Purposed. To determine the location and activity of renin-angiotensin system (RAS) components in the developing rat retina and whether the RAS influences retinal vascularization.

Methods. Transgenic Ren-2 rats, which overexpress the RAS, and Sprague-Dawley (SD) rats were studied at postnatal day (P)1, P7, P14, P21, and P90. Immunohistochemistry was performed for angiotensinogen, prorenin, angiotensin II (Ang II), and the angiotensin type 1 (AT1) and 2 (AT2) receptors. Retinal active renin and prorenin were measured by radioimmunoassay, and the density of angiotensin-converting enzyme (ACE) by autoradiography. At P1 to P7, Ren-2 and SD rats were administered either the ACE inhibitor lisinopril (10 mg/kg per day, intraperitoneally [IP]) or the AT1 receptor antagonist losartan (10 mg/kg per day, IP), and vessel length and density were measured.

Results. At all time points, RAS components were localized to blood vessels and cells in the ganglion cell layer. At P1, Ang II and both the AT1 and AT2 receptors were on hyaloid vessels. ACE binding increased in intensity from P1 to P90. Retinal renin was mainly activated and was 5- to 15-fold higher in Ren-2 than in SD rats. In Ren-2 rats, the growing vasculature extended farther into the retinal periphery than in SD rats and was unchanged with either lisinopril or losartan. Vascular density was increased in the periphery of Ren-2 rats compared with SD rats and was reduced with lisinopril but not with losartan.

Conclusions. In the developing rat retina, a complete RAS is mainly found in blood vessels and cells in the ganglion cell layer, where it may influence the early stages of vascularization. (Invest Ophthalmol Vis Sci. 2005;46:1069–1077) DOI:10.1167/iovs.04-0885

Recognized as a major systemic endocrine system, the renin-angiotensin system (RAS) is involved in the regulation of blood pressure and electrolyte homeostasis, with circulating renin originating primarily from the juxtaglomerular cells of the kidney. Active renin, derived from the inactive precursor prorenin, cleaves the inactive substrate angiotensinogen to angiotensin I (Ang I). This is subsequently cleaved by angiotensin-converting enzyme (ACE) to form the effector molecule of the system, angiotensin II (Ang II). Recently, a substantial amount of work has provided evidence for local RAS in a variety of tissues, including the reproductive tract, adrenal glands, and thymus. Evidence of an ocular RAS is also considerable, with findings that all components of the system are not only localized but also are expressed in adult eye tissues of humans, rats, and other mammals.6–10 These data, taken together with the findings that Ang-I and -II levels in the eye are higher than in plasma,10,11 indicate that there is local production of Ang II in the eye.

Ang II exerts its actions primarily through two receptor subtypes: the Ang II type 1 (AT1) and 2 (AT2) receptors. Virtually all the biological actions of Ang II are mediated through the AT1 receptor, and including blood pressure regulation, cell growth, angiogenesis, and growth factor induction.12,13 In retinal endothelial cells, Ang II stimulates proliferation via the AT1 receptor, which involves upregulation of the potent angiogenic, vascular permeability, and endothelial cell survival factor vascular endothelial growth factor (VEGF).12,14,15 The functional role of the AT2 receptor is not fully understood, and there is evidence that it may oppose the actions of the AT1 receptor.15 In addition, the AT2 receptor has been reported to have pro-, anti-, or no angiogenic effects.15,16–18 High expression of this receptor subtype in fetal and neonatal tissue, with relatively low or absent levels in adult tissues19 has led to the hypothesis that the AT2 receptor may be involved in the regulation of cell growth and differentiation in developing organs.15

The presence of the constituents of the RAS in the eye implies a physiological function of the system. Indeed, it is thought that Ang II contributes to the regulation of the ophthalmic circulation20,21 and to the control of aqueous humor dynamics and intraocular pressure.22 The localization of Ang II within various neuronal cell types in the retina6,10,23 has also led to the hypothesis that Ang II acts as a neuromodulator within the eye, and electrophysiological studies have suggested a functional role for the RAS in the visual system.24–26 Furthermore, the potent angiogenic- and growth factor-inducing properties of Ang II12,27–30 have implicated this molecule in the pathogenesis of ocular angiogenesis in experimental diabetes34 and in models of oxygen-induced retinopathy.32–34

The developing retina is characterized by glial migration and subsequent vascularization.75–77 Currently, no studies have been undertaken to examine the contribution of the RAS to angiogenesis in the developing retina. Because the standard laboratory rat displays relatively low tissue renin and angiotensin, we chose to study the transgenic m(Ren-2)27 rat (Ren-2).38 Derived from the insertion of the murine Ren-2 gene into the genome of the Sprague-Dawley (SD) rat, the Ren-2 rat displays elevated renin and Ang II in tissues except the kidney, high plasma prorenin similar to the human phenotype, and fulminating hypertension. The Ren-2 rat has facilitated the study of the extrarenal RAS in various tissues, including the retina.1,3,5,31–39 Our first objective was to evaluate the location of RAS components in the developing retina of Ren-2 and SD rats and to make comparisons to the mature eye. Second, we sought to determine whether the RAS influences the early stages of vascularization in the immature retina. This was assessed in neonatal Ren-2 and SD rats after blockade of the RAS with the ACE inhibitor lisinopril and the AT1 receptor antagonist losartan.
METHODS

Animals

Study 1: Characterization of the RAS in the Developing Rat Retina. Transgenic Ren-2 rats homozygous for the Ren-2 gene and SD rats were studied on P1, P7, P14, P21, and P90. Rats were housed in the Biological Research Facility of the University of Melbourne in a 12-hour light–dark cycle, with temperature of 21°C and free access to standard rat chow (GR2: Clark-king and Co., Gladesville, New South Wales, Australia).

Study 2: Developmental Retinal Angiogenesis and RAS Blockade. At P1, homozygous Ren-2 rat and SD pups were randomized to the following groups: untreated control (vehicle), the ACE inhibitor lisinopril (10 mg/kg per day) and the AT$_1$ receptor antagonist losartan (10 mg/kg per day). All agents were administered at the same time of day by intraperitoneal injection with sterile saline (pH 7.4), as the vehicle. The injection volume was 100 μL. Agents were administered from postnatal day (P1) to P7. The doses of lisinopril and losartan were based on previous studies in oxygen-induced retinopathy, where they reduced retinal angiogenesis.32

In both studies 1 and 2, homozygous Ren-2 mothers were withdrawn from maintenance antihypertension therapy (lisinopril, 10 mg/kg in drinking water) 3 weeks before mating and continued without antihypertensive therapy during pregnancy and the weaning period. Mothers were allowed drinking water ad libitum. All investigations adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the guidelines of the University of Melbourne’s Animal Experimentation Ethics Committee.

Study 1: Characterization of the RAS in the Developing Rat Retina

Immunohistochemistry. On P1, P7, P14, P21, and P90, rats were killed by anesthetic overdose (Nembutal, 60 mg/kg body weight, intraperitoneally [IP]; Boehringer-Ingelheim, North Ryde, NSW, Australia). Eyes were fixed in Bouin’s fixative (Pathtech Diagnostics Pty. Ltd., Melbourne, Victoria, Australia) for approximately 24 hours and then immersion in 20% hydrogen peroxide in methanol for 10 minutes and another 5 minutes each was followed by incubation with a solution of 20% hydrogen peroxide in methanol for 10 minutes and another rinse with PBS (1 × 5 minutes). The sections were then incubated for 30 minutes with biotinylated swine-antimouse-rabbit linker antibody (Dako Corp., Carpinteria, CA) diluted 1:90 in 0.1 M PBS for 1 hour, and then rinsed with 0.1 M PBS (1 × 5 minutes). Sections were then incubated for 45 minutes with avidin-biotin peroxidase complex (equal volumes of each component diluted 1:200 in 0.1 M PBS; Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and liquid diaminobenzidine substrate-chromogen solution (Dako Corp.) was used as a chromogen for immunoperoxidase staining. The sections were then rinsed in tap water, stained in Mayer’s hematoxylin, differentiated in Scott’s tap water, dehydrated in alcohol, cleared (Histolene; Fronee Pty. Ltd., Riverstone, NSW, Australia) and mounted (DPX medium; BDH Laboratories, Poole, UK). Six to eight randomly chosen sections per eye and six to eight eyes from each animal group were used.

The primary antibodies were angiotensinogen, prorenin, Ang II, and the AT$_1$ and AT$_2$ receptors. The polyclonal rabbit anti-renin antibody was diluted 1:1000 with 0.1 M PBS. The angiotensinogen antibody was a gift from Conrad Sernia (Department of Physiology and Pharmacology, University of Queensland, Australia). The monoclonal rabbit anti-mouse prorenin antibody is targeted at 13 amino acids in the prosequence (PSVREELERGV). The prorenin antibody was used at dilutions of 1:750 to 1:1500 in 0.1 M PBS and was a gift from Geoff Tregear (Howard Florey Institute, Melbourne, Australia). The polyclonal guinea pig anti-human Ang II antibody (Peninsula Laboratories Inc., Belmont, MA), was used at dilutions of 1:750 to 1:1500 in 0.1 M PBS. The polyclonal antibody to the AT$_1$ receptor (sc 1175) and to the AT$_2$ receptor (sc 9040; Santa Cruz Biotechnology, Santa Cruz, CA), were diluted 1:200 in 0.1 M PBS. Confirmation of specific antibody labeling was achieved in sections of rat liver (angiotensinogen) and kidney and ovary (prorenin, Ang II and AT$_1$, and AT$_2$ receptors).

ACE Autoradiography. A separate group of Ren-2 and SD rats were killed by anesthetic overdose at P1, P7, P14, P21, and P90. Encuclated eyes were embedded in optimal cutting temperature compound (Cryomatrix; Sakura Finetechnical Co., Ltd., Tokyo, Japan), immersed in isopentane, snap frozen in liquid nitrogen and stored at −80°C. Sections were cut at 20 μm on a cryostat at −20°C, thaw mounted onto glass slides precoated with 3-amino-propyltriethoxysilane, dehydrated under reduced pressure at 4°C overnight, and stored at −80°C. A tyrosol derivative of the ACE inhibitor lisinopril, 351A was radioiodinated with $^{125}$I, using chloramine T and purified by Sephadex C25 chromatography. The binding properties and use of this radioligand have been extensively described.41 Five randomly chosen sections per eye and six to eight eyes from each animal group were used. Brain and kidney sections were used as controls, as ACE has been localized in these tissues. Sections were brought to room temperature and preincubated for 15 minutes in sodium phosphate buffer (150 mM NaCl, 10 mM Na$_2$HPO$_4$) containing 0.2% bovine serum albumin (BSA, pH 7.4). They were then transferred to fresh buffer containing 0.3 Ci/mL of $^{[125}$I]351A for 1 hour at room temperature. For the determination of nonspecific binding, parallel incubations were performed in the presence of 1 nM EDTA (BDH Laboratory Supplies). After incubation, the sections were transferred through four successive 1-minute washes in ice-cold fresh buffer (excluding BSA) and dried under a stream of cold air. Dry slides were placed in cassettes, including a set of radioactivity standards, and exposed to x-ray film (Agfa-Scopix CR3; Agfa-Geaera, Melbourne, Australia) for 3 days, and the films were then developed (Department of Radiology, Austin Hospital, Heidelberg, Victoria, Australia).

Autoradiographs for radioligand binding were quantitated by computerized image analysis (Analytical Imaging Station; Imaging Research, Ontario, Canada). Developed films were placed on a uniformly illuminating fluorescent light box and images captured with a digital camera (Fujix HC-2000; Fuji, Tokyo, Japan). Radioactive standards were fitted to calibration curves, and the optical density of each pixel of digitized image converted into disintegration per minute of 1$^{25}$I per square millimeter of tissue. Nonspecific binding was negligible. Sections of rat kidney and brain served as the positive control.

Enzyme Kinetic Renin Assay. A separate group of Ren-2 and SD rats were killed by anesthetic overdose at P1, P7, P14, P21, and P90. Total renin (prorenin plus active renin) and active renin were estimated in eyes by an established technique.3,5,31-52 Prorenin is derived as total renin minus prorenin. Rats were anesthetized (60 mg/kg body weight IP; Nembutal; Boehringer-Ingelheim), eyes enucleated, and the lenses and vitreous removed. To estimate total renin, right eyes were snap frozen in 0.1 M PBS at pH 7.4. To estimate active renin, left eyes were snap frozen in a 30% protease inhibitor solution containing 25 mM N-ethyl maleimide, 20 mM EDTA, and 100 mM benzamidine. All samples were stored at −20°C and later thawed, homogenized, and refrozen twice before assay by an enzyme kinetic method with hog renin (National Standards Laboratory, London, UK) as the reference.
standard and 24-hour nephrectomized rat plasma as the angiotensino-
gen substrate. Duplicate samples were incubated for 1 hour at 37°C
(pH 7.4), and the renin present in the samples was estimated by
immunoassay of generated Ang I referenced against the amount of Ang
I generated by 2 × 10^−6 Goldblatt units (GU) of hog renin. Total
renin was assayed by using tissue incubated without inhibitors after
activation of prorenin by trypsin treatment (2 mg/ml for 10 minutes
on ice).

**Study 2: Developmental Retinal Angiogenesis and RAS Blockade**

**Fluorescence Histochemistry on Retinal Wholemounts.** At P7, Ren-2 and SD rats were killed by anesthetic overdose and retinas dissected and fixed for 2 hours in 4% paraformaldehyde (BDH Laboratory Supplies) diluted in 0.1 M PBS. The retinas were then permeabilized overnight in 0.1 M PBS containing 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 5% NGS. These were subsequently incubated with the endothelial cell marker FITC Griffonia (Ban-
deiraea) simplicifolia lectin B4 (Vector Laboratories, Burlingame, CA),
diluted 1:80 in 0.1 M PBS containing 0.5% Triton X-100 (PBS/Triton
X-100 solution), for 6 hours at room temperature. Retinas were washed
for 15 minutes with PBS/Triton X-100 solution and stored overnight on
a circular shaker at 4°C in clean PBS/Triton X-100 solution. Retinas were subsequently washed again (four times for 15 minutes each)
with PBS/Triton X-100 solution at room temperature and then incu-
bated for 3 hours at room temperature with FITC-streptavidin (Dako
Corp.) diluted 1:100 in PBS/Triton X-100 solution. This process was
followed by six 15-minute washes with PBS/Triton X-100. Retinas
were then flat mounted on clean glass slides with fluorescent
mounting medium (Dako Corp.) and stored flat in the dark at 4°C for
1 week.

**Quantitation of Vessel Density.** Low-power images of each
quadrant of the flatmounted retinas, encompassing the area from the
optic disc to the peripheral edge of the section, were captured with
a digital camera (Spot; SciTech Pty. Ltd., Presto, Victoria, Australia)
attached to a reflected fluorescence microscope (BX52; Olympus,
Tokyo, Japan) with a narrow-band excitation filter for detection of
FITC fluorescence. Retinal vascular density was evaluated with appro-
ciately calibrated computerized image analysis (Analytical Imaging
Station; Imaging Research), a technique based on methodology previ-
ously described.42 Vascular density in each region (central, middle,
and peripheral retina) was determined by measuring the proportional area
of fluorescein labeled vessels in two 200 × 200-μm sampling fields.
Sampling fields were chosen to ensure they were not bisected by a
major artery or vein. From the optic disc, the central retina was defined
as a distance of up to 1.25 mm, the middle retina between 1.25 and 2.5
mm, and the peripheral retina beyond 2.5 mm. Six to eight retinas
were analyzed per animal group.

**Quantitation of Vessel Length.** Low-power images used for the
quantitation of retinal vessel density were also used in the quanti-
tation of vessel length, which was evaluated with appropriately cali-
ibrated computerized image analysis (Analytical Imaging Station; Im-
ing Research), based on methodology previously described.43 Briefly,
vessel length was measured from the optic disc to the edge of the
vascular front in all four retinal quadrants, and an average value was
taken for each retina. Six to eight retinas were analyzed per animal
group.

**Statistical Analysis**

Statistical analysis was performed using one-way ANOVA with post hoc
analysis by the Student-Newman Keuls test for multiple comparison of
individual mean values. Data for the Ang II receptor autoradiography
were log transformed.

**Results**

**Study 1: Characterization of the RAS in the Developing Rat Retina**

**Body Weights.** The mean ± SEM body weights of Ren-2 and SD rats were similar during development and in adulthood. Body weights were recorded at P1 (Ren-2, 7.5 ± 0.6 g; SD, 7.5 ± 0.9 g), P7 (Ren-2, 14.0 ± 1.5 g; SD, 16.5 ± 1.9 g), and P90 (Ren-2, 311.3 ± 7.2 g; SD, 321.8 ± 11.9 g).

**Angiotensinogen, Prorenin, Ang II, and AT1 and AT2 Receptor Localization.** Angiotensinogen protein labeling was localized to the cytoplasm of cells in the ganglion cell layer (GCL) and to blood vessels in the region of the GCL and inner limiting membrane (ILM) at all time points in both Ren-2 (Fig.
1) and SD rats (Fig. 1). Only in Ren-2 rats was angiotensinogen also localized to the somas of cells in the middle of the inner nuclear layer (INL) at P14, P21, and P90 (Figs. 1C–E).

Prorenin protein was detected at all time points in the cytoplasm of cells of the GCL and in blood vessels in the GCL and ILM of both rat strains (Fig. 2). From P14, weak labeling for prorenin was also detected in the outer plexiform layer (OPL) and the region of the outer limiting membrane (OLM; Figs. 2C–E).

Ang II immunolabeling was detected in cells and blood vessels of the GCL and ILM. The intensity and distribution of Ang II immunolabeling was similar in both rat strains at all time
points studied (Fig. 3). At P1, Ang II labeling was also detected in hyaloid vessels in the vitreous cavity of both Ren-2 and SD rats.

Specific immunolabeling for the AT1 and AT2 receptors was observed in blood vessels in the GCL and ILM and in the cytoplasm of cells in the GCL (Fig. 4). At P1, immunolabeling for the AT1 and AT2 receptors was also present in the hyaloid vessels in the vitreous cavity of both rat strains (Figs. 4C, 4C).

**ACE Radioligand Binding.** ACE binding in Ren-2 and SD rat eyes was relatively low at P1, and the intensity of binding progressively increased with time (Fig. 5). This increase was in parallel with the relative optical density of ACE in the retinas of both rat strains (Fig. 5). ACE binding from P7 and P90 was higher in SD rats than in Ren-2 rats.

**Retinal Renin Levels.** Overall, Ren-2 rats displayed 5- to 10-fold higher renin levels in retinas than did SD rats (Fig. 6). In Ren-2 rats, similar amounts of prorenin and active renin were detected at P1, P7, and P14; however, active renin was ele-

vated at P21 and P90. Prorenin levels did not alter across time in the Ren-2 rat. In SD rat retinas, active renin predominated and increased without further change after P1. In SD rat reti-

nas, prorenin levels were generally very low and below the detection limit of the assay at P7, P21, and P90. These findings are consistent with previous studies in SD rat tissues.1,5

**Study 2: Developmental Retinal Angiogenesis and RAS Blockade**

**Distance of the Retinal Vasculature from the Optic Disc in the Immature Retina.** In SD rats at P7, the distance of the growing edge of the retinal vasculature from the optic disc was similar in all groups (Figs. 7, 8). In control transgenic Ren-2 rats at P7, the growing edge of the vasculature extended farther into the retinal periphery than in control SD rats, and this was unchanged with either lisinopril or losartan (Figs. 7, 8).

**Vascular Density in the Immature Retina.** At P7, in control Ren-2 rats, the density of isolectin-labeled blood vessels was similar in the central, middle, and peripheral retina (Fig.
9A). Lisinopril reduced vascular density in the peripheral retina of Ren-2 rats, and there was no effect of losartan in any region of the retina (Fig. 9A). At P7, in control SD rats, vascular density in the peripheral retina was reduced compared with that in the
central and mid regions of control SD rat retinas and compared with all regions of the retina in Ren-2 rats (Fig. 9B). In SD rats, neither lisinopril nor losartan had any effect on vascular density in any region of the retina (Fig. 9B).

**DISCUSSION**

The present study is the first to demonstrate the presence of all components of the RAS in the developing rat retina. From as early as P1, angiotensinogen, prorenin, Ang II, and the AT₁ and AT₂ receptors were largely confined to the inner retina in both the vasculature and cells in the GCL. Ang II is a known proangiogenic growth factor that promotes the growth of retinal endothelial cells by a VEGF-dependent mechanism. Support for its role in pathologic retinal angiogenesis comes from studies in our laboratory and other investigations showing that blockade of the RAS attenuates new blood vessel growth in retinopathy of prematurity and endothelial cell proliferation in diabetic rats. Indeed, such studies have shown that blockade of the RAS reduces overexpression of the retinal

**FIGURE 1.** Angiotensinogen protein immunolabeling in 3-µm paraffin-embedded sections of transgenic Ren-2 and SD rat retinas counterstained with Mayer’s hematoxylin. Shown are Ren-2 rat retinas at P1 (A), P7 (B), P14 (C), P21 (D), and P90 (E) and SD rat retinas (F–J) at the same time points, respectively. In both Ren-2 and SD rats, labeling (brown) for angiotensinogen was present in blood vessels (double arrows) in the ILM and GCL and in cells of the GCL (single arrow) at all time points. In addition, at P14, P21, and P90 in Ren-2 rats, labeling was observed in the somas of the cells in the middle of the differentiated INL (arrowhead). Negative controls (K) were sections of rat liver incubated with NGS instead of primary antiserum and showed no positive labeling. Positive controls (L) were sections of rat liver that showed specific angiotensinogen labeling in hepatocytes (arrowhead). Magnification, ×150. Scale bar, 50 µm.

**FIGURE 2.** Prorenin protein immunolabeling in 3-µm paraffin-embedded sections of transgenic Ren-2 and SD rat retinas counterstained with Mayer’s hematoxylin. Shown are Ren-2 rat retinas at P1 (A), P7 (B), P14 (C), P21 (D), and P90 (E) and SD rat retinas (F–J) at the same time points, respectively. In both rat strains and at all time points, prorenin immunolabeling was observed in blood vessels of the ILM (double arrows) and in cells of the GCL (single arrow). From P14 to P90, prorenin was also detected in the OPL (**) and in the OLM (**). Magnification, ×250. Scale bar, 50 µm.
The present study provides new evidence that the RAS has a similar role in the early stages of developmental angiogenesis in the Ren-2 rat retina. We have reported that adult transgenic Ren-2 rats that overexpress the RAS in extrarenal tissues have higher levels of ocular active renin than do adult SD rats. We now report a similar finding in the developing retina of transgenic Ren-2 rats. This increased level of renin was accompanied by more extensive development of the peripheral retinal vasculature at P7 than in age-matched SD rats. Further support for an angiogenic role for angiotensin is provided by the reduction in vascular density in the immature peripheral retina of Ren-2 rats after ACE inhibition. Of interest is that there was no effect on either vascular length or density with AT1 receptor blockade in either rat strain. This may be due to the higher density of AT1 receptors compared with AT2 receptors in the early stages of retinal development, and the suggested role of the AT2 receptor in organogenesis.

Gene expression studies have identified that a tissue-based RAS exists in the adult eye. Renin, angiotensinogen, and ACE mRNA have been detected in retinal pigment epithelium/choroid and whole neural retina samples from human eyes. In situ hybridization and immunohistochemical studies have identified the retinal cell types in which RAS components are located. In the inner retina of the adult rat, mouse, rabbits, and humans, the RAS is found in blood vessels, neurons, and...
In the present study, RAS components were located in similar sites from as early as P1 and persisted through retinal development and into adulthood. The finding of RAS components in blood vessels of the immature rat retina and Ang II and AT1 and AT2 receptor protein in hyaloid vessels (a transiently existing network of capillaries that nourish the developing lens and retina) suggests a role for the RAS in vascularization of the developing retina. With regard to ACE, previous studies have reported immunoreactivity in retinal vessels and in blood vessels in other organs. In the present study, autoradiographic analysis showed retinal ACE to increase with retinal matura
tion, with highest levels in the adult at P90. In general, Ren-2 rats had slightly lower levels of retinal ACE than did SD rats. This was most notable at P7 and P90. The reason for this strain difference is probably the overexpression of the retinal RAS in Ren-2 rats, which has the effect of suppressing tissue ACE levels. Our previous studies support this finding, as adult Ren-2 rats have reduced levels of plasma ACE compared with SD rats. It should be noted that in several species, ACE increases over the perinatal period in lung, kidneys, heart, and aorta. The reason for the increase in ACE with tissue development is not precisely known, but could relate to the extent of vascularization. For instance, it has been suggested that the postnatal increase in pulmonary ACE expression in lambs may partly reflect the increase in capillary surface area associated with growth.

In the present study, RAS components were localized to cells in the GCL, which could represent ganglion and displaced amacrine cells. RAS components also exist in macroglial Müller cells. Together, these findings suggest that the angiotensin produced in these cells exerts a paracrine effect on the neighboring vasculature. Indeed, neurons and glia in the retina are structurally aligned with the retinal vasculature, and contribute to the epiretinal membranes that form in diabetic retinopathy. Alternatively, the retinal RAS may influence neuronal activity (reviewed in Ref. 54). Of interest is that blockade of RAS modulates the a- and b-waves of the electroretinogram. ACE may also have a role in neuronal development in the retina. In chicken retina that lack blood vessels, ACE activity and gene expression increase in the developing embryo retina and peak transiently postpartum. This developmental up-regulation of ACE coincides with the period of synaptogenesis in the chicken retina, leading to the suggestion that ACE may be involved in the fine tuning of the neuronal retinal network and/or the metabolism of neuropeptides, such as substance P.

**Figure 5.** Representative computer-generated color autoradiographs of specific ACE radioligand binding in transgenic Ren-2 and SD rat eyes. Shown is high (red), moderate (yellow and green) and low (blue) ACE binding in Ren-2 rat retinas at P1 (A), P7 (B), P14 (C), P21 (D), and P90 (E) and in SD rat retinas (F-J) at the same time points, respectively. ACE binding was relatively low in eyes from P1 (A, F) and began to increase at P7 (B, G), with progressively higher binding at P14 (C, H) and P21 (D, I), peaking in the mature P90 (E, J) eye. Positive controls are sections of kidney (K) and brain (L). Scale bar, 1000 μm. Quantitation of ACE radioligand binding in developing Ren-2 and SD rat eyes is shown graphically. Data are the mean ± SEM of results in six to eight eyes per group. # SD rat; # Ren-2 rat. *P < 0.05 compared with all SD groups; #P < 0.05 between Ren-2 and SD at a given time point; †P < 0.05 compared with all other time points for the Ren-2 rat; and ‡P < 0.05 compared with all Ren-2 groups.

**Figure 6.** Active renin (A) and prorenin (B) levels in retinas of transgenic Ren-2 and SD rats. Data are the mean ± SEM of results in 8 to 10 eyes. # SD rat; # Ren-2 rat. *P < 0.01 comparing active renin between Ren-2 and SD rats; #P < 0.05 comparing active renin with that in Ren-2 rats at P1, P7, and P14; †P < 0.01 comparing prorenin between Ren-2 and SD rats.
The role of Ang II in the developing retina is unknown. The present study is the first to examine whether the RAS participates in the development of the rat retinal vasculature during the postnatal period. The transgenic Ren-2 rat has been extensively used by our laboratory to examine the location of RAS in tissues such as ovary, thymus, adrenal gland, and prostate and to determine its possible role in disease at these sites.1,3,5,39 In particular, when diabetes is induced in the Ren-2 rat it causes upregulation of the RAS in specific cell types in the kidney and has pathologic effects, including severe glomerulosclerosis and tubulointerstitial disease, and in the eye, endothelial cell proliferation in the retina and iris.31,57,58 In the present study, enzyme renin, the rate-limiting enzyme in the RAS cascade responsible for the liberation of Ang I from angiotensinogen, increased with retinal development and was mostly present in an activated form. Notably, the level of renin and prorenin was approximately 5 to 15 times higher in the eyes of transgenic Ren-2 rats from P1 to P90 than in age-matched SD rats. Indeed, very low levels of prorenin were found in the retinas of SD rats, so that at P7, P21, and P90 prorenin levels were below the detection level of the enzyme kinetic assay, a finding consistent with results showing tissue prorenin to be extremely low in SD rats.1,5 The overexpression of renin in the developing retina of Ren-2 rats is associated with more extensive vascularization. In the normal rat, the retinal vasculature develops between P0 and P18 and extends from the optic disc to the outermost edge.

FIGURE 7. Wholemounts of transgenic Ren-2 and SD rat retinas at P7 showing immunolabeling with the endothelial cell marker isolectin. (A) Control Ren-2; (B) Ren-2 treated with the ACE inhibitor lisinopril; (C) Ren-2 treated with the AT1 receptor antagonist losartan; (D) control SD; (E) SD treated with lisinopril; and (F) SD treated with losartan. In control Ren-2 rats (A) the retinal vasculature extended farther from the optic disc toward the periphery than in control SD rats (D). In both Ren-2 and SD rats the distance from the optic disc was unchanged with either lisinopril or losartan. Arrows: the growing edge of the vasculature in the peripheral retina. Magnification, ×40. Scale bar, 250 μm.

FIGURE 8. Length of the vasculature from the optic disc to the retinal periphery in transgenic Ren-2 and SD rats at P7. Each value represents the mean ± SEM of results in six to eight retinas per group. ***P < 0.001 between Ren-2 and SD rats.

FIGURE 9. Vascular density per field in central, middle, and peripheral regions of the retina in transgenic Ren-2 (A) and SD (B) rats at P7. In control Ren-2, the density of isolectin labeled blood vessels was similar in all regions of the retina. In control SD rats, vascular density in the peripheral retina was reduced compared with all regions of Ren-2 retina and the central and middle regions of SD retina. Lisinopril reduced vascular density in the periphery of Ren-2 rat retina. P < 0.05 compared with all regions and groups in Ren-2 retina. *P < 0.05 compared with the central and mid retina in SD rats and all regions of retina in Ren-2 rats.
of the peripheral retina. In transgenic Ren-2 rats at P7, the growing edge of the vasculature extended farther, to the retinal periphery, and was denser in the periphery than in SD rats, indicating that upregulation of the retinal RAS promotes retinal vascular development.

Blockade of the RAS is commonly used to determine the contribution of Ang II to disease, including angiogenesis. In neonates with oxygen-induced retinopathy, ACE inhibition reduces pathologic retinal angiogenesis in both Ren-2 and SD rats when administered from P11 to P18. In the present study, ACE inhibition administered from P1 to P7 had an anti-angiogenic effect in Ren-2 rats, reducing vascular density in the peripheral retina, although not affecting the length of the growing vasculature from the optic disc to the periphery. The reasons for the lack of an antiangiogenic response with ACE inhibition in the developing retina of SD rats is not clear but could be due to a number of factors. SD rat tissues have been reported to have a reduced RAS compared with Ren-2 rats and may be less sensitive to RAS blockade than Ren-2 rats. For instance, in Ren-2 rats, very low-dose RAS blockade normalizes systolic blood pressure and reduces fibrinotic and angiogenic disease in both normal and diabetic tissues.

Therefore, although the dose of the ACE inhibitor lisinopril used in the present study reduced pathologic angiogenesis in SD rats with oxygen-induced retinopathy, a higher dose may be needed to influence vasculogenesis in the growing SD retina.

In the present study, AT1 receptor blockade had no effect on vessel length or density in the retinas of either Ren-2 or SD rats when administered from P1 to P7, perhaps because of the higher abundance of AT1 receptors in this period, particularly at P1. The contribution of the AT2 receptor to the early stages of retinal angiogenesis is difficult to assess because of the lack of AT2 receptor antagonists that do not necessitate concomitant administration. Previous in vivo studies using the AT2 receptor antagonist, PD123319, have shown that continuous administration with miniosmotic pumps is necessary to ligate the AT2 receptor. In studies such as the present one, it is not technically feasible to insert miniosmotic pumps into P1 rats. Nevertheless, an angiogenic effect of AT2 receptor blockade has been reported in slightly older rats with oxygen-induced retinopathy, and the AT2 receptor is associated with organ development being highly expressed in fetal and developing tissues and less abundant in the adult.

In summary, this study demonstrates that a retinal RAS is present in the vasculature and neurons of the inner retina of the rat from as early as P1. Overexpression of the RAS in transgenic Ren-2 rats is associated with augmented vascularization in the developing retina which can be reduced with ACE inhibition. AT1 receptor antagonism does not influence developmental angiogenesis in the rat retina, which may be due to the predominance of the AT2 receptor and its reported role in organogenesis. Further studies are needed to elucidate the contribution of the RAS and its receptors to angiogenesis and neuroregulation both in development and disease.

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References


