The Effects of the CXCR4 Antagonist, AMD3465, on Human Retinal Vascular Endothelial Cells (hRVECs) in a High Glucose Model of Diabetic Retinopathy

Di Wu, Li Jin, Hongshuang Xu

Background: High blood glucose levels in diabetes result in retinal angiogenesis, which is the key feature of diabetic retinopathy. This study aimed to investigate the effects of the CXCR4 antagonist, AMD3465, on human retinal vascular endothelial cells (hRVECs) in vitro.

Material/Methods: Cell viability and the protein expression levels of CXCR4 and stromal cell-derived factor 1 (SDF-1) were evaluated in high glucose (HG)-treated human retinal vascular endothelial cells (hRVECs). The cell counting kit 8 (CCK-8) assay, the colony formation assay, immunofluorescence, and Western blot were used to investigate the effects of AMD3465 on hRVEC cell viability, colony formation, cell proliferation, and expression of CXCR4 and SDF-1. Cell apoptosis and angiogenesis were assessed by flow cytometry and Western blot.

Results: Treatment with high glucose reduced the viability of hRVECs and increased the protein expression levels of CXCR4 and SDF-1. Following treatment with AMD3465, the colony formation capacity and cell proliferation in hRVECs increased, and there was a significant reduction in apoptosis rate compared with the untreated cells. AMD3465 significantly reduced the expression of angiogenesis-associated proteins, including ICAM1, VCAM1, VEGF, and AngII. AMD3465 significantly reduced the protein expression levels of TNF-α, IL-1β, NF-κB, and p-p65.

Conclusions: The CXCR4 antagonist, AMD3465, reduced apoptosis of HG-treated hRVECs in an in vitro model of diabetic retinopathy.

MeSH Keywords: Diabetic Retinopathy • Protective Agents • Receptors, CXCR4

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Background

Worldwide, diabetes mellitus is a common chronic disease [1]. Type 2 diabetes mellitus (T2DM) is associated with complications that include long-term damage to vital organs, including the eyes, kidneys, nerves, and blood vessels [2]. Diabetic retinopathy is associated with chronic diabetes mellitus and is the main ocular complication of the disease [3,4]. Previous studies have shown that diabetic retinopathy can be caused by biochemical changes, apoptosis, inflammation, oxidative stress, and angiogenesis [5–7]. Among these factors, angiogenesis is one of the most detrimental events responsible for the progression of diabetic retinopathy. The formation of new blood vessels from the existing vasculature generates anatomical and physiological changes that can result in blindness [8].

CXCR4 is a chemokine receptor that selectively binds to the CXC chemokine stromal cell-derived factor 1 (SDF-1), or CXCL12 [9]. CXCR4 is associated with chemotaxis, invasion, angiogenesis, and cell proliferation [10]. The SDF-1/CXCR4 axis takes part in many physiological processes, including immune cells homoeostasis and cell trafficking [11]. This axis contributes to the activation of tip cells and microglia in retinal angiogenesis [12]. Also, the expression of CXCR4 participates in angiogenesis associated with increased expression of SDF-1 in injured tissues [13]. Because CXCR4 is a target for several diseases, there is therapeutic potential for novel CXCR4 antagonists. AMD3465 is a CXCR4 antagonist that has been shown to inhibit tumor cell growth in a mouse model by the downregulation of glycogen synthase kinase-3 (GSK3) and decreased angiogenesis [14]. GSK3 is an important factor involved in the signaling pathways to regulate angiogenesis [15]. Also, another CXCR4 antagonist, AMD3100, has been shown to reduce the expression of different angiogenic factors and to cause a significant reduction in tumor growth and invasion [16,17]. However, although there is considerable knowledge concerning the function of the CXCR4 antagonist, AMD3465, in tumor angiogenesis, few studies have identified its relationship with diabetic retinopathy. Therefore, this study aimed to investigate the effects of the CXCR4 antagonist, AMD3465, on human retinal vascular endothelial cells (hRVECs) treated with high levels of glucose.

Material and Methods

Cell culture and establishment of a high glucose (HG)-treated human retinal vascular endothelial cell (hRVEC) model of diabetic retinopathy

Human retinal vascular endothelial cells (hRVECs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in 5% CO2. The normal group (cells cultured with 5.5 mmol/L glucose), the low group (cells cultured in 20 mmol/L glucose), the middle group (cells cultured in 80 mmol/L glucose), and the high group (cells cultured in 120 mmol/L glucose) were studied to determine the optimum concentration of glucose to be used in the following experiments. The hRVECs were divided into three groups: the control group (cells cultured with 5.5 mmol/L glucose), the model group (cells cultured in 80 mmol/L glucose), and the model+AMD3465 group (cells cultured in 80 mmol/L glucose and 2 μM of AMD3465).

Cell viability assay

The hRVEC cells were seeded in 96-well plates at 4×10^3 cells per well with glucose at different concentrations and incubated for 72 h. Cell viability was measured using a cell counting kit 8 (CCK-8) assay kit (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer’s protocol. Absorbance was measured at 450 nm using a microplate spectrophotometer.

Colony formation assay

The cells were seeded in 12-well plates at 5×10^4 cells/mL with glucose (80 mmol/L) and AMD3465 (2 μM). After incubation for 10 days, the plates were stained with 0.5% crystal violet (Wako, Osaka, Japan) in 4% paraformaldehyde for 5 minutes to count the colony numbers. The cell group containing more than 50 cells was identified as a colony. Each assay was repeated five times.

Immunofluorescence staining

The hRVEC cells were fixed on glass slides with 4% paraformaldehyde for 15 min and incubated in PBS with 0.1% Triton X-100 for 20 min to permeabilize the cells. After blocking using 5% dried skimmed milk powder in 0.01 M PBS, the cells were incubated with an antibody to Ki67 (SAB4501880) (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C. After washing three times in PBS, the cells were then incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA) for 2 h at room temperature. The sections were coverslipped, and the fluorescence staining was detected with an Olympus FV1000 confocal laser scanning microscope (Olympus, Shinjuku, Tokyo, Japan).

Western blot

The hRVEC cells were lysed with ice-cold RIPA buffer. After centrifugation at 12000 rpm for 10 min, the supernatant was collected and the concentration of protein was determined using a BCA protein assay reagent kit (Beyotime, Shanghai, China). The proteins (15 μg) were separated by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene
fluoride (PVDF) membrane. Then membrane was blocked with 5% dried skimmed milk powder for 2 h on a horizontal shaking table. The PVDF membranes were then incubated overnight at 4°C with the following primary antibodies to: CXCR4 (1: 500) (ab1670; Abcam, Cambridge, MA, USA); SDF-1 (1 µg/ml) (ab18919; Abcam, Cambridge, MA, USA); ICAM1 (1: 1000) (ab20; Abcam, Cambridge, MA, USA); VCAM1 (1: 1000) (ab98954; Abcam, Cambridge, MA, USA); VEGF (1: 2000) (ab53465; Abcam, Cambridge, MA, USA); Angll (1: 1000) (ab236317; Abcam, Cambridge, MA, USA); CDK2 (5 µg/ml) (ab6433; Abcam, Cambridge, MA, USA); cyclin E (1: 2000) (ab71535; Abcam, Cambridge, MA, USA); p21 (1: 3000) (ab227443; Abcam, Cambridge, MA, USA); Bcl2 (1: 500) (ab196495; Abcam, Cambridge, MA, USA); Bax (1: 500) (ab53154; Abcam, Cambridge, MA, USA); TNF-α (1: 2000) (ab6671; Abcam, Cambridge, MA, USA); IL-1β (1: 1000) (ab2105; Abcam, Cambridge, MA, USA); NF-κB (1: 1000) (ab194729; Abcam, Cambridge, MA, USA); p65 (1: 1000) (ab16502; Abcam, Cambridge, MA, USA); p-p65 (1: 1000) (ab28856; Abcam, Cambridge, MA, USA); and GAPDH (1: 2000) (ab6671; Abcam, Cambridge, MA, USA). The membranes were incubated with a horseradish peroxidase (HRP)-labeled secondary antibody (Abcam, Cambridge, MA, USA). The results were visualized with an ECL detection kit. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the density of the bands.

Flow cytometry

Apoptosis assays were performed using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Biosciences Pharmingen, San Jose, CA, USA). Briefly, after incubation for 48 h, the hRVEC cells were harvested and washed with cold PBS and resuspended at a concentration of 1×10⁶ cells/ml. Apoptotic cells were analyzed using flow cytometry. Statistical analysis was performed using Flowjo software (Tree Star, Ashland, OR, USA).

Statistical analysis

Data were analyzed using SPSS version 20.0 software. The results were expressed as the mean ± standard deviation (SD). Student’s t-test was used for comparison between the two groups, and one-way analysis of variance (ANOVA) was performed to compare three or more groups. A P-value <0.05 was considered to be statistically significant.

Results

The effect of high glucose on the viability of human retinal vascular endothelial cells (hRVECs)

To determine the appropriate concentration of glucose for this study, we first tested the viability of hRVECs with different concentrations of glucose. Glucose at 5.5 mmol/L was used to simulate the condition of hyperglycemia in vitro. The results of the cell counting kit 8 (CCK-8) assay showed that high glucose reduced the viability of hRVECs in a dose-dependent manner (Figure 1).

The effect of high glucose on protein expression of CXCR4 and SDF-1 in hRVECs

To investigate the effect of high glucose on CXCR4 and SDF-1 in hRVECs, Western blot analysis was performed. As shown in Figure 2, high glucose increased the protein expression of CXCR4 and SDF-1 in a dose-dependent manner when compared with the normal group. Based on this finding, glucose at a concentration of 80 mmol/L was chosen for the subsequent experiments.

The CXCR4 antagonist, AMD3465, promoted the proliferation of high glucose (HG)-treated hRVECs

To determine whether AMD3465 could affect cell proliferation of hRVECs under HG conditions, immunofluorescence staining, the colony formation assay, and Western blot were used. The results showed that the expression of Ki67 was significantly lower in the in vitro model than the control. In contrast to the model, the level of Ki67 was increased in hRVECs treated with AMD3465 (Figure 3A). The colony formation assay showed that colony numbers were significantly reduced under high glucose conditions compared with the control, while AMD3465 enhanced the ability of hRVECs to form colonies. Also, there was no significant difference in the colony numbers between the control group and the model+AMD3465 (Figure 3B). Western blot was used to determine the expression of proliferation-associated proteins in HG-treated hRVECs. High glucose levels significantly reduced the expression of CDK2 and cyclin E and increased the expression of p21 compared with...
Figure 2. High glucose increased the protein levels of CXCR4 and SDF-1 human retinal vascular endothelial cells (hRVECs) in a dose-dependent manner. Data are expressed as the mean ± standard deviation (SD). * P<0.05, ** P<0.01, *** P<0.001 versus control.
the control, while AMD3465 treatment had the opposite results (Figure 3C). These results indicated that AMD3465 facilitated the proliferation of HG-treated hRVECs.

**AMD3465 inhibited the apoptosis of HG-treated hRVECs**

Flow cytometry and Western blot were used to evaluate the effect of AMD3465 on apoptosis of HG-treated hRVECs. The results showed that high glucose significantly increased the apoptosis rate of HG-treated hRVECs compared with the control. However, AMD3465 treatment significantly reduced cell apoptosis (Figure 4A, 4B). The expression of the pro-apoptotic protein Bax was found to be increased, and the anti-apoptotic protein Bcl-2 was reduced in hRVECs with high glucose treatment, while AMD3465 treatment showed the opposite results (Figure 4C). These results showed that AMD3465 exerted an inhibitory effect on the apoptosis of HG-treated hRVECs.

**The effect of AMD3465 on endothelial cell function and angiogenesis in HG-treated hRVECs**

To determine whether AMD3465 affected endothelial cell function and angiogenesis, cell adhesion molecules and angiogenesis-related proteins were studied. The results of Western blot analysis showed that the protein levels of ICAM1, VCAM1, VEGF, and AngII in HG-treated hRVECs were significantly increased. The protein levels were decreased in HG-treated hRVECs when also treated with AMD3465 (Figure 5). These results showed that AMD3465 improved endothelial cell function, but inhibited angiogenesis in HG-treated hRVECs.

**AMD3465 protected HG-treated hRVECs by inhibiting the NF-κB signaling pathway**

To explore the potential mechanism of AMD3465 in protecting HG-treated hRVECs, we detected the NF-κB signaling pathway by Western blot analysis. The results showed that the expression of TNF-α, IL-1β, NF-κB, and p-p65 were significantly increased in HG-cultured hRVECs compared with the control. AMD3465 treatment reduced the expression of TNF-α, IL-1β, NF-κB, and p-p65. The protein expression of p65 was unchanged in the three groups (Figure 6). These data showed that AMD3465 protected HG-treated hRVECs partly by inhibiting the NF-κB signaling pathway.

**Discussion**

The findings from the present study of an in vitro model of diabetic retinopathy that used high glucose (HG)-treated human retinal vascular endothelial cells (hRVECs), showed that the CXCR4 antagonist, AMD3465, promoted endothelial cell proliferation, improved endothelial cell function, and inhibited apoptosis and angiogenesis. These findings indicated that...
AMD3465 protected hRVECs from the effects of high glucose by inhibiting the NF-κB signaling pathway.

High glucose can disrupt the physiology of normal human endothelial cells [18–21]. High glucose induces hyperosmolarity, which promotes angiogenesis and diabetic retinopathy [22,23]. Also, hypoxia induced by high glucose in retinal tissues stimulates angiogenesis by modulating the balance of pro-angiogenic and anti-angiogenic mediators, leading to retinal neovascularization [24–26]. Retinal neovascularization is the hallmark of diabetic retinopathy [27]. The SDF-1α/CXCR4 signaling axis is associated with many of these endogenous processes and has a critical role in promoting angiogenesis [28]. A previous study showed that the SDF-1/CXCR4 axis contributed to vasculogenesis and the migration of inflammatory cells by the recruitment of bone marrow-derived endothelial progenitor cells (EPCs) [29]. Another study showed that Danshensu promoted angiogenesis after myocardial infarction in rats and reduced apoptosis induced by high glucose in human retinal vascular endothelial cells (hRVECs). (A) Flow cytometry evaluated cell apoptosis in high glucose (HG)-treated hRVECs. (B) Western blot was used to examine the levels of apoptosis-related proteins, Bax and Bcl-2. Data are expressed as the mean ± standard deviation (SD). *** P<0.001 versus control; * P<0.05, *** P<0.001 versus the model.

Due to the over-activation of the SDF-1/CXCR4 axis induced by high glucose, a previous study showed that VEGF and EGFR levels were increased, which resulted in angiogenesis and apoptosis [32]. AMD3465 is a specific antagonist of CXCL12/CXCR4-mediated interactions, including ligand binding and receptor activation [33]. CXCR4 blockade has been shown to result in eosinophil recruitment in granuloma formation and to inhibit immune activation of T-cell in lymphoid tissue [34]. Recent studies have shown that AMD3465 inhibited breast cancer cell migration in vitro and prevented breast cancer cell growth and metastasis in vivo [14]. CXCR4 antagonists have been shown to significantly inhibit the proliferation, migration, and invasion of SW780 cells [35]. Our results indicated that AMD3465 promoted cell proliferation and inhibited cell apoptosis in HG-treated hRVECs, which is inconsistent with previous reports. We speculated that AMD3465 might regulate the proliferation of normal cells and malignant cells through different signaling pathways, but further studies should be performed to confirm this. Also, AMD3465 has previously been shown to inhibit angiogenesis in breast cancer by abolishing the phosphorylation of glycogen synthase kinase-3 (GSK3), which promotes angiogenesis and tumor progression [14]. Another CXCR4 antagonist, AMD3100, prevented the development of HPS via suppressing pulmonary angiogenesis in CBDL rats and reducing pulmonary C-Kit-positive cells [36]. Our results showed that
AMD3465 significantly reduced the expression of the angiogenesis-related proteins, VEGF, and AngII, in high glucose (HG)-treated hRVECs. These findings suggested that AMD3465 protected HG-exposed hRVECs by regulating cell proliferation, apoptosis, and angiogenesis.

Previous studies have shown that diabetes mellitus could disrupt the pathways that regulate the physiology of NF-κB, and that high glucose can activate several signaling pathways, including JNK, P38, MAPK, and NF-κB. Several pathological changes induced by high glucose lead to overactivation of NF-κB, such as accumulation of advanced glycation end products and...
oxidative stress [8,21]. This overactivation is responsible for the progression of angiogenesis during diabetic retinopathy. Therefore, we speculated that the NF-κB signal pathway plays critical roles in the progression of diabetic retinopathy. In recent studies, diabetic retinopathy could be prevented by inhibiting the activation of NF-κB in a rat model of HG-induced diabetic retinopathy treated with naringin [37], resolvin D1 [38], and resveratrol [39]. In the present study, the findings showed that AMD3465 treatment effectively inhibited the activation of NF-κB in HG-treated hRVECs to protect retinal endothelial cells from apoptosis and angiogenesis, but the underlying mechanisms remain to be determined.

Figure 6. The CXCR4 antagonist, AMD3465, exerted its effects in high glucose (HG)-treated human retinal vascular endothelial cells (hRVECs) by regulating NF-κB activation. Western blot, using specific antibodies, studied the effects of AMD3465 on the protein levels of TNF-α, IL-1β, NF-κB, and p-p65 in the NF-κB signaling pathway. GAPDH was used as an internal reference. Data are expressed as the mean ± standard deviation (SD). *** P<0.001 versus control; * P<0.05, ** P<0.01 versus the model.

Conclusions

This study aimed to investigate the effects of the CXCR4 antagonist, AMD3465, on human retinal vascular endothelial cells (hRVECs) in vitro. The findings from this study showed that treatment with AMD3465 could inhibit high glucose (HG)-induced apoptosis by reducing the level of Bax and Bcl-2 as well as promoting the proliferation of HG-induced hRVECs. AMD3465 suppressed HG-induced angiogenesis by reducing the activity of VEGF and AngII and inhibited the NF-κB signaling pathway. These findings indicated that AMD3465 might have therapeutic potential in the prevention of the progression of diabetic retinopathy.

Competing interests

None.
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