission from 9.

In addition to the proteolytic activation of prorenin, nonproteolytic activation of prorenin, which was recently demonstrated *in vitro* (7) and *in vivo* (8), has attracted growing attention as an activator of tissue RAS causing organ damage. In the mechanism of nonproteolytic activation, when the prorenin-binding proteins interact selectively with the handle region of the prorenin prosegment, prorenin undergoes conformational change with exposure of the active center and obtains enzymatic bioactivity of renin without cleavage of the prorenin prosegment or change in molecular weight (Figure 1A). Although AT1-R blockade was shown to suppress retinal inflammation and neovascularization (1, 2), the precise mechanism for activating ocular RAS was not defined. Recently, we have elucidated the role of (pro)renin receptor as a trigger to activate tissue RAS in retinal inflammation and neovascularization (9, 10).

3. PRORENIN AND (PRO)RENIN RECEPTOR

3.1. Prorenin

Prorenin, the precursor of renin, was considered to have little or no bioactivity for many years, although its circulating level is 10 times higher than that of renin (11). Prorenin is known to be produced in various organs including the kidney, brain, testis, ovary and vascular endothelium. Also in the eye, prorenin was found to be present in the human surgical samples (12, 13) and in the rodent retina (14, 15). Vitreous aspirates from patients with proliferative diabetic retinopathy contained the increased levels of prorenin (13).

3.2. (Pro)renin receptor

(Pro)renin receptor is a recently identified transmembrane protein consisting of 350 amino acids. (Pro)renin receptor interacts with prorenin to exert renin activity through the conformational change of the prorenin molecule instead of the conventional proteolysis of the prorenin prosegment basically achieved by processing enzymes such as cathepsin B. Since the membrane-bound (pro)renin receptor is reported to exist in the heart, brain, placenta, liver, pancreas and kidney but not in the circulation (16), the nonproteolytic activation of prorenin is hypothesized to play a critical role in the activation of tissue, but not circulatory, RAS.

3.3. Nonproteolytic activation of prorenin

Nonproteolytic activation of prorenin was originally observed under acidic pH or low temperature *in vitro* (11, 17, 18), which are called acid-activation and cryo-activation, respectively. However, these experimental phenomena have never been found *in vivo*. Our recent report (8, 19) indicated the importance of interaction of (pro)renin receptor with the handle region of the prorenin prosegment for *in vivo* nonproteolytic activation of prorenin (Figure 1A) and revealed the association of nonproteolytic activation with pathogenesis in the kidney. When rats with streptozotocin-induced diabetes received a peptide with the structure of the handle region of the prorenin prosegment (handle-region peptide [HRP], Figure 1B) as a decoy for (pro)renin receptor, it potently suppressed the progression of diabetic nephropathy by inhibiting the nonproteolytic activation of prorenin and the subsequent upregulation of the RAS in the kidney. We have suggested the importance of this novel receptor-associated prorenin (RAP) system as an organ-specific RAS enhancer in disease, because the RAS, independently of soluble processing enzymes, is locally upregulated by nonsoluble, membrane-bound (pro)renin receptor in target organs. In stroke-prone spontaneously hypertensive rats, cardiac fibrosis developed together with activation of the RAS (20). Systemic HRP administration led to significant suppression of cardiac fibrosis, suggesting the involvement of nonproteolytic activation of prorenin with the pathogenesis in the heart (20).

3.4. Handle region peptide (HRP)

3.4.1. Rat HRP

Figure 1B shows the prosegment of rat prorenin. To cover the handle region (position 11-15) (7) , we designed a decapeptide, NH2-RILLKKMPSV-COOH, as an HRP of rat prorenin and purified it by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column (8). The purity and retention time of HPLC was 97.6% and 26.2 minutes, respectively. The mass of the product was 1,185.7, and similar to the theoretical mass value (1,186.0). The specific inhibitory action of HRP against prorenin activation was recently confirmed by utilizing recombinant rat prorenin and COS-7 transfectant cells expressing rat (pro)renin receptor (accession number AB188298 in the

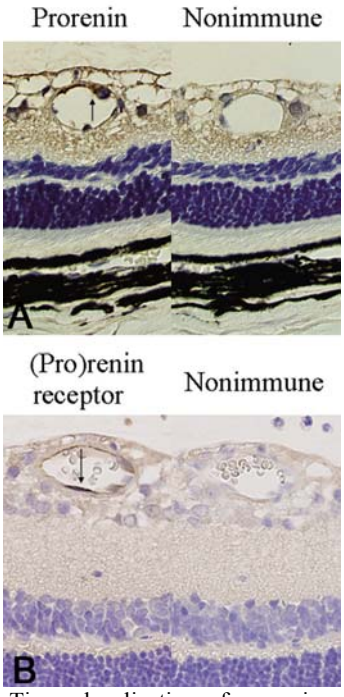

Figure 2. Tissue localization of prorenin and (pro)renin receptor and in EIU eyes (A-B). Positive staining for prorenin and (pro)renin receptor on the retinal vessels (arrows in A-B). Reproduced with permission from 9.

DNA Databank of Japan) (8), which was originally cloned by Nguyen *et al* (16).

3.4.2. Mouse HRP and control peptide (CP)

To cover the mouse handle region (position 11-15) (7), a decapeptide, NH2-IPLKKMPS-COOH, as an HRP of mouse prorenin was designed and purified it by high-pressure liquid chromatography (HPLC) on a C-18 reverse-phase column. The purity and retention time of HPLC was 97.4 % and 6.8 minutes, respectively. The mass of the product was 913.4 and similar to the theoretical mass value (913.2). The specific inhibitory action of HRP against nonproteolytic activation of prorenin in mice was confirmed in our recent *in vivo* data (21). As a negative control for HRP, a control peptide (CP), NH_2-MTRLSAE-COOH, was also prepared, which corresponds to positions 30 to 36 of the prorenin prosegment.

4. PATHOLOGIC ROLES OF PRORENIN AND (PRO)RENIN RECEPTOR IN THE EYE

4.1. Ocular inflammation

4.1.1. Endotoxin-induced uveitis (EIU) model

EIU is an animal model of acute ocular inflammation induced by the administration of lipopolysaccharide (LPS), a component of gram-negative bacterial outer membranes (22-24). Since uveitis frequently leads to severe vision loss and blindness with retinal vasculitis, retinal detachment and glaucoma, it is important to further elucidate the mechanisms in the development of ocular inflammation. LPS enhances the expression of various inflammatory mediators, such as interleukin (IL) -6 (24, 25), tumor necrosis factor (TNF)-α (26) and MCP-1 (27), which contribute to the development of EIU, resulting in the breakdown of blood ocular barrier and in leukocyte infiltration. For the first step of leukocyte infiltration, cell adhesion to vascular endothelium is essential, in which adhesion molecules play important roles (28). Among various adhesion molecules, ICAM-1 and its counter receptor ß2 (CD18) integrins (i.e., LFA-1 and Mac-1) are important for the development of EIU (28-30). Although EIU was originally utilized as a model mimicking anterior uveitis, increasing evidence shows EIU as having inflammation in the posterior segment of the eye with recruitment of leukocytes adhering to the retinal vasculature and infiltrating into the vitreous cavity (31, 32).

4.1.2. EIU and RAS

Recently, several studies demonstrate the diverse biological functions of angiotensin II as a modulator of angiogenesis, vascular remodeling and inflammation (3-5, 33, 34). As an inflammatory mediator, angiotensin II enhances vascular permeability via prostaglandins and VEGF (3), and contributes to the recruitment of inflammatory cells by inducing chemokines and adhesion molecules (4, 5). Moreover, angiotensin II directly induces the proliferation and differentiation of inflammatory cells per se (6). AT1-R blockade is reported to attenuate such inflammatory processes effectively (3-5). EIU is prevented by suppressing inflammatory mediators including IL-6, TNF-α, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and MCP-1 (26, 27, 35-39). However, it was not clear whether AT1-R blockade is effective in reducing ocular inflammation.

Our recent report (2) showed that AT1-R signaling plays an important role in ocular inflammation in EIU. EIU was induced in C57BL/6 mice by a single intraperitoneal injection of 150 μg LPS. The AT1-R antagonist, telmisartan, was administered intraperitoneally at the dose of 10 mg/kg daily for 5 days until the injection of LPS. Immunohistochemistry demonstrated that retinal vessels were positive for AT1-R. In mice with EIU, retinal AT1-R mRNA and protein levels were significantly increased compared with normal controls. Twenty-four hours after LPS administration, EIU animals also showed significant increases in the numbers of inflammatory cells infiltrating into the anterior chamber and adhering to the retinal vessels together with retinal ICAM-1 levels. Administration of telmisartan to EIU mice resulted in significant suppression of retinal ICAM-1 expression and leukocyte adhesion and infiltration compared to vehicle treatment. Protein concentration in the aqueous humor of telmisartan-treated EIU mice tended to be lower than that of vehicle-treated EIU mice, but the difference was not statistically significant. These results demonstrate AT1-R signaling blockade inhibited retinal ICAM-1 upregulation and leukocyte adhesion and infiltration in the EIU model, suggesting the potential use of an AT1-R antagonist as a therapeutic agent to reduce ocular inflammation (2).

4.1.3. Pathologic roles of prorenin and (pro)renin receptor in EIU

We have demonstrated the expression of prorenin (Figure 2A) and (pro)renin receptor (Figure 2B) in retinal vessels in rats with endotoxin-induced uveitis and the association of the (pro)renin receptor with ocular

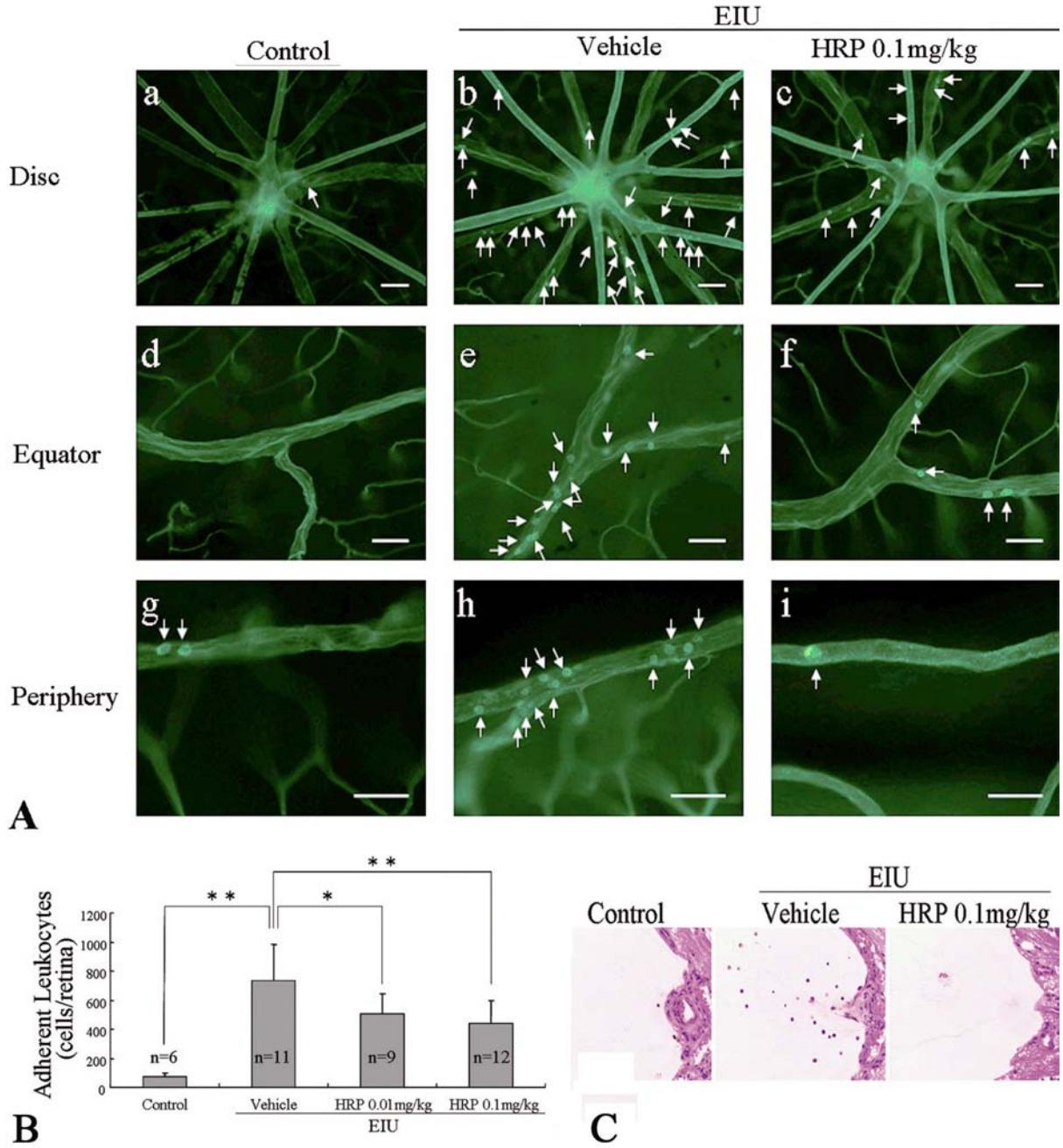

Figure 3. Effects of HRP on leukocyte adhesion and infiltration. (A) Flatmounted retinas from normal rats (a, d, g), vehicle-treated EIU rats (b, e, h) and HRP-treated EIU rats (c, f, i). Vehicle-treated EIU rats showing increased adherent leukocytes (arrows) to the retinal vasculature compared with normal animals. Systemic treatment with HRP led to the suppression of leukocyte adhesion. Scale bar = 100 μm. (B) The graph showing the numbers of retinal adherent leukocytes. HRP-treated EIU rats showing significantly fewer adherent leukocytes than vehicle-treated animals. The results represent the mean ± SD. $P < 0.05$, $P < 0.01$ by Mann-Whitney test. (C) Leukocyte infiltration into the vitreous cavity. The number of leukocytes around the optic disc, which increased in EIU rats compared with normal controls, were suppressed by the treatment with HRP. Reproduced with permission from 9.

inflammation (9). To inhibit the (pro)renin receptor-mediated upregulation of the RAS, HRP, a decoy peptide binding to (pro)renin receptor, is intraperitoneally administered 24 hours before and immediately after the injection of LPS. Systemic treatment with HRP resulted in dose- and time-dependent inhibition of the leukocyte adhesion (Figure 3A, B) and infiltration (Figure 3C) and the protein leakage (Figure 4), all of which were increased by the induction of EIU. Retinal mRNA expression and protein levels (Figure 5) of ICAM-1, MCP-1 and IL-6,

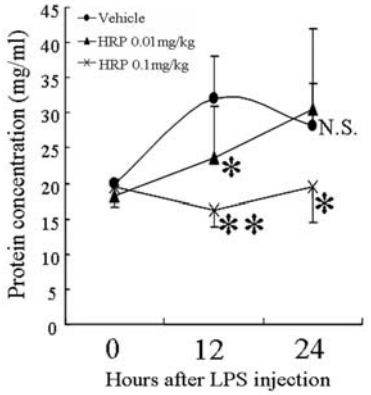

Figure 4. Effects of HRP on anterior uveitis. Systemic treatment with HRP resulted in dose- and time-dependent inhibition of protein leakage into the anterior chamber. The results represent the mean ± SD; n=6-11. $^{\square}P < 0.05$, $^{\square\square}P < 0.01$ by Mann-Whitney test. Reproduced with permission from 9.

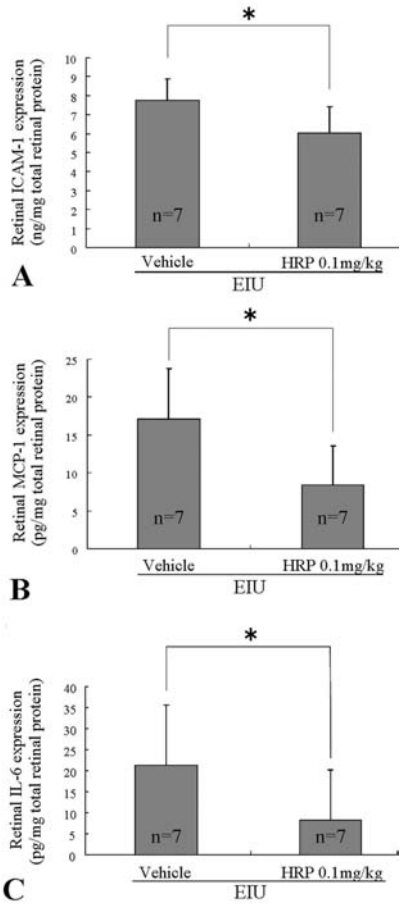

Figure 5. Effects of HRP on protein levels of retinal ICAM-1 (A), MCP-1 (B), and IL-6 (C). Retinal protein levels of ICAM-1 (A), MCP-1 (B), and IL-6 (C) in vehicle-treated EIU rats were significantly reduced by the treatment with HRP. The results represent the mean ± SD. $^{\square}P < 0.05$ by Mann-Whitney test. Reproduced with permission from 9.

induced in rats with EIU, were also significantly suppressed with application of HRP.

Leukocyte adhesion to the vessel walls is an important process of inflammation. When leukocytes are recruited to inflammatory sites, adhesion molecules play essential roles in the first step of inflammation. ICAM-1 and its counter receptor ß2 (CD18) integrins (i.e., LFA-1 and Mac-1) regulate leukocyte-endothelial interaction in the pathogenesis of EIU (28-30). During the development of EIU, ICAM-1 is upregulated and expressed on vascular endothelial cells of the iris and the ciliary body shortly after LPS injection (29). Additionally, several studies demonstrate that treatment with an anti-ICAM-1 antibody significantly inhibited the development of EIU (29, 30). In our study (9), retinal ICAM-1 upregulation in EIU was suppressed after treatment with HRP (Figure 5A). This is likely to result from the suppression of the RAS, activated in EIU with AT1-R upregulation (2), following HRP-induced inhibition of nonproteolytic activation of prorenin. Recent *in vivo* and *in vitro* data (2, 5, 40) showed that angiotensin II signaling induces ICAM-1 expression via AT1-R. Collectively, the suppression of retinal inflammation observed in our study depends in part on HRP-induced inhibition of ICAM-1 via the RAS downregulation. Our recent data (8) showed that HRP administration resulted in the inhibition of nephropathy in rats with streptozotocin-induced diabetes, while ICAM-1 deficiency was also protective against diabetic nephropathy in db/db mice (41).

Besides ICAM-1, various chemical mediators are involved in the pathogenesis of EIU. We showed that HRP treatment led to the suppression of EIU-induced inflammation-related molecules, including ICAM-1, IL-6 and MCP-1 (Figure 5). Pro-inflammatory effects of angiotensin II are attributable to its induction of these inflammation-related molecules, most of which are downstream products of nuclear factor (NF)-κB, a transcription factor which promotes the gene expression of various inflammatory cytokines (42). AT1-R downstream signaling is known to lead to the activation of NF-κB (43, 44). LPS-induced inflammation is mediated by the activation of NF-κB (42). Indeed, ocular inflammation is suppressed by administration of an NF-κB inhibitor in EIU (45). Taken together, anti-inflammatory effects of HRP are likely to result from suppressed gene expression of NF-κB-induced molecules via the inhibition of AT1-R signaling. These previous findings, in accordance with our data, suggest that HRP affects not only ICAM-1-mediated leukocyte adhesion but also various inflammatory processes.

These results demonstrate that nonproteolytically activated prorenin plays a significant role in the development of ocular inflammation in the EIU model. Our study suggests the potential use of HRP as a therapeutic agent to reduce ocular inflammation (9).

4.2. Ocular neovascularization

4.2.1. Retinal neovascularization

Retinal neovascularization is a hallmark of vision-threatening retinal diseases, including diabetic retinopathy

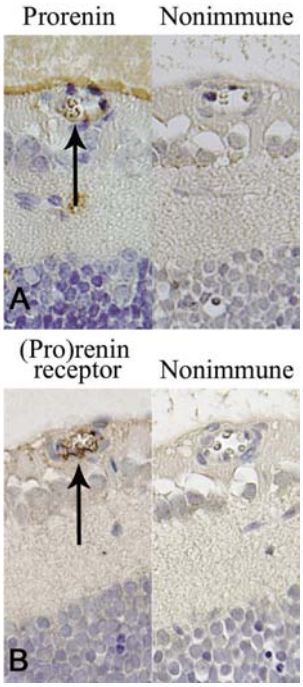

Figure 6. Immunohistochemical staining for prorenin and (pro)renin receptor in ischemic retinopathy eyes (A, B). Retinal vessels were positive for prorenin and (pro)renin receptor (arrows in A, B). The immunoreactivity was diminished in the negative control sections, where the primary antibodies were replaced with nonimmune IgG. Reproduced with permission from 10.

and retinopathy of prematurity, which are major causes of blindness in adults and children, respectively. There are two distinctly different types of retinal neovascularization, physiologic versus pathologic, both of which are induced by retinal ischemia. In the former, new vessels grow systematically in the retina to compensate for the retinal ischemia, whereas in the latter, retinal new vessels ectopically invade the transparent vitreous, which originally lacks the vasculature. Because simultaneous prevention of both types of retinal neovascularization causes retinal ischemia to be untreated, ophthalmologists await the establishment of new therapy that selectively targets pathologic neovascularization, while sparing compensatory physiologic neovascularization. The molecular and cellular mechanisms differentiating pathologic from physiologic retinal neovascularization have been recently highlighted (46-48). The influx of inflammatory cells at the growing tip of new vessels is likely to be a critical step of changing the direction of retinal neovascularization from intraretinal to extraretinal growth.

4.2.2. Retinal neovascularization and RAS

Recent reports have suggested that RAS plays a key role in various inflammatory processes including not only the expression of chemokines and adhesion molecules for the recruitment of inflammatory cells, but also the differentiation and proliferation of inflammatory cells per se (5, 6, 49, 50). RAS blockade seems to be a useful strategy for the improvement of inflammation-related pathologies, such as pathologic retinal neovascularization. Actually, ACE inhibition was shown to suppress the progression of human diabetic retinopathy to its proliferative (angiogenic) stage (51). AT1-R blockade suppressed retinal neovascularization in a murine model of ischemic retinopathy (52), which supports the result of the clinical trial. Recently, we elucidated the mechanisms in the anti-angiogenic effects of AT1-R inhibition in a murine model of ischemic retinopathy, focusing on the inflammatory mechanisms that promote pathologic, but not physiologic, retinal neovascularization (1).

C57BL/6 neonatal mice were reared in 80% concentration of oxygen from postnatal (P) day 7-12, followed by room-air breeding to P17 to induce ischemia-initiated retinal neovascularization (i.e. a murine model of ischemic retinopathy). To investigate the anti-inflammatory and anti-angiogenic effects of AT1-R blockade, animals were intraperitoneally treated with telmisartan. Vessels in human fibrovascular tissues as well as murine retinas were immunoreactive for AT1-R. Pathologic, but not physiologic, retinal neovascularization was significantly suppressed in telmisartan-treated mice compared with vehicle-treated animals. The number of adherent leukocytes was also significantly reduced together with retinal ICAM-1 levels in the telmisartan-treated group than in the control group. No statistical difference was detected in retinal VEGFR-2 levels between the two groups, whereas retinal VEGFR-1 levels in the temisartan-treated group were significantly lower than in the vehicle-treated group. Our study suggests that AT1-R signaling blockade leads to the selective suppression of pathologic, but not physiologic, retinal neovascularization via the inhibition of the inflammatory processes related to the pathologic neovascularization (1).

4.2.3. Pathologic roles of prorenin and (pro)renin receptor in retinal neovascularization

Using the rodent model of ischemic retinopathy which was recently shown to be mediated by the RAS (1), our recent report elucidated that nonproteolytically activated prorenin plays a significant role in retinal neovascularization, and that HRP as a decoy for (pro)renin receptor is anti-angiogenic in the eye (10). After 80% oxygen exposure, vehicle (phosphate-buffered saline), CP (1.0mg/kg) or HRP (1.0mg/kg) was intraperitoneally applied for 5 days in normoxia (21% oxygen) following the hyperoxic exposure (P12-16). The degree of retinal neovascularization and the number of adherent leukocytes were evaluated on P17.

Retinal vessels in ischemic retinopathy eyes were positive for prorenin (Figure 6A), which is consistent with the data from the normally developing retina (15) , and (pro)renin receptor was also present in the vessels (Figure 6B). Pathologic, but not physiologic, retinal neovascularization was significantly attenuated in HRP-treated mice compared with vehicle- or CP-treated animals (Figure 7A, C, D). The number of adherent leukocytes was also significantly reduced (Figure 7B, E). This is supported

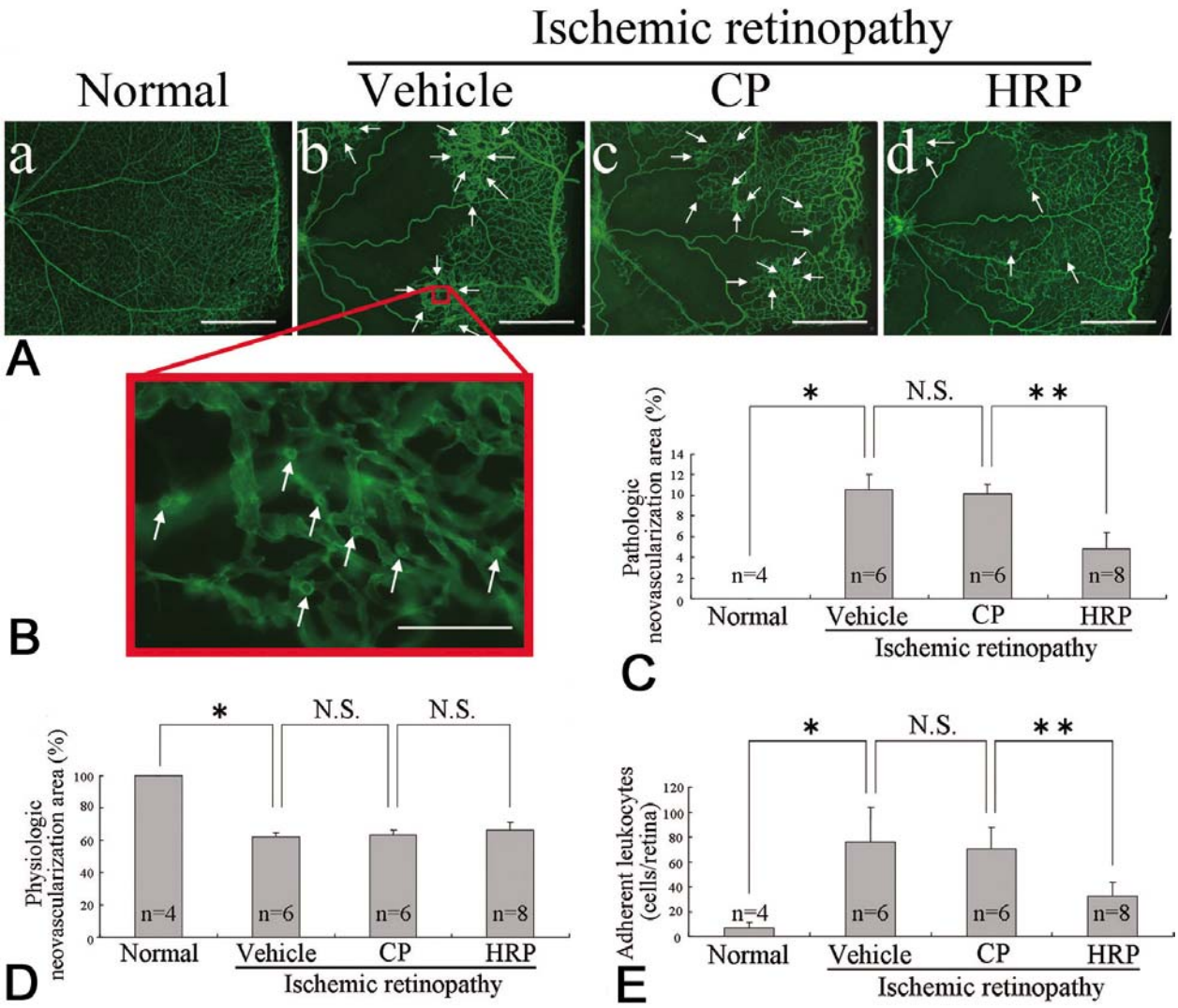

Figure 7. (A) Flatmounted retinas from P17 normal mice (a), ischemic retinopathy mice with pathologic neovascular buds (arrows in b, c) and HRP-treated retinopathy mice showing decreased pathologic neovascularization (arrows in d) and intact physiologic neovascularization compared to vehicle- or CP-treated animals (b, c). Scale bars: 200μm. (B) Adherent leukocytes (arrows) accompanied by pathologic neovascularization. Scale bar: 100μm. (C) Effects of HRP on pathologic neovascularization. HRP-treated retinopathy mice showing significantly less pathologic neovascularization than vehicle- or CP-treated animals. (D) Effects of HRP on physiologic neovascularization in ischemic retinopathy. HRP-treated mice exhibited no significant ($P > 0.05$) difference in physiologic neovascularization compared to vehicle- or CP-treated animals. (E) Effects of HRP on leukocyte adhesion. HRP-treated retinopathy mice showing significantly fewer adherent leukocytes than vehicle- or CP-treated mice. The results represent means $\pm$ SD. *$P < 0.05$, **$P < 0.01$ by Mann-Whitney test. Reproduced with permission from 10.

in part by previous reports showing that the RAS inhibitors, including an ACE inhibitor and both AT1-R and AT2-R blockers, suppressed retinal neovascularization, although no mechanistic explanation was presented concerning inflammatory processes associated with pathologic neovascularization (34, 53).

We have recently proposed that ischemia-induced retinal neovascularization, when it becomes pathologic, involves inflammation (46-48). A previous immunohistochemical study pointed out the infiltration of macrophages in fibrovascular tissues excised at vitrectomy for proliferative diabetic retinopathy (54), indicating a possible link between retinal neovascularization and inflammation. In an animal model of ischemic retinopathy, pathologic, but not physiologic, neovascularization was shown to be preceded and accompanied by the adhesion of inflammatory monocytes to the retinal vasculature (46). When clodronate-liposome, a reagent that induces apoptosis specifically to monocyte/macrophage-lineage cells, was used, pathologic retinal neovascularization was suppressed without any substantial effects on physiologic neovascularization (46). In addition, other reports have suggested the proangiogenic role of inflammatory

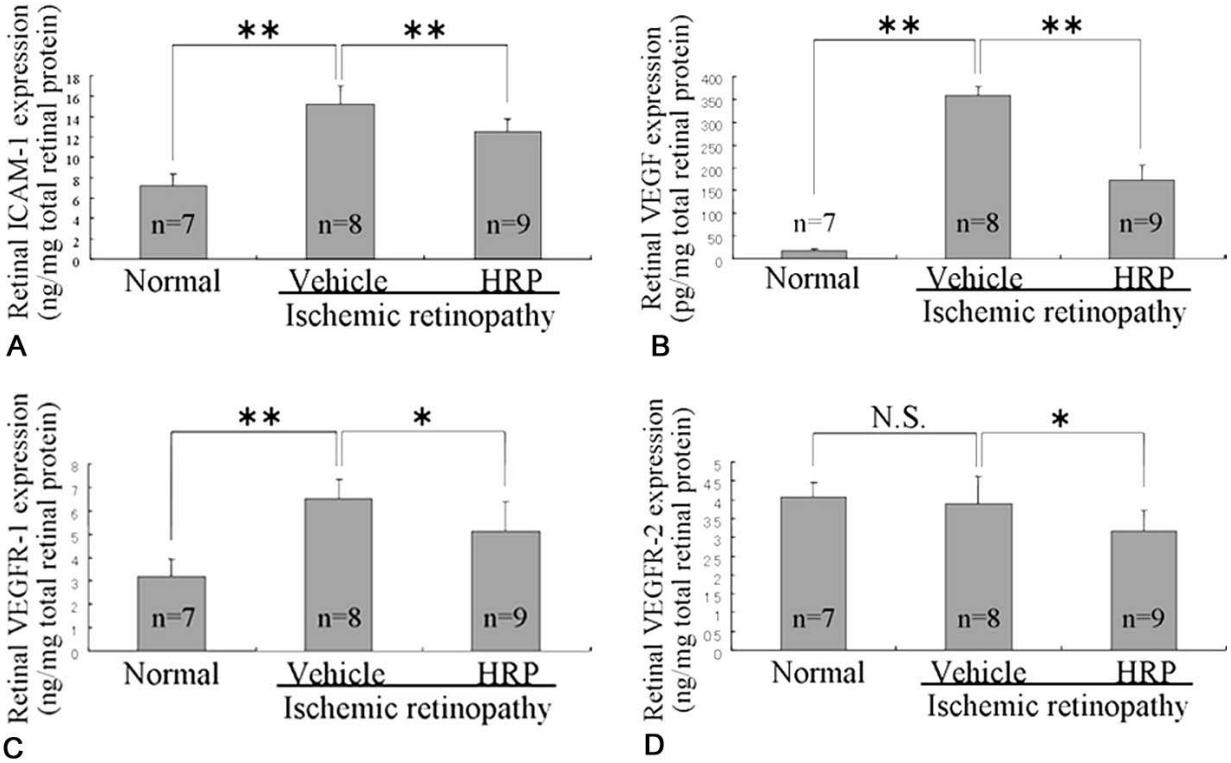

Figure 8. ELISA analyses for retinal ICAM-1, VEGF, VEGFR-1 and VEGFR-2. (A-D) Retinal protein levels of ICAM-1, VEGF, VEGFR-1 and VEGFR-2 were significantly lower in HRP -treated retinopathy group than in vehicle-treated retinopathy group. The results represent means ± SD. *P < 0.05, **P < 0.01 by Mann-Whitney test. Reproduced with permission from 10.

monocytes and macrophages in murine ischemic retinopathy. Intravitreally infiltrating macrophages adjacent to the pathologic new vessels express and produce VEGF in the animal model (55). Neutralizing antibodies against MCP-1 and macrophage inflammatory protein (MIP)-1α were shown to reduce pathologic retinal neovascularization and inflammation (56). Therefore, inflammatory monocytes are likely to disrupt the direction of physiologic neovascularization, triggering pathologic retinal neovascularization.

Retinal mRNA expression and protein levels (Figure 8) of ICAM-1, VEGF, VEGFR-1 and VEGFR-2 in ischemic retinopathy were also significantly suppressed with application of HRP. A previous study with donor eyes demonstrated that diabetic retinas had increased levels of ICAM-1 immunoreactivity in the vessels as well as infiltrating leukocytes, compared with normal retinas (57). In a rodent model of diabetes, ICAM-1-dependent leukocyte adhesion is enhanced in the early stage (58, 59), and various retinal pathologic conditions related to long-term diabetes have been shown to be mediated by ICAM-1 (60). *In vitro*, angiotensin II was shown to induce the expression of ICAM-1 on vascular endothelial cells and promote leukocyte-endothelial adhesion (5). In accordance with the *in vitro* data, HRP-treated animals exhibited the decreased retinal ICAM-1 mRNA expression and production as well as the suppressed leukocyte adhesion to the retinal vessels, reasonably resulting in the inhibition of inflammation-related pathologic neovascularization.

VEGF has two cognate receptors called VEGFR-1 and VEGFR-2 (61-64). VEGF-mediated endothelial cell mitogenic activity was shown to depend not on VEGFR-1, but on VEGFR-2 (64, 65). VEGFR-2 blockade in the retinopathy model was reported to suppress both pathologic and physiologic neovascularization (66), suggesting a major role of the VEGF-VEGFR-2 system in retinal neovasuclarization. Angiotensin II induced the *in vitro* expression of VEGF and VEGFR-2 mRNA on cultured bovine retinal vascular cells, enhancing VEGF-induced angiogenic activity (67, 68). In our study, HRP application to mice with ischemic retinopathy caused substantial (52%) and modest (18%) decrease in retinal production of VEGF and VEGFR-2, respectively (Figure 8B, D). Accordingly, the inhibition of pathologic neovascularization is likely attributed to the HRP-induced suppression of the VEGF signaling. Interestingly, HRP administration led to selective suppression of VEGF165, the pathologic isoform capable of inducing inflammation-related pathologic neovascularization in ischemic retinopathy (46, 69). Reasonably, the reduced protein level of residual VEGF following HRP treatment, still higher than the physiologic level (Figure 8B), is thought to be sufficient for promoting physiologic neovascularization.

Our result shows that inhibition of nonproteolytic activation led to the significant decrease in retinal VEGFR-1 levels in ischemic retinopathy. Shih *et al.* (70) showed that VEGFR-1 signaling activated by placenta growth factor (PlGF)-1 led to suppression of hyperoxia-induced vaso-obliteration, and suggested the possibility of VEGFR-1-mediated prevention of pathologic retinal neovascularization secondary to the decreased extent of retinal ischemia. They also described that PlGF-activated signaling of VEGFR-1 did not affect any of three types of vaso-proliferation (i.e. physiologic neovascularization during normal retinal development, physiologic neovascularization after hyperoxia-induced ischemia, or pathologic neovascularization after hyperoxia-induced ischemia). In our study, we applied HRP to mice with retinopathy during the proliferative stage after the phase of hyperoxia-induced vaso-obliteration. Reasonably, our administration of HRP did not affect the extent of avascular area formation in the retinopathy mice. Since vaso-proliferation after the ischemic phase depends not on VEGFR-1 (70), but on VEGFR-2 (66), VEGFR-1 downregulation on vascular endothelial cells is thought to have little or no effect on retinal neovascularization. In contrast, VEGFR-1 is well known to be expressed on inflammatory leukocytes including monocytes (69, 71, 72). The HRP-induced decrease in retinal VEGFR-1 seen in our study, therefore, is compatible with and explained at least in part by the suppression of VEGFR-1-bearing inflammatory leukocytes adherent to the retinal vasculature.

Our findings suggest that nonproteolytic activation of prorenin selectively promotes pathologic, but not physiologic, retinal neovascularization through the inflammatory processes related to pathologic neovascularization (10).

5. PERSPECTIVE

Hypertension is an important risk factor for the progression of diabetic retinopathy (73-75). Strict blood pressure control with an ACE inhibitor for hypertensive patients with diabetic retinopathy resulted in significant suppression of retinopathy progression (76), indicating a possible role of the circulatory RAS in the ocular pathogenesis. However, diabetic retinopathy is well known to progress in normotensive, as well as hypertensive, patients. Treatment with an ACE inhibitor for normotensive patients with diabetic retinopathy also resulted in significant suppression of progression to proliferative retinopathy (51), suggesting the contribution of the tissue RAS in the pathogenesis of retinal neovascularization. It is notable, however, that there are indeed a large number of normotensive patients with diabetic retinopathy who have the potential risk of hypotension caused by antihypertensive agents. Our recent reports showed that HRP administration to streptozotocin-induced diabetes inhibited the development of diabetic nephropathy via suppression of the tissue RAS in the kidney without affecting the circulatory RAS or systemic blood pressure (8, 19). Also in the eye, diabetic patients with proliferative retinopathy had high concentration of prorenin (13). Reasonably, nonproteolytic activation of prorenin is suggested to play an important role in the regulation of the tissue RAS in the eye with retinal inflammation and neovascularization. Targeting nonproteolytically activated prorenin may prove to be useful as a novel therapeutic strategy for vision-threatening proliferative retinopathies.

6. ACKNOWLEDGMENTS

This work was supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology (grant-in-aid for scientific research no. 18791296 to S.S.).

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Key Words: Renin-angiotensin system, Prorenin, (Pro)renin receptor, Ocular inflammation, Ocular neovascularization, Review

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