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Activation of the Wnt Pathway Plays a Pathogenic Role in Diabetic Retinopathy in Humans and Animal Models

Ying Chen,^{*†‡} Yang Hu,^{*†‡} Ti Zhou,^{*†§}
Kevin K. Zhou,^{*†‡} Robert Mott,^{*†‡} Mingyuan Wu,^{†‡}
Michael Boulton,[¶] Timothy J. Lyons,^{†‡}
Guoquan Gao,[§] and Jian-xing Ma^{*†‡}

From the Departments of Cell Biology,^{*} and Medicine,[†] and the Harold Hamm Oklahoma Diabetes Center,[‡] University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; the Department of Biochemistry,[§] Zhongshan University, Guangzhou, China; and the Department of Anatomy and Cell Biology,[¶] University of Florida, Gainesville, Florida

Although Wnt signaling is known to mediate multiple biological and pathological processes, its association with diabetic retinopathy (DR) has not been established. Here we show that retinal levels and nuclear translocation of β -catenin, a key effector in the canonical Wnt pathway, were increased in humans with DR and in three DR models. Retinal levels of low-density lipoprotein receptor-related proteins 5 and 6, coreceptors of Wnts, were also elevated in the DR models. The high glucose-induced activation of β -catenin was attenuated by aminoguanidine, suggesting that oxidative stress is a direct cause for the Wnt pathway activation in diabetes. Indeed, Dickkopf homolog 1, a specific inhibitor of the Wnt pathway, ameliorated retinal inflammation, vascular leakage, and retinal neovascularization in the DR models. Dickkopf homolog 1 also blocked the generation of reactive oxygen species induced by high glucose, suggesting that Wnt signaling contributes to the oxidative stress in diabetes. These observations indicate that the Wnt pathway plays a pathogenic role in DR and represents a novel therapeutic target. (*Am J Pathol* 2009, 175:2676–2685; DOI: 10.2353/ajpath.2009.080945)

Diabetic retinopathy (DR), the leading cause of blindness in the working age population, represents a common concern in types 1 and 2 of diabetes mellitus (DM).¹ Accumulating evidence suggests that DR is a chronic inflammatory disorder.² Retinal inflammation is believed

to play a causative role in vascular leakage, which can lead to diabetic macular edema, and in retinal neovascularization (NV). It has been shown that levels of soluble intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 are significantly higher in the vitreous from patients with proliferative diabetic retinopathy than in nondiabetic vitreous.^{3,4} Increased ICAM-1, vascular cell adhesion molecule-1, and e-selectin levels were found in the serum from patients with diabetic microangiopathy.^{5–7} In diabetic animal models, increased retinal ICAM-1 expression is believed to be responsible for leukocyte adhesion or leukostasis and increased vascular permeability. Leukostasis is believed to contribute to capillary nonperfusion and local ischemia, which subsequently induces the overexpression of vascular endothelial growth factor (VEGF).^{8–11} Increased VEGF levels are responsible for the retinal vascular leakage and retinal NV.^{12,13} Recent studies have indicated that oxidative stress, induced by hyperglycemia, contributes to retinal inflammation in diabetes.^{14,15} However, the pathogenic mechanisms by which diabetes and oxidative stress induce inflammation are not certain at the present time.

Wnts are a group of secreted, cysteine-rich glycoproteins, which bind to a coreceptor complex of frizzled (Fz) receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) and regulate expression of a number of target genes through an intracellular signaling pathway, namely the Wnt pathway.¹⁶ In the absence of Wnt ligands, β -catenin, a down-stream effector of the canonical Wnt pathway, is phosphorylated by a protein

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Y.C. and Y.H. contributed equally to this work.

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Address reprint requests to Jian-xing Ma, 941 Stanton L. Young Blvd., BSEB 328B, Oklahoma City, OK 73104. E-mail: jian-xing-ma@ouhsc.edu.

complex containing glycogen synthase kinase-3 β . The phosphorylated β -catenin is constantly degraded, to prevent its accumulation. On binding of certain Wnts to the Fz-LRP5/6 coreceptors, phosphorylation of β -catenin is inhibited, which prevents the degradation of β -catenin and results in its accumulation.¹⁷ β -catenin is then translocated into the nucleus, associates with T-cell factor for DNA binding, and regulates expression of target genes including VEGF.^{18–20}

LRP5/6 are known to play a critical role in Wnt/ β -catenin signaling.^{21–23} On binding with Wnt ligands, LRP6 dimerizes with Fz receptor, which is the first and essential step in activation of the Wnt pathway. The cytoplasmic domain of LRP6 has multiple modular phosphorylation sites, and phosphorylation of LRP6 is an essential event for activation of the canonical Wnt pathway, as the phosphorylation of LRP6 promotes the recruitment of the scaffold protein Axin, and thus, activates the canonical Wnt signaling pathway.^{24,25}

Recent evidence indicates that the canonical Wnt pathway plays a role in angiogenesis.^{26,27} Extensive studies have shown that the Wnt pathway up-regulates nuclear factor κ B, signal transducer and activator of transcription 3 and a number of inflammatory factors, and thus, plays a key role in inflammation.^{28,29} The present study investigated the possible role of the Wnt signaling pathway in DR by using human donor eyes, diabetic animal models, and cultured cells.

Materials and Methods

Human Tissue

Normal and diabetic eyes fixed in 10% neutral buffered formalin (NBF) within 12 hours postmortem and were obtained from National Diseases Research Interchange (Philadelphia, PA) with full ethical approval for use in research. Diabetic eyes were categorized according to a standardized protocol.³⁰

Animals

Akita mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Brown Norway rats were purchased from Charles River (Wilmington, MA). Care, use, and treatment of all animals in this study were in strict agreement with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology.

Isolation and Culture of Bovine Retinal Capillary Endothelial Cells and Pericytes

Bovine retinal capillary endothelial cells (RCEC) and pericytes were isolated from bovine eyes, as described by Grant and Guay³¹ with some modifications. At passage 3 or 4, the purity of the cells in culture was determined. The identity of RCEC was confirmed by a characteristic cobblestone morphology and the incorporation of acetylated

low-density lipoprotein labeled with a fluorescent probe, Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Biomedical Technologies, Inc; Stoughton, MA). Purity of the pericyte culture was determined by immunostaining using a fluorescein isothiocyanate-conjugated antibody specific to α -smooth muscle actin (Sigma; St. Louis, MO).

Induction of Diabetes in Rats

Experimental diabetes was induced by an intraperitoneal injection of streptozotocin (STZ) (50 mg/kg in 10 mmol/L of citrate buffer; pH 4.5) into anesthetized Brown Norway rats (8 weeks of age) after an overnight fast. Age-matched control rats received an injection of citrate buffer alone for nondiabetic control. Blood glucose levels were measured 48 hours after the STZ injection and monitored weekly thereafter. Only the animals with glucose levels >350 mg/dl were considered diabetic.

The Oxygen-Induced Retinopathy Model and Analysis of Retinal NV

The oxygen-induced retinopathy (OIR) model was induced in Brown Norway rats as described previously.³² Quantification of preretinal vascular cells was described by Smith et al.³³ Briefly, the eyes of eight rats from each group at postnatal day 18 (P18) were enucleated, fixed with 10% formaldehyde, sectioned, and then stained with H&E. The nuclei of vascular cells on the vitreal side of the retina were counted under a light microscope in a double-blind study. Ten sagittal sections from each eye were examined, and cell numbers were averaged in each group of animals. The average number of preretinal vascular nuclei was compared with that in the control group by using Student's *t*-test.

Retinal Angiography with High-Molecular-Weight Fluorescein

Rats at P18 were anesthetized with 10 mg/kg xylazine plus 75 mg/kg ketamine i.p. and perfused with 50 mg/ml high molecular weight fluorescein isothiocyanate-dextran (molecular weight 2×10^6 ; Sigma) via intraventricular injection as described by Smith et al.³³ The animals were immediately euthanized. The eyes were enucleated and fixed with 4% paraformaldehyde in PBS for 10 minutes. The retina was then separated from the eyecup and fixed with 4% paraformaldehyde for 3 hours. Several incisions were made to the retina, which was flat-mounted on a gelatin-coated slide. The vasculature was then examined under a fluorescence microscope (Axioplan2 Imaging; Carl Zeiss; Jena, Germany).

Immunohistochemistry

Immunohistochemistry was performed as described.³⁴ The primary antibodies specific for LRP5/6 (Abcam; Cambridge, MA) and hypoxia-inducible factor-1 α (HIF-

1 α) (Santa Cruz Biotechnology; Santa Cruz, CA) were used at a dilution of 1:200, and antibody for β -catenin (Cell Signaling Technology, Danvers, MA) at a dilution of 1:300. The secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, Inc; West Grove, PA) and Texas Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory) at a dilution of 1:200.

Measurement of Reactive Oxygen Species Generation

Cellular oxidative stress was determined by measuring intracellular reactive oxygen species (ROS) generation.^{35,36} Briefly, the treated and untreated cells at a density of 2×10^6 cells/ml were incubated with freshly prepared 5-(and 6-)chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂-DCF-DA) at 37°C in the dark. The CM-H₂-DCF-DA-loaded cells were rinsed twice in PBS and analyzed immediately by fluorometer at 488 nm excitation and 530 nm emission. Data were expressed as fluorescence intensity in arbitrary units from the average of three separate experiments.

Intravitreal Injection of Dickkopf Homolog 1

Briefly, animals were anesthetized with a 50:50 mix of ketamine (100 mg/ml) and xylazine (20 mg/ml), and their pupils were dilated with topical application of phenylephrine (2.5%) and tropicamide (1%). A sclerotomy was created approximately 0.5 mm posterior to the limbus with a blade, and a glass injector (~33 gauge) connected to a syringe filled with 3 μ l of Dickkopf homolog 1 (DKK1) with desired concentrations or bovine serum albumin (BSA) was introduced through the sclerotomy into the vitreous cavity.

Soluble ICAM-1 Enzyme-Linked Immunosorbent Assay

A commercial soluble ICAM-1 (sICAM-1) enzyme-linked immunosorbent assay kit (R&D Systems, Inc; Minneapolis, MN) was used to measure sICAM-1 levels in mouse retinal tissues, following the manufacturer's protocol. The samples from mouse tissues were diluted 10 times to ensure that the sICAM-1 concentration fell within the range of the sICAM-1 standard curves.

Western Blot Analysis

The same amounts of retinal proteins from each rat or mouse were used for Western blot analysis using specific primary antibodies for each protein and blotted with a horseradish peroxidase-conjugated secondary antibody.³⁴ The signal was developed with a chemiluminescence detection kit (ECL; Amersham International; Piscataway, NJ). Blots were then stripped and reblotted with an antibody specific for β -actin.

The primary antibodies used are specific for LRP5/6 (Santa Cruz Biotechnology) and HIF-1 α (Santa Cruz Biotechnology) at a dilution of 1:500, and the anti- β -catenin antibody (Cell Signaling Technology) at a dilution of 1:3000.

Vascular Permeability Assay

Vascular permeability was quantified by using Evans blue as a tracer as described previously.³⁷

Results

The Wnt Pathway Is Activated in the Retina of Human Patients with DR

To evaluate the activation status of the Wnt pathway in the retina of diabetic patients, we have measured retinal levels of total β -catenin, an essential effector of the canonical Wnt pathway. Ocular sections from six diabetic donors with nonproliferative diabetic retinopathy (NPDR) and those from five nondiabetic donors were stained for β -catenin by using immunohistochemistry. Under the same development intensity, there was a statistically significant increase in β -catenin staining intensity in the inner retina from the donors with NPDR, while there were

Figure 1. Activated Wnt signaling in the human retina with DR. Retinal sections from five non-DM donors and six diabetic donors with NPDR were immunostained with an antibody for β -catenin. The signal was developed with the diaminobenzidine method (brown color). Representative retinal images from two non-DM (A and B) and two NPDR donors (C and D) showed more intensive signals of β -catenin in the inner retina and increased β -catenin staining in the nuclei of the retinal cells from the DM-NPDR donors, compared with that from the non-DM subjects. Scale bar = 20 μ m. **E:** β -catenin signal was quantified by using morphometric analysis software and expressed as arbitrary units (mean $\pm$ SD). ** $P < 0.01$.

only basal levels of β -catenin signal in the retina from the nondiabetic donors (Figure 1, A–E). Moreover, immunohistochemistry showed that the donors with NPDR had more intensive β -catenin signals (brown color) in the nuclei of the retinal cells, compared with that in the nondiabetic donors (Figure 1), suggesting increased nuclear translocation of β -catenin in the retinas from patients with NPDR.

Activation of the Wnt Pathway in the Retinas of Akita Mice, STZ-Induced Diabetic Rats, and Rats with OIR

To confirm the activation of the Wnt pathway in the retina of DR animal models, we have measured β -catenin levels in the retinas from Akita mice, a genetic model of type-1 diabetes, STZ-induced diabetic rats, and in OIR rats, a model of ischemia-induced retinal NV. As shown by Western blot analysis, β -catenin levels were elevated in the retinas from Akita mice at the age of 16 weeks, compared with that in their nondiabetic littermates (Figure 2A). Similarly, retinal β -catenin levels were also elevated in STZ-diabetic rats at 16 weeks after the induction of diabetes, compared with age-matched nondiabetic rats (Figure 2B). In rats at the age of postnatal day 16 (P16) under constant normoxia, low levels of β -catenin were detected in the retina, while the OIR rats at the same

age showed dramatically increased β -catenin levels in the retina (Figure 2C).

To identify the cellular location of the β -catenin accumulation, ocular sections from the eyes of Akita mice, STZ-diabetic rats, OIR rats, and their respective controls were stained with an antibody specific for β -catenin by using immunohistochemistry. More intensive β -catenin signals (green color) were detected in the inner retinas of the Akita mice, STZ-diabetic rats, and OIR rats, when compared with their respective controls (Figure 2, D–Q). Increased β -catenin signals in the nuclei of retinal cells were also observed in the diabetic animals and OIR rats (Figure 2, D–Q).

Increased Retinal Levels of LRP5/6 in Diabetic and OIR Rats

To further assess the activation status of the Wnt pathway, retinal levels of LRP5/6, coreceptors in the Wnt pathway, were measured by Western blot analysis. The results showed that retinal levels of LRP6 were higher in the retinas from STZ-induced diabetic rats at 16 weeks following the onset of diabetes than that in nondiabetic controls (Figure 3A). Similarly, retinal LRP6 levels were also elevated in the retinas from OIR rats at the age of P16, compared with age-matched normoxia controls (Figure 3B).

Figure 2. Increased β -catenin levels in the retinas of Akita mice, STZ-induced diabetic rats, and OIR rats. The retinas from Akita mice at 16 weeks of age, STZ-DM rats at 16 weeks following the STZ injection, OIR rats at the age of P16, and age-matched nondiabetic or normoxic controls were used for Western blot (A–C) and immunohistochemistry (D–Q) analyses of β -catenin. A–C: The same amount (50 μ g) of retinal proteins from each animal was blotted with an antibody specific for β -catenin. The membranes were stripped and reblotted with an antibody for β -actin. Each lane represents an individual animal. D–Q: Representative retinal sections from Akita mice (G–I) and their nondiabetic littermates (D–F), STZ-DM rats (M–O) and non-DM rats (J–L), OIR rats (Q), and age-matched normal rats maintained under constant normoxia (P) were immunostained with an antibody for β -catenin. F, I, L, and O: The nucleus was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (colored red) and merged with β -catenin signal. Red arrows (in I and O) indicate the nuclei showing green or orange color as a result of increased β -catenin signal in the nuclei of diabetic retinas, while the white arrows (in F and L) point to nuclei (red color) in nondiabetic retinas. Scale bar = 20 μ m.

Figure 3. Up-regulated expression of LRP5/6 in the retinas of STZ-diabetic and OIR rats. **A** and **B**: The same amount of retinal proteins (100 μ g) from three STZ-induced diabetic rats 16 weeks after the onset of diabetes and age-matched nondiabetic rats (**A**), and four OIR rats and normal rats at age of P16 (**B**) was used for Western blot analysis using an antibody specific for LRP5/6 (Santa Cruz Biotechnology). The same membranes were stripped and reblotted with an antibody for β -actin. **C–F**: Retinal sections from STZ-diabetic rats (**D**) and non-DM controls (**C**), and those from OIR rats (**F**) and their normoxic controls (**E**) were immunostained with the antibody against LRP5/6 (green). The nuclei were counterstained with DAPI (red). Original magnification, $\times 400$.

Immunohistochemical analysis in ocular sections showed increased LRP5/6 signals in the inner retina of STZ-induced diabetic rats (green color in Figure 3, C and D), compared with nondiabetic controls. In OIR rats, the more intensive LRP5/6 signals were detected primarily in the retinal vasculature (Figure 3, E and F).

Hypoxia and Oxidative Stress Are Responsible for the Wnt Pathway Activation in Diabetes

To identify the cause for the Wnt pathway activation in diabetes, we evaluated the effects of hypoxia and hyperglycemia, known pathogenic factors of DR, on Wnt signaling *in vitro*. As shown by Western blot analysis, exposure of primary RCEC to hypoxia (2% oxygen) for 14 hours increased the total β -catenin levels (Figure 4A), indicating that hypoxia is a causative factor for the Wnt

Figure 4. Induction of Wnt signaling by hypoxia and oxidative stress. **A**: RCEC were exposed to 2% oxygen and normoxia for 14 hours. Levels of total β -catenin were determined by Western blot analysis using the same amount (50 μ g) of total proteins from each sample and normalized to β -actin levels. Note that the blot represents two independent experiments. **B–D**: RCEC were treated with low glucose (LG; 5 mmol/L glucose and 25 mmol/L mannitol, **B**), high glucose (HG, 30 mmol/L glucose, **C**), and high glucose plus 10 μ mol/L aminoguanidine (HG+AG, **D**) for 24 hours. The subcellular distribution of β -catenin was revealed by immunocytochemistry by using the antibody for β -catenin. **E**: The same amount of isolated nuclear proteins (50 μ g) from each of the above groups was blotted with an antibody for β -catenin and normalized to TATA box-binding protein (TBP) levels.

pathway activation in the retina of the diabetic and OIR models.

RCEC were also exposed to 30 mmol/L glucose for 24 hours, in the presence and absence of 10 μ mol/L aminoguanidine, which is known to have anti-oxidant activities. The subcellular distribution of β -catenin in RCEC was determined by using immunocytochemistry. In the cells cultured under the low glucose medium (5 mmol/L glucose and 25 mmol/L mannitol), β -catenin was distributed primarily in the cytosol and membrane, and was undetectable in the nuclei (green color in Figure 4B). The high glucose medium induced β -catenin nuclear translocation (Figure 4C), suggesting that high glucose alone is sufficient to activate the Wnt pathway. Under the same condition, aminoguanidine inhibited the nuclear translocation of β -catenin induced by high glucose (Figure 4D). Consistently, Western blot analysis using isolated nuclear proteins showed that nuclear levels of β -catenin were elevated in the RCEC exposed to the high glucose me-

Figure 5. DKK1 ameliorates retinal inflammation, vascular leakage, and NV, and inhibits ROS generation. **A:** Various doses of purified DKK1 were injected into the vitreous of the right eye of STZ-diabetic rats at 16 weeks following the onset of diabetes, and the same amounts of BSA were injected into the contralateral eyes for controls. Soluble ICAM-1 concentrations in the retina were measured by enzyme-linked immunosorbent assay, normalized by total protein concentrations, and expressed as ng per mg of proteins (means $\pm$ SD, $n = 3$). **B:** Purified DKK1 was injected into the vitreous of the right eye (1.2 μ g/eye) of STZ-diabetic rats at 16 weeks following the onset of diabetes, and the same amounts of BSA were injected into the contralateral eyes for controls. Retinal vascular leakage was measured 48 hours after the injection by using Evans blue as a tracer, normalized by total protein concentrations, and expressed as μ g of Evans blue per mg of retinal proteins (means $\pm$ SD, $n = 4$). **C and D:** At the age of P14, the OIR rats received an intravitreal injection of DKK1 (1 μ g/eye) into the right eye and the same amount of BSA into the contralateral eyes. The retinas were harvested at P16, and the same amount of retinal proteins (20 μ g) was loaded for Western blot analysis by using antibodies specific for COX2 (**C**) and VEGF (**D**), and normalized by β -actin levels. **E:** OIR rats at P14 received an intravitreal injection of DKK1 at doses as indicated. Retinal vascular leakage was measured at P16 by using Evans blue as a tracer, normalized by total protein concentrations, and expressed as μ g of Evans blue per mg of retinal proteins (means $\pm$ SD, $n = 3$). * $P < 0.05$. **F–J:** OIR rats received an intravitreal injection of 2 μ g/eye DKK1, and BSA into the contralateral eyes at age of P14. At P18, retinal vasculature was visualized by fluorescein angiography on the whole-mounted retina from the eyes injected with BSA (**F** and **G**) and injected with DKK1 (**H** and **I**). Original magnification: $\times 12.5$ (**F** and **H**); $\times 100$ (**G** and **I**). **J:** Pre-retina vascular cells were counted on cross ocular sections from the eyes injected with BSA and DKK1 (means $\pm$ SD, $n = 5$).

dium, compared with those in the low glucose medium. Aminoguanidine blocked the high glucose-induced increase of nuclear β -catenin levels, suggesting that oxidative stress is responsible for the high glucose-induced activation of the Wnt pathway (Figure 4E).

Blockade of the Wnt Pathway Ameliorates Retinal Inflammation, Vascular Leakage, and NV in DR Models

To further establish the causative role of the Wnt pathway activation in DR, we blocked the Wnt pathway activation in the retinas of the DR models by using DKK1, a specific inhibitor of the Wnt pathway. An intravitreal injection of different doses of purified DKK1 into STZ-diabetic rats

reduced retinal soluble ICAM-1 levels in a dose-dependent manner, when compared with that in the contralateral eyes injected with the same amounts of BSA, suggesting that Wnt signaling is responsible for retinal inflammation in diabetic rats (Figure 5A). To evaluate the role of Wnt signaling in retinal vascular leakage in diabetic rats, purified DKK1 was injected into the vitreous of the right eye (1.2 μ g/eye) of STZ-diabetic rats at 16 weeks following the onset of diabetes, and the same amounts of BSA into the contralateral eyes for control. Retinal vascular leakage was measured 48 hours after the injection by using Evans blue as a tracer, and normalized by total retinal protein concentrations. Consistently, vascular permeability assays showed that retinal vascular leakage was significantly decreased in the eyes

injected with DKK1 in diabetic rats, compared with that in the contralateral eyes injected with the same dose of BSA (Figure 5B).

We have also blocked the Wnt pathway by injection of DKK1 (1.0 $\mu\text{g}/\text{eye}$) into OIR rats at age P14. Two days after the injection, expression of pro-inflammation factor such as COX2 and permeability factor VEGF was significantly down-regulated (Figure 5, C and D). Consistently, vascular permeability assays showed that retinal vascular leakage was significantly decreased in the eyes injected with 1 $\mu\text{g}/\text{eye}$ DKK1, compared with that in the contralateral eyes injected with the same dose of BSA (Figure 5E).

To evaluate the role of Wnt signaling in the ischemia-induced retinal NV, DKK1 was injected into the vitreous of OIR rats at the age of P14. The retinal vasculature was visualized by fluorescein angiography in whole-mounted retina at P18. The DKK1 injection induced apparent decreases of neovascular areas and tufts, compared with the contralateral eyes injected with BSA (green color in Figure 5, F–I). Retinal NV was quantified by counting preretinal vascular cells, which showed significant decreases in preretinal vascular cells in the DKK1-injected eyes compared with that in the contralateral eyes injected with BSA (Figure 5J).

Blockade of Wnt Signaling Attenuates the High Glucose-Induced HIF-1 Activation and ROS Generation

HIF-1 activation is known to play a crucial role in the overexpression of VEGF and retinal NV in DR. Here we examined whether the role of Wnt signaling is through HIF-1. Cultured RCEC were exposed to 30 mmol/L glucose in the presence and absence of different concentrations of DKK1 for different durations, with 5 mmol/L glucose and 25 mmol/L mannitol as negative controls, and 1 μg of tumor necrosis factor (TNF)- α as the positive control. As shown by immunocytochemistry using an anti-HIF-1 α antibody, DKK1 inhibited the HIF-1 α nuclear translocation, a key step in its activation, induced by the high glucose medium (green color in Figure 6, A–D).

As oxidative stress is believed to be a key pathogenic factor in DR,³⁸ we evaluated the effect of Wnt signaling on ROS generation induced by high glucose and TNF- α . As shown by ROS measurement, both TNF- α and the high glucose medium (30 mmol/L) significantly increased ROS production in RCEC, compared with the low glucose medium. DKK1 showed a dose-dependent (6.25 to 100 nmol/L) reduction of ROS generation induced by TNF- α and high glucose. At high concentrations (50 and 100 nmol/L), DKK1 decreased the ROS generation to the same extent as that of 10 $\mu\text{mol}/\text{L}$ of aminoguanidine (Figure 6E).

Discussion

The Wnt signaling pathway has been shown to regulate multiple biological and pathological processes.^{39–41}

Figure 6. The Wnt pathway contributes to the oxidative stress and HIF-1 activation. **A–D:** DKK1 inhibits HIF-1 α activation: Primary RCEC were exposed to 5 mmol/L glucose and 25 mmol/L mannitol (**A**), 30 mmol/L glucose (**B**), TNF- α (**C**), and 30 mmol/L glucose with 1 $\mu\text{g}/\text{ml}$ DKK1 (**D**) for 4 hours. HIF-1 α nuclear translocation was determined by using immunocytochemistry with an anti-HIF-1 α antibody. Scale bar = 50 μm . **E:** RCEC were exposed to low glucose (5 mmol/L glucose plus 25 mmol/L mannitol) or high glucose (30 mmol/L glucose), or 1 $\mu\text{g}/\text{ml}$ TNF- α in the absence or presence of various concentrations of DKK1 (6.25 to 100 nmol/L). Aminoguanidine (AG; 10 $\mu\text{mol}/\text{L}$) was used as a positive control. Intracellular ROS generation was measured and expressed as fluorescent unit per well (means $\pm$ SD, $n = 3$).

However, the association of the Wnt pathway with DR has not been reported previously. The present study demonstrates for the first time that the Wnt pathway is activated by oxidative stress and hypoxia in DR in humans and animal models. Furthermore, we have shown that blockade of Wnt signaling with a specific inhibitor of the Wnt pathway ameliorates retinal inflammation, vascular leakage, and NV in the DR models, suggesting that the Wnt pathway plays a causative role in DR. Therefore, these observations have established a new pathogenic role for the Wnt pathway.

β -catenin is an essential down-stream effector in the canonical Wnt pathway.⁴² Our studies using human ocular sections revealed increased retinal levels of β -catenin and enhanced nuclear translocation, a key step in the activation of β -catenin, in the inner retinal cells in patients with DR, compared with that in nondiabetic donors. The location of the β -catenin activation in the inner retina correlates with the pathological changes in DR. The activation of Wnt signaling in the retina with NPDR, which manifests inflammation and vascular leakage but lacks of

NV, suggest that the Wnt activation can occur at early stages of DR, before the proliferative stages.

To confirm the activation of the Wnt pathway in the retina with DR, we examined retinal β -catenin levels in three animal models of DR. STZ-induced diabetes is a commonly used type-1 diabetic model. Akita mouse is a genetic model of type-1 diabetes. Both of the models have been shown to develop retinal inflammation and vascular leakage but not retinal NV, and thus, are NPDR models.^{43,44} Western blot analysis and immunohistochemistry both showed that total levels of β -catenin were higher in the retinas of STZ-diabetic rats than in the age-matched nondiabetic controls. Similarly, Akita mice also had increased β -catenin levels compared with their nondiabetic littermates. The results from these diabetic models suggest that the activated Wnt pathway correlates with retinal inflammation and vascular leakage.

OIR is a commonly used model of ischemia-induced retinal NV. Although it is not a diabetic model, the pathological features of this model, such as preretinal NV, vascular leakage, and overexpression of HIF-1 and VEGF in the retina, resemble that of proliferative diabetic retinopathy. Thus, OIR is commonly accepted as a proliferative diabetic retinopathy model. In OIR rats, β -catenin levels were also increased in the inner retina. These results suggest a potential role of the Wnt pathway in ischemia-induced retinal NV.

LRP5/6 are closely related coreceptors of Wnt ligands.⁴² To confirm the activation of the Wnt pathway in DR, we have measured the retinal levels of LRP5/6 in the DR models. Western blot analysis and immunohistochemistry both showed that the retinal levels of LRP5/6 were elevated in the STZ-induced diabetic and OIR models. In contrast, no significant changes of the Fz receptor levels were detected in the retina of both of the models (Supplemental Figure S1, see <http://ajp.amjpathol.org>). Together with the β -catenin accumulation in these models, these results demonstrate that the Wnt pathway is overactivated in DR.

DR is a complex and multifactorial disorder.^{45,46} It has been shown that hypoxia and hyperglycemia are the major pathogenic factors.^{46,47} To identify the cause for the Wnt pathway activation in diabetes, we assessed the impacts of hypoxia and high glucose on Wnt signaling. In cultured retinal endothelial cells, hypoxia and high glucose medium induced the accumulation of β -catenin and its nuclear translocation. These experiments suggest that hypoxia and hyperglycemia are causative factors for the Wnt pathway activation in diabetes.

Oxidative stress induced by hyperglycemia has been shown to be a key pathogenic factor for retinal inflammation and vascular injury.^{46,48} To test the role of the oxidative stress in the Wnt pathway activation induced by high glucose, we used aminoguanidine as it has antioxidant activities. Both immunocytochemical and Western blot analyses showed that the β -catenin nuclear translocation induced by high glucose medium can be attenuated by aminoguanidine alone, which suggests that the oxidative stress induced by high glucose is a direct cause of the Wnt pathway activation in DR.

The Wnt pathway is known to be activated under many pathological conditions.^{49–52} To establish the causative role of activated Wnt signaling in DR, we blocked the Wnt pathway in the DR models by using DKK1, a specific peptide inhibitor of the Wnt pathway. DKK1 is known to bind to coreceptors LRP5/6 with high specificity and affinity, and block the dimerization of LRP5/6 with the Fz receptor, an essential step in Wnt pathway activation. In the DR models, an intravitreal injection of DKK1 alone is sufficient to mitigate retinal inflammation as it blocks the overexpression of pro-inflammatory factors such as ICAM-1 and COX-2. Similarly, DKK1 also reduced retinal vascular leakage and ameliorated the ischemia-induced retinal NV. These results indicate that blockade of the Wnt pathway alone is sufficient to ameliorate DR. Further, activation of the Wnt pathway alone without high glucose in cultured cells was sufficient to induce VEGF expression (Supplemental Figure S2, see <http://ajp.amjpathol.org>). These results suggest that the Wnt pathway activation plays a causative role in DR. This conclusion is consistent with previous observations in other tissues that the Wnt pathway mediates inflammation and angiogenesis.^{53–57}

To elucidate the mechanism by which Wnt signaling mediates DR, we evaluated the effects of the Wnt pathway in oxidative stress. In cultured endothelial cells, ROS generation was significantly elevated by high glucose and by TNF- α , an inflammatory factor. Blocking Wnt signaling with DKK1 inhibits the ROS generation induced by high glucose medium and TNF- α . These results suggest that the pathogenic role of the Wnt pathway in DR may be via induction of oxidative stress and subsequently induction of inflammation in the retina. It remains to be elucidated how Wnt signaling mediates ROS generation in diabetes.

In summary, our study provides the first evidence showing that the Wnt pathway activation is a novel pathogenic mechanism for DR in both human patients and in animal models. Thus, the Wnt pathway represents a new target for pharmaceutical intervention of DR. Our study also suggests that DKK1 has therapeutic potential in the treatment of DR.

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