Renin-Containing Müller Cells of the Retina Display Endocrine Features

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Purpose. An ocular renin-angiotensin system has been implicated in the proliferation of retinal blood vessels and blindness in diabetes mellitus. Its cellular basis has not been established. The objective was to identify sites of renin synthesis, secretion, and processing in eyes from humans, BALB/c mice, Sprague-Dawley rats, and a hypertensive transgenic rat model (mREN-2) that displays amplified extrarenal renin synthesis.

Methods. Paraffin sections of eyes were incubated with antisera to renin protein, prorenin, vimentin, and Müller cells. Enzyme kinetic renin assay was performed on extracts of whole eyes (excluding lens and vitreous) and comparisons made with adrenal glands and kidneys. For detection of renin mRNA, retinas were separately pooled from BALB/c and Swiss mice.

Results. In normal rodent and autopsy human eyes, labeling for renin, vimentin, and Müller cell protein was observed in the cytoplasm of all macroglial Müller cells, with renin labeling most obvious in endfeet closely apposed to retinal blood vessels. Prorenin labeling was not detected. Less intense renin labeling, again without prorenin, was seen in nonpigmented ciliary epithelium of rodents. In transgenic (mREN-2) rat eyes, renin and prorenin labeling of Müller cells and nonpigmented ciliary epithelium were intense. Prorenin was localized to the posterior region of Müller cells but only sparsely to endfeet in rodent retinas, and renin was present only in an active form in amounts one third that of one adrenal. Renin mRNA was readily detected. In human retina, renin was present in active and pro-forms, and the total amount was approximately one fiftieth that of adrenal.

Conclusion. Renin is synthesized in the retina and is specifically localized to the macroglial Müller cells. Nonpigmented ciliary epithelium also contains renin. The presence of prorenin in the posterior part of the Müller cell, with active renin throughout but notably in endfeet in apposition to retinal capillaries, suggests directional processing of renin. These findings are consistent with earlier suggestions that retinal neovascularization may be associated with Müller cell dysfunction. Invest Ophthalmol Vis Sci. 1995;36:1450-1458.

Renin is secreted primarily from the kidney into blood where it cleaves a protein substrate, resulting in the formation of angiotensin peptides with vasoconstrictor, ion transport, and aldosterone-stimulating activity. Angiotensin II (ANG II) also stimulates angiogenesis and promotes hypertrophy and hyperplasia in vascular smooth muscle cells. Renin mRNA has been demonstrated in extrarenal tissues including brain, adrenal gland, ovary, and eye. ANG I and ANG II have been detected in ocular tissues, and prorenin, the precursor of renin, increases in ocular vitreous fluid of patients with proliferative diabetic retinopathy. Indeed plasma prorenin has been shown to be a sensitive marker of diabetic retinopathy, leading to the proposition that through increased activity of a local renin-angiotensin system, ANG II production in the diabetic eye may become a causative factor in neovascularization. Prorenin also has been localized to the ciliary body, but, again, functional aspects are unclear. A necessary initial step toward elucidating the function of an ocular renin-angiotensin system would be to characterize its cellular distribution and to study its processing. For this purpose, immunocytochemical techniques have been
applied to localize renin and prorenin in the normal eyes of humans, mice, and rats. Eyes from transgenic (mREN-2) rats (TG Ren-2) also were studied because this hypertensive model displays amplified extrarenal expression of renin in cells that normally express renin, including the eye.\textsuperscript{15,16}

\section*{METHODS}

\subsection*{Tissue Fixation}

This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Three male BALB/c mice (20 to 25 g), three male Sprague-Dawley rats (200 to 250 g), three male homozygous TG Ren-2 rats (200 to 250 g) maintained on the angiotensin converting enzyme inhibitor lisinopril (10 mg/kg body weight), and three female heterozygous TG Ren-2 rats (200 to 250 g) that had never been administered angiotensin converting enzyme inhibitor were anesthetized with Nembutal (60 mg/kg body weight; Boehringer Ingelheim, Artarmon, Australia) and were prepared for vascular perfusion.\textsuperscript{17} The abdominal aorta was cannulated with a 26-gauge (mice) or 18-gauge (rats) needle filled with Hank’s balanced salt solution (CSL, Melbourne, Australia). Vascular perfusion was begun at 200 mm Hg using 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.6 for 10 minutes. The sections were incubated for 18 hours at 4°C with either a polyclonal anti-mouse renin protein antiserum (gift of Professor P. Corvol, INSERM U36, Paris, France). Polyclonal mouse and rat renin prosequence antiserum,\textsuperscript{17} six monoclonal anti-Müller cell antisera (4D6, 3F11, 4H11D6, 4C8, 3F8, and 3F3; gift of Dr. Z. Dreher, University of Sydney, Australia), an antivimentin antiserum (Boehringer Mannheim, Mannheim, Germany), and normal sera also were used. Sections of eye were treated with a 0.05% proteinase K in PBS (Sigma, St. Louis, MO) for 30 minutes at 37°C before incubation with the primary antisera. After thorough rinsing with PBS (3 × 5-minute changes), the sections were flooded with a solution of 3% hydrogen peroxide in methanol for 10 minutes. After rinsing with PBS (2 × 5 minutes), the sections were incubated with either biotinylated goat anti-rabbit immunoglobulin or biotinylated goat anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) diluted 1:200 with PBS. The sections were rinsed with PBS (2 × 5 minutes) and incubated with avidin–biotin peroxidase complex (Vector, Burlingame, CA) (10 μl of each component in 2 ml of PBS). After rinsing with PBS (2 × 5 minutes), the sections were incubated in 0.05% diaminobenzidine–0.05% hydrogen peroxide in PBS at pH 7.6 for 10 minutes. The sections were rinsed in tap water for 5 minutes, stained in Mayer’s hematoxylin, differentiated in Scott’s tap water, dehydrated in alcohol, cleared in D-limonene, and mounted in Di-butyl phthalate xylene.

For sections of eye and adrenal gland, the renin protein and prosequence antisera were applied at a dilution of 1:50 to 1:600, whereas for kidney sections, a dilution of 1:1000 to 1:10,000 was used. Müller cell antisera were applied to sections at a dilution of 1:50 to 1:200. The vimentin antiserum was used at a dilution of 1:10. All antisera were diluted in PBS.

\subsection*{Antiserum Specificities}

The mouse and rat renin protein and prosequence antisera specifically label juxtaglomerular cells of the kidney, the adrenal cortex, and granulated convoluted tubules of the mouse submandibular gland.\textsuperscript{17,18} The specificities of the mouse, rat, and human renin protein and the mouse and rat prosequence antisera were established by absorbing their respective peptides and incubating them on paraffin sections of kidney and eye. Immunolabeling of juxtaglomerular cells in kidney and Müller cells in retina was abolished. The renin protein antiserum is raised against an oligopeptide and reacts with its epitope in the prosequence. The prosequence antisera reacts only with the proform. The Müller cell antisera each label part of the Müller cell cytoplasm.\textsuperscript{19} For comparison, sections were incubated with anti-sheep albumin and transthyretin antisera (Dakopatts). These antisera do not specifically label Müller cells. The vimentin antiserum labeled endothelial cells and vascular smooth muscle cells in eye, kidney, and brain.

\subsection*{Immunohistochemistry}

Three-micrometer sections of tissue were incubated for 20 minutes with normal goat serum diluted 1:10 with 0.1 M phosphate-buffered saline (PBS) at pH 7.4. The sections were incubated for 18 hours at 4°C with either a polyclonal anti-mouse renin protein antiserum,\textsuperscript{17} a polyclonal anti-rat renin fusion protein antiserum,\textsuperscript{18} or a monoclonal anti-human renin protein antiserum (gift of Professor P. Corvol, INSERM U36, Paris, France)
Renin Assay

Eyes (excluding lens and vitreous), adrenal glands, and kidneys were removed from anesthetized (60 mg/kg Nembutal [Boehringer Ingelheim], intraperitoneally) male BALB/c mice (n = 6, 20 to 25 g) and male Sprague-Dawley rats (n = 6, 200 to 250 g). For estimations of total renin, the left eye, adrenal gland, and kidney from each animal were separately snap frozen in either 150 µl (eyes) or 1 ml (adrenal glands and kidneys).
were microdissected from the orbit and separately Renin Gene Expression formation of ANG I from ovine angiotensinogen is cut equatorially at the ora serrata, and the anterior kidsneys) of 0.1 M phosphate buffer at pH 7.4. For estimations of active renin, the right eye and adrenal gland of each animal were snap frozen in protease inhibitors (8.3 mM N-ethylmaleimide, 6.7 mM ethylendiaminetetraacetic acid, 30 mM benzamidine). All samples were thawed and frozen twice, homogenized, and assayed for renin by an enzyme kinetic method using hog renin as the reference standard (National Standards Laboratory, London, UK) and 24-hour ne-

![image](https://via.placeholder.com/150)

**FIGURE 1.** Three-micrometer paraffin sections of mouse, rat, and human retinas counterstained with Mayer’s hematoxylin. ilm = inner limiting membrane; ipl = inner plexiform layer; mnl = inner nuclear layer; opl = outer plexiform layer; onl = outer nuclear layer; olm = outer limiting membrane; v = vitreous; c = capillary. (a) BALB/c mouse retina. Magnification, ×210. Scale bar = 48 μm. (b) Sprague-Dawley rat retina. Magnification, ×210. Scale bar = 48 μm. (c) Human retina. Magnification, ×250. Scale bar = 38 μm. (a,b,c) Intense labeling for renin protein is seen beneath the ilm in Müller cell endfeet (arrows). Arising from the endfeet, fine processes labeled for renin protein extend through the ipl (double arrows) and surround nuclei in the inl. Intense labeling is seen in the opl, between photoreceptor nuclei in the onl and in the region of the olm (asterisk). (d) Oblique section of BALB/c mouse retina labeled for renin protein. Endfeet of Müller cells labeled for renin protein (arrows) make numerous contacts with retinal capillaries. Ganglion cell nuclei are not labeled. Magnification, ×1280. Scale bar = 8 μm. (e) BALB/c mouse retina. Endfeet of Müller cells (arrows) at the vitread surface are labeled with the anti- Müller cell antiserum 4D6. Magnification, ×530. Scale bar = 19 μm. (f) BALB/c mouse retina. Müller cell processes are labeled for vimentin (arrow). Magnification, ×330. Scale bar = 30 μm.

Renin in Müller Cells of the Retina

**RESULTS**

**Immunohistochemistry**

Renin protein antisera labeled retinas in a similar manner in eyes from BALB/c mice, Sprague-Dawley
rats, and humans (Figs. 1a, 1b, 1c). There was intense labeling for renin protein in discrete areas beneath the inner limiting membrane. Aiming from these areas, fine processes labeled for renin protein traversed the inner plexiform layer and surrounded nuclei in the inner nuclear layer (Müller, amacrine, and horizontal cell nuclei). Numerous processes in the outer plexiform layer were labeled, and labeling was seen between photoreceptor nuclei and in the region of the outer limiting membrane. At the region of the inner limiting membrane, intense labeling for renin protein was seen in discrete areas that contacted the anterior surface of the retina and made numerous contacts with retinal blood vessels (Fig. 1d).

The only retinal cell type extending from the inner limiting membrane to the outer limiting membrane is the Müller cell, suggesting this single type to be the location of renin. To confirm this, we incubated sections of eye with other Müller cell-labeling antibodies. The Müller cell antiserum labeled the retina in a manner similar to the renin protein antiserum, with labeling predominant in Müller cell endfeet (Fig. 1e). The vimentin antiserum also exhibited a similar pattern of labeling (Fig. 1f), with Müller cell processes in the inner plexiform and inner nuclear layers most obvious. There was no specific labeling of the eye in sections incubated with normal sera instead of the primary antiserum (not shown). Weak renin protein labeling was seen in nonpigmented ciliary epithelium of BALB/c mice and Sprague–Dawley rat eyes. Other retinal cells and other regions of human and rodent eyes were not specifically labeled for renin protein. No specific labeling for prorenin was seen in any part of human, BALB/c mice, or Sprague–Dawley rat eyes.

Transgenic (mREN-2) Rat

In eyes of TG Ren-2 rats, labeling for renin protein was more intense in Müller cells than in human and rodent eyes (Fig. 2a), with labeling more obvious in the outer plexiform layer and between photoreceptor nuclei. Labeling for prorenin was detected in the outer plexiform layer and between photoreceptor nuclei of TG Ren-2 rats (Fig. 2b). Prorenin labeling of Müller cell endfeet was observed (Fig. 2b). Intense labeling for renin protein and prorenin was seen in the nonpigmented ciliary epithelium of the ciliary body (Figs. 2c, 2d). No other areas of the eye were specifically labeled for renin protein or prorenin in TG Ren-2 rats.

Renin Assay

In BALB/c mice and Sprague–Dawley rats, renin in the eye and adrenal gland was in an active form, with prorenin at or below the detection limit (Table 1). In mice, active renin in the perfused eye (40 ± 7 μGU) was approximately one third that of an adrenal gland (120 ± 40 μGU) and one hundred thousandth that of a kidney (3.35 × 10⁶ ± 0.4 × 10⁶ μGU). Similar renin levels were found in rat tissues. Flushing retinal blood vessels with buffer (perfused eyes) to remove blood did not alter the renin levels. In human retinas, total renin was approximately one fiftieth that of adrenal (Table 1).

Renin Gene Expression

Figure 3 shows that renin mRNA was detected in the microdissected retinas of BALB/c and Swiss mice and had a size of 200 base pairs, corresponding to that of kidney renin mRNA. As the starting sources, 7.5 μg of total kidney RNA and 15 μg of retinal RNA were used.

DISCUSSION

The major new finding of this study is the immunohistochemical localization of renin to Müller cells of human, mouse, and rat retinas. Confirmation that the site of renin labeling was Müller cells was achieved by using specific antisera. Species specific Müller cell antisera have been used in previous studies to define their morphology and distribution. Antivimentin antiserum was also used and is considered to label all Müller cells in mammalian retinas. Müller cells are macroglial cells involved in various physiological functions in the retina, including metabolism of neurotransmitters, active uptake of potassium, and removal of carbon dioxide from the retinal extracellular space. Cytoplasmic processes extend from the nucleus in the inner nuclear layer posteriorly to the outer limiting membrane, whereas anteriorly cytoplasmic processes thicken to become endfeet beneath the inner limiting membrane. We observed intense labeling for renin protein in the endfeet of Müller cells, which made numerous contacts with retinal blood vessels. The endfeet provide a basement membrane that fuses with the basement membrane of smooth muscle cells, pericytes, and endothelial cells of the vessel walls. Müller cells are, therefore, in an ideal position to transport metabolites to the retinal vasculature.

Using the polymerase chain reaction, we detected renin mRNA in mouse and rat retinas. Previously, reported renin, angiotensin converting enzyme (ACE), and angiotensinogen mRNA in human retinas. While in situ hybridization and immunogold studies have not yet been reported, the present morphologic and biosynthetic evidence is consistent with synthesis of renin within Müller cells and its possible transport to endfeet on retinal capillaries. Relevant to this possibility, other components of the renin–angiotensin system have been found on or near the retinal vasculature. ANG II binding sites have been identified on bovine and human retinal blood vessels, which constrict in response to exogenously administered ANG II. Also, reported that in
FIGURE 2. Three-micrometer paraffin sections of transgenic (mREN-2) rat eye labeled for renin protein and prorenin. Counterstained with Mayer's hematoxylin. ilm = inner limiting membrane; ipl = inner plexiform layer; inl = inner nuclear layer; opl = outer plexiform layer; onl = outer nuclear layer; olm = outer limiting membrane; v = vitreous. (a) Labeling for renin protein in Müller cells (arrows) is more intense than in BALB/c mouse, Sprague-Dawley rat, and human retinas. Magnification, X330. Scale bar = 30 μm. (b) Labeling for prorenin is most obvious in the opl, between photoreceptor nuclei (arrows). The section is only lightly counterstained to reveal labeling of Müller cell endfeet at the ilm. Labeling for renin protein (c) and prorenin (d) in nonpigmented ciliary epithelium of the ciliary body. Magnification, X210. Scale bar = 48 μm.

Anterior uveal tract, retina, and retinal pigment epithelium plus choroid, ANG I and ANG II levels were higher than plasma, indicating that ANG I and ANG II are produced locally in ocular tissues. Amacrine cells and possibly Müller cell bodies have been shown to be immunolabeled for ANG II, and ACE has been found on retinal blood vessels.

Labeling for prorenin has been reported in the nonpigmented ciliary epithelium (NPCE) of the human eye, and angiotensinogen and ACE have been localized to the ciliary body. In situ synthesis of prorenin with processing and secretion of renin in NPCE have not been proven, although prorenin labeling has been identified on NPCE and in adjacent vitre-
TABLE 1. Total, Active, and Prorenin Levels in Eye, Adrenal Gland, and Kidney of Mice and Rats and Human Retina and Adrenal Gland

<table>
<thead>
<tr>
<th>Tissues</th>
<th>n</th>
<th>Total Renin</th>
<th>Active Renin</th>
<th>Prorenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male BALB/c mice (μGU) eye</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not perfused</td>
<td>5</td>
<td>52 ± 8</td>
<td>45 ± 5</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Buffer perfused</td>
<td>6</td>
<td>50 ± 5 (12)</td>
<td>40 ± 7 (12)</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Inhibitor perfused</td>
<td>6</td>
<td>130 ± 20</td>
<td>120 ± 40</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 3.35 × 10⁶</td>
<td>* 0.40 × 10⁶</td>
<td>*</td>
</tr>
<tr>
<td>Male Sprague–Dawley rat (μGU)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye (retina, choroid, sclera)</td>
<td>5</td>
<td>30 ± 10</td>
<td>20 ± 5</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>6</td>
<td>130 ± 30</td>
<td>100 ± 20</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>Kidney (not perfused)</td>
<td>6</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 5.7 × 10⁶</td>
<td>* 0.24 × 10⁶</td>
<td>*</td>
</tr>
<tr>
<td>Human (μIU) Retina</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(some vitreous attached)</td>
<td>1</td>
<td>34.52</td>
<td>24.48</td>
<td>10.04</td>
</tr>
<tr>
<td>Retina (no vitreous attached)</td>
<td>1</td>
<td>17.56</td>
<td>7.66</td>
<td>9.90</td>
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<tr>
<td>Adrenal Gland</td>
<td>1</td>
<td>1848</td>
<td>536</td>
<td>1912</td>
</tr>
</tbody>
</table>

n = number of animals. Numbers of individual eyes assayed are shown in brackets where this differs from n. Values are mean ± SEM.

Except for the two "not perfused" groups, blood was removed from tissues by whole body vascular perfusion.

* Separate estimations of total renin and prorenin from active renin in kidney were not attempted.

† The value for renin in rat kidneys is from our previously published work, assuming the male 200 g rat kidney to weigh 1 g.

In mice and rats: Goldblatt Hog Units/organ.
In humans: WHO International Units/organ.

FIGURE 3. Detection of renin mRNA by polymerase chain reaction. Lane 1, BALB/c mouse retina; lane 2, Swiss mouse retina; lane 3, negative control; lane 4, pBR322 HaeIII digested marker; lane 5, BALB/c mouse kidney; lane 6, Swiss mouse kidney.

Biochemically measurable renin in the retina (excluding ciliary body, lens, and vitreous) was active renin, with the total amount per eye only one hundred thousandth that of a kidney. It seems unlikely that locally produced ocular renin could contribute significantly to plasma levels of either active or prorenin because the total renin content of BALB/c mouse eye, Sprague–Dawley rat eye, and human retina was respectively only 1/500, 1/16, and 1/6 that of 1 ml of plasma.37–39

It was important to apply these techniques to the TG Ren-2 rat model in which the entire mouse Ren-2 renin gene, with its normal regulatory segments, has been added to the rat genome, resulting in enhanced expression in those extrarenal tissues that normally express the rat renin gene.15,16 In the NPCE of TG Ren-2 rats, renin labeling was more intense than in normal rodents, and intense prorenin labeling was detected. Prorenin labeling was also present in the posterior region of Müller cells but only occasionally in Müller cell endfeet, whereas labeling for renin extended throughout the cell cytoplasm and was most intense in endfeet. This polarity is consistent with renin processed rapidly in the direction of retinal blood vessels, but immunogold and in situ hybridization studies will be required to confirm that renin is synthesized in and secreted from Müller cells and NPCE.

The normal role that ANG II might play in the retina includes regulation of intraocular blood flow and pressure because administration of renin inhibitors and ACE inhibitors to the eye lowers intraocular pressure. ANG II is also a growth factor in vascular smooth muscle cells and has powerful angiogenic actions. In this regard, the recently established association between increased amounts of prorenin in the blood of patients...
Renin in Müller Cells of the Retina

with diabetic proliferative retinopathy is of great interest.\(^{11,12}\) Proliferative diabetic retinopathy is characterized by the ingrowth of new blood vessels to the retina and vitreous that eventually results in impaired vision and blindness.\(^{8}\) Prorenin, the precursor of renin, increases in plasma with the onset of proliferative retinopathy in patients with diabetes.\(^{11,12}\) and prorenin levels in vitreous have been found to be higher in patients with diabetic retinopathy than in patients with spontaneous retinal detachment unrelated to diabetes.\(^{10}\) These circumstances are consistent with increased renin production in the eyes of people with diabetes, which, through ANG II formation, has been proposed to play a role in the pathogenesis of neovascularization. A feature of proliferative diabetic retinopathy is the formation of epiretinal membranes that cause impaired vision and tractional retinal detachment.\(^{10}\) Müller cells and astrocytes have been identified as components of diabetic epiretinal membranes,\(^{44,45}\) and Müller cells have been shown to induce endothelial cells to form blood–brain barrier properties in the rat eye.\(^{46}\) These reports have led to the proposal that Müller cells play an active role in proliferative diabetic retinopathy. The presence of renin within these cells is consistent with paracrine renin–angiotensin system involvement in this process.

**Key Words**

renin, angiotensin, Müller cells, retina, proliferative diabetic retinopathy.

**Acknowledgments**

The authors thank Darren Kelly, Bronwyn Rees, David Robinson, and Kerrie Nicholsohn for expert technical assistance. Human eyes were obtained with the assistance of Dr. Graham Pullock and Mr. Greg Allen from the Lions Eye Bank, Victoria, Australia.

**References**