The Pathogenic Role of the Canonical Wnt Pathway in Age-Related Macular Degeneration

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PURPOSE. The authors' previous studies showed that the Wnt signaling pathway is activated in the retinas and retinal pigment epithelia of animal models of age-related macular degeneration (AMD) and diabetic retinopathy (DR). The purpose of this study was to investigate the role of the canonical Wnt pathway in pathogenesis of these diseases.

METHODS. The Wnt pathway was activated using the Wnt3a-conditioned medium and adenovirus expressing a constitutively active mutant of β -catenin (Ad-S37A) in ARPE19, a cell line derived from human RPE. Ad-S37A was injected into the vitreous of normal rats to activate the Wnt pathway in the retina. Accumulation of β -catenin was determined by Western blot analysis, and its nuclear translocation was revealed by immunocytochemistry. Inflammatory factors were quantified by Western blot analysis and ELISA. Oxidative stress was determined by measuring intracellular reactive oxygen species (ROS) generation and nitrotyrosine levels.

RESULTS. The Wnt3a-conditioned medium and Ad-S37A both increased β -catenin levels and its nuclear translocation in ARPE19 cells, suggesting activation of the canonical Wnt pathway. Activation of the Wnt pathway significantly upregulated the expression of VEGF, NF- κ B, and TNF- α . Further, Ad-S37A induced ROS generation in a dose-dependent manner. Wnt3a also induced a twofold increase of ROS generation. Intravitreal injection of Ad-S37A upregulated the expression of VEGF, ICAM-1, NF- κ B, and TNF- α and increased protein nitrotyrosine levels in the retinas of normal rats.

CONCLUSIONS. Activation of the canonical Wnt pathway is sufficient to induce retinal inflammation and oxidative stress and plays a pathogenic role in AMD and DR. (*Invest Ophthalmol Vis Sci.* 2010;51:4371–4379) DOI:10.1167/iovs.09-4278

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Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries.¹ In the United States, approximately 1.75 million people have AMD, and another 7 million people are at risk for it. The prevalence of AMD is predicted to double in the next decade without efficient prevention and treatment.² AMD is a multifactorial disease,¹ and the most established risk factors are advanced age, cigarette smoking, diet, and race.³

Although the pathogenesis of AMD has not been fully elucidated, inflammation has been shown to play a pathogenic role in AMD.^{4–6} Hageman⁷ presented the integrated hypothesis on the role of AMD and proposed that drusen are associated with localized inflammatory responses.⁴ Recent studies^{8–10} found that a common variant in the complement factor H gene is strongly associated with AMD. Activation of the complement system can produce proinflammatory responses, release chemokines to mediate the recruitment of inflammatory cells, and increase capillary permeability.^{11,12} Accumulating evidence strongly suggests that inflammation in the retina and the retinal pigment epithelium (RPE) plays an important role in the development of AMD.

Vascular endothelial growth factor (VEGF) has been shown to play a critical role in the pathogenesis of choroidal neovascularization (NV) in AMD, and anti-VEGF therapies have shown beneficial effects on wet AMD.^{13–18} Elevated tumor necrosis factor (TNF)- α levels have been found in neovascular membranes of eyes with AMD.¹⁹ New studies have shown that membrane complement regulatory proteins and complement factor B can be upregulated by TNF- α .^{20,21} Another study shed a light on the anti-TNF- α treatment of AMD, which provided in vivo evidence of a pathogenic link of locally produced or acting TNF- α to neovascular AMD.²² In addition to VEGF and TNF- α , intercellular adhesion molecule (ICAM)-1 is constitutively expressed in the RPE and is important for leukocyte adherence.^{23,24} A recent case study showed that increases in circulating levels of sICAM-1 precede the development of visually significant AMD in women.²⁵

Oxidative stress is considered another important contributor to AMD.^{26–28} Oxidative stress can induce RPE cell death, which leads to the impairment of RPE function,^{29–31} and some antioxidants can ameliorate the cell death and dysfunction of RPE.^{32–34} SOD knockout animals have been shown to develop some features of AMD,^{35,36} and the inflammation induced by oxidative damage can initiate AMD.³⁷ Therefore, oxidative stress is considered a key causative factor of inflammation and AMD.

Diabetic retinopathy (DR) is another leading cause of acquired blindness.³⁸ Similarly, it was well documented that retinal inflammation and oxidative stress play indispensable roles in the development of DR.³⁹ VEGF has been established as an important pathogenic factor of DR. ICAM-1 levels have been found to be increased in the vitreous and sera of DR patients.^{40–42}

The Wnt signaling pathway is a multifunctional pathway that regulates cell proliferation and differentiation, apoptosis, stem cell maintenance, and carcinogenesis.^{43–46} Wnt ligands are secreted cysteine-rich glycosylated proteins that bind to a receptor complex comprising the frizzled (fz) receptors and the low-density lipoprotein receptor-related protein (LRP) 5 or LRP6. Binding with this receptor complex by Wnts leads to inactivation of the “destruction complex,” which is composed of glycogen synthase kinase (GSK)-3 β , Axin, and APC (adenomatous polyposis coli), prevents the proteasomal degradation of the transcriptional factor β -catenin, and promotes its accumulation and nuclear translocation. Once β -catenin translocates to the nucleus, it associates with the T-cell factor and regulates the expression of the Wnt target genes, including VEGF and inflammatory factors.^{47–52}

A previous study reported that the very low-density lipoprotein receptor (VLDLR) gene knockout results in subretinal NV.⁵³ Our studies showed that *Vldlr*^{−/−} mice develop retinal inflammation, vascular leakage, and visual impairment, suggesting that *Vldlr*^{−/−} mice represent a good model of AMD.⁵⁴ A recent study demonstrated that *Vldlr*^{−/−} mice also develop oxidative stress in the retina.⁵⁵ Further, we have shown that the canonical Wnt pathway is activated in the RPE and retina in *Vldlr*^{−/−} mice, correlating with retinal inflammation, oxidative stress, and subretinal NV.⁵⁶ A genetic study has associated LRP6 and VLDLR with AMD in human patients.⁵⁷

Meanwhile, our recent study has shown that Wnt pathway activation also plays a pathogenic role in retinal inflammation and NV in DR in human patients and in diabetic animal models.⁵⁸ These findings suggest that the Wnt pathway activation may represent a common pathway mediating inflammation and oxidative stress in both DR and AMD. The purpose of the present study was to prove that activation of β -catenin alone is sufficient to cause retinal inflammation and oxidative stress, similar to that in AMD and DR.

METHODS

Materials and Antibodies

Conditioned media containing Wnt3a (Wnt3a-CM) were prepared from mouse L cells (ATCC) stably expressing Wnt3a. Control conditioned media were obtained from parental L cells (Ctrl-CM).

Antibodies against β -catenin (H-102, sc-7199, rabbit polyclonal) and VEGF (sc-152, rabbit polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas those against TNF- α (ab9739, polyclonal), NF- κ B (ab16502, polyclonal), nitrotyrosine (ab61392, mouse monoclonal), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab9482), β -actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Abcam (Cambridge, MA). An anti-histone-3 antibody was from Cell Signaling (Danvers, MA), FITC-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratory, Inc. (West Grove, PA), and anti-His-tag antibody was from Upstate (Billerica, MA).

Construction of Adenovirus Expressing Constitutively Active Mutant of β -Catenin (Ad-S37A)

Human β -catenin cDNA clone (pCMV-SPORT6) was purchased from Invitrogen (Carlsbad, CA). A six-Histidine tag (His-tag) was fused to the C terminus of β -catenin using polymerase chain reaction. The β -catenin/His-tag construct was cloned into the pcDNA3.1 vector (Invitrogen) at the *KpnI* and *XbaI* sites. Mutagenesis of serine37 to Ala was performed using a site-directed mutagenesis kit (Quick-Change; Stratagene, La Jolla, CA). Then the S37A mutant/His-tag of β -catenin was cloned into the pShuttle-CMV vector (Invitrogen) at the *KpnI* and *XbaI*

sites. The recombinant Ad-S37A virus was generated using an adenoviral vector system (AdenoVator; Qbiogene, Irvine, CA), purified using a virus purification kit (Adeno-X; Clontech, Mountain View, CA), and titered using a rapid titer kit (Adeno-X; BD Biosciences, San Jose, CA).

Cell Culture

ARPE19, a cell line derived from human RPE, was purchased from ATCC (Manassas, VA) and cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics. Cells were cultured to 50% to 80% confluence and infected with virus. Confluent culture cells were starved in DMEM containing 2% heat-inactivated FBS and 1% antibiotics overnight and then exposed to 50% Ctrl-CM or Wnt3a-CM.

Transfection

ARPE19 cells were seeded in four-well glass slide chambers at 1×10^4 /well and incubated overnight. The cells were transfected with 0.2 μ g DNA of a plasmid expressing the mutant β -catenin fused with GFP (S37A-GFP) or a plasmid expressing GFP only in optimal medium (Invitrogen) using a transfection agent (Fugene 6; Roche, Indianapolis, IN). After 72 hours in culture, the transfected cells were washed twice with PBS and fixed with 4% formaldehyde in PBS for 20 minutes. Slides were prepared with a mounting medium containing 4',6-diamidino-2-phenylindole (cat. no. H-1200; Vector Laboratories, Burlingame, CA) to counterstain the nucleus.

Immunocytochemistry

ARPE19 cells grown on four-well glass slide chambers to 60% confluence were infected with Ad-S37A or Ad- β -gal control virus for 48 hours, washed twice with phosphate-buffered saline (PBS), and fixed with 4% formaldehyde in PBS for 20 minutes. Then the cells were rinsed three times with PBS and permeabilized for 20 minutes with 0.01% Triton X-100 in PBS. After thorough washes, the cells were incubated for 30 minutes in PBS containing 3% bovine serum albumin (BSA) and 10% normal goat serum and then incubated with the anti- β -catenin antibody in blocking buffer overnight at 4°C. The cells were thoroughly rinsed with PBS followed by staining with FITC-conjugated secondary antibody (1:200 dilution in blocking buffer; 2 hours). Slides were prepared with a mounting medium containing 4',6-diamidino-2-phenylindole (cat. no. H-1200; Vector Laboratories) to counterstain the nucleus.

Nuclear Extract Preparation

Nuclear extracts were prepared with a nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

NF- κ B p65 Transcription Factor Activation Assay

To quantify NF- κ B activation, the DNA-binding activity of p65, a subunit of NF- κ B, was assayed using the NF- κ B Consensus Oligonucleotide Agarose Conjugates (sc-2505AC; Santa Cruz Biotechnology). In this assay, an oligonucleotide containing the DNA binding motif of NF- κ B (5'-GGGACTTCC-3') was provided as agarose conjugates for precipitation of NF- κ B. Whole cell lysates (1 mg) were added to the washed agarose conjugates in binding buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μ g/mL poly dI-dC) and incubated with rotation overnight at 4°C. An aliquot was saved from each sample for Western blot analysis of β -actin as input control. Then the mixture was centrifuged at 12,000 rpm for 5 minutes at 4°C. After three washes with the binding buffer, the pellet was resuspended in SDS-PAGE sample buffer, boiled for 90 seconds, and centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was analyzed by Western blot analysis.

Measurement of Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) generation was measured using 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂-DCF-DA; Molecular Probes, Carlsbad, CA) as a probe according to the manufacturer's protocol. Briefly, the treated and untreated cells at a density of 1×10^5 cells/mL were incubated with freshly prepared CM-H₂-DCF-DA at 37°C for 30 minutes in the dark. The cells were rinsed twice in PBS and analyzed by fluorometer at 488 nm excitation and 530 nm emission.

Animals

Brown Norway rats were purchased from Harlan (Indianapolis, IN). Care, use, and treatment of all animals in this study were in strict agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the guidelines for the care and use of laboratory animals set forth by the University of Oklahoma.

Intravitreal Injection of Adenovirus

Briefly, rats were anesthetized with a 50:50 mix of ketamine (20 mg/mL) and xylazine (4 mg/mL), and pupils were dilated with topical application of phenylephrine (2.5%) and tropicamide (1%). A sclerotomy was created approximately 1 mm posterior to the limbus with a self-made blade, and a 33-gauge needle connected to a microinjector filled with 5 μ L virus (1×10^{10} IFU/mL) was introduced through the sclerotomy into the vitreous cavity.

ELISA for sICAM-1

The eyecups, including the retinas and RPE, were dissected, homogenized, and centrifuged at 5000 rpm for 5 minutes. The total protein concentration in the supernatant was measured using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). A commercial sICAM-1 ELISA kit (R&D Systems, Inc., Minneapolis, MN) was used

to measure sICAM-1 levels according to the manufacturer's instruction and was normalized by total protein concentrations in the retina.

Western Blot Analysis

Cells were washed with cold PBS and harvested using the RIPA lysis buffer (Santa Cruz Biotechnology). The eyecups, including the retina and RPE, were dissected from animal eyes and sonicated in cold PBS. The total protein concentration was measured using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). The whole retinal lysate was resolved by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dry milk in Tris-buffered saline Tween-20 (TBST) for 1 hour, the membrane was incubated overnight at 4°C with a primary antibody. After washing with TBST, the membrane was incubated with the appropriate secondary antibody for 2 hours. The membrane was again washed with TBST, and immunoblots were developed with the enhanced chemiluminescent reagents from Pierce according to the manufacturer's instruction. Densitometry was performed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) and normalized by β -actin levels.

RESULTS

Wnt3a Increased β -Catenin Levels and Induced Expression of Inflammatory Factors

To establish the pathogenic role of the canonical Wnt pathway in ocular inflammation, ARPE19 cells were exposed to 50% Wnt3a-CM, with 50% Ctrl-CM for control. The activation of Wnt signaling was determined by measuring total levels of β -catenin, an essential effector of the canonical Wnt pathway, because its accumulation is the key step in the activation of the canonical Wnt pathway. As shown by Western blot analysis,

FIGURE 1. Wnt3a-conditioned medium elevated β -catenin levels and upregulated the expression of inflammatory factors. (A) ARPE19 cells were treated with 50% Ctrl-CM or Wnt3a-CM for 12 hours. Levels of total β -catenin were determined by Western blot analysis using 50 μ g total proteins from each sample. The blot is representative of three independent experiments. (B) Quantification of total β -catenin by densitometry from three independent experiments, normalized by β -actin levels and expressed as a percentage of that in cells treated with Ctrl-CM (mean $\pm$ SD). * $P < 0.05$. (C) Cells were treated with 50% Ctrl-CM and Wnt3a-CM for 24 hours. Cellular levels of VEGF and TNF- α were determined by Western blot analysis using 50 μ g total proteins from each sample. (D, E) VEGF and TNF- α expression was quantified by densitometry from three independent experiments, normalized by β -actin levels and expressed as a percentage of that in the cells treated with Ctrl-CM (mean $\pm$ SD). * $P < 0.05$.

Wnt3a-CM increased β -catenin levels significantly, compared with its control (Figs. 1A, 1B), suggesting the activation of the canonical Wnt pathway by Wnt3A. In the same cells, VEGF and TNF- α levels were elevated by Wnt3a-CM, compared with those treated with Ctrl-CM (Figs. 1C-E), suggesting that Wnt signaling induces an inflammatory response in the RPE cells.

A Constitutively Active Mutant of β -Catenin Increased Total β -Catenin Levels and Its Nuclear Translocation

To confirm the effect of Wnt signaling, the Wnt pathway was also activated by a constitutively active mutant of β -catenin in which the phosphorylation site S37 was substituted by Ala (S37A). ARPE19 cells were infected with Ad-S37A and with adenovirus expressing β -galactosidase (Ad- β -gal) at the same multiplicity of infection (MOI) as control. Forty-eight hours after viral infection, β -catenin levels were determined by Western blot analysis, which showed that total β -catenin levels were increased significantly in cells infected with Ad-S37A compared with those infected with Ad- β -gal (Figs. 2A, 2B). To distinguish the recombinant β -catenin mutant from endogenous β -catenin expression, the infected cell lysates were also blotted with an antibody for the His-tag. The expression of the S37A mutant was detected only in cells infected with Ad-S37A and not in cells infected with Ad- β -gal (Fig. 2A).

To determine the nuclear translocation of β -catenin, the subcellular distribution of β -catenin in ARPE19 cells was determined by immunocytochemistry using an anti- β -catenin antibody. In control cells infected with Ad- β -gal, β -catenin was distributed mainly in the membrane pool and cytosol and was undetectable in the nuclei (Figs. 2C1, 2C2). Ad-S37A induced an apparent increase of β -catenin in the nuclei (Figs. 2D1, 2D2). Further, the increased nuclear translocation of the β -catenin mutant was confirmed using a plasmid expressing S37A-GFP, the S37A mutant fused with GFP. As shown by fluorescence microscopy, S37A-GFP was detected primarily in the nuclei, suggesting the nuclear translocation of mutant β -catenin (Figs. 2E, 2F). Moreover, β -catenin levels in isolated nuclei were determined by Western blot analysis, which showed that nuclear β -catenin levels were increased in cells infected with Ad-S37A compared with those infected with Ad- β -gal (Figs. 2G, 2H).

The Constitutively Active Mutant of β -Catenin Induced Expression of Inflammatory Factors

Although it is evident that inflammation plays a role in the progression of AMD and DR, the signaling pathway that mediates the inflammation in AMD and DR has not been identified. To investigate whether activation of the canonical Wnt pathway induces the expression of inflammatory factors, ARPE19 cells were infected with Ad-S37A and the control virus at the

FIGURE 2. Constitutively active mutant of β -catenin increased total β -catenin levels and its nuclear translocation. ARPE19 cells were infected with Ad- β -gal or Ad-S37A at the same MOI for 48 hours. **(A)** Total cellular proteins (50 μ g from each sample) were sequentially immunoblotted with antibodies for His-tag, β -catenin, and β -actin. Blots are representatives of three independent experiments. **(B)** Total β -catenin levels were quantified by densitometry and normalized by β -actin levels, and expressed as percentage of control cells infected by Ad- β -gal (mean $\pm$ SD; $n = 3$). * $P < 0.05$. **(C1, D1)** Immunostaining of the infected cells using an antibody specific for β -catenin to reveal the subcellular localization of β -catenin. **(C2, D2)** β -Catenin signal in **(C1, D1)** merged with DAPI staining of the nucleus. *Light blue*: colocalization of β -catenin and DAPI staining in the nuclei in **D2**. **(E1, F1)** ARPE19 cells were transfected with a plasmid expressing GFP **(E1)** or S37A fused with GFP **(F1)**. **(E2, F2)** GFP signals (green) in **E1** and **F1** were merged with DAPI staining (blue). Images are the representatives from three independent experiments. **(G, H)** Nuclei were isolated, and nuclear β -catenin levels were determined by Western blot analysis using 50 μ g nuclear proteins **(G)** and were quantified by densitometry, normalized by histone-3 level, and expressed as percentage of the control **(H)** (mean $\pm$ SD; $n = 3$). * $P < 0.05$.

FIGURE 3. Constitutively active mutant of β -catenin induced overexpression of inflammatory factors. (A, C, E) ARPE19 cells were infected with Ad- β -gal and Ad-S37A at the same MOI for 48 hours. Levels of VEGF (A), TNF- α (C), and NF- κ B (E) in the cells were determined by Western blot analysis using 50 μ g total proteins from each sample. Each blot is a representative of three independent experiments. (B, D, F) Quantification of VEGF, TNF- α , and NF- κ B by densitometry, normalized by β -actin levels, and expressed as percentage of the control. (G, H) NF- κ B was precipitated with an oligonucleotide containing NF- κ B-binding sites. Precipitated proteins were blotted with the anti-NF- κ B antibody (G). Precipitated NF- κ B levels were quantified by densitometry and normalized by β -actin levels in the samples before precipitation (H). All values are mean $\pm$ SD ($n = 3$). * $P < 0.05$.

same MOI for 48 hours. As shown by Western blot analysis, expression of VEGF, TNF- α , and NF- κ B was upregulated significantly in cells infected with Ad-S37A compared with those infected with the control virus (Figs. 3A–F). To further confirm the activation of NF- κ B, we measured the DNA-binding activity of NF- κ B p65. As shown by coprecipitation with NF- κ B-binding oligonucleotide, the DNA-binding activity of NF- κ B was enhanced in cells infected with Ad-S37A compared with those infected with the control virus (Figs. 3G, 3H).

Activation of the Wnt Pathway Induced ROS Generation and Protein Nitration

ROS is regarded as a major stimulus for the release of inflammatory cytokines,⁵⁹ and oxidative damage to the RPE has been shown to have a role in the development of certain pathologic features of AMD.³⁵ To investigate whether activation of the canonical Wnt pathway is responsible for the induction of ROS generation in the RPE, ARPE19 cells were infected with Ad- β -gal or Ad-S37A at the same MOI or exposed to Ctrl-CM and in Wnt3a-CM. As shown by ROS measurement, Ad-S37A induced significant ROS generation in an MOI-dependent manner. At MOI 16, Ad-S37A induced a fourfold increase in ROS generation compared with the control virus (Fig. 4A). Similarly, Wnt3a-CM also induced a twofold increase of ROS generation in ARPE19 cells (Fig. 4B).

Because nitrotyrosine is one of the markers used to assess oxidative stress,⁶⁰ nitrotyrosine levels in cellular proteins were examined by Western blot analysis. Results showed increased

protein nitration when the canonical Wnt pathway was activated either by Ad-S37A or by Wnt3a-CM (Figs. 4C, 4D), suggesting that the activation of the canonical Wnt pathway alone can induce oxidative stress that might be responsible for the induction of inflammatory factors.

The Constitutively Active Mutant of β -Catenin Increased Retinal Levels of β -Catenin and Inflammatory Factors in Normal Rats

To investigate whether activation of the canonical Wnt pathway alone induces retinal inflammation and oxidative stress, normal Brown Norway rats at age 6 weeks were injected intravitreally with Ad- β -gal or Ad-S37A (5×10^7 IFU/eye). As shown by Western blot analysis 14 days after the injection, total β -catenin levels were increased by threefold in the retinas injected with Ad-S37A compared with those injected with Ad- β -gal (Fig. 5). In the same retinas, levels of VEGF, TNF- α , and NF- κ B were elevated significantly by Ad-S37A over that in the control eyes injected with Ad- β -gal, as shown by Western blot analysis (Figs. 6A–F). Similarly, sICAM-1 concentrations were 1.5-fold higher in the retinas injected with Ad-S37A than in those injected with Ad- β -gal (Fig. 6I). Similar to the in vitro results, Ad-S37A also significantly increased 3-NT levels in the retina compared with that of control virus (Figs. 6G, 6H). Together, these results suggested that activation of the canonical Wnt pathway alone is sufficient to induce inflammation and oxidative stress in the retina.

FIGURE 4. ROS generation and protein nitration induced by Wnt signaling. (A, B) ARPE19 cells were infected with Ad-β-gal or Ad-S37A at the MOI indicated for 48 hours (A) or treated with 50% Ctrl-CM and Wnt3a-CM for 12 hours (B). Intracellular ROS generation was measured using CM-H2DCFDA as a probe. Values are mean $\pm$ SD ($n = 3$). ** $P < 0.01$ compared with cells infected with Ad-β-gal. (C) Levels of 3-nitrotyrosine (3-NT) were determined by Western blot analysis using 40 μ g total proteins from each sample and normalized by β -actin levels. (D) Nitrotyrosine was quantified by densitometry from three independent experiments and expressed as a percentage of that in the control cells (mean $\pm$ SD). * $P < 0.05$.

DISCUSSION

Our previous studies showed that VLDLR was a negative regulator of the Wnt signaling pathway,⁵⁶ and VLDLR gene knock-out resulted in activation of the Wnt pathway in the retina and RPE. The activation of the Wnt pathway correlates with subretinal NV and retinal inflammation in the *Vldlr*^{-/-} mice.⁵⁴ Moreover, our recent study⁵⁸ has shown that the canonical Wnt signaling pathway was also activated in the retina from human and animal models with DR. These observations inspired us to hypothesize that activation of the canonical Wnt pathway is responsible for retinal inflammation and is indirectly involved in AMD. Our in vitro and in vivo results both showed that activation of the Wnt pathway alone induced retinal inflammatory factors and oxidation, thus providing evidence supporting a potential role of the canonical Wnt pathway in AMD.

β -Catenin plays a central role in the canonical Wnt pathway. In the absence of Wnt ligands, β -catenin is phosphorylated by GSK-3 β and is constantly degraded. Once activated by certain Wnt ligands, β -catenin phosphorylation ceases, and β -catenin accumulates in cytoplasm and then translocates into the nucleus and induces target gene transcription.⁴⁷⁻⁵² Four phosphorylation sites in β -catenin at serine (S)/threonine (T) residues (S33, S37, T41, S45) are conserved from *Drosophila* to human,⁶¹ and the degradation-resistant mutation S37A is the most common aberration in tumors.⁶²⁻⁶⁴ Therefore, we used the S37A mutant in this study to achieve constitutive activation of the canonical Wnt pathway.

Wnt3a is one of the Wnt ligands that activate the canonical Wnt pathway.⁴⁶ Because the purified Wnt3a is unstable, the

Wnt3a conditioned medium is commonly used for the activation of the canonical Wnt pathway in in vitro experiments. Here we showed that the Wnt3a-conditioned medium and S37A mutant both increased total β -catenin levels and induced β -catenin nuclear translocation. As a result, both S37A and Wnt3a induced the overexpression of inflammatory factors. Further, activation of the Wnt pathway also induced oxidative stress, as shown by ROS and nitrotyrosine measurements.

The RPE has metabolic and supportive functions that are significant for maintenance of the blood-retinal barrier, participation in the visual cycle, phagocytic uptake, and degradation of photoreceptor outer segment. Dysfunction of the RPE is the initial event in the development process of AMD.¹ The RPE constitutively produces VEGF, which is a well-established angiogenic and proinflammatory factor.^{65,66} VEGF has been shown to play a key role in NV and vascular leakage in AMD. Thus, our in vitro studies used an RPE cell line.

Inflammation is commonly believed to be a pathogenic factor in AMD.⁴⁻⁶ Cousins reported that AMD patients with the highest TNF- α expression develop more choroidal NV (CNV).⁶⁷ TNF- α is expressed in experimental models and human CNV membranes in macrophages, RPE, and vessels.¹⁸ In addition, ICAM-1 is expressed in CNV tissue in human surgical specimens and the laser-induced murine CNV model.¹⁸ However, the signaling pathway mediating the inflammatory factor overexpression in AMD has not been well established. Our results showed herein that the Wnt3a and the constitutively active mutant of β -catenin both significantly induced the expression of VEGF, TNF- α , and ICAM-1. Consistently, our previous studies demon-

FIGURE 5. Constitutively active mutant of β -catenin induced β -catenin accumulation in the retina. (A) Normal Brown Norway rats at 6 weeks of age received intravitreal injection of Ad- β -gal and Ad-S37A (5×10^7 IFU/eye). Two weeks after the injection, the retina was dissected, and the levels of total β -catenin were determined by Western blot analysis using 50 μ g total proteins from each retina and normalized by β -actin levels. Each lane represents an individual animal. (B) Quantification of β -catenin expression by densitometry. * $P < 0.05$. Values are mean $\pm$ SD ($n = 3$).

strated that elevated proinflammatory factors include VEGF, TNF- α , and ICAM-1 levels in *Vldlr*^{-/-} mice, which have overactive canonical Wnt pathway in the retina and

RPE.^{54,56} Further, activation of the Wnt pathway alone in normal animals was sufficient to induce overexpression of these pro-inflammatory factors. Therefore, our results suggest that the canonical Wnt pathway mediates the inflammation in AMD.

The VEGF gene is a known target genes regulated by β -catenin.^{68,69} In contrast, there is no direct evidence showing that Wnt/ β -catenin directly regulates TNF- α and ICAM-1 gene transcription. The mechanism by which Wnt signaling induces the expression of TNF- α and ICAM-1 remains to be studied.

Substantial evidence indicates that oxidative stress is involved in the pathogenesis of AMD.^{26–28} ROS is considered a potent stimulus for the release of cytokines⁵⁹ and can activate transcription factor NF- κ B, which upregulates the expression of proinflammatory factors.^{70,71} To elucidate the mechanism by which the canonical Wnt pathway mediates inflammation, we evaluated the effects of the canonical Wnt pathway on oxidative stress. In cultured RPE cells, cellular ROS generation and nitrotyrosine level, a hallmark of oxidative stress, were significantly elevated by Wnt3a and the constitutively active mutant of β -catenin. Similarly, nitrotyrosine levels were significantly higher in the retina and the RPE injected with Ad-S37A than in those injected with the control virus. It is likely that the ROS generation induced by activation of the canonical Wnt pathway contributes to the overexpression of inflammatory factors through the NF- κ B pathway. Meanwhile, it is reported that VEGF and TNF- α can stimulate the production of ROS.⁷² Therefore, it is possible that ROS induced by activation of the canonical Wnt pathway upregulates inflammatory factors, and, in turn the overexpression of inflammatory factors intensifies the formation of ROS, forming a vicious circle that exacerbates the pathol-

FIGURE 6. Constitutively active mutant of β -catenin-induced expression of inflammatory factors and protein nitration in the normal retina. (A, C, E, G) Normal rats at 6 weeks of age received intravitreal injection of Ad- β -gal and Ad-S37A (5×10^7 IFU/eye). Two weeks after the injection, the retina and RPE complex were dissected, and levels of VEGF, TNF- α , NF- κ B, and 3-NT were determined by Western blot analysis using 50 μ g total proteins from each sample and normalized by β -actin levels. Each lane represents an individual animal. (B, D, F, H) Levels of VEGF, TNF- α , NF- κ B, and 3-NT were quantified by densitometry and expressed as a percentage of that in the eyes injected with the control virus. * $P < 0.05$. Values are mean $\pm$ SD ($n = 3$). (I) Soluble ICAM-1 (sICAM-1) concentrations in the retina were measured using an ELISA kit, normalized by total protein concentrations, and expressed as ng/mg of retinal proteins (mean $\pm$ SD; $n = 3$).

ologies of AMD. Therefore, the complex interactions between oxidative stress, inflammation and the canonical Wnt pathway warrant further investigation.

Intravitreal injection of Ad-S37A resulted in Wnt pathway activation in the inner retina (data not shown). Similarly, Wnt pathway activation was detected in the retinas of *Vldlr*^{-/-} mice, which are considered a new AMD model.^{53,54,56} It is likely that activation of the Wnt pathway in the retina induces the overproduction of angiogenic and inflammatory factors that subsequently induce inflammation and NV in the subretinal space. A recent linkage and allelic association study using a family-based association dataset and an independent case control dataset revealed significant associations of genetic variations in the VLDLR and LRP6 genes with AMD.⁵⁷ Together with this study, our findings suggest that the Wnt pathway represents a novel pathogenic pathway in AMD and a potential drug target for the treatment of AMD.

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