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Adeno-Associated Virus Overexpression of Angiotensin-Converting Enzyme-2 Reverses Diabetic Retinopathy in Type 1 Diabetes in Mice

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Angiotensin-converting enzyme (ACE)-2 is the primary enzyme of the vasoprotective axis of the renin angiotensin system that regulates the classic renin angiotensin system axis. We aimed to determine whether local retinal overexpression of adenoassociated virus (AAV)—ACE2 prevents or reverses diabetic retinopathy. Green fluorescent protein (*GFP*)—chimeric mice were generated to distinguish resident (retinal) from infiltrating bone marrow—derived inflammatory cells and were made diabetic using streptozotocin injections. Retinal digestion using trypsin was performed and acellular capillaries enumerated. Capillary occlusion by GFP^+ cells was used to measure leukostasis. Overexpression of ACE2 prevented (prevention cohort: untreated diabetic, 11.3 ± 1.4; ACE2 diabetic, 6.4 ± 0.9 per mm^2) and partially reversed (reversal cohort: untreated diabetic, 15.7 ± 1.9; ACE2 diabetic, 6.5 ± 1.2 per mm^2) the diabetes-associated increase of acellular capillaries and the increase of infiltrating inflammatory cells into the retina ($F4/80^+$) (prevention cohort: untreated diabetic, 24.2 ± 6.7; ACE2 diabetic, 2.5 ± 1.6 per mm^2; reversal cohort: untreated diabetic, 56.8 ± 5.2; ACE2 diabetic, 5.6 ± 2.3 per mm^2). In both study cohorts, intracapillary bone marrow—derived cells, indicative of leukostasis, were only observed in diabetic animals receiving control AAV injections. These results indicate that diabetic retinopathy, and possibly other diabetic microvascular complications, can be prevented and reversed by locally restoring the balance between the classic and vasoprotective renin angiotensin system. *(Am J Pathol 2016, 186: 1688—1700; http://dx.doi.org/10.1016/j.ajpath.2016.01.023)*

Microvascular complications of diabetes constitute a number of painful and debilitating conditions. Previously, we found that the bone marrow is susceptible to diabetic neuropathy,[1,2] which leads to denervation of stromal cells and altered secretion of growth factors and cytokines that ultimately shifts hematopoiesis toward a disproportionate increase of proinflammatory monocytes.[3,4] Consequently, monocytes are increased in the circulation and subsequently in tissues subject to diabetic complications, including retina, kidney, and brain.[5] Diabetic retinopathy (DR), the most common diabetic microvascular complication and the leading cause of blindness in working adults, is characterized by inflammation, leukostasis, and microangiopathy. Current treatment options are symptomatic and suboptimal, and prevention strategies are limited to aggressive regulation of blood pressure, lipids, and glucose.[6] However, studies suggest that manipulation of the systemic and local retinal renin angiotensin system (RAS) may offer new and powerful therapeutic options.

The levels of almost all proinflammatory, classic RAS components [prorenin, renin, angiotensinogen, angiotensin-converting enzyme (ACE)-1, angiotensin II (Ang II), and the Ang II type 1 receptor (AGTR1)] are significantly

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elevated in ocular tissues of diabetic patients[7,8] and animals.[9,10] Accordingly, systemically administered ACE1 inhibitors and AGTR1 antagonists attenuate retinal microvascular disease in diabetic rodents by decreasing vascular hyperpermeability, acellular capillaries, and the expression of angiogenic factors, such as vascular endothelial growth factor.[9,11] Despite the clear success of systemic RAS blockade in diabetic rodents, systemic suppression in humans has led to only marginal improvement. AGTR1 blockade decreased the incidence of DR in individuals with type 1 diabetes and improved mild and moderate DR in those with type 2 diabetes. However, AGTR1 blockade did not influence DR progression or DR at the more advanced stages once ischemic changes were present.[12,13] The most widely proposed explanation for this shortcoming is that the blood-retinal barrier limits access of therapeutic concentrations of systemically administered pharmaceuticals, such that suppression of the classic RAS in ocular tissues fails to occur.[10] From these data, the notion has emerged that restoring the balance between the classic RAS and its endogenous counterbalance, the vasoprotective RAS, represents a viable strategy for controlling DR in humans. The crucial enzyme in this axis is ACE2, which converts the vasoconstrictive and proinflammatory peptide, Ang II, into the vasodilatory and anti-inflammatory peptide, angiotensin-(1-7) [Ang(1-7)], which mediates its effects primarily through the receptor Mas.[14] Importantly, ACE1 inhibitors do not block the effects of ACE2.[15]

Thus, restoring the local retinal RAS balance, by bolstering the vasoprotective RAS, may diminish oxidative stress and chemokine production, decreasing inflammatory cell infiltration and preventing retinal vasodegeneration, the key histologic characteristic of DR. In this study, we asked whether local retinal overexpression of ACE2 could prevent or reverse the histologic features of DR in a mouse model of type 1 diabetes and whether a decrease in resident and bone marrow–derived inflammatory cells in the retina would occur.

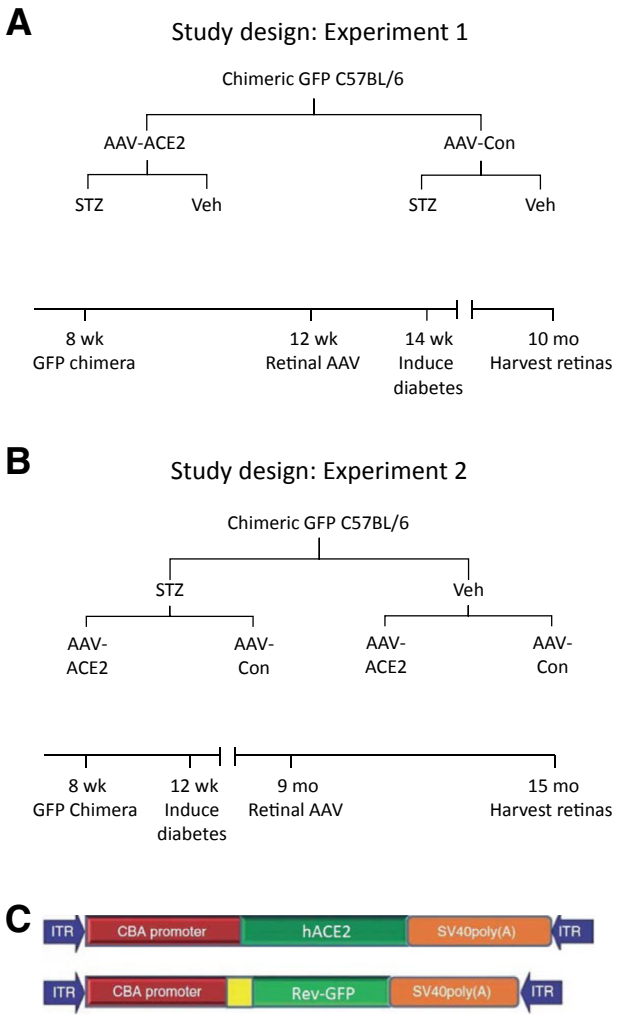

Figure 1 Experimental approach. **A:** Prevention cohort. **B:** Reversal cohort. **C:** Maps of the adenoassociated virus (AAV) vectors expressing the human *ACE2* gene (hACE2) and the control (Con) vector expressing a reverse sequence for green fluorescent protein (GFP). ACE2, angiotensin-converting enzyme; CBA, chicken β-actin promoter; ITR, inverted terminal repeat; STZ, streptozotocin; Veh, vehicle. Adapted from Verma et al.[10]

Materials and Methods

Experimental Approach

The experimental paradigm is shown in Figure 1A for the prevention cohort and in Figure 1B for the reversal cohort. Briefly, the prevention cohort consisted of C57BL/6 mice that underwent bone marrow transplantation with hematopoietic progenitor cells from GFP^+ mice. These GFP^+-chimeric mice received an intravitreal injection with adenoassociated virus (AAV)–ACE2 or AAV-reverse-*GFP* (control vector) and were subsequently made diabetic with streptozotocin (Figure 1A). This approach allowed us to test the hypothesis that retinal overexpression of ACE2 improves the diabetic phenotype of the retina, characterized by increased acellular capillaries and increased inflammatory cell expression. The reversal cohort tested the ability of retinal overexpression of ACE2 to reverse preexisting disease by administering streptozotocin 6 months before AAV injections (Figure 1B). Both studies used the same end points. The AAV constructs are shown in Figure 1C.

Generation of GFP-Chimeric Mice

We used two timeline sequences that enabled us to evaluate a prevention role (Figure 1A) and a reversal role (Figure 1B) for ACE2 overexpression. To determine whether retinal overexpression of ACE2 prevents the inflammatory bone marrow cell influx into the diabetic retina, C57BL/6-*GFP*-chimeric mice were generated. Male C57BL/6 mice ($n = 7$ per group), which were obtained commercially from The Jackson Laboratory (Bar Harbor, ME), were housed five per cage with unrestricted access to food and water with a 12:12 light:dark cycle. All procedures that involved mice were performed in accordance with the guidelines of the