Angiopoietin 2 Induces Pericyte Apoptosis via α3β1 Integrin Signaling in Diabetic Retinopathy

Sung Wook Park1,2, Jang-Hyuk Yun3,4, Jin Hyoung Kim1, Kyu-Won Kim5, Chung-Hyun Cho3,4#

4#, Jeong Hun Kim1,2,6#

S.W.P. and J.-H.Y. contributed equally to this work.

#These authors are regarded as co-corresponding authors.

1Fight against Angiogenesis-Related Blindness Laboratory, Biomedical Research Institute, Seoul National University Hospital, Seoul 110-744, Korea

2Department of Biomedical Sciences, College of Medicine, Seoul National University, Seoul 110-799, Korea

3Department of Pharmacology and Ischemic/Hypoxic Disease Institute, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea

4Cancer Research Institute, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea

5Department of Pharmacy, Seoul National University, Seoul 151-742, Korea

6Department of Ophthalmology, Seoul National University Hospital, Seoul 110-744, Korea

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*Correspondence:

Jeong Hun Kim, M.D., Ph.D.
Fight against Angiogenesis-Related Blindness (FARB) Laboratory, Biomedical Research Institute, Seoul National University Hospital and Department of Biomedical Sciences and Ophthalmology, College of Medicine, Seoul National University, 103 Daehak-ro, Jongno-gu, Seoul, 110-744, Korea
Phone: 82-2-2072-2438; Fax: 82-2-741-3187; E-mail: steph25@snu.ac.kr

Chung-Hyun Cho, Ph.D.
Department of Pharmacology and Ischemic/Hypoxic Disease Institute and Cancer Research Institute, College of Medicine, Seoul National University, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Korea
Phone: +82-2-740-8283 Fax: +82-2-745-7996; E-mail: iamhyun@snu.ac.kr

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Abstract

Pericyte loss is an early characteristic change in diabetic retinopathy. Despite accumulating evidences that hyperglycemia induced angiopoietin 2 (Ang2) has a central role in pericyte loss, the precise molecular mechanism has not been elucidated. This study was to investigate the role of Ang2 in pericyte loss in diabetic retinopathy. We demonstrated that pericyte loss occurred with Ang2 increase in the diabetic mice retina, and the source of Ang2 could be endothelial cell. Ang2 induced pericyte apoptosis via p53 pathway under high glucose while Ang2 alone did not induce apoptosis. Integrin, not Tie-2 receptor, was involved for Ang2 induced pericyte apoptosis under high glucose as an Ang2 receptor. High glucose changed integrin expression pattern which increased integrin α3 and β1 in pericyte. Furthermore, in vitro, Ang2 induced pericyte apoptosis was effectively attenuated via p53 suppression by blocking integrin α3 and β1. In vivo, while intravitreal injection of Ang2 induced pericyte loss in C57/BL6 mice retina, intravitreal injection of anti-integrin α3 and β1 antibodies attenuated Ang2 induced pericyte loss. Taken together, Ang2 induced pericyte apoptosis under high glucose via α3β1 integrin. Glycemic control or blocking Ang2/integrin signaling could be a potential therapeutic target to prevent pericyte loss in early diabetic retinopathy.
INTRODUCTION

Diabetic retinopathy (DR) is the leading cause of the visual loss in working-aged people and the most common microvascular complication in the diabetic patients despite of the recent improvement in the management of DR via glycemic control and photocoagulation (1). Both the macular edema (leakage) and neovascularization (angiogenesis) cause severe vision loss in DR (2), and loss of pericyte is one of the earliest and most characteristic changes of DR (3).

In DR, pericyte plays important role in two major clinical reasons. First, pericyte enwraps endothelial cells to keep the integrity of inner blood-retinal barrier (BRB) with the role of microvascular autoregulation (4). Thus, pericyte loss could weaken inner BRB even when endothelial cells are intact, and can lead to capillary instability as well as vascular leakage in macular edema. Second, microaneurysm and neovascularization occur in proliferating endothelial cells at the site of pericyte loss (5). As pericyte loss is early diabetic change, it would be beneficial to prevent pericyte loss for primary prevention of DR.

Although pericyte loss is important in early DR, the mechanism by which hyperglycemia leads to pericyte loss remains largely unknown. However, Ang2 plays a critical role in pericyte loss in DR. Hyperglycemia causes pericyte apoptosis and ultimately pericyte loss (6-8). Ang2 increases in vitreous of patients with proliferative DR (9). In addition, Ang2 is upregulated by hyperglycemia in diabetic retina and endothelial cells (10-12). Ang2 induces
pericyte loss in normal mice retina and Ang2 overexpressing mice (10,13). Thus, we postulated that hyperglycemia increases Ang2, which in turn induces pericyte apoptosis in DR.

The role of Ang2 in pericyte loss has been studied (10,11,13-15). Both apoptosis and migration are suggested mechanisms of pericyte loss by Ang2. However, the precise mechanism by which Ang2 induces pericyte loss has not been fully elucidated yet. Ang2 has been known to bind to the endothelial specific TieB2 tyrosine receptor, with similar affinity to Ang1 (16). Ang2 acts as autocrine manner in the angiogenesis. This endothelial cell-derived antagonistic ligand of the vessel maturation and remodeling controls Ang1-Tie-2 signaling axis (17). Although it has been postulated that Ang2 naturally binds to Tie-2 receptor in pericyte as Ang-Tie system (11,13), it is not clear whether Tie-2 indeed serves as a receptor for Ang2 induced pericyte loss. Recently, it was found that integrin mediates PDGF-BB-induced pericyte loss in tumor vessels (18). Also, Ang2 binds to integrin and regulates angiogenesis through integrin signaling (19). Thus, we hypothesized that Ang2 induces pericyte apoptosis via integrin signaling.

In this study, we demonstrated that Ang2 induced pericyte apoptosis via p53 pathway under high glucose. Interestingly, integrin, not Tie-2 receptor, was important for Ang2 induced pericyte apoptosis under high glucose. High glucose increased integrin α3β1 in pericyte. Furthermore, our results showed Ang2 induced pericyte apoptosis was effectively attenuated by blocking integrin α3β1 in vitro and in vivo. Taken together, Ang2 induced pericyte
apoptosis via α3β1 integrin signaling in diabetic retinopathy.
RESEARCH DESIGN AND METHODS

Cell cultures

Human umbilical vein endothelial cells (HUVECs, Lonza), human retina microvascular endothelial cells (HRMECs, ACBRI), human brain astrocytes (ACBRI), and human pericytes (Promo cell) were maintained in EBM-2, M199 medium, DMEM with 20% FBS, and pericyte media containing growth factors (Promo cell), respectively. All cells were cultured at 37°C in an incubator with a humidified atmosphere of 95% O₂ and 5% CO₂.

Reagents and antibodies.

Recombinant mouse and human Ang2, human integrin α3β1, and PE-conjugated anti-Tie-2 and mouse IgG antibodies were purchased from R&D systems; anti-Bcl-2 family antibodies, EPITOMICS; anti-phospho-p53, anti-PARP, anti-cleaved caspase-3, anti-integrin β1 antibodies, Cell signaling Technology; anti-p53, anti-Tie1, peroxidase-conjugated secondary antibodies, Santa Cruz Biotechnology; Anti-Tie2 antibody and H-Gly-Arg-Gly-Asp-Ser-OH (GRGDS) peptide, Millipore; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Sigma-Aldrich; FITC-conjugated annexin V/propidium iodide assay kit, BD Biosciences; anti-NG2, anti-integrin α3β1, anti-Ang2 antibodies, Abcam; TUNEL fluorescein kit, Roche. Anti-integrin α1 (clone FB12, MAB1973), anti-integrin α3 (clone P1B5, MAB1952), anti-integrin β1 (clone 6S6, MAB2253), and α integrin blocking and IHC kit (α1-
6, v) were purchased from Millipore and used for functional blocking. siRNAs for p53 were purchased from Bioneer (Daejeon, Korea).

Animals.

All animal experiments in this study were in strict agreement with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Seoul National University Animal Care and Use Committee. Eight-week-old, pathogen-free male C57BL/6J mice were purchased from Central Lab. Animal Inc. After, 8 hours fast, diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ; Sigma) at a concentration of 180 mg/kg body weight in 10 mmol/l citrate buffer (pH 4.5). Age-matched controls received citrate buffer only. Mice with blood glucose levels > 300 mg/dl 4 days after STZ-injection were deemed diabetic. Diabetic and nondiabetic mice were killed 6 months after diabetes induction, and eyes were collected under deep anesthesia and immediately frozen at -80°C for ELISA, or fixed in 4% paraformaldehyde for retinal digestion. Glucose levels and body weight were monitored consecutively, and glycated hemoglobin was determined before sacrifice.

Retinal digest preparations.

Vascular preparations of whole-mount retinas were performed using a trypsin digestion
technique. Briefly, after at least 24 hours fixation in 4% paraformaldehyde, retinas were incubated in water for 1 hour. Then, retinas were digested in 2.5% trypsin (Gibco) at 37°C for 1 hour. After removal of inner limiting membrane carefully, the retinal vessels were isolated by careful irrigation with filtered water. The retinal digest samples were dried and stained with periodic acid and Schiff base for 15 minutes and hematoxylin.

**Morphological quantification of pericyte and acellular capillary.**

To determine numbers of retinal pericytes and acellular capillary, retinal digest preparations (n=7-8) were analyzed. Pericytes were identified according to the morphology and relative location to capillary. Total numbers of pericytes were counted in 10 randomly selected areas (magnification 400x) in the middle one-third of the retinal capillary area. Number of pericytes and acellular capillaries were standardized to the capillary area (numbers of cells or acellular capillaries per mm² capillary area). The capillary area was calculated using NIS-elements AR 3.2 program (Nikon). Samples were evaluated in a masked fashion.

**Intravitreal injection of Ang2 and anti-integrin antibodies.**

Normal six-week-old male mice were used. One microliters containing 100 ng of Ang2 and 500 ng of anti-integrin α3 (clone P1B5) or anti-integrin β1 (clone 6S6) was injected intravitreally under deep anesthesia. Sterile PBS was injected for control. After 10 days,
retinas were subjected to digestion preparation.

**Transferase-mediated dUTP nick-end labeling (TUNEL) assay and immunofluorescence.**

Retinal digestion preparations were incubated with anti-rabbit NG2 antibody (1:100) and TUNEL fluorescein kit. Nuclei were counter stained with 4’, 6-Diamidino-2-Phenylindole (DAPI). TUNEL and NG2-double positive cells were evaluated with a fluorescence microscope (Nikon, Tokyo, Japan).

**Cell viability assay.**

In all experiments, 2.5x10^4 cells were seeded into 96-well plates. After 24 hours, cells were treated with Ang1 (300 ng/ml), Ang2 (300 ng/ml) under normal glucose (5 mM glucose), high glucose (25 mM glucose), and high mannitol (5 mM glucose plus 20 mM mannitol) as an osmotic control for 48 hours. Cell viability was determined by MTT assay according to the manufacturer’s instructions. Three independent experiments were performed for each experimental condition.

**FACS analysis**

In order to evaluate the apoptosis, 5x10^5 cells were treated with Ang2 (300 ng/ml) under normal glucose, high mannitol, and high glucose at 37℃ for 48 hours. To determine the
effect of integrin blocking, anti-integrin blocking antibodies (5 µg/ml) was treated 1 hour before the addition of Ang2. The cells were harvested and washed two times in PBS. Cells were stained with FITC annexin-V and PI for 15 minutes, and analyzed by flow cytometry. Annexin V positive/PI negative cells were determined to apoptotic.

In order to evaluate Tie-2 expression, pericytes and HUVECs (1×10^6) were suspended in the complete media (25 µl) for each experiment. PE-conjugated anti-Tie2 antibody was added to the each sample at 4°C for 1 hour. PE-conjugated mouse IgG antibody was used as a control.

**Quantitative RT-PCR**

All RNA was collected and isolated from cells using RNeasy Plus Mini kit (Qiagen). cDNAs were prepared from RNAs (1µg) using 2.5 µM oligo-dT primers, 1mM dNTPs, and MuLV reverse transcriptase. qPCR assays were performed in qPCR mastermix for SYBR Green PCR Master MIX (Applied Biosystems) using 7900HT real-time PCR (Applied Biosystems). Reaction conditions were 50 cycles of 95°C for 5s and 60°C for 20s for qPCR. Quantitative real-time PCR was performed using the following primers: Ang2 (forward: 5’-ACTGTGTCCCTCTTCCACCAC-3’ and reverse: 5’-GGATGTTTAGGGTGTTTGCTTT-3’); Tie-2 (forward: 5’-GCTTGCTCCTTCTGGAAGGTCTTGCTTT-3’ and reverse: 5’-CGCCACCAGAGGCAAT-3’) (20); Tie-1 (forward: 5’-AGAACCTAGCCTCCAAGATT-
3’ and reverse: 5’-ACTGTAGTTCCAGGGACTCAA-3’; ITGA1 (forward: 5’-GTTCTACCTGGGAGTATT-3’ and reverse: 5’-AACCTTGCTTGAGGAGCA-3’); ITGA3 (forward: 5’-AGGGACCTTCAGTTCA-3’ and reverse: 5’-TGTTGCTTGCTGATTGAGGCA-3’); ITGB1 (forward: 5’-GAGGGTTGCCCTCCAGA-3’ and reverse: 5’-GCTTGAGCTTCTCTGCTGTT-3’); β-actin (forward: 5’-GCCGCCAGCTCACCACAT-3’ and reverse: 5’-TCGATGGGACTTCAGAAG-3’). A mean quantity was calculated from triplicate qPCR for each sample and it was normalized to the control gene.

For Tie-2 RT-PCR, different primers were used as following (Forward: 5’-TGTTCTGTGCCAAGGGCTG-3’ and reverse: 5’-CAGTGGGGTGCTTTCAA-3’). PCR products were separated on 1% agarose gels and visualized using SYBR® Safe DNA gel stain (Invitrogen) under UV transillumination.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation, Ang2 (500 ng), integrin α3β1 (500 ng), and anti-integrin α3β1 antibodies (2ug) were incubated with G-sepharose beads at 4°C overnight (19). Immune complexes were collected by centrifugation and washed with buffer 3 times. For immunoblotting, cells were harvested and lysed in RIPA buffer with a protease inhibitor cocktail. Protein lysates were resolved by SDS polyacrylamide gel and transferred onto
nitrocellulose membrane. The membranes were incubated with primary antibodies (1:1000) at 4°C overnight and secondary antibodies (1:5000) at room temperature for 1 hour. The membranes were incubated with enhanced chemiluminescent substrate (Pierce) and exposed to film.

**Statistical Analysis**

Statistical analyses were performed using the standard two-tailed Student t-test assuming unequal variances, and *p<0.01 was considered statistically significant. Quantitative data are given as mean±SD. Figures are depicted as mean±SE.
RESULTS

The number of retinal capillary pericyte is decreased in the diabetic mice retina.

The numbers of the pericyte and the acellular capillaries in retinal digestion were compared between mice with 6-month STZ-induced diabetes and age-matched nondiabetic control mice (Fig. 1A). The number of retinal capillary pericyte was significantly decreased in the STZ-induced diabetic mice retina (932.6±70.3) compared to that of the control group (1341.8±55.9, p<0.001) (Fig. 1B). On the other hand, the number of retinal acellular capillary was significantly increased in the STZ-induced diabetic mice retina (93.0±22.5) compared to that of the control group (28.1±11.5, p<0.001) (Fig. 1C). Table 1 shows the metabolic and physical parameters of the experimental groups.

Ang2 is increased in diabetic retina and the source of Ang2 could be endothelial cells.

The effect of hyperglycemia on Ang2 expression in STZ-induced diabetic retina was evaluated by qRT-PCR. In diabetic retinas at 6 months, Angpt2 mRNA increased 1.87 fold compared to non-diabetic normal retina (p=0.004) (Fig. 2A). Also, Angpt1 mRNA and Vegfa mRNA increased 2.54 fold (p=0.004) and 1.6 fold (p=0.027), respectively (Supplementary Fig. 1A, B).

Next, to determine the source of Ang2 in diabetic retina, we examined the effects of high glucose on in vitro ANGPT2 mRNA transcription by qRT-PCR in three major components of
Ang2 plays synergistic role in pericyte apoptosis under high glucose condition.

We determined the effect of Ang2 on the cell viability and apoptosis of pericyte under high glucose. Ang2 alone did not affect cell viability in pericyte (97.6±3.6%, p=0.221). High glucose reduced cell viability in pericyte (89.4±7.9%, p=0.020) (Fig. 3A). Interestingly, Ang2 aggravated cell death under high glucose in pericyte (72.4±2.9%, p=0.002) (Fig. 3A). Next, pericyte apoptosis was assessed by annexin-V/PI flow cytometric analysis. Of importance, the number of apoptotic pericyte was increased under high glucose (7.7±0.3%, p<0.001) compared that of the control group (2.4±0.4%). Furthermore, high glucose induced pericyte apoptosis was significantly aggravated by Ang2 (25.9±0.4%, p<0.001) while Ang2 alone did not induced apoptosis (2.7±0.1%, p=0.298) under normal glucose (Fig. 3B and Supplementary Fig. 2A).

blood retinal barrier: HRMEC, pericyte, and astrocyte. In HRMEC, high glucose increased ANGPT2 mRNA level more than 1.5-fold (1.52±0.09, p=0.003) compared with normal glucose (Fig. 2B). On the other hand, high mannitol, an osmotic control, did not increase ANGPT2 mRNA (1.21±0.13, p=0.094) in HRMEC (Fig. 2B). Also, high glucose did not increase ANGPT2 mRNA in pericyte (1.13± 0.22, p=0.418) and astrocyte (1.13± 0.22, p=0.418), respectively (Fig 2B and C). These data demonstrate that Ang2 increases in diabetic retina and the source of an Ang2 increase is retinal microvascular endothelial cells.
Next, we determined the effect of Ang2 on the cell viability and apoptosis of HRMEC under high glucose condition. Ang1 and Ang2 increased cell viability in HRMEC under high glucose (114.4±8.8%, p=0.014, and 109.5±2.3%, p=0.040, respectively) (Fig. 3C). As expected, apoptotic cell population was significantly decreased by Ang2 in HRMEC (Fig. 3D, and Supplementary Fig. 2B). These data suggested that Ang2 plays synergistic role in pericyte apoptosis under high glucose condition and protective effect on endothelial cell.

**Ang2 induces pericyte apoptosis via the p53 pathway under high glucose condition.**

Western blot studies confirmed that Ang2 induced apoptosis pathway with increase of Bax, cleaved PARP, and cleaved caspase-3 under high glucose, but not under normal glucose (Fig. 4A). Next, we aimed to identify the mechanism that mediates Ang2 induced pericyte apoptosis under high glucose. We found that Ang2 induced p53 phosphorylation (Fig. 4B) and subsequently p53 accumulation (Fig. 4C) under high glucose. Then, to determine the role of the p53 pathway for the observed Ang2-mediated pericyte apoptosis, we treated pericytes with either control siRNA or two different p53 siRNAs. The p53 siRNAs effectively down-regulated p53 expression (Fig. 4D), and they also attenuated Ang2 induced pericyte apoptosis under high glucose (Fig. 4E). Interestingly, Ang2 phosphorylated ERK, but not Akt (Fig. 4F). This ERK phosphorylation by Ang2 was inhibited by PD98059 (ERK inhibitor), and ERK inhibitor attenuated Ang2 induced p53 phosphorylation (Fig. 4G). These data suggest that
Ang2 induces pericyte apoptosis via the p53 pathway under high glucose condition.

*Integrin, not Tie-2, is important for Ang2 induced pericyte apoptosis under high glucose as an Ang2 receptor.*

To determine whether Tie-2 receptor is related with Ang2 induced pericyte apoptosis, western blot analysis (Fig. 5A) and RT-PCR (Fig. 5B) for Tie-2 and Tie-1 were performed on lysates obtained from HUVEC and pericyte. Interestingly, pericyte did not express Tie-2 or Tie-1 while HUVEC expressed Tie-2 and Tie-1. These data were confirmed by quantitative RT-PCR. *TIE2* mRNA and *TIE1* mRNA levels were significantly lower in pericyte than HUVEC (Fig. 5C). Compared to HUVEC with Tie-2 expression, pericyte did not express Tie-2 in FACS analysis (Fig. 5D).

To test whether or not integrins may serve as receptors for Ang2 in pericyte apoptosis under high glucose, we incubated pericyte under high glucose for 15 minutes with Ang2 and GRGDS peptides, which can inhibit integrins that bind RGD sequence (21). GRGDS (0.5 mg/ml) attenuated Ang2 induced p53 phosphorylation (Fig. 5E). This result showed that integrin signaling is involved in Ang2 induced p53 phosphorylation. Of importance, these data suggested that integrin, not Tie-2, is important for Ang2 induced pericyte apoptosis under high glucose as an Ang2 receptor.
High glucose increases integrin α1, α3, and β1 in pericyte.

As shown in Fig.3C and Fig. 5E, Ang2 induced pericyte apoptosis via integrin receptor under high glucose, but not under normal glucose. We hypothesized that high glucose preconditioned pericyte susceptible to Ang2 by changing the integrin pattern. To determine which integrin subunit is responsible for Ang2 induced pericyte apoptosis, we screened the integrin α subunits (α1-6 and αv). Integrin α1 and α3 blocking antibodies attenuated Ang2 induced p53 phosphorylation (Fig. 6A).

Next, to determine whether high glucose changes the integrin expression pattern, we performed quantitative RT-PCR and western blot studies for α1, α3, and β1. The choice was made because integrin α1, α3 can form the heterodimer with only integrin β1 (22). High glucose increased ITGα1 (1.86-fold and 2.20-fold, p<0.01) (Fig. 6B), ITGα3 (1.50-fold, 1.83-fold, p<0.01) (Fig. 6C), and ITGβ1 (1.50-fold, 1.43-fold, p<0.01) (Fig. 6D) mRNA levels for 24 hours and 48 hours, respectively. In addition, high glucose increased integrin α1, α3, and β1 expression (Fig. 6E). However, the integrin α1 was rarely expressed compared to integrin α3. In this regard, we performed co-immunoprecipitation assay to show direct binding of Ang2 to integrin α3β1. Indeed, Ang2 directly bound to integrin α3β1 (Fig. 6F).

Ang2 induced pericyte apoptosis is inhibited by suppression of integrin α3β1.

From the result of integrin expression in pericyte under high glucose, integrin α1β1 or α3β1...
were supposed to be the possible receptor of Ang2. To determine whether Ang2 induced pericyte apoptosis through integrin α1β1 or α3β1, pericyte was incubated under 25 mM high glucose with Ang2 and anti-integrin α1, α3, and β1 antibodies for 48 hours. Interestingly, Ang2 induced pericyte apoptosis under high glucose was attenuated by anti-integrin α3 and β1 antibodies, not by anti-integrin α1 antibody (Fig. 7A, and Supplementary Fig. 3A). These results were confirmed for p53 expression on western blot analysis (Fig. 7B). Ang2 significantly increased p53 expression under high glucose (2.7±0.2, p<0.01), but it was significantly attenuated by anti-integrin α1, α3, and β1 antibodies (1.5±0.2, 1.0±0.1, and 0.7±0.1, respectively, p<0.01).

Based on in vitro experiments, we next intravitreously injected 100ng Ang2 with/without 500 ng anti-α3 or 500 ng anti-β1 antibodies to normal mice. Ten days after intravitreal injection, the isolated retinae were digested with trypsin for pericyte evaluation (Fig. 7C). In vivo, intravitreal injection of Ang2 induced pericyte loss in the retina compared to that in PBS injected control mice (1047±52 and 1401±109 cells/mm², p<0.001). Ang2 induced pericyte loss was significantly attenuated by anti-α3 or anti-β1 antibodies (1354±148 and 1380±66 cells/mm², p<0.001) (Fig. 7D). In addition, Ang2 induced TUNEL and NG2 double positive pericytes were decreased in anti-α3 or anti-β1 antibodies injected mice (Fig. 7E). These in vivo data suggest that Ang2 induced pericyte loss via apoptotic mechanism in retina. Overall, these data demonstrated that integrin α3 and β1 important for Ang2 induced pericyte
apoptosis via p53 pathway.
Discussion

In this study, we demonstrated that pericyte loss occurred with Ang2 increase in the diabetic mice retina (10,11,13). Previously, Tie-2 was known to be related with Ang induced pericyte survival and recruitment (11). However, there was no direct evidence that Ang2 induced pericyte apoptosis via Tie-2. Unlike the previous study (11), pericyte did not express Tie-2 mRNA or protein in our study. While Ang2 alone did not induce apoptosis in vitro (11), Ang2 induced pericyte apoptosis under high glucose via p53 pathway (Fig. 4). Thus, we postulated that Ang2 would induce Tie-2 independent apoptosis in pericyte. Accumulating evidence support the role of integrin in Ang2 activity in endothelial cells (19). However, little is known about Ang2/integrin system in the pericyte. Thus, we focused on the integrin receptors on pericytes.

Integrins are cell adhesion molecules that are expressed on the surface of endothelial cells and pericytes. The contribution of endothelial and mural cell integrins to angiogenesis has been studied. Pericytes express integrins including: the collagen receptors, α1β1, α2β1; the laminin receptors, α3β1, α6β1, α6β4, α7β1; the fibronectin receptor, α4β1, α5β1; and the osteoponin receptors, α8β1, α9β1 (22). Cytokines and extracellular matrix changes integrin subtypes in pericyte (23). Based on the differential response to Ang2 in pericyte depending on glucose concentration (Fig. 3B and 4A), we hypothesized that high glucose could change integrin subtype more susceptible to Ang2. During quantitative RT-PCR array for screening
integrin subtype change response to high glucose, only ITGα1, ITGα2, ITGα3, and ITGβ1 mRNA increased over 1.5-fold among ITGα1, ITGα2, ITGα3, ITGα4, ITGα5, ITGα6, ITGα7, ITGαv, and ITGβ1 mRNA (data not shown). In addition, basal ITGα3 mRNA was over 6 times higher than ITGα1 or ITGα2 mRNA in pericyte even under normal glucose condition. Thus, we concluded that integrin α3 and β1 are both predominant and high glucose inducible integrins in pericyte. The proportion of integrin α1 in pericyte is too minor to be responsible for Ang2 induced pericyte apoptosis (Fig. 6E and 7B). In addition, many integrins, including αvβ3, α5β1, αIibβ3, αvβ6, and α3β1 recognize the tripeptide Arg-Gly-Asp (RGD) in their ligands (24). On the other hand, integrin α1β1 recognizes a configuration of residues formed by arginine and aspartic acid residues, not RGD. Attenuation of p53 by GRGDS supported that Ang2 induced pericyte apoptosis was effectively attenuated by blocking integrin α3β1 (Fig. 5E and 7B). Integrin receptors induced by hyperglycemia in the diabetic retinopathy could make the pericyte more susceptible to the Ang2, in turn, lead to the pericyte apoptosis by Ang2/Integrin signaling pathway.

The laminin receptor α3β1 integrin is expressed in vascular endothelial cells and acts to suppress pathological angiogenesis in the retina and other organs (25,26). However, a role for integrin α3β1 in pericyte is yet to be determined (22). Inhibition of β1 integrin induced a rounded morphology of the pericytes, suggesting pericyte adhesive properties were affected or that these cells were undergoing apoptosis (27). In this study, we suggested that integrin
α3β1 was important for Ang2 induced apoptosis. This is notably different from classical concept that Tie-2 mediates the effect of Ang2 as an Ang2 receptor. Absence of Tie-2 in human pericyte used in this study was confirmed by various experiments including RT-PCR with two different primers, western blot, and FACS analysis (Fig. 5A-D). In addition, both Ang1 and Ang2 can directly bind to some integrins and thereby signal in the absence of Tie-2 (28-31). Thus, we suggested that integrin mediated Ang2 induced apoptosis at least in Tie-2 negative pericyte.

Previous studies have shown that the primary source of Ang2 is vascular endothelial cells (32-35). High glucose increases Ang2 mRNA in HRMEC (36). Endothelial cell is regarded as potential source of Ang2 in the retina by transient rapid release from Weibel-Palade body and chronic upregulation of Ang2 (32,37,38). Chronic hyperglycemia upregulates Ang2 in the diabetic retina (36,39) and Ang2 upregulation is causally involved in the pathogenesis of pericyte loss in diabetic retinopathy (10). Hyperglycemia induces pericyte loss not only by increasing Ang2 in endothelial cells, but also changing integrin subtype prone to Ang2 in pericytes. Our data support that glycemic control is important to prevent Ang2 induced pericyte loss in early diabetic retinopathy. Glycemic control is the effective treatment to reduce the progression of diabetic retinopathy (40-42).

We showed that Ang2 induced pericyte apoptosis under high glucose via p53 pathway. Furthermore, in vitro and in vivo, Ang2 induced pericyte apoptosis was effectively attenuated
by blocking integrin α3β1. Integrin α3 and β1 blockers reduced p53 signaling by Ang2. Ser-15 of p53 is phosphorylated by a mitogen-activated protein kinase-dependent pathway (ERK1/2), and this step is required for apoptosis to occur (43).

It has been suggested that hyperglycemia causes pericyte loss by apoptosis (6-8). In this study, we postulated that hyperglycemia induced Ang2 overexpression in endothelial cells, in turn, caused apoptotic cell death of pericytes. In an animal model of diabetic retinopathy, high glucose was induced by STZ-injection as early as 3 days. Then, Ang2 increased upon 3 months with pericyte loss, and finally acellular capillary at 6 months (10,44). The mechanism by which capillaries become acellular is largely unknown. Apoptosis preceding the formation of acellular capillaries in retinae from diabetic rats and humans is one mechanism by which endothelial cells could be eliminated from the diabetic capillary (8). Retinal capillary coverage with pericytes is crucial for the survival of endothelial cells. Loss of pericytes is related to the increase of acellular capillaries from hyperglycemic injury (5). Based on our data, Ang2 did not directly induce endothelial cell death (Fig. 3C and D). Thus, we postulated that acellular capillary could be endothelial cell death secondary to pericyte loss by Ang2.

In conclusion, we demonstrated that pericyte loss occurred with Ang2 increase in the diabetic mice retina. Ang2 induced pericyte apoptosis via p53 pathway under high glucose. High glucose increased integrin α3β1 in pericyte. Interestingly, integrin was involved in Ang2 induced pericyte apoptosis. Furthermore, Ang2 induced pericyte apoptosis was effectively
attenuated by blocking integrin α3β1 both in vitro and in vivo. Taken together, Ang2 induced p53-dependent pericyte apoptosis via α3β1 integrin signaling in diabetic retinopathy. We suggest that glycemic control or blocking Ang2/integrin signaling could be a potential therapeutic target to prevent pericyte loss in early diabetic retinopathy.
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The authors confirm that there are no conflicts of interest relevant to this article.

S.W.P. and J.-H.Y. performed the experiments and wrote the manuscript. Ji.H.K. and K.-W.K. analyzed the data and reviewed the manuscript. Je.H.K. and C.-H.C. designed the study and critically revised the paper. Je.H.K. is the guarantor of this work, as such, takes full responsibility for the work including the integrity and accuracy of the data, and the decision to submit and publish the manuscript.
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Table 1. Metabolic and physical parameters of the STZ-induced diabetic and age-matched non diabetic control mice at 6 months.

<table>
<thead>
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<th>Non-diabetic</th>
<th>Diabetic</th>
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<tr>
<td>Body weight (g)</td>
<td>32.46±3.22</td>
<td>18.97±0.69</td>
<td>&lt;0.001</td>
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<tr>
<td>Blood glucose (mmol/l)</td>
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<td>32.83±1.34</td>
<td>&lt;0.001</td>
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<tr>
<td>HbA1c (%)</td>
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<tr>
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Figure Legends

Figure 1: The number of retinal capillary pericyte is decreased in the diabetic mice retina. Pericytes were identified in retinal digest preparations by morphologic criteria (shape, staining intensity, and relative position in the capillary), and quantitated in 6 month streptozotocin (STZ) induced diabetic mice and age-matched controls. (A) Representative examples of retinal digest preparations of nondiabetic and diabetic mice after 6 months of diabetes are shown. Arrows indicate pericytes. Arrowheads indicate acellular capillaries. (B and C) The number of pericytes (B) and acellular capillaries (C) were normalized to the area of capillaries (mm²) in which they were counted. A: periodic acid-Schiff (PAS) and hematoxylin-stained retinal digest preparations; original magnification 400x. Scale bar=20 μm. B and C: n=8 mice in each group. Bar graph represents mean±SE, Student t-test: *p<0.01

Figure 2: Ang2 is increased in diabetic retina and the source of Ang2 could be endothelial cells. (A) Angpt2 mRNA level was determined in 6 month streptozotocin (STZ)-induced diabetic mice retina by qRT-PCR and normalized to Rn18s mRNA. Ang2 expression increased in 6 month STZ-induced diabetic mice retina. (B, C, and D) ANGPT2 mRNA transcription is induced by high glucose in HRMEC (B), not in pericyte (C) or astrocyte (D). HREMC, pericyte, and astrocyte were incubated for 48 hours under 25 mM high glucose and 20mM mannitol + 5mM glucose, as an osmotic control. ANGPT2 mRNA transcription were
assessed by qRT-PCR. Actin was used as an internal control. *ANGPT2*-mRNA levels were normalized to *ACTIN* mRNA and reported as fold induction compared to cells exposed to 5mM glucose. *A*: n=6 mice in each group. Bar graph represents mean±SE, Con; age-matched normal mice, DM; streptozotocin induced diabetic mice. Student t-test: *p<0.01, #p>0.05

**Figure 3: Ang2 plays synergistic role in pericyte apoptosis under high glucose condition.**

The effects of Ang2 on the cell viability and apoptosis of pericyte and HRMEC under high glucose condition were determined. Both pericyte and HRMEC were incubated for 48 hours with/without Ang1 (300 ng/ml) or Ang2 (300 ng/ml) under high glucose (25 mM). (*A, C*) Cell viability was assessed by MTT assay. Ang2 induced cell death under high glucose in pericyte (*A*), but not in HRMEC (*C*). (*B, D*) Pericyte and HRMEC were stained with annexin-V FITC and propidium iodide and analyzed by flow cytometry. Cell apoptosis was expressed as the percentage of apoptotic cells in total cell populations. (*B*) High glucose induced pericyte apoptosis and Ang2 aggravated that apoptosis. (*D*) Ang2 showed protective effect on HRMEC apoptosis. Bar graph represents mean±SE of three independent experiments. NG; 5mM glucose, HM; 5mM glucose + 20mM mannitol, HG; 25mM glucose. Student t-test: *p<0.01

**Figure 4: Ang2 induces pericyte apoptosis via the p53 pathway under high glucose condition.** (*A*) Western blot analysis for Bax, Bcl-2, Bcl-xL, cleaved caspase-3, and cleaved
PARP were performed on lysates obtained from pericyte treated with Ang2 (300 ng/ml) under 5mM glucose, 5mM glucose + 20mM mannitol, and 25 mM glucose for 48 hours. (B) Western blot analysis for phospho-p53 (Ser15) was performed on lysates obtained from pericyte treated with Ang2 for 15, 30, and 60 minutes under 25 mM high glucose. (C) Western blot analysis for p53 was performed on lysates obtained from pericyte treated with Ang2 for 24 and 48 hours under 25 mM high glucose. (D) After the pericytes transfection with either control siRNA or p53 siRNA, western blot analysis for p53 was performed on cell lysates with Ang2 for 48 hours under 25 mM high glucose. β-tubulin was used as a loading control. Data represent three independent experiments. (E) Apoptotic cell counts were assessed by FACS analysis 48 hours after Ang2 treatment in siRNA transfected pericytes. Bar graph represents mean±SE of three independent experiments. Student t-test: *p<0.01. (F) Ang2 was treated for 5, 15, 30, and 60 minutes under 25mM high glucose. Phospho-ERK, ERK, phosphor-Akt, and Akt were determined by western blot. (G) Pericytes were preincubated with either Wortmannin (1 µM) or PD98059 (20 µM) for 1 hour, and treated with Ang2 for 15 minutes under high glucose. Phospho-p53, p53, phospho-ERK, ERK, phosphor-Akt, and Akt were determined by western blot. β-tubulin was used as a loading control.

**Figure 5: Integrin, not Tie-2, is important for Ang2 induced pericyte apoptosis under high glucose as an Ang2 receptor.** Western blot (A) and RT-PCR (B) for Tie-2 and Tie-1
expression were performed on lysates obtained from HUVEC and pericyte. β-tubulin was used as an internal control. (C) TIE2 and TIE1 mRNA transcriptions were assessed by quantitative RT-PCR. Actin was used as an internal control. TIE2 (C) and TIE1 (D) mRNA levels were normalized to ACTIN mRNA and reported as fold induction compared to HUVEC. (D) Both HUVEC and pericyte were analyzed by flow cytometry for Tie-2 expression. (E) Western blot analysis for phospho-p53 (ser15) and p53 were performed on lysates obtained from pericyte treated with Ang2 (300 ng/ml) or RGDS (0.5 mg/ml) under 25 mM high glucose for 15 minutes. β-tubulin was used as a loading control. Data represent three independent experiments.

**Figure 6: High glucose increases integrin α1, α3, and β1 in pericyte.** (A) Western blot analysis for phospho-p53 (Ser15) and p53 were performed on lysates obtained from pericyte treated with Ang2 (300 ng/ml) or various integrin blocking antibodies (5 µg/ml, α1, α2, α3, α4, α5, α6, and αv) under 25 mM high glucose for 15 minutes. β-tubulin was used as a loading control. Data represent three independent experiments. (B, C, and D) Pericyte were incubated under 25 mM high glucose for 24 and 48 hours. ITGa1 (B), ITGa3 (C), and ITGb1 (D) mRNA transcriptions were assessed by quantitative RT-PCR. Actin was used as an internal control. ITGa1, ITGa3, and ITGb1 mRNA levels were normalized to actin mRNA and reported as fold induction compared to control. (E) Western blot analysis for integrin α1, α3, and β1 were

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performed on lysates obtained from pericyte incubated under 25 mM high glucose for 48 hours. β-tubulin was used as a loading control. Data represent three independent experiments. Student t-test: *p<0.01. (F) After incubation of Ang2 (500 ng), integrin α3β1 (500ng), and integrin α3β1 antibody, immune complexes were co-immunoprecipitated to show direct binding of Ang2 to integrin α3β1. Then, they were analyzed for Ang2 and integrin β1. Same amounts of recombinant Ang2 and integrin α3β1 were used as an input.

**Figure 7: Ang2 induced pericyte apoptosis is inhibited by suppression of integrin α3β1.**

(A, B) Pericyte was incubated under 25 mM high glucose with Ang2 (300 ng/ml) and anti-integrin α1, α3, and β1 antibodies (5 µg/ml) for 48 hours. (A) Pericyte apoptosis was analyzed by FACS and expressed as the percentage of apoptotic cells in total cell populations. (B) Western blot analysis for p53 was performed on lysates obtained from pericyte. β-tubulin was used as a loading control. Data represent three independent experiments. (ImageJ quantitation; n = 3, mean±SD, 2-tailed Student’s t test, *p<0.01 compared to Ang2 treatment.)

(C) Ang2 (100 ng) with/without anti-α3 or anti- β1 antibodies (500 ng) were intravitreously injected to normal mice. Ten days after intravitreal injection, eyes were enucleated and the isolated retinae were digested with trypsin for pericyte evaluation. Pericytes were identified in periodic acid-Schiff (PAS) and hematoxylin-stained retinal digest preparations by morphologic criteria. Representative examples of retinal digest preparations are shown.
Arrows indicate representative pericytes. (D) The number of pericytes was normalized to the area of capillaries (mm$^2$) in which they were counted. (E) Retinal digest preparations were immunostained with NG2 (red), TUNEL (green), and DAPI (blue). White arrows indicate TUNEL positive pericytes. C and E: original magnification 400x. Scale bar=20µm. C: n=8 mice in each group. Bar graph represents mean±SE, Student t-test: *p<0.01
Diabetes

180x99mm (300 x 300 DPI)
**A**

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<th>(-)</th>
<th>(+)</th>
<th>(+)</th>
<th>(+)</th>
<th>(+)</th>
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</table>

**B**

**ITG α1**

![Graph showing ITG α1 expression over time](image)

**C**

**ITG α3**

![Graph showing ITG α3 expression over time](image)

**D**

**ITG δ1**

![Graph showing ITG δ1 expression over time](image)

**E**

**Integrin α1**

**Integrin α3**

**Integrin β1**

**β-tubulin**

![Graph showing integrin expression](image)

**F**

<table>
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<tr>
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<th>IB: Integrin β1</th>
<th>IB: Ang2</th>
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<td>Ang2 + α3β1</td>
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180x199mm (300 x 300 DPI)
Supplementary Figure Legends

Supplementary Figure 1: Angpt1 and Vegfa mRNA expression in diabetic retina. (A and B) Angpt1 and Vegfa mRNA in 6 month streptozotocin-induced diabetic mice retina were assessed by qRT-PCR and normalized to Rn18s mRNA. n=6 mice in each group. Bar graph represents mean±SE, Con; age-matched normal mice, DM; 6 month streptozotocin-induced diabetic mice, Student t-test: *p<0.01.

Supplementary Figure 2: Ang2 induced cell apoptosis under high glucose were determined by flow cytometry. Cells were incubated for 48 hours under 25 mM high glucose or 5 mM glucose + 20 mM mannitol as an osmotic control, or 5 mM glucose. Cells were treated with Ang2 (300 ng/ml). Representative results from flow cytometric analysis of annexin-V FITC/propidium iodide stained pericytes (A) and HUVEC (B) are shown. The results shown represent one from three independent experiments.

Supplementary Figure 3: Ang2 induced apoptosis under high glucose were determined in the p53 siRNA treated pericytes by flow cytometry. Cells were incubated for 48 hours under 25 mM high glucose. Cells were treated with Ang2 (300 ng/ml). Representative results from flow cytometric analysis of annexin-V FITC/propidium iodide stained pericytes are shown. The results shown represent one from three independent experiments.
Supplementary Figure 4: Ang2 induced pericyte apoptosis after treatment of anti-integrin α1, α3, and β1 antibodies were determined by flow cytometry. Cells were incubated for 48 hours under 25 mM high glucose. Cells were treated with Ang2 (300 ng/ml) and anti-integrin α1, α3, and β1 antibodies (5 µg/ml). Representative results from flow cytometric analysis of annexin-V FITC/propidium iodide stained pericytes are shown. The results shown represent one from three independent experiments.
<Pericyte under HG>

Con          Ang2

Control siRNA

Propidium Iodide

p53 siRNA (1)

p53 siRNA (2)

Annexin V

88x150mm (300 x 300 DPI)
&nbsp;