Diabetic retinopathy (DR) is a microvascular complication of diabetes and a leading cause of vision loss in working-age adults in developed countries. During diabetes, hyperglycemia and oxidative stress upregulates a major angiogenic factor, vascular endothelial growth factor (VEGF-A or VEGF), which induces retinal neovascularization, vascular leakage, and perhaps macular edema (1,2). DR is also known as a chronic inflammatory disorder. During the early stage of DR, proinflammatory proteins, such as intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α (TNF-α), are upregulated, and increased leukostasis is observed (3–5). These early pathologic changes are associated with upregulation of VEGF (6–8).

Clinical observations that elevated VEGF levels are associated with DR have led to intensive studies on VEGF action over the past decade, and have resulted in anti-VEGF treatments as a major therapeutic strategy for DR. Surprisingly the role of VEGF in the pathogenesis of DR has not been well investigated at the cellular level. Since VEGF may be required for the maintenance and survival of retinal neurons (9–12), revealing cellular mechanism of VEGF actions becomes necessary to the safety and effectiveness of anti-VEGF therapies.

In the retina, VEGF is mainly expressed in Müller cells (13), endothelial cells (14), astrocytes (15), retinal pigment epithelium (RPE) (16), and ganglion cells (17). At present, the in vivo function of VEGF produced by these retinal cell-types remains largely uninvestigated. Although the role of retinal Müller cell-produced VEGF in DR was probed a decade ago (13), its function in the disease is unclear. An in vitro study demonstrated that Müller cells produce a large amount of VEGF in response to hypoxia (14). This has led to speculation that Müller cells are a major source of VEGF in DR and a major cellular target for the treatment of the disease. To determine the role of Müller cell-derived VEGF and dissect the cellular mechanism of DR, we generated conditional VEGF knockout (KO) mice by mating floxed VEGF mice with transgenic mice expressing Cre recombinase in retinal Müller cells (18,19), with a Cre/lox-based conditional gene KO approach (20). Using these conditional VEGF KO mice, we recently demonstrated that Müller cell-derived VEGF contributed significantly to ischemia-induced retinal neovascularization (21). This report describes our investigation of the role of Müller cell-derived VEGF in retinal inflammation and vascular leakage that occurs as a consequence of diabetes.

**RESEARCH DESIGN AND METHODS**

**Treatment of conditional VEGF knockout mice.** All animal experiments followed the guidelines established by the ARVO Statement for the Use of...
Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees. Conditional VEGF KO mice were generated by mating floxed VEGF mice with transgenic mice expressing Cre recombinase in retinal Müller cells, as described previously (18,19,21). Doxycycline was administered through the drinking water of pregnant mice at a concentration of 10 mg/kg in 5% sucrose from embryonic day 15 to postnatal day 1. To minimize the influence of genetic background, littersmates were used as controls in this study. PCR analysis of tail DNA was performed for genotyping mice using primer pairs (5'-CTT GCC CTA CAA GTA CAC TCT -3') and b (5'-TCC GTA CGA CCA ATT TAT GAG-3') to detect a 108-bp product for the wild-type (WT) allele and a 148-bp product for the floxed VEGF allele (21). PCR products were visualized in 5% agarose gel in 0.5x TBE buffer from embryonic day 15 to postnatal day 1. To minimize the influence of genetic background, littersmates were used as controls in this study. PCR analysis of tail DNA was performed for genotyping mice using primer pairs (5'-CTT GCC CTA CAA GTA CAC TCT -3') and b (5'-TCC GTA CGA CCA ATT TAT GAG-3') to detect a 108-bp product for the wild-type (WT) allele and a 148-bp product for the floxed VEGF allele (21). PCR products were visualized in 5% agarose gel in 0.5x TBE buffer. Mice were necropsied on day 18 postnatal day 1. The total number of ganglion nuclei in H&E-stained retinal sections was used to represent ganglion cell density.

**Statistical analysis**. All results that required statistical analysis were expressed as the mean ± SEM. Statistical significance was evaluated by Student’s t test with P value < 0.05 considered significant.

**RESULTS**

**Preparation of diabetic conditional VEGF knockout mice.** In an effort to generate human VMD2 promoter controlled tetracycline-inducible RPE-specific Cre mice (28), we identified a mouse line displaying Cre function predominantly localized to the retinal Müller cells (29). Further characterization demonstrated that this mouse line is feasible for conditional gene expression in Müller glia (19). The transgenic Cre mice (cre+) were mated with mice carrying a floxed VEGF (Vegf) allele (18) to generate the conditional Müller glial VEGF KO mice. Using primary cultures derived from the conditional VEGF KO mice, we determined that there was a 66.5% reduction of VEGF expression in Müller cells (21). Immunoblotting assays demonstrated a significant decrease (43.2%) in VEGF expression in the retina of conditional VEGF KO mice (cre+Vegf) compared with WT (cre VEGF) controls (Fig. 1A), confirming that Müller cells are a major source of VEGF in the retina.

Diabetes was induced by STZ-injection in conditional VEGF KO mice and WT controls. Blood glucose levels and average body weights of mice at the time of experiments are shown in Table 1. A significant elevation of blood glucose level and loss of body weight were observed in diabetic groups compared with nondiabetic controls (P < 0.001) 2 and 6 months after induction of diabetes. No significant changes in blood glucose levels or body weight were observed between conditional VEGF KO and WT mice under normal or diabetic conditions (Table 1), indicating that disruption of Müller cell-derived VEGF did not affect blood glucose levels or body weight. Under diabetic stress, expression of VEGF increased dramatically (2.16-fold) in WT mice (Fig. 1A). In contrast, disruption of Müller cell-derived VEGF resulted in a 51.4% reduction in VEGF (Fig. 1A). This result was supported by immunohistochemical analysis in conditional VEGF KO mice subjected to diabetic stress (Fig. 1C–D). As HIF-1α is upregulated in diabetic retinas and contributes to increased levels of VEGF (30), we verified the state of HIF-1α in conditional VEGF KO mice by measuring the expression of HIF-1α in diabetic retinas. Our results demonstrated a greater than twofold upregulation of HIF-1α (Fig. 1B) in diabetic retinas of both WT and conditional VEGF KO mice, which is consistent with a previous observation (31). However, no significant difference was observed in HIF-1α levels between WT and conditional VEGF KO mice, suggesting that the loss of Müller cell-derived VEGF did not alter diabetes-induced HIF-1α accumulation.

**Retinal inflammatory responses.** Leukostasis, a major parameter for inflammation and early pathologic changes in DR, was quantified by FITC-conjugated ConA staining assay 2 months after inducing diabetes (3). Under normal conditions, the number of adherent leukocytes in the retinal microvasculature of WT and conditional VEGF KO mice was negligible (data not shown). The number of adherent leukocytes in the retinal microvasculature of diabetic WT mice was elevated significantly and increased...
threefold compared with diabetic conditional VEGF KO mice (Fig. 2A–C).

To verify whether disrupting Müller cell-derived VEGF significantly reduced retinal inflammation, we examined the expression of proinflammatory markers ICAM-1 and TNF-α by immunoblotting. Retinas from diabetic WT mice demonstrated a greater than twofold increase in the levels of both ICAM-1 and TNF-α compared with those from nondiabetic animals (Fig. 2D–E). No apparent increases in ICAM-1 and TNF-α were observed in diabetic conditional VEGF KO mice. These results suggest that the loss of Müller cell-derived VEGF significantly reduces inflammation in diabetic retinas.

The transcription factor NF-κB is activated in diabetes.

### Table 1

**Body weights and blood glucose levels of normal and diabetic mice**

| Group       | Body weight (g) | Blood glucose (mg/dl) |
|-------------|-----------------|-----------------------|
|             | 2 months | 6 months | 2 months | 6 months |
| Diabetic WT | 21.46 ± 1.60*  | 23.03 ± 2.35*        | 461.43 ± 66.66* | 497.71 ± 6.05* |
| Diabetic KO | 20.86 ± 1.22*  | 23.29 ± 0.63*        | 432.86 ± 65.15* | 480.86 ± 50.65* |
| Nondiabetic WT | 28.17 ± 2.28  | 32.43 ± 1.66         | 178.14 ± 33.35 | 184.14 ± 22.44 |
| Nondiabetic KO | 28.04 ± 2.08  | 31.86 ± 2.53         | 199.29 ± 24.28 | 176.14 ± 12.23 |

Data are means ± SE, n = 20–30. *P < 0.001 compared with nondiabetic WT or KO.
and plays a major role in diabetes-induced early pathologic changes in DR (32,33). To determine the relationship between Müller cell-derived VEGF and NF-κB in diabetic retinas, we investigated the level and phosphorylation state at serine 276 of NF-κB in diabetic retinas, and plays a major role in diabetes-induced early pathologic changes. To determine the relationship between Müller cell-derived VEGF and NF-κB in diabetic retinas, we investigated the level and phosphorylation state at serine 276 of NF-κB p65 subunit, as phosphorylation of this subunit increases transcriptional activity of NF-κB (34). Although there was no detectable difference in the overall level of NF-κB p65 subunit between diabetic conditional VEGF KO and control mice, loss of Müller cell-derived VEGF resulted in a twofold decrease in phosphorylated NF-κB p65 in the retinas 2 months after diabetes (Fig. 2F), suggesting that Müller cell-derived VEGF regulates NF-κB transcriptional activity in DR.

Retinal vascular histopathology and leakage. To determine the role of Müller cell-derived VEGF in vascular leakage, we performed immunoblotting for extravascular albumin content in the retinas and vitreous from PBS-perfused mice 6 months after inducing diabetes. The extravascular albumin content in diabetic WT mice was 2.5-fold higher than that in nondiabetic WT mice (Fig. 3A). However, there was a 58.9% reduction of extravascular albumin in the retinas and vitreous of diabetic conditional VEGF KO mice (Fig. 3A). Using FITC-labeled albumin, we visualized and quantified vascular leakage in conditional VEGF KO mice 6 months after inducing diabetes (Fig. 3B–F). Compared with diabetic WT mice, diabetic conditional VEGF KO mice had significantly fewer areas of leaked FITC-labeled albumin (arrows in Fig. 3C and E) in their retina. Computer-assisted quantitative analysis demonstrated a similar level of reduction (61.5%) of FITC-labeled albumin leakage in diabetic conditional VEGF KO mice compared with diabetic WT controls (Fig. 3F).

To determine the mechanism of Müller cell-derived VEGF in diabetes-induced vascular leakage, we examined the expression of occludin and ZO-1, two important tight junction proteins. Although diabetes caused 39.2% and 58.1% decreases of retinal occludin and ZO-1 in WT mice, virtually no change in retinal occludin and ZO-1 contents were observed in diabetic conditional VEGF KO mice (Fig. 4A and B). Compared with WT controls, the diabetic conditional VEGF KO mice demonstrated 59.7% and 130.3% increases of occludin and ZO-1, respectively, in their retinas (Fig. 4A–B). This result was confirmed by immunohistochmical analysis of ZO-1 in retinal vessels (Fig. 4C–F). Our results suggest that the loss of Müller cell-derived VEGF significantly reduced diabetes-induced retinal vascular leakage by attenuating the depletion of occludin and ZO-1.

To assess retinal vascular lesions, we quantified the number of acellular capillaries in trypsin digestion assay and examined basement membrane thickness by TEM in conditional VEGF KO mice 6 months after inducing diabetes. The diabetic conditional VEGF KO mice had significantly fewer acellular capillaries than diabetic WT controls (Fig. 5A–C). However, we did not observe significant differences in basement membrane thickness between diabetic conditional VEGF KO and WT mice and between diabetic and nondiabetic mice (Fig. 5D–F).

Morphometric and ultrastructural analyses. To determine if the loss of Müller cell-derived VEGF in the condi-
Dr. (13). Because of the lack of appropriate animal models, cells have long been suspected as a major contributor to diabetes. The origins of pathogenic VEGF become paramount. Müller major therapeutic strategy for DR, revealing the cellular role in retinal neuron survival and choriocapillaris fenestrations (9–12) and anti-VEGF treatments have become a crucial for BRB function, we measured retinal tight junction proteins occludin and ZO-1 (Fig. 4) to determine how increases in Müller cell-derived VEGF might result in diabetes-induced vascular leakage. Our result that diabetes-induced depletion of occludin and ZO-1 was attenuated in conditional VEGF KO mice supports a role for Müller cell-derived VEGF in diabetes-induced reorganization of tight junctions (37–39). Our results also suggest that reducing Müller cell-derived VEGF attenuates capillary acellularity (Fig. 5A–C), a relatively early vascular lesion in diabetic rodent retinas. Since VEGF is a survival factor for endothelial cells, one might expect that reducing VEGF levels might accelerate endothelial loss and the development of acellular capillaries in diabetic retinas, which is contrary to our observation. However, our result is consistent with that in a study demonstrating an increase in acellular capillaries in transgenic mice overexpressing VEGF165 from rod photoreceptors (40). As inflammation may play a central role in developing pathologic changes, including acellular capillaries in DR (41), we speculate that the diabetes-induced inflammatory responses may play a more prominent role in endothelial death and capillary acellularity and that the reduced inflammatory responses in diabetic conditional VEGF KO mice may attenuate the development of acellular capillaries. Furthermore, as we did not disrupt VEGF in endothelial cells, autocrine endothelium-derived VEGF signaling could play a role in promoting endothelial survival and in reducing capillary acellularity in diabetic conditional VEGF KO mice. Based on our results, we cannot conclude if the Müller cell-derived VEGF plays a role in the thickening of basement membranes, as we did not observe significant change in this process between diabetic mice and nondiabetic controls 6 month after STZ-injection (Fig. 5A–C). This result is in agreement with a previous observation in similar aged diabetic and nondiabetic mice (42).

Leukostasis, adhesion of leukocytes to the vascular wall, is increased in the diabetic retina and results in retinal vascular occlusion (5). The diabetes-induced leukostasis is mediated via upregulation of ICAM-1 and its ligand CD18 (3). Disruption of ICAM-1 and CD18 in mice results in decreases in both leukostasis and vascular permeability (41). It has been shown that VEGF increases expression of ICAM-1 in endothelial capillaries and intra-}

**DISCUSSION**

VEGF is produced by multiple cellular origins in the retina and has paracrine activity. To date, no in vivo study has convincingly demonstrated the cellular origin of VEGF induced in DR. Since VEGF has been suggested to play a role in retinal neuronal survival and choriodcapillaris fenestration (9–12) and anti-VEGF treatments have become a major therapeutic strategy for DR, revealing the cellular origins of pathogenic VEGF becomes paramount. Müller cells have long been suspected as a major contributor to DR (13). Because of the lack of appropriate animal models (35), in vivo function of Müller cell-derived VEGF in DR remained unaddressed until recently when we developed an inducible Cre/lox system to delete VEGF from Müller cells (19).

Increased VEGF levels in DR are responsible for blood-retina barrier (BRB) breakdown and lead to vascular leakage (36). However, the causative role of Müller cell-derived VEGF in diabetes-induced BRB breakdown has not been established. In this study, we demonstrate that the loss of Müller cell-derived VEGF significantly inhibits diabetes-induced vascular leakage (Fig. 3). Since the integrity of tight junction proteins in endothelial cells is crucial for BRB function, we measured retinal tight junc-

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**FIG. 3.** Analysis of retinal vascular leakage in conditional VEGF KO mice 6 months after the onset of diabetes. A: Immunoblotting analysis of retinal extravascular albumin in conditional VEGF KO mice. B and E: Microscopic images of retinal flat-mounts (B and C) and enlarged areas (D and E) showing fluorescently labeled albumin (arrows) in diabetic retinas of WT and conditional VEGF KO mice after injection with FITC-labeled albumin. Scale bars represent 100 μm. F: Computer-assisted quantitative analysis of FITC-labeled albumin in diabetic retinas of conditional VEGF KO mice. Error bar: SEM. ***P < 0.001. Loss of Müller cell-derived VEGF caused a significant reduction in diabetes-induced retinal vascular leakage.
vitreal injection of VEGF upregulates the level of retinal ICAM-1 (8). TNF-α is an inflammatory cytokine which is upregulated in diabetic retinas. It has also been reported that an inhibitor of TNF-α is capable of reducing diabetes-induced leukostasis and BRB breakdown (41) and that anti-VEGF treatment inhibited the expression of TNF-α (43). In the current report, we demonstrate that disrupting Müller cell-derived VEGF inhibits diabetes-induced leukostasis and overexpression of ICAM-1 and TNF-α (Fig. 2), early pathologic changes in DR. We also demonstrated that disruption of Müller cell-derived VEGF caused a significant attenuation of diabetes-induced depletion of tight junction proteins occludin and ZO-1. ns, not significant.

FIG. 4. Analysis of tight junction protein expression in retinas of conditional VEGF KO mice 6 months after diabetes. **A** and **B**: Immunoblotting and quantification of tight junction protein occludin and ZO-1 in retinas of diabetic conditional VEGF KO mice. Error bar: SEM. ***P < 0.001. **C**–**F**: Confocal microscopic analysis of immunostained tight junction protein ZO-1 in retinal vasculature (**C** and **D**) from the diabetic retinas of conditional VEGF KO mice. Scale bars represent 50 μm. Loss of Müller cell-derived VEGF caused a significant attenuation of diabetes-induced depletion of tight junction proteins occludin and ZO-1. ns, not significant.
throughout this study. Very low levels of retinal inflammation and vascular leakage and the inability for other retinal VEGF-producing cells to compensate the loss of VEGF in diabetic conditional VEGF KO mice only suggest that Müller cell-derived VEGF is a primary source of VEGF involved in the diabetes-induced retinal inflammation and vascular leakage. However, we cannot exclude the possibility that VEGF derived from other retinal cells contrib-

**FIG. 5.** Retinal vascular histopathology in the conditional VEGF KO mice 6 months after inducing diabetes. A–C: Analysis of acellular capillaries (arrowheads) with trypsin digestion in diabetic retinas of the conditional VEGF KO mice and WT controls. Error bar: SEM. ***P < 0.001. Scale bar in B represents 50 μm. Scale bars in D–F represent 1 μm. D–F: Representative TEM images for basement membrane thickness in retinal vascular capillaries. Although the number of acellular capillaries was significantly decreased in diabetic conditional VEGF KO mice, no apparent change in the thickness (arrowheads) of basement membrane was observed among all mice examined.

**FIG. 6.** Retinal morphology and morphometry in conditional VEGF KO mice 6 months after onset of diabetes. A and B: Morphology of H&E-stained retinal sections. C: Thickness of outer nuclear layer, inner nuclear layer, and total retina. D and E: Representative TEM images showing no detectable defects in diabetic retina of conditional VEGF KO mice. F: Average number of ganglion cells. Scale bar equals 50 μm in B and 1 μm in D and E. No detectable changes in retinal thickness, numbers of ganglion cells, and retinal ultrastructure were observed in diabetic conditional VEGF KO mice. Ch, choroid; CC, choriocapillaris; GC, ganglion cells; INL, inner nuclear layer; ns, not significant; ONL, outer nuclear layer; P, photoreceptors.
utes to diabetes-induced retinal inflammation and vascular leakage in the presence of Müller cell-derived VEGF, which may serve as a key to the “pathologic threshold.”

In this study, HIF-1α expression was upregulated in the retinas of diabetic conditional VEGF KO mice and WT controls, which is consistent with a previous finding that HIF-1α is increased in diabetic retina and that inducing HIF-1α contributes to the increased levels of VEGF (30). However, loss of Müller cell-derived VEGF did not alter the expression of HIF-1α in diabetic retina (Fig. 1B). This result indicates that the phenotypic changes observed in the conditional VEGF KO mice are not attributed to the altered expression of the upstream regulator HIF-1α. Therefore, the reduction of diabetes-induced retinal inflammation and vascular leakage in the conditional VEGF KO mice appears to be a direct consequence of reduced VEGF expression. This result may suggest that HIF-1α is a potential target for a more stringent therapeutic strategy to treat DR, which is supported by a recent finding that inhibition of HIF-1α has an effect on ischemia-induced vascular leakage (46).

We demonstrated that disrupting Müller cell-derived VEGF did not cause significant changes in retinal morphology/morphometry in diabetic conditional VEGF KO mice 6 months after STZ-injection (Fig. 6), suggesting that the reduction in VEGF level may not be sufficient to cause accelerated retinal neuron loss within the time frame. Therefore, the phenotypic changes described herein can be directly attributed to the disruption of Müller cell-derived VEGF, not the loss of retinal neurons. Since Cre expression in retinal ganglion cells is negligible (19), the reduced retinal inflammation and vascular leakage in our conditional VEGF KO mice is not likely caused by disruption of VEGF in ganglion cells. The inability for ganglion cells to compensate for the loss of Müller cell-produced VEGF in conditional VEGF KO mice may suggest that the major function of ganglion cell-derived VEGF is not pathogenic, and is perhaps important for neuronal survival under stress conditions (10,11). A significant decrease of VEGF produced by retinal endothelial cells under hyperglycemia suggests a minor role for endothelial-cell–derived VEGF in the pathogenesis of DR (47). The role of the RPE-derived VEGF in DR is intriguing. As the RPE barrier is tighter than the endothelial barrier, the significance of the RPE-derived VEGF/outer BRB breakdown in DR has not been focused sufficiently. Although the RPE-derived VEGF has been suggested to play a role in RPE-choriocapillaris interaction (9), RPE-derived soluble VEGF may regulate outer BRB function (37). The breakdown of the RPE barrier has been observed in diabetic patients (48). Some DR patients with no significant changes in the endothelial barrier have eventually demonstrated a severe outer BRB breakdown (49). However, the significance of the RPE-derived VEGF and RPE barrier dysfunction in overall pathologic changes in DR remains largely uninvestigated, which is caused by difficulties in distinguishing the leakage from each BRB. With the development of cell-specific gene expression systems, a better understanding of the outer BRB dysfunction in DR is likely to emerge shortly.

In summary, the results from the current investigation and a previous study demonstrating a significant role for Müller cell-derived VEGF in retinal neovascularization (21) suggest that the Müller cell-derived VEGF plays a causative role in major pathologic changes in DR, including retinal neovascularization, vascular leakage, vascular lesions, and inflammation. Therefore, Müller cells are a primary cellular source for signals that mediate pathologic changes in DR. As VEGF is a target for clinical trials to treat DR, this information is valuable to the design of the delivery mechanisms for anti-VEGF therapies.

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