Retinal Neovascularization Is Prevented by Blockade of the Renin-Angiotensin System


Abstract—Both angiotensin II and vascular endothelial growth factor are angiogenic agents that have recently been implicated in the pathogenesis of proliferative diabetic retinopathy. In this study, retinal neovascularization was examined in a model of retinopathy of prematurity with the use of neonatal transgenic (mRen-2)27 rats, which overexpress renin in tissues, and Sprague-Dawley rats. Blockers of the renin-angiotensin system were administered during the neovascularization period. The ACE inhibitor lisinopril and the angiotensin type 1 receptor antagonist losartan both increased retinal renin levels and prevented inner retinal blood vessel growth. Quantitative in situ hybridization revealed that the expression of vascular endothelial growth factor and its type 2 receptor in the inner retina and proliferating blood vessels were increased in rats with retinopathy of prematurity. Lisinopril reduced both retinal vascular endothelial growth factor and its type 2 receptor mRNA in retinopathy of prematurity rats, whereas losartan had no effect. It is predicted that agents that interrupt the renin-angiotensin system may play an important role as retinoprotective agents in various forms of proliferative retinopathy. (Hypertension. 2000;36:1099-1104.)

Key Words: renin ■ growth substances ■ angiotensin ■ renin-angiotensin system

Angiogenesis is the major feature of several retinal pathologies including proliferative diabetic retinopathy and retinopathy of prematurity (ROP). In these conditions, retinal neovascularization has catastrophic effects on vision leading to hemorrhage, edema, and ultimately, blindness. The factors that stimulate retinal blood vessel growth have not been fully defined, but circumstantial evidence indicates that this involves not only angiogenic cytokines such as vascular endothelial growth factor (VEGF) but also vasoactive hormones such as angiotensin II (Ang II). Recently, in a multicenter trial, the EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus (EUCLID) study group reported that ACE inhibition (ACEI) was associated with a reduction in proliferative diabetic retinopathy, providing a potential clinical role for suppression of the renin-angiotensin system (RAS) in preventing and treating retinal neovascularization.

The hypothesis that an ocular RAS is involved in the development of proliferative diabetic retinopathy is supported by evidence that all components of the RAS are present in the retina and that Ang II, the effector molecule of this system, is angiogenic. We have localized renin to the macroglial Müller cell, making this the likely site for pathophysiological processes involving the retinal RAS. The Müller cell is also the site of synthesis of the potent angiogenic factor VEGF and its tyrosine kinase receptors. There is evidence of an association between VEGF, the RAS, and retinal neovascularization because both VEGF and prorenin increase in the vitreous of patients with proliferative diabetic retinopathy, and Ang II increases VEGFR-2 receptor mRNA in retinal endothelial cells.

This study sought to determine if the retinal renin-angiotensin and VEGF systems influence vessel growth in a rat model of ROP and whether RAS blockade can prevent retinal neovascularization. Comparisons were made with the transgenic (mRen-2)27 rat, which exhibits enhanced renin expression in many tissues including the retina.

Methods

Retinopathy of Prematurity (ROP) Model

All experimental procedures adhered to the guidelines of the Australian National Health and Medical Research Council. Newborn Sprague-Dawley (SD) and homozygous transgenic Ren-2 rats and their mothers were housed in an incubator regulated at 80±2% O₂ and <2% CO₂, with medical grade O₂ and industrial grade air. The gas composition of the chamber was monitored every 4 to 8 hours by an ML 205 gas analyzer and chart recorder (MacLab/2E System, AD Instruments Pty Ltd) while an air flow rate of ~2.5L/min guarded against increases in metabolically produced CO₂ and falls in O₂ tension. The rats were housed in the incubator for 11 days. Retinal neovascularization was induced by exposure to room air for 7 days.
after the time in oxygen. Adult homozygous Ren-2 rats are usually maintained on ACEI for blood pressure control. This drug was withdrawn from the hypertensive mothers 3 weeks before mating. All rats were allowed free access to water and standard rat chow (GR2, Clark-King & Co) and exposed to 12-hour light/dark cycles.

SD and Ren-2 rats (n=6 rats/group) were subjected to the following protocols: ROP shams (newborn pups housed in room air for 18 days), ROP (newborn pups housed in 80±2% O2 for 11 days and then in room air for 7 days), and ROP rats administered either the ACEI lisinopril (Zeneca Pharmaceuticals, 10 mg/kg body wt IP daily) or the angiotensin type 1 (AT1) receptor blocker losartan (Merck, 10 mg/kg body wt IP daily) from days 11 to 18. At day 18, rats were anesthetized for the collection of tissues (60 mg/kg body wt IP Nembutal, Bohringer Ingelheim).

**Retinopathy and Quantification of Blood Vessels**

Eyes were enucleated, fixed for 2 hours in Bouin’s fixative, dehydrated in alcohol, and then embedded in paraffin for sectioning at 90° to the optic nerve. Each eye was serially sectioned at 3 μm (~500 sections/eye), and every 10th section was stained with hematoxylin and eosin to examine retinal morphology. The number of blood vessel profiles (BVPs) in the inner retina were evaluated in a double-masked fashion in 3 randomly chosen sections. The inner retina comprised the inner limiting membrane (ILM), the ganglion cell layer (GCL), and the inner plexiform layer (IPL). A BVP was defined as an endothelial cell or a blood vessel with a lumen. Because it was unclear whether vessels in the vitreous originated from the inner retina, they were excluded from the BVP index. Each section was projected onto a screen by means of a closed-circuit camera. A stereological test grid measuring 150×150 mm and divided into 16-U areas was superimposed onto each image, and an automatic stage was used to advance across the entire retina by means of an unbiased counting frame. BVPs within ≥500-U areas were counted per eye.

**Renin Assay**

Both eyes from each rat were enucleated, and lenses and vitreous were removed. Active renin was measured in the left eye and total renin (trypsin activated) in the right eye by means of an enzyme kinetic method. Prorenin was derived as total minus active renin. Plasma was obtained by cardiac puncture from anesthetized rats before the rats were killed and assayed for active renin and prorenin.

**VEGF and VEGFR-2 Gene Expression**

Riboprobes were synthesized from cDNAs encoding mouse VEGF and VEGFR-2 (Dr S. Stacker, Ludwig Institute, Melbourne, Australia). The cDNAs were cloned into pGEM 4Z (Promega) and linearized with HindIII to produce antisense probes with SP6 RNA polymerase. Gene expression of VEGF and VEGFR-2 in retina were evaluated by 2 different techniques as previously described. Briefly, in the first method, densitometry was performed on autoradiographs of the entire retina (n=6 retina per group). In the second approach, dark field images were obtained from 3-μm paraffin sections of retina. The area of the inner retina occupied by autoradiographic grains was determined in 6 sections from each rat.

**Statistical Analysis**

Data were analyzed by ANOVA followed by a Fisher’s post hoc comparison, with a value of P<0.05 considered statistically significant. Because retinal renin levels were not normally distributed, these data were analyzed after logarithmic transformation.

**Results**

**Histology**

In shams, BVPs in the inner retina were observed in the ILM and IPL (Figure 1A), and none extended into the vitreous. By contrast, in ROP rats, all eyes exhibited BVPs protruding into the vitreous from the ILM (Figure 1B). Numerous BVPs were observed in the IPL that appeared tortuous and branched. Hemorrhage and inflammatory cells were present in the vitreous and inner retina of some eyes. In SD and Ren-2 ROP rats treated with either losinopril or losartan (Figure 1, C, D, G and H), the inner retina appeared similar to shams, although occasional vessels protruded into the vitreous of losinopril-treated ROP rats.

**Retinal Blood Vessel Profile Index**

There was no significant difference in the number of BVPs per unit area of inner retina between SD and Ren-2 ROP shams (Figure 2). In contrast, BVPs were increased in both
SD and Ren-2 ROP groups compared with shams. Furthermore, Ren-2 ROP rats had significantly more BVPs than did SD ROP rats. Lisinopril and losartan reduced the number of BVPs in both SD ROP and Ren-2 ROP rats to the level of ROP shams.

**Eye and Plasma Renin**

The results are presented in the Table. In ROP shams, total renin was ∼6 times higher in Ren-2 than in SD rats. ROP increased retinal total renin in both SD and Ren-2 rats and was further increased with lisinopril and losartan. In both rat strains, the rise in total renin with RAS blockade was mainly due to active renin, particularly in SD ROP rats. The plasma of Ren-2 ROP sham rats contained more total renin than SD ROP shams, but it was mainly prorenin. Plasma active renin in SD and Ren-2 shams was not different, and ROP did not alter their levels. ROP increased plasma prorenin moderately in Ren-2 ROP rats. With lisinopril and losartan treatment, plasma total renin increased in both SD ROP and Ren-2 ROP rats, with SD ROP containing mainly active renin and Ren-2 ROP similar amounts of active renin and prorenin.

**Quantitative In Situ Hybridization Microscopy**

In shams, VEGF (Figure 3, A and B) and VEGFR-2 (not shown) expression were detected in the ILM, GCL, inner nuclear layer (INL), and retinal pigment epithelium. With ROP, the intensity of both VEGF (Figure 3, C and D) and VEGFR-2 (not shown) mRNA were increased, and expression was also observed on vessels protruding into the vitreous. In ROP rats treated with lisinopril, labeling for VEGF (Figure 3, E and F) and VEGFR-2 (not shown) was reduced to almost undetectable levels. Losartan did not alter the intensity or distribution of VEGF (Figure 3, G and H) or VEGFR-2 (not shown) mRNA in ROP compared with untreated ROP rats. Quantification of optical density in autoradiographs of VEGF (Figure 4A) and VEGFR-2 (Figure 4B) and cellular expression of VEGF (Figure 4C and VEGFR-2 (Figure 4D) in dark-field micrographs confirmed the qualitative assessment of probe distribution. No differences in the distribution of VEGF and VEGFR-2 expression were observed between rat strains.

**Discussion**

This study implicates the local ocular RAS in the pathogenesis of retinal neovascularization in ROP, as evidenced by increased retinal renin levels and prevention of vascular growth by both ACEI and AT1 receptor blockade. In addition, in the Ren-2 rat, which phenotypically exhibits enhanced retinal active renin levels, the vessel profiles were increased more than for SD rats with induction of the hypoxia-mediated retinal injury. Of particular significance was the ability of the RAS blockers to suppress retinal neovascularization in both Ren-2 and normal SD rats. Together, these findings suggest that endogenous retinal Ang II is involved in the retinal neovascularization process.

Identification of RAS components including angiotensinogen, ACE, and renin expression within the eyes of humans, rats, and other species has provided evidence for a local RAS.3–7 The detection of retinal Ang II receptors by

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**Eye (Excluding Vitreous and Lens) and Plasma Renin Levels of Sprague-Dawley and Transgenic (mRen-2)27 Rats After Retinopathy of Prematurity and Sham Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Eye Renin, µGU/eye</th>
<th>Plasma Renin, mGU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Renin</td>
<td>Active Renin</td>
</tr>
<tr>
<td>SD ROP sham</td>
<td>5.7±0.6*</td>
<td>4.5±0.7*</td>
</tr>
<tr>
<td>SD ROP</td>
<td>16.6±5.1†</td>
<td>10.9±3.0†</td>
</tr>
<tr>
<td>SD ROP+LIS</td>
<td>750.3±133.6‡</td>
<td>750.3±133.6‡</td>
</tr>
<tr>
<td>SD ROP+LOS</td>
<td>1163.8±325.0§</td>
<td>1163.8±325.0§</td>
</tr>
<tr>
<td>Ren-2 ROP sham</td>
<td>47.1±2.6</td>
<td>36.2±1.2</td>
</tr>
<tr>
<td>Ren-2 ROP</td>
<td>220.6±55.1†</td>
<td>78.0±20.0†</td>
</tr>
<tr>
<td>Ren-2 ROP+LIS</td>
<td>990.7±123.1</td>
<td>569.6±65.5</td>
</tr>
<tr>
<td>Ren-2 ROP+LOS</td>
<td>2327.1±330.6</td>
<td>1519.0±392.2§</td>
</tr>
</tbody>
</table>

SD indicates Sprague-Dawley; Ren-2, transgenic (mRen-2)27; LIS, lisinopril; and LOS, losartan.

Renin values are presented as mean±SEM; n=6 retina/group. *P<0.05 vs Ren-2 ROP sham. †P<0.01 vs respective ROP sham. ‡P<0.01 vs SD ROP. §P<0.001 vs respective ROP. ||P<0.05 vs Ren-2 ROP. \\P<0.01 vs SD ROP+lisinopril and SD ROP+losartan. **P<0.05 vs respective ROP.
levels in bovine eyes are higher than in plasma. Previously, the presence of a functioning intraretinal RAS. Local production of Ang II is also suggested by the finding that retinal Ang II levels in bovine eyes are higher than in plasma.4 Previously, we reported that renin protein exists in normal rodent and human retina mainly in the form of active renin, with localization to the Müller cell.3 The present study provides evidence that after ROP and RAS blockade, retinal renin could indicate that in the developing eye, renin may be stored and not important for the induction of normal vasculogenesis. Only after ROP in both Ren-2 and SD rats were BVPs increased and accompanied by a substantial rise in ocular active renin. The larger rise in BVPs in Ren-2 ROP rats compared with SD ROP rats may be explained by the release of increased amounts of stored active renin forming Ang II after the hypoxic stimulus. Whether circulating Ang II itself might be elevated and contributes to the retinal neovascularization process associated with ROP remains to be investigated, but this would seem unlikely on the basis of the observation for plasma active renin discussed above. With respect to a role for blood pressure in the stimulation of retinal blood vessel growth in the Ren-2 ROP rat, no association between hypertension and retinal neovascularization has been reported in humans19 or the spontaneously hypertensive rat.20

Many reports have indicated that ACEI retards the cellular and fibrointerstitial damage in conditions such as diabetic nephropathy.13 Similar studies in the eye have been limited, although the recent report by the EUCLID study group2 suggests that ACEI with lisinopril slows the progression of proliferative diabetic retinopathy in patients. Our present findings, together with previous observations on the retinal RAS3 and the demonstration of angiogenic properties of Ang II, provide strong support at the cellular level for anticipating a beneficial action from RAS blockade in retinal conditions displaying neovascularization.

VEGF, a potent vessel permeability and angiogenic factor, is implicated in blood vessel growth.21 VEGF binds to high-affinity tyrosine kinase receptors VEGFR-1 and VEGFR-2, also known as flt-1 and flk-1, respectively.22 These two receptors have different functions, with VEGFR-2 viewed to induce alterations in cell morphology, actin reorganization, chemotaxis, and mitogenesis, whereas VEGFR-1 lacks such effects.22 VEGF expression is induced by hypoxia and is considered to be the stimulus in neovascularizing eye pathologies such as proliferative diabetic retinopathy and ROP.10,21,23 To evaluate the associated roles of the renin-angiotensin and VEGF systems in retinal neovascularization, we chose a rodent model of ROP. It is well established that exposure of neonatal animals to a high oxygen environment results in cessation of vessel growth in the inner retina, whereas subsequent exposure to room air is presumed to cause tissue hypoxia, which leads to upregulation of retinal VEGF and neovascularization.20 Using quantitative in situ hybridization, we found that VEGF and VEGFR-2 mRNA are increased with ROP in the inner retina and in proliferating retinal vessels of both SD and Ren-2 rats. This is consistent with previous findings in ROP models21,23 and has been observed in the eyes of patients with proliferative diabetic

**Figure 3.** Paired bright and dark-field photomicrographs showing VEGF gene expression in 3-μm paraffin sections of SD rat retina. RPE indicates retinal pigment epithelium; v, vitreous. Counterstain is hematoxylin and eosin; magnification ×150. Scale bar is ~67 μm. VEGF expression is detected throughout inner retina in ROP shams (A and B). With ROP (C and D), VEGF expression is increased and is evident on retinal vessels (arrow). In ROP rats treated with lisinopril (E and F), hybridization signal was not detected; in ROP rats treated with losartan (G and H), signal was intense throughout inner retina.
retinopathy. The appropriateness of this model to other forms of retinal neovascularization, such as occurs in diabetes, requires further evaluation. There is increased VEGF and VEGFR-2 expression in experimental diabetic retinopathy, and our previous findings suggest a decrease in retinal VEGF mRNA with ACEI in rodent diabetes.

The sequence and interaction of growth factor actions in retinal angiogenesis has yet to be fully explored; however, the ability of Ang II to increase VEGF mRNA in human renal glomerular mesangial cells and the finding that losartan inhibits this process suggest that an increase in retinal Ang II with tissue hypoxia may be the initiating event in the rat ROP model. To our knowledge, there have been no previous studies evaluating this concept in the eye. Otani and colleagues have reported that Ang II enhances VEGF mRNA and VEGF-induced cell growth in cultured bovine retinal endothelial cells. In the present study, retinal neovascularization was associated with a rise in retinal renin, VEGF, and VEGFR-2 mRNA. These findings, together with the observation that ACEI prevented neovascularization and virtually abolished VEGF and VEGFR-2 mRNA, suggest that Ang II may potentiate or initiate VEGF-induced neovascularization. Unlike Otani et al and in contrast to our findings with ACEI, AT1 receptor blockade, although preventing retinal neovascularization in ROP, failed to suppress expression of VEGF and VEGFR-2. This suggests that although Ang II appears to act as the primary effector molecule in retinal neovascularization in ROP, VEGF-2 expression may
not be primarily influenced by the AT1 receptor but may be
mediated by other pathways such as the angiotensin type 2
receptor or cytokines. It may also be possible that VEGF is
not essential for neovascularization in the ROP model.
Indeed, 98% inhibition of VEGF with soluble VEGF receptor
chimeric proteins in a murine model of ischemic retinopathy
resulted in only a partial reduction (56%) in retinal neo-
vascularization.27 However, a pivotal role for VEGF in the
growth factor pathway leading to ROP should not be under-
estimated; a recent study reported that inhibition of VEGF
receptor kinase activity blocked retinal neovascularization in
the ROP model.28

Our findings imply that the retinal RAS plays an important
role in the pathogenesis of neovascularization in the ROP
model. This information provides a rationale for the use of
agents that interrupt the RAS in the prevention of prolifera-
tive retinopathy related to retinal hypoxia and possibly
ischemia.

Acknowledgments

This study was supported by Merck Research Laboratories, the
Diabetes Australia Research Trust, the National Health and Medical
Research Council of Australia, and Juvenile Diabetes Foundation
International. Dr Richard Gilbert is the recipient of a Career
Development Award from the Juvenile Diabetes Foundation Inter-
national. The authors thank Alison Cox for technical assistance.

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Hypertension. 2000;36:1099-1104
doi: 10.1161/01.HYP.36.6.1099

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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