Chapter 19

Gene Therapy for Diabetic Retinopathy – Targeting the Renin-Angiotensin System

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1. Introduction

1.1. Diabetic retinopathy clinical features and current treatment options

The prevalence of diabetes has been continuously increasing for the last few decades and it is being recognized as a worldwide epidemic [1]. Diabetic retinopathy (DR) is the most common diabetic microvascular complication, and despite recent advances in therapeutics and management, DR remains the leading cause of severe vision loss in people under age of sixty [2-4]. The prevalence of DR increases with duration of diabetes, and nearly all individuals with type 1 diabetes and more than 60% of those with type 2 have some form of retinopathy after 20 years [5-7].

Diabetic retinopathy (DR) is characterized by the development of progressive pathological changes in the retinal neuro-glial cells and microvasculature. The earlier hallmarks of diabetic retinopathy include breakdown of the blood-retinal barrier (BRB), loss of pericytes, thickening of basement membrane, and the formation of microaneuryms, which are outpouchings of capillaries [8]. BRB breakdown results in increased vascular permeability and leakage of fluid into the macula causing macular edema, another significant cause of vision loss in those with diabetes. With the progression of diabetic retinopathy, hemorrhage, macular edema, cotton wool spots, all signs of retinal ischemia, and hard exudates, the result of precipitation of lipoproteins and other circulating proteins through abnormally leaky retinal vessels become increasingly apparent. More severe and later stages of diabetic retinopathy, known as proliferative diabetic retinopathy (PDR), is char-

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acterized by pathological neovascularization. Vision loss can occur from vitreous hemorrhage or from tractional retinal detachment [8, 9].

Despite recent developments in the pharmacotherapy of DR, treatment options for patients with DR are still limited. Laser photocoagulation, the primary treatment option for patients with PDR, is still considered gold standard therapy for the treatment of PDR. Although this treatment slows the loss of vision in those with PDR, it does not represent a cure, and is in itself a cell destructive therapy. Corticosteroids and anti-VEGF agents have shown promising results with regard to prevention of neovascularization, but remain limited in use due to their short-duration effects. More importantly, none of these agents have been able to substitute for the durability and effectiveness of laser mediated panretinal photocoagulation in preventing vision loss in the late stages of DR.

1.2. RAS and diabetic complications

The renin-angiotensin system (RAS) plays a vital role in the cardiovascular homeostasis by regulating vascular tone, fluid and electrolyte balance, and in the sympathetic nerve system. Angiotensin II (Ang II), a peptide hormone of RAS, has been known to regulate a variety of hemodynamic physiological responses, including fluid homeostasis, renal function, and contraction of vascular smooth muscle [10]. In addition, Ang II is capable of inducing a multitude of non-hemodynamic effects, such as the induction of reactive oxygen species (ROS), cytokines, and the stimulation of collagen synthesis [11-14]. Most of the pathophysiological actions of Ang II are mediated via activation of Ang II type 1 receptors (AT1R), G protein–coupled receptors (GPCRs) that couple to many signaling molecules, including small G proteins, phospholipases, mitogen-activated protein (MAP) kinases, phosphatases, tyrosine kinases, NADPH oxidase, and transcription factors to stimulate vascular smooth muscle cell growth, inflammation, and fibrosis [11, 15, 16]. Dysregulation of RAS has been implicated in a number of major cardiovascular and metabolic diseases, including endothelial dysfunction, atherosclerosis, hypertension, renal disease, diabetic complications, stroke, myocardial infarction and congestive heart failure [17, 18]. RAS blockade produces beneficial cardiovascular and renal effects in numerous clinical trials [19-21].

1.3. Recent advances in RAS research

Recent discoveries have revealed that the RAS hormonal signaling cascade is more complex than initially conceived with multiple enzymes, effector molecules, and receptors that coordinately regulate the effects of the RAS. Recent studies have identified additional peptides with important physiological and pathological roles, new enzymatic cascades that generate these peptides and more receptors and signaling pathways that mediate their function [22, 23].

Discovery of angiotensin-converting enzyme 2 (ACE2) has resulted in the establishment of a novel axis of the RAS involving ACE2/Ang-(1-7)/Mas [24-27]. ACE2, like ACE, is a zinc-metallopeptidase, exhibiting approximately 42% amino acid identity with ACE in its catalytic domain. However, unlike somatic ACE, ACE2 only contains a single catalytic site and func-

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tions as a carboxymonopeptidase, cleaving a single C-terminal residue from peptide substrates, thus ACE2 is able to cleave Ang II to form Ang (1-7). Ang (1-7), a biologically active component of the RAS [28-30] binds to a G-protein coupled receptor, Mas receptor [31], and plays a counter-regulatory role in the RAS by opposing the vascular and proliferative effects of Ang II [32]. A current view of RAS consists of at least two axis with counteracting biologic effects (Figure 1).

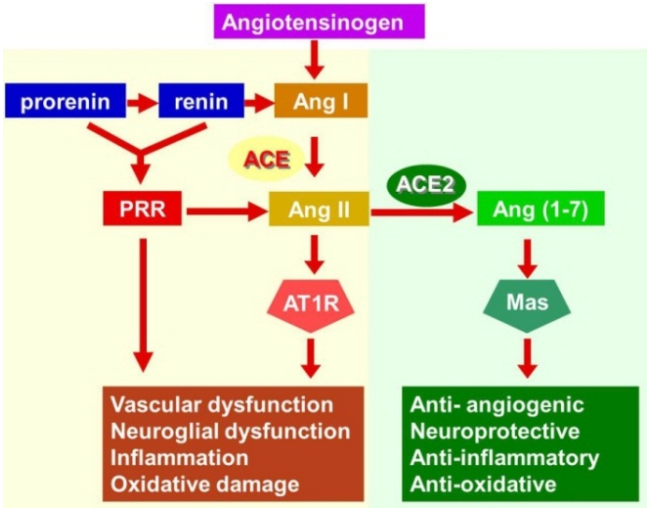

Figure 1. Schematic diagram depicting the key components of the Renin Angiotensin System. Angiotensinogen is cleaved by renin to form angiotensin I (Ang I). Angiotensin converting enzyme (ACE) converts Ang I into Angiotensin II (Ang II) the main effector peptide of the RAS. Ang II elicits is cellular effects by activating the main receptor, Angiotensin II receptor 1 (AT1R), as well as other receptors (not shown). Angiotensin II-converting enzyme 2 (ACE2), a recently discovered component of RAS, cleaves Ang II to form Angiotensin (1-7) (Ang 1-7), which activate Mas receptor to produce counteracting effects mediated by Ang II. All these components are expressed locally in various cell types in the eye, regulating metabolism, cell survival, and other local neuronal-vascular and immune-modulating functions in the retina.

This vasoprotective axis of RAS counteracts the traditional proliferative, fibrotic, proinflammatory and hypertrophic effects of the ACE/Ang II/AT1R axis of the RAS [24]. The importance of the vasodeleterious axis of the RAS [ACE/angiotensin II (Ang II)/ AT1R] in cardiovascular disease, as well as in diabetes and diabetic complications, is well established since ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs) are leading therapeutic strategies [20, 33-35]. However, the impact of the vasoprotective axis of the RAS remains poorly understood [24, 36-38]. The concept that shifting the balance of the RAS towards the vasodilatory axis by activation of ACE2 or its product, Ang-(1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis [24, 39-43]. Indeed, ACE2/Ang-(1-7) activation is now considered to be a critical part of the beneficial actions of ACEi and ARB drugs [24, 36].

1.4. Tissue RAS in end-organ damage

The classical (endocrine) RAS has been traditionally regarded as systemic hormonal system. Ang II is formed from liver-synthesized angiotensinogen via a series of proteolytic cleavage events. Circulating Ang II activates AT1 and AT2 receptors in various tissues, such as the brain, adrenal and vascular tissues to modulate cardiovascular and hydromineral homeostasis.

However, most components of RAS have also been identified in essentially every organ including kidney, heart, liver, brain, adipose tissue, reproductive tissue, hematopoietic tissue, immune cells and eye, and increasing evidence supports the existence of tissue- specific RAS that exerts diverse physiological effects locally and independently of circulating Ang II [44-46]. These tissue- specific paracrine, intracrine andautocrine actions of RAS may contribute to end-organ damage in many pathological conditions including diabetic complications and maybe the basis for the reported limited beneficial effects of RAS blockade.

2. Ocular RAS in pathogenesis of diabetic retinopathy

Increasing evidence continues to implicate the involvement of the local renin-angiotensin-system (RAS) in retinal vascular dysfunctions. Various components of RAS have been detected in the different cell types of the eye (Table 1).

RAS components	Retinal Localization	Reference
Angiotensinogen	Retinal microvasculature, RGCs, RPE	[47, 48]
Angiotensin I	Aqueous, vitreous, and subretinal fluid	[49]
Angiotensin II	Aqueous, vitreous, and subretinal fluid, RGCs, retinal endothelial cells and photoreceptors	[49-51]
Angiotensin 1-7	Muller cells	[50]
Renin	Muller cells and vitreous fluid	[52, 53]
Renin receptor	Retinal microvasculature, microglia, astrocytes, RGCs, RPE	[54-58]
ACE	Muller cells, RGCs, retinal endothelial cells, photoreceptors, and vitreous	[51, 59-61]
ACE2	Retina	[50]
AT1R	Muller cells, retinal blood vessels, photoreceptors and RGCs	[50, 51]
AT2R	Muller cells, nuclei of some inner, nuclear layer neurons, and ganglion cells	[50]
Mas receptor	RGCs, retinal microvasculature, microglia, subset of astrocytes	unpublished results

GC: retinal ganglion cells; RPE: retinal pigment epithelium.

Table 1. All components of RAS are expressed locally in the eye.

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Hyperglycemia has been shown to directly stimulate angiotensin gene expression via the hexominase pathway, thus contributing to increased Ang II synthesis [62]. Elevated levels of renin, prorenin, and Ang II have been found in patients with DR. In fact, ACE inhibitors and angiotensin receptor blockers (ARBs) have been shown to improve diabetes-induced vascular, neuronal, and glial dysfunction [61, 63-66]. Recent clinical studies have also clearly demonstrated the beneficial effects of RAS inhibition in both type 1 and type 2 diabetic patients with retinopathy [67-71]. Despite these positive outcomes, RAS blockers are not completely retinoprotective and retinopathy still progresses to more advanced stages. This could be attributed to the existence of local Ang II formation and that current therapeutic agents are unable to cross the blood-retina barrier (BRB) in a concentration sufficient to influence the local RAS in the eye. In addition, increasing evidence suggests that Ang II can be generated via multiple pathways, many of which may not be blocked by classic inhibitors of ACE [72-75]. Furthermore, additional components of RAS that contribute to end-organ damage, such as receptors for renin and prorenin (PRR), have been recently identified [76]. Activation of prorenin/PRR signaling pathway can initiate the RAS cascade independent of Ang II [76].

Ang II may contribute to development and progression of DR by several mechanisms. First, Ang II has been shown to increase VEGF expression directly via activation of AT1R signaling and indirectly by PCK activation [77] to enhance the role of VEGF induced vascular permeability and angiogenesis. Treatment with ACE inhibitors reduces vitreous levels of VEGF and attenuates VEGF-mediated BRB breakdown [78, 79]. Second, Ang II, mediated via AT1R, also contributes to diabetes-induced retinal inflammation by activation of nuclear factor-κβ signaling pathway within retinal endothelial cells [80, 81] leading to the release of inflammatory cytokines which perpetuates the inflammatory cycle. Pro-inflammatory cytokines, chemokines and other inflammatory mediators play an important role in the pathogenesis of DR [82, 83]. These lead to persistent low-grade inflammation, the adhesion of leukocytes to the retinal vasculature (leukostasis), breakdown of BRB and neovascularization with subsequent sub-retinal fibrosis or disciform scarring [84-88]. Third, Ang II may contribute to increased oxidative stress in diabetic retina. Ang II induces reactive oxygen species (ROS) production by activation of NADPH oxidases [89], which has been implicated in diabetic complications [90, 91]. Ang II also induces mitochondrial ROS production, which further stimulate of NADPH oxidases leading to vicious cycle and contributing tissue damage [92, 93].

Fourth, Ang II may also contribute to neuronal dysfunction induced by diabetes [94]. Receptors for Ang II are also expressed in the inner retinal neurons (Table 1). Ang II induced AT1R signaling may cause neuronal dysfunction by reducing the synaptophysin protein in the synaptic vesicles [94].

3. Protective role of the ACE2/Ang1-7-Mas axis of RAS in diabetic complications

The discovery of ACE2- mediated degradation of Ang II into the protective peptide Ang 1-7 thereby negatively regulating the classic RAS, has instigated stimulated interest regarding the potential of ACE2 as a therapeutic target [88, 89], and strategies aimed at enhancing

ACE2 action may have important therapeutic potential for cardiovascular disorders as well as for diabetic complications [40, 95-99]. Ang (1-7) has been shown to prevent diabetes-induced cardiovascular dysfunction [100] and nephropathy [101]. The protective effect of Ang 1-7 signaling is at least in part mediated by direct inhibition of diabetes-induced ROS production due to elevated NADPH oxidase activity [101, 102] and reduction in PPAR-gamma and catalase activities [102]. Adenovirus mediated gene delivery of human ACE2 in pancreas improved fasting blood glucose, beta-cell dysfunction and apoptosis occurring in type 2 diabetes mouse model [103]. The importance of ACE2 as a negative regulator of RAS in diabetic complications is supported by the facts that ACE2 deficiency exacerbates diabetic complications [104, 105] and enhancing ACE2 action counteracts the deleterious effects of Ang II and produces protective effects [96-99, 106].

3.1. Diabetes induced changes in the expression of the retinal RAS genes in the mouse retina during the progression of diabetes

We have previously shown that diabetes induced by STZ treatment in $eNOS^{-/-}$ mice results in more severe, accelerated retinopathy than diabetes in untreated $eNOS^{+/+}$ animals [107]. Thus it became critical to compare retinal mRNA levels of the RAS genes in control and diabetic animals during the progression of diabetes. We observed significant (3-10 fold) increases in the mRNA levels of the vasodeleterious axis of the RAS (angiotensinogen, renin, pro/renin receptor, ACE and AT1 receptor subtypes) following STZ treatment (Figure 2) [108]. In contrast, there was ~ 30% reduction in ACE2 mRNA following an initial stimulatory response. As a result the ACE/ACE2 mRNA ratio was increased by 10-fold, while AT1R/Mas ratio was increased by 3-fold following one month of diabetes (Figure 2). These observations were our initial indication that DR is associated with a shifting balance of the retinal RAS towards vasodeleterious axis.

3.2. Enhancing ACE2/Ang1-7-Mas axis by AAV-mediated gene delivery

3.2.1. Characterization of AAV vectors expressing ACE2 and Ang-(1-7)

AAV vector expressing the secreted form of human ACE2 was constructed under the control of the chicken-beta-actin (CBA) promoter (Figure 3A). This secreted form of ACE2 has been previously characterized and shown to be active enzymatically [109]. Since Ang-(1-7) peptide contains only 7 amino acids and small peptides are usually difficult to express in mammalian cells, we designed an expression construct in which the Ang-(1-7) peptide is expressed as part of the secreted fusion GFP protein, and is subsequently cleaved upon secretion into the active peptide. Expression of the fusion sGFP-FC-Ang-(1-7) is under the control of the CBA promoter in the AAV vector (Figure 3A) and was confirmed by tranfecting HEK293 cells using this plasmid DNA (Figure 3B). To ensure that the fusion protein was indeed secreted, proteins isolated from the culture supernatants as well as cell lysates from transfected, sham-transfected or untransfected cells were analysized by western blotting (Figure 3B). Mass spectrometry analysis of Ang (1-7) peptide in supernatant samples of HEK293 cells transfected with the sGFP-FC-Ang-(1-7) plasmid DNA was also performed. The Ang-(1-7) peptide is detectable in supernatant isolated from cells transfected with sGFP-FC-Ang-(1-7) plasmid DNA, but not detectable

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in samples isolated from un-transfected cells, or cells transfected with the control plasmid expressing only the cytoplasmic GFP protein (data not shown). Intravitreal administration of AAV-Ang-(1-7) resulted in a robust transduction of retinal cells primarily within the inner retinal layer (Figure 3C-F). This was associated with an increase in both cellular and secreted Ang-(1-7) (Figure 3G-H). Similarly, ACE2 protein level was increased in the retina following transduction with AAV-ACE2 (Figure 3G).

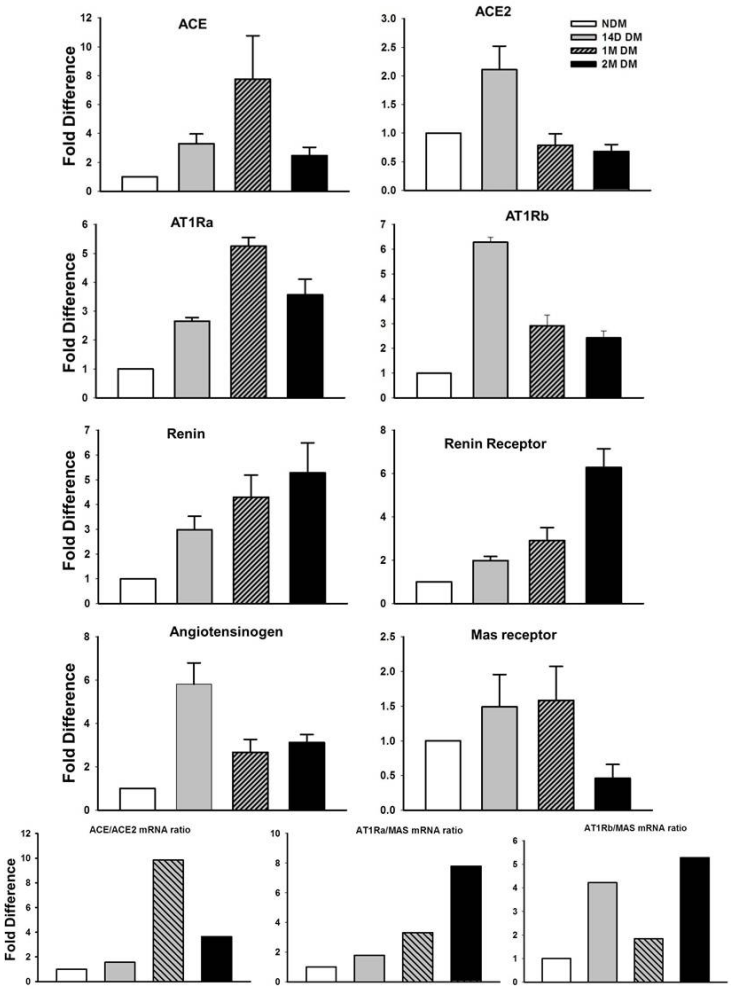

Figure 2. Real-time RT-PCR analysis of retinal mRNA for renin-angiotensin system genes. Values represent fold difference compared to age matched non-diabetic retinal samples for each gene at each time point (14 day and 1 month after induced diabetes). DM: diabetic. NDM: non-diabetic. At least 4 eyes were analyzed at each time point. *p<0.01 (versus NDM group). (From [108] with permission of Mol. Therapy).

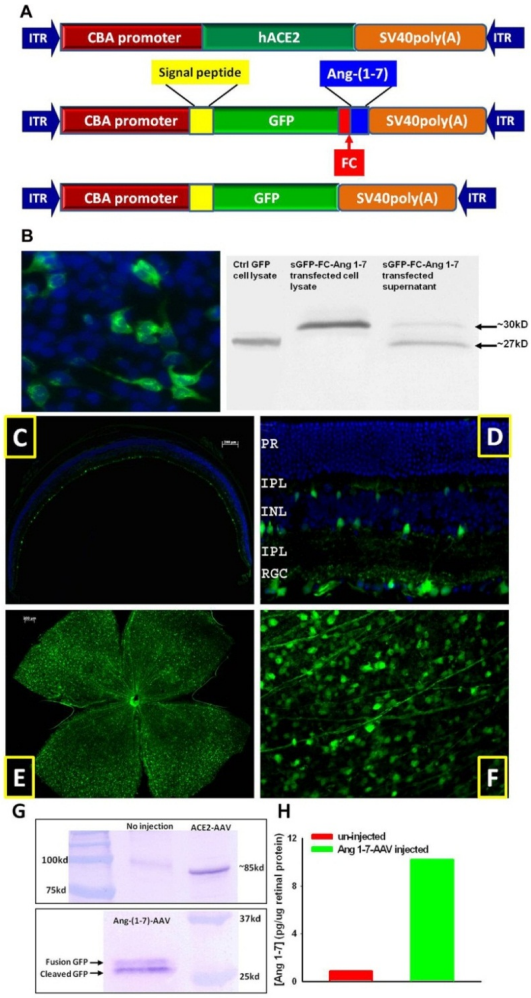

Figure 3. Construction and characterization of AAV vectors expressing ACE2 and Ang-(1-7).A: Maps of the AAV vector expressing the human ACE2 gene (hACE2) and the AAV vector expressing Ang-(1-7) gene. The Ang-(1-7) peptide is expressed as part of fusion protein, and cleaved in vivo upon secretion at the furin cleavage (FC) site. ITR: inverted terminal repeat; CBA: CMV- chicken-β-actin promoter. A control vector contains the coding region for the secreted GFP without the Ang-(1-7) peptide coding sequence. B: Expression and cleavage of the fusion protein. In cultured HEK293 cells transfected with the plasmid sGFP-FC-Ang-(1-7), or infected with AAV-sGFP-FC-Ang-(1-7), there was robust expression of GFP as expected. Proteins isolated from cell lysates contained a single protein band with molecular weight ~30 kd, as predicted for the precursor (fusion protein), but culture supernatants contained two protein bands (30kd and a 27kd), indicating that the secreted protein is cleaved at the furin cleavage site as predicted. C-F: Transduction of mouse retina with AAV vector expressing sGFP-FC-Ang-(1-7) and hACE2. A single intravitreal injection of 1µl AAV vector (10^9 vg/eye) resulted in efficient transduction of inner retinal cells, primarily retinal ganglion cells. C. Low magnification of cross section of a mouse eye that received AAV2-sGFP-FC-Ang-(1-7) injection. D. Higher magnification of the same eye. E. A retinal whole mount showing GFP expression. F. Higher magnification of the same retinal whole mount. G: Western blot of proteins isolated from an uninjected eye and an eye injected with AAV2-ACE2 (top)

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and AAV2-sGFP-FC-Ang-(1-7) (bottom) compared to a molecular weight standard (right lane). H: Ang-(1-7) peptide levels in the retina with and without AAV-sGFP-FC-Ang-(1-7) injection. There was more than a 10-fold increase in Ang-(1-7) peptide level detected by using an Ang-(1-7) specific EIA kit (Bachem, San Carlos, CA) in retinas receiving injection of AAV-sGFP-FC Ang-(1-7). PR: photoreceptor; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC: retinal ganglion cells. (From [108] with permission of Mol. Therapy).

3.2.2. Ocular gene delivery of ACE2/Ang-(1-7) via the AAV vector in the retina results increased ACE2 activities and Ang-(1-7) peptide levels

Diabetes induced more than a 5-fold increase in ACE activity in the retinas of eNOS$^{-/-}$ mice, whereas ACE2 activity was relatively unchanged (Figure 4A). AAV2-ACE2 injected retinas show more than a two-fold increase in ACE2 enzymatic activity (Figure 4A) and this is associated with a reduced level of Ang II and increased Ang-(1-7) peptide level (Figure 4B), but has only a marginal effect on ACE activity (Figure 4A). Injection of AAV2-Ang-(1-7) has no effect on ACE2 activity, but significantly decreased ACE activity (Figure 4A).

We also determined Ang II and Ang-(1-7) peptide levels using a commercial EIA kit (Bachem, San Carlos, CA). STZ induced diabetes resulted in more than a 2-fold increase in Ang II levels whereas the Ang-(1-7) level was unchanged in the retinas of eNOS-/- mice (Figure 4B). This increase of Ang II was completely normalized in retinas injected with AAV-ACE2 but was unchanged in retinas injected with AAV-Ang-(1-7) vector (Figure 4B).

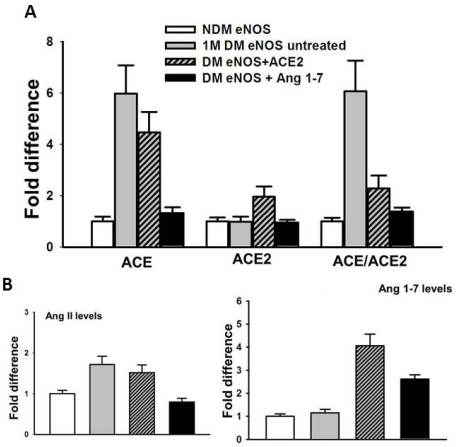

Figure 4. ACE, ACE2 activities and angiotensin peptide levels in the mouse retina.A: ACE and ACE2 enzymatic activities and ACE/ACE2 ratios in non-diabetic (NDM), 1 month diabetic (1M DM), and 1 month diabeticeNOS-/- mouse retinas treated with AAV-ACE2/Ang-(1-7). Values are expressed as fold differences compared with age-matched non-diabetic group. *p<0.01 (versus untreated DM group, N=6/group). B: Ang II and Ang-(1-7) peptide levels in non-diabetic (NDM), 1 month diabetic (1M DM), and 1 month diabetic eNOS-/- retinas treated with AAV-ACE2/Ang-(1-7), measured by ELISA using a commercial kit. *p<0.01 (versus untreated DM group). Values represent fold difference compared with age-matched non-diabetic group. Three retinas were pooled for each measurement, each measurement was done in duplicates, and three separate pools were averaged for each group. (From [108] with permission of Mol. Therapy).

3.3. Protective role of ACE2/Ang (1-7) AAV gene delivery in mouse model of DR

3.3.1. Enhanced ACE2/Ang1-7 expression in the retina reduced diabetes-induced retinal vascular leakage

We investigated if elevated expression of retinal ACE2 or Ang-(1-7) would overcome the vasodeleterious effect of the ACE/AT1R axis and prevent the development of diabetes-induced retinopathy. Effects of increased ACE2 and Ang-(1-7) expression on retinal vascular permeability were evaluated by FITC-labeled albumin extravasations and quantified by measuring its fluorescence intensity in serial sections from non-diabetic, untreated, ACE2 treated diabetic eNOS$^{-/-}$ mice and Ang 1-7 treated diabetic eNOS$^{-/-}$ mice. Induction of diabetes for 2 month in eNOS$^{-/-}$ mice resulted in a 2-fold increase in vascular permeability. This pathophysiology was significantly reduced in diabetic retinas which received ACE2/Ang-(1-7) vector treatments (Figure 5), but not in the retinas receiving control vector containing the coding sequence for secreted GFP without Ang-(1-7) or ACE2 (data not shown).

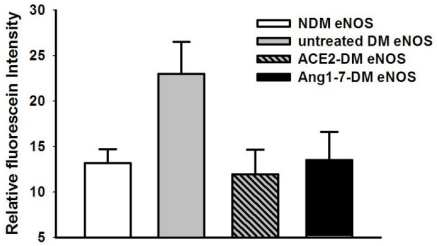

Figure 5. Effects of ocular treatments with ACE2 and Ang-(1-7)-AAV2 on retinal vascular permeability in diabetic eNOS$^{-/-}$ mice. Retinal vascular permeability was evaluated by FITC-labeled albumin extravasations and quantified by measuring the fluorescence intensity in serial sections from eNOS$^{-/-}$ mice at 1 month after induced diabetes. Data are presented as mean ± SD from 6 eyes in each group. *p<0.01 (versus untreated DM group). NDM: non-diabetes; DM: diabetes. (From [108] with permission of Mol. Therapy).

3.3.2. Increased expression of ACE2 and Ang1-7 resulted in reduced ocular inflammation in diabetic retina

Diabetes-induced ocular inflammation, as demonstrated by increased infiltrating CD45 positive macrophages and activation of CD11b positive microglial cells, was significantly reduced in eyes treated with ACE2 and Ang-(1-7) expression vectors (Figure 6).

3.3.3. Increased ACE2/Ang1-7 expression reduced the number of acellular capillaries in the diabetic retina

Induction of diabetes for 2 month in eNOS-/- mice resulted in a >10-fold increase in the formation of acellular capillaries that was significantly reduced in diabetic retinas which received ACE2/Ang-(1-7) vector treatments (Figure 7). Furthermore, increasing the level of ACE2 also prevented basement membrane thickening in diabetic eNOS$^{-/-}$ retina (Figure 8).

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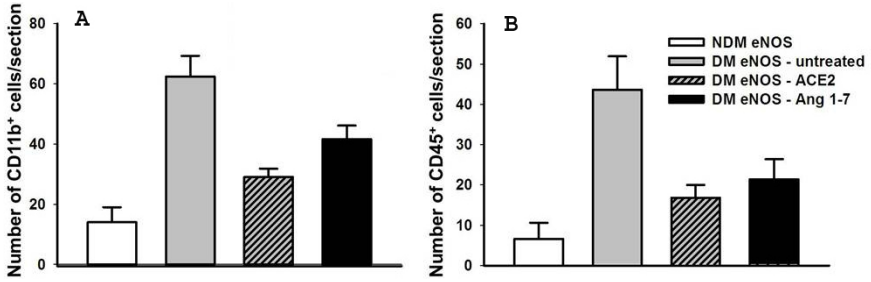

Figure 6. Intravitreal administration of ACE2 or Ang-(1-7)-AAV reduces diabetes-induced ocular inflammation. A. Quantification of CD45positive inflammatory cells in the retinas from untreated non-diabetic, ACE2 treated and Ang-(1-7) treated diabetic eNOS$^{-/-}$ mouse retinas at 1 month after induced diabetes or the equivalent age in untreated controls. B. Quantification of CD11b positive inflammatory cells in the retinas from untreated non-diabetic, ACE2 treated and Ang-(1-7) treated diabetic eNOS$^{-/-}$ mouse retinas at 1 month after induced diabetes or the equivalent age in untreated controls. N=4 for each group. *p<0.01 (versus untreated DM group). (From [108] with permission of Mol. Therapy).

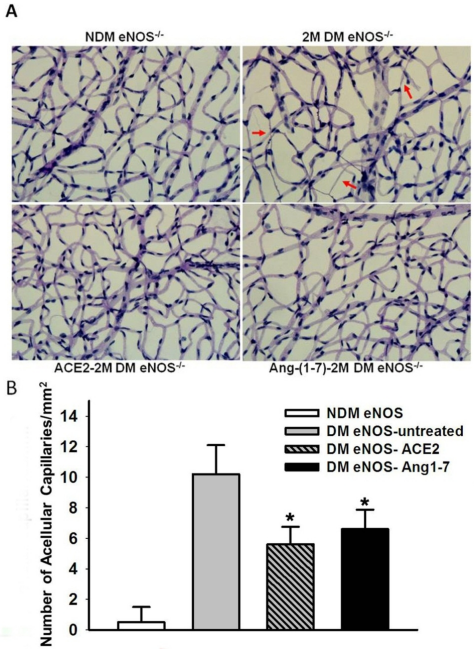

Figure 7. Evaluation of acellular capillary formation in untreated and AAV-ACE2/Ang-(1-7) treated retinas of diabetic mice. Treatments with ACE2 and Ang 1-7 vectors in the diabetic eNOS-/- mouse retinas reduced acellular capillaries. A: Representative images of trypsin-digested retinal vascular preparations from untreated non-diabetic eNOS-/-, ACE2 and Ang-(1-7) treated diabetic eNOS$^{-/-}$ mouse retinas (2 months after induced diabetes or the equivalent age in untreated controls. Arrows indicate the acellular capillaries. B. Quantitative measurements of acellular capillaries. The values on Y-axis represent the number of acellular capillaries per mm^2 retina. NDM: non-diabetes; DM: diabetes. N=6. *p<0.01 (versus untreated DM group). (From [108] with permission of Mol. Therapy).

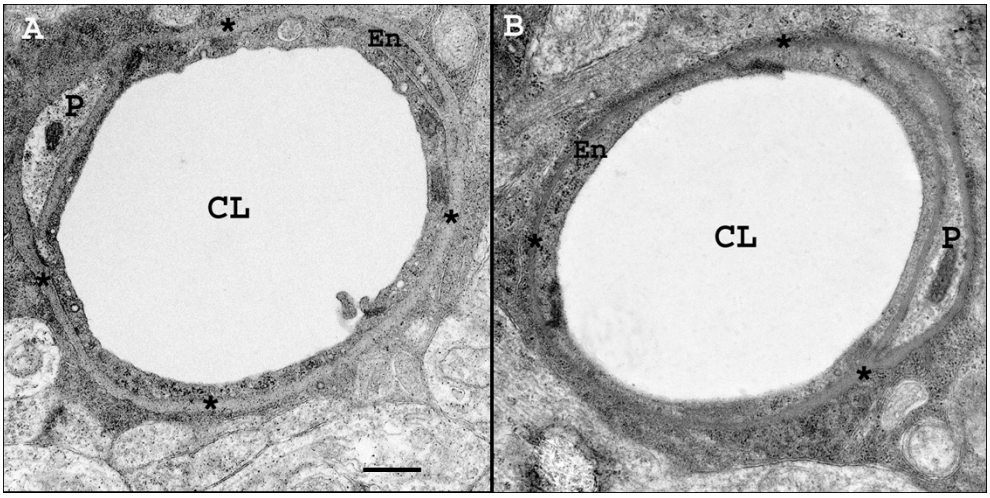

Figure 8. Transmission electron micrographs of retinal capillaries from a untreated 2 month diabetic eNOS$^{-/-}$ mouse eye (A), and an eye that received AAV-ACE2 treatment 2 weeks before STZ-induction of diabetes (B). CL: capillary lumen; En: endothelial cell; P: pericyte; * indicates the capillary basement membrane. Scale bar = 500nm. We have previously shown that the basement membranes of retinal capillaries from the diabetic eNOS$^{-/-}$ animals at two months after STZ induction of diabetes was significantly thicker than those from age-matched, non-diabetic animals [107]. The thickening of the basement membrane was prevented in the AAV-ACE2 treated eyes (73.81+17nm, versus 95.72+20 nm in untreated DM eye).

3.4. Protective role of ACE2/Ang (1-7) AAV gene delivery in a rat model of DR

3.4.1. Increased ACE2/Ang1-7 expression reduced the number of acellular capillaries in the diabetic rat retina

We also used STZ-induced diabetic SD rats as an additional animal model of diabetes to provide conceptual validation. We observed more than a 5-fold increase in the number of acellular capillaries in STZ-induced diabetic rat retinas at 14 month of diabetes. This increase was almost completely prevented by gene delivery of either ACE2 or Ang-(1-7) (Figure 9).

3.4.2. Increased expression of ACE2/Ang-(1-7) reduces oxidative damage in diabetic retina

Diabetes and its complications are associated with increased oxidative stress. We assessed oxidative damage measuring the levels of thiobarbituric acid-reactive substances (TBARs, is a marker for oxidative damage [110]) in the retina). Diabetes induced a significant increase in TBARs (Figure 10A) in eNOS$^{-/-}$ mouse retinas (Figure 10A). This increase is completely prevented by AAV-ACE2 or Ang-(1-7) treatment. Similar results were also obtained in SD rat retinas (Figure 10B).

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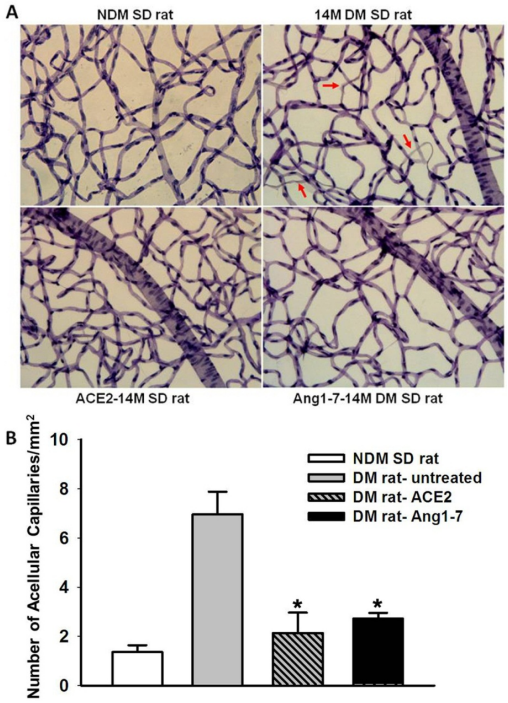

Figure 9. Evaluation of acellular capillary formation in untreated and ACE2/Ang-(1-7) AAV2 vector treated retinas of diabetic SD rats. (A) Representative images of trypsin-digested retinal vascular preparations from non-diabetic SD rat, untreated, ACE2 and Ang-(1-7) treated diabetic SD rat retinas (14 months after induced diabetes). (B) Quantitative measurements of acellular capillaries. Values on Y-axis represent the number of acellular capillaries per $mm^2$of retina. NDM: non-diabetes; DM: diabetes. N=6. *p<0.01(versus untreated DM group). (From [108] with permission of Mol. Therapy).

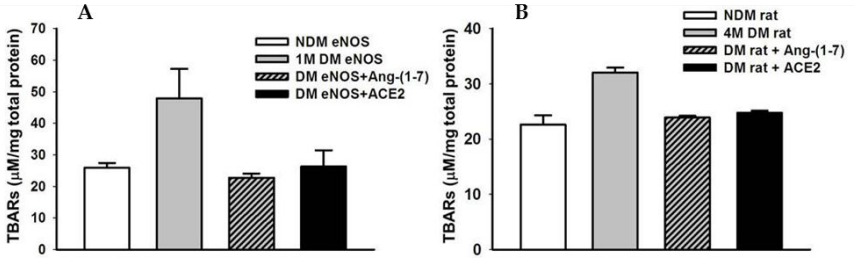

Figure 10. TBARs levels in eNOS-/- mouse retinas (A) and SD rat retinas (B). Diabetes resulted in increased TBARs levels in both $eNOS^{-/-}$ mouse retinas at1 month of diabetes and SD rat retinas at 4 months of diabetes. These increases were prevented by AAV-ACE2/Ang-(1-7) treatments. NDM: non-diabetes; DM: diabetes. N=6/group. *p<0.01(vs untreated DM). (From [108] with permission of Mol. Therapy).

3.5. Possible mechanisms of protective action of ACE2/Ang (1-7) in diabetic retina

We demonstrate that all the genes within the RAS are expressed in the retina, consistent with various previous reports (reviewed in [111] and references therein), and the expression levels of genes in the vasoconstrictive arm of RAS (renin, ACE, AT1R) are highly elevated in diabetic retinas, whereas there is initial increase in the expression of genes in the vascodilative axis (ACE2 and MAS) earlier in diabetes that attenuate over time with the progression of diabetes, thus tipping the balance towards more vasoconstrictive, proinflammatory, hypertrophic effects of RAS mediated by ACE/Ang II/AT1R axis. This is associated with increased ACE activity and Ang II levels in diabetic retinas, whereas ACE2 activity and Ang-(1-7) levels are not significantly changed, while the mRNA levels for ACE2 and Mas receptor are reduced under these conditions.

Furthermore, we show that enhanced expression of either ACE2 or Ang-(1-7) via AAV vector mediated gene delivery in the retina prevents diabetes-induced retinal vascular permeability, thickening of basement membrane, retinal inflammation, formation of acellular capillaries, and oxidative damage in both mouse and rat models of diabetic retinopathy. More importantly, these beneficial effects occur in the absence of systemic control of glucose, blood pressure, which is elevated in eNOS-/- mice [107], and other diabetic complications [112], suggesting that local RAS activation plays a significant role of pathogenesis of diabetic retinopathy, and can be modulated locally to restore the balance between the two counter-acting arms by enhancing the ACE2/Ang-(1-7)/MAS axis. These observations provide conceptual support that enhancing ACE2/ Ang-(1-7) axis maybe an effective strategy for the treatment of DR.

Although various components of RAS have been detected in retina, our study is the first to examine the expression levels of all known RAS genes during the progression of diabetes in the $eNOS^{-/-}$ mice, which exhibit accelerated retinopathy [107]. We show that increased expression of genes in the vasoconstrictive, proinflammatory axis of RAS (ACE, AT1R, renin, renin receptor) occur early, 14 days after STZ-induced diabetes. We have previously shown that increased retinal vascular permeability and gliosis are already detectable at this time point in diabetic $eNOS^{-/-}$ mouse retina, suggesting that local hyperactivity of the deleterious axis (ACE/Ang II/AT1R) may contribute to these pathological changes. We also measured ACE and ACE2 activities in diabetic $eNOS^{-/-}$ mouse retina. In contrast to a previous report which showed that ACE enzyme activity was decreased, whereas ACE2 enzyme activity was increased in diabetic rat retinas [113], we found that ACE activity is highly increased in diabetic retinas, whereas ACE2 activity remains unchanged. This discrepancy may be due to the difference in animal models or the time points at which these assays were performed.

The importance of the vasodeleterious axis of the RAS (ACE/ Ang II/ AT1R) in cardiovascular disease, as well as in diabetes and diabetic complications, is well established since ACE inhibitors (ACEi) and angiotensin receptor blockers (ARBs) are leading therapeutic strategies [20, 33-35]. However, the impact of the vasoprotective axis of the RAS remains poorly understood, particularly in the eye. The concept that shifting the balance of the RAS towards the vasodilatory axis by activation of ACE2 or its product, Ang-(1-7) is beneficial has been supported by many studies in cardiac, pulmonary, and vascular fibrosis [24, 36-38]. We

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show that increased expression of either ACE2 or Ang-(1-7) is protective in both eNOS$^{-/-}$ mouse and rat models of diabetic retinopathy. However the action of ACE2 and Ang-(1-7) may be different. The protective effect of ACE2 may result from reduced Ang II, by catalyzing its conversion to Ang-(1-7), thus increasing the level of Ang-(1-7), or combination of both. Indeed, in the AAV-ACE2 treated retina diabetes-induced elevation of Ang II is reduced and this is associated with an increased level of Ang-(1-7). On other hand, the fact that increased Ang-(1-7) expressed from AAV vector in the retina is also protective and that the Ang II level remained high in AAV-Ang-(1-7) treated retinas suggest that Ang-(1-7) can produce physiological responses that direct counteract these of Ang II, consistent with well-established effects of Ang-(1-7) [114].

It is interesting to note that ACE2 over-expression resulted in reduced Ang II and increased Ang-(1-7) levels as expected, but has no effect on ACE activity. However, over-expression of Ang-(1-7) had no effect on endogenous ACE2 activity, but significantly reduced ACE activity. Paradoxically, despite reduced ACE activity in AAV-Ang-(1-7) treated retinas, Ang II levels remained high. It is possible that other enzymes/pathways may be involved in Ang II formation in addition to ACE. One such candidate is chymase, which has been detected in vascular systems and other tissues including eye [115]. Another candidate is the receptor for prorenin and renin (pro/renin). It has been recently demonstrated that binding of pro/renin to its receptor, pro/renin receptor (PRR), causes its prosegment to unfold, thereby activating prorenin so that it is able to generate angiotensin peptides that stimulate the Ang II-dependent pathway [76]. Considering the fact that retina contains high level of prorenin, and its level is further increased in patients with diabetic retinopathy [52], this pathway likely contributes to increased Ang II level under diabetic conditions. The existence of multiple pathways for Ang II formation at the tissue level may explain the limited beneficial effects of classic RAS blockers, and may also lend support for thtoe notion that enhancing the protective axis of RAS (ACE2/Ang-(1-7)/Mas) may represent a more effective strategy for treatment of diabetic retinopathy and other diabetic complications.

AAV vector mediated gene therapy for ocular diseases has been studied in animal models for more than a decade. Reports focusing on retinal therapy include a wide variety of retinal degenerative animal models of corresponding human retinopathies, as well as the therapeutic effects of AAV-vector mediated expression of neuroprotective, anti-apoptotic, and anti-angiogenic agents in the retina [116]. In view of recent clinical trials in which AAV delivered RPE65 gene led to restoration of vision in human patients and other reports on successful trials on treatment of ocular diseases and inherited immune deficiencies (reviewed in [117] and references therein), gene therapy has emerged as promising approach and may become a standard treatment option for a wide range of diseases in the future. In particular, when considering that the diabetic individual experience this serious ocular complication for decades, a therapeutic strategy that is long-lasting and does not require patient compliance is particularly desirable. Thus, the delivery of ACE2 and/or Ang-(1-7) could serve as a novel gene therapeutic target for DR in combination with existing strategies to control hyperglycemic and insulin resistance states.

4. Summary

All genes of the RAS are locally expressed in the retina, establishing the existence of an intrinsic retinal RAS. It is clear that the expression of genes of the vasoconstrictive/pro-inflammatory/ proliferative/fibrotic (i.e., vasodeleterious) axis (ACE/Ang II/AT1R) is highly elevated, while the vasoprotective axis [ACE2/Ang-(1-7)/Mas] is decreased in the diabetic retina. We have demonstrated that increased expression of ACE2 or Ang-(1-7), two key members of the vasoprotective axis, via AAV-mediated gene delivery to the retina attenuates diabetes-induced retinal vascular pathology. Moreover, these beneficial effects of gene transfer occur without influencing the systemic hyperglycemic status. Thus, strategies enhancing the protective ACE2/Ang-(1-7) axis of RAS could serve as a novel therapeutic target for DR.

5. Implications and future challenges

Hyperactivity of RAS, resulting in elevated concentrations of the principal effector peptide Ang II, is central to pathways leading to increased vascular inflammation, oxidative stress, endothelial dysfunction and tissue remodeling in variety of conditions including heart failure, stroke, renal failure, diabetes and its associated complications including DR. As a result, RAS inhibitors are one of the first-line therapeutic agents for treating patients with cardiovascular diseases, metabolic syndrome, diabetes and diabetic complications. Ang II blockade has shown to be antiangiogenic [66, 118, 119], anti-inflammatory [120] and improves retinal function [65], and indeed Ang II blockade therapy for retinopathy is in several clinical trials [67, 68, 121]. Despite the clear beneficial effects of RAS blockers (ACE inhibitors [ACEi] and angiotensin receptor blockers [ARBs]) [70, 71, 122], end-organ damage still ensue in patients with diabetes. Overwhelming evidence now supports the notion thatactivation of RAS at tissue levels contributes to the development and progression of diabetic complications including DR, independent of circulating RAS regulation. However the precise molecular and cellular mechanisms as to how retinal RAS contributes to the development and progression of DR remain to be elucidated. Recent studies have also revealed the evolving complexity of RAS with a myriad cellular and intracellular pathways leading to formation of Ang II, as well as Ang II- independent signaling pathways resulting in hyperactivity of tissue RAS. The physiological implications of many of these components are still not well understood and new antagonists/agonists specific to these new components remain to be discovered. Nevertheless, our results clearly demonstrate that enhancing the protective axis of RAS (ACE2/Ang1-7/Mas) locally may be a better strategy for counteracting the effects of the pathological RAS activation than present systemic approaches. Furthermore, since AAV vector mediated gene delivery has been shown to be safe, and improve vision for extended periods of time after a single administration in several clinical trials, enhancing the endogenous protective axis of RAS (ACE2/Ang1-7/Mas) by local gene delivery, in combination with combination with existing strategies to control hyperglycemic and insulin resist-

ance states may represent a better strategy for preventing and treating diabetic complications such as diabetic retinopathy.

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