Original Article
Apoptotic effect of matrix metalloproteinases 9 in the development of diabetic retinopathy

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Abstract: Objective: To explore the potential regulatory mechanism of MMP9 in the development of DR. Methods: Plasmids pcDNA-MMP9 and pcDNA-Ang2 were transfected into primary rat retinal Müller cells (RMCs) using Lipofectamine 2000. Cell viability and apoptosis were respectively determined by MTT assay and flow cytometry. Moreover, the interaction between MMP9 and Ang2 was explored. Besides, RMCs were treated with MMP-9 under normal glucose and high glucose condition for 2d. Besides, the expression levels of apoptotic proteins, like MMP9, Ang2, Bax2, Bcl2, cleaved PARP and cleaved caspase3 were determined by Western blot. Results: The cell viability of siRNA-MMP9 group was significantly increased while decreased in MMP9 overexpression group when compared to control group, respectively. The apoptotic cells in MMP9 overexpression group significantly increased while decreased in siRNA-MMP9 group when compared with control group. MMP9 expression was significantly regulated by Ang2 whereas no significant changes occurred in Ang2 expression when MMP9 expression changed. Moreover, MMP9 expression in HG group significantly increased while there were no significant differences between NG group and control group. Besides, the expression of Bax2, Bcl2, cleaved PARP and cleaved caspase3 in HG group increased while there were no significant differences between NG group and control group. Conclusion: Our findings indicate that MMP9 may play an important role via inducing cell apoptosis in the development of DR via regulating by Ang2 or targeting apoptotic proteins, such as Bax2, Bcl2, cleaved PARP and cleaved caspase3.

Keywords: Diabetic retinopathy, matrix metalloproteinases 9, angiopoietin 2, apoptosis

Introduction

Diabetic retinopathy (DR) is a common complication of diabetes and continues to be a leading cause of blindness in working-age worldwide. It is characterized by retinal microvascular dysfunction and finally vision loss [1]. The clinical hallmarks of DR are increased vascular permeability, macular edema, vascular microaneurysms and vascular proliferation [2]. Early detection of DR is critical in preventing visual loss, but regrettfully, current management strategy, such as laser photocoagulation, is hampered by the fact that this disease is generally asymptomatic at early stages and only target advanced stages of disease [3]. Analysis of specific genetic alterations on molecular mechanisms will help the development of targeted therapies.

Several candidate genes have been implicated in the pathogenesis of DR via complex interactions of environmental, genetic, and epigenetic factors [4]. Recently, the secretion of several matrix metalloproteinases (MMPs), especially MMP9, is shown to play an important role in the pathogenesis of DR [5, 6]. MMP9 can be activated by increasing oxidative stress and affecting mitochondrial apoptotic machinery [7]. MMP-9 is also induced or repressed by a variety of cytokines and growth factors in diabetic retinal vasculopathy and neuropathy [8]. Moreover, MMP-9 is observed to up-regulate in retinal microvascular cells cultured under high glucose conditions [9-11]. Besides, MMP-9 is proved to be downstream of H-Ras and may induce the apoptosis of retinal capillary cells in the development of DR via activation of the Raf-1/mitogen activated kinase (MEK)/extracellular sig-
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nal-regulated kinase (ERK) [12]. Despite these, the molecular mechanism by which MMP-9 contributes to the development of DR is not fully understood.

In the current study, we used plasmid transfection method to overexpress and suppress MMP9 and angiopoietin 2 (Ang2) in primary rat retinal Müller cells (RMCs), respectively. Cell viability and apoptosis after plasmid transfection was determined using MTT assay and flow cytometry analysis. Moreover, the interaction of MMP9 with Ang2 was explored. Besides, cells were treated with different concentration of glucose and the expression of several apoptotic proteins were determined by western blot. Our study aimed to explore the potential mechanism of MMP9 underlying the development of DR and provide a new insight to identify novel targets for inhibiting the development of this disease.

Materials and methods

Cell culture

The primary RMCs culture was prepared as follows. Briefly, enucleated eyes from postnatal day 5-7 Wistar rats were incubated in Dulbecco’s Modified Eagle Medium (DMEM) overnight. Eyecups were transferred to DMEM (containing 0.1% Trypsin and 70 U/ml collagenase) and incubated in CO2 incubator at 37°C for 1 h. The retina was dissected out from retinal pigment epithelium (RPE) and ciliary epithelium with care to avoid contamination. The retina was then dissociated into small aggregates and incubated in DMEM containing 10% fetal bovine serum (FBS, Welgene Ltd.) for 8-10 days. The purified population of RMCs attached to the bottom of the dish was collected after removing floating retinal aggregates and debris. RMCs were then trypsinized and cultured in DMEM containing 10% FBS for another 5 days to get a further purified population.

Plasmid transfection

Plasmids pcDNA-MMP9 and pcDNA-Ang2 were purchased form the American Type Culture Collection (ATCC, Rockville) and were transfected into RMCs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as instructed by the manufacturer. On the other hand, cells were transfected with antisense or siRNA against MMP9 and Ang2 (Qiagen) using the same method. Cells continued to incubate with normal media for 4 h and were then harvested.

Cell viability assay

Cell viability was determined using MTT assay. Briefly, cells were seeded in 96-well plates at a density of 5 × 10^3 cells/well for 24 h. Subsequently, 10 μL MTT (5 mg/ml) was added to each well at 0 h, 24 h, 48 h, 72 h and 96 h after transfection and incubated for another 4 h. Each experiment was performed in triple. The resulting formazan crystals were dissolved in 100 μL DMSO, and the optical density (OD) at 570 nm was measured using an automatic microplate reader (BioTek Instruments, Inc.). Finally, cell viability of each treated group at different time points was calculated.

Apoptosis assay by flow cytometry analysis

Cell apoptotic rate was determined using fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kits (KeyGEN Biotech, Nanjing, China) using flow cytometry analysis. Cells were collected by trypsinization, washed twice with ice-cold PBS and resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/ml. Afterwards, 5 μl FITC Annexin V and 5 μl PI were mixed with 100 μl of cells, and continued to incubate for 15 min. Finally, the cells were then sent out at 488 nm by flow cytometry (BD, USA) and analyzed with the BD FACS calibur™ system (BD, USA).

Cell treatment with different concentration of glucose

Total 4 × 10^5 cells were seeded into 96-well plates. After 24 h, cells were treated with MMP-9 (300 ng/mL) under normal concentration of glucose (NG, 5 mmol/L glucose), high concentration of glucose (HG, 20 mmol/L glucose) for 2 d. Cells without any treatment were set as control.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was firstly extracted using the Trizol reagent (Invitrogen, Burlington, ON, Canada) following the manufacturer’s instructions. The concentration and purity of the total RNA were detected with a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) and then
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reversely transcribed into cDNA with the PrimeScriptTM RT Master Mix Kit (Takara, RR036A). Quantitative real-time PCR assays were carried out using SYBR Green real-time PCR MasterMix under ABI 7500 RT-PCR amplification equipment (Applied Biosystems, CA, USA). Human actin was used as internal control. The PCR reaction consisted of 1 cycle at 95°C for 15 s followed by 45 cycles at 95°C for 5 s and at 60°C for 30 s. The primer sequences were as follows: 5’-GCACCACCAACATCAC-3’ (sense), 5’-ACCACAACTCGTCATCGTC-3’ (antisense) for MMP-9 (284 bp), 5’-GGCGGCACCCATGTACCCT-3’ (sense), 5’-AGGGGCCGGACTGTCATACT-3’ (antisense) for actin (202 bp). Each reaction was performed in triplicate. The relative expression level of MMP-9 was normalized based on the expression level of actin and calculated using the comparative threshold (Ct) cycle (2^ΔΔCt).

Preparation of cell lysates and Western blot analysis

Western blot was performed to examine the expression levels of MMP9, Ang2, Bax2, Bcl2, cleaved PARP and cleaved caspase3. Briefly, cells were washed twice with ice-cold PBS and then lysed with RIPA buffer containing protease inhibitors (BestBio, Shanghai, China) on ice for 1 h. Total protein concentration was examined by BCA assay (Beyotime, Haimen, China). Equal amounts of protein (30 μg) were separated on 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocked in TBS-0.05% Tween-20 (TBST) containing 3% BSA, the membrane was incubated with an appropriate antibody at 4°C overnight. After washed three times with TBST, the membrane was then incubated with the second antibody conjugated with horseradish peroxidase (HRP) at 25°C for 1 h. The blots were developed by enhanced chemiluminescence kit (Millipore). Finally, the expression level of these proteins was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistics analysis

All the data are presented as the mean ± SD. The statistical analyses were performed using T-test or one-way ANOVA as appropriate, followed by Bonferroni’s test for multiple comparisons using SPSS 20.0 and GraphPad Prism 5.0. A value of $P \leq 0.05$ was considered statistically significant.

Results

The relationship of MMP9 expression with cell viability

MTT analysis showed the cell viability in an experimental period of 96 h after transfection. As shown in Figure 1, the cell viability gradually increased with the increase of transfected time and showed significant differences of transfected groups at 72 h after transfection compared with control group. Moreover, the cell viability of siRNA-MMP9 group was higher than the control group, while the cell viability of MMP9 overexpression group was significantly lower than the control group.

Detection of cell apoptosis

Flow cytometry analysis showed the percentage of apoptotic cells. The results showed that the apoptotic cells in MMP9 overexpression group significantly increased while that in siRNA-MMP9 group obviously decreased when compared with that in control group (Figure 2).

The interaction between MMP9 and Ang2

To explore the interaction of MMP9 with Ang2, we performed Western blot to determine Ang2 expression in MMP9 overexpression group and siRNA-MMP9 group, as well as to determine

Figure 1. The viability of primary rat retinal Müller cells at different times after transfection determined by MTT assay.
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MMP9 expression in Ang2 overexpression group and siRNA-Ang2 group. The results showed that there were no significant differences of Ang2 expression in MMP9 overexpression group and siRNA-MMP9 group when compared with that in control group, respectively (Figure 3A). Notably, MMP9 expression in Ang2 overexpression group significantly increased and obvious decreased in siRNA-Ang2 group when compared with that in control group (P < 0.05, Figure 3B), respectively, indicating MMP9 was a downstream target of Ang2.

Analysis of MMP9 expression under different concentration of glucose

The mRNA and protein expression level of MMP9 under different concentration of glucose were respectively determined by qRT-PCR and western blot analysis. Similar results were obtained that MMP9 expression in HG group significantly increased compared with that in control group (P < 0.05), while there were no significant differences between NG group and control group (Figure 4).

Figure 2. The apoptosis of primary rat retinal Müller cells determined by flow cytometry.

Figure 3. The expression level of MMP9 and Ang2 in primary rat retinal Müller cells of different groups. Error bars indicate means ± SD and *indicates significant difference compared with control group (P < 0.05).
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Western blot analysis showed the expression of apoptotic proteins, such as Bax2, Bcl2, cleaved PARP and cleaved caspase3, in HG group and NG groups, respectively. The results showed the expression of Bax2, Bcl2, cleaved PARP and cleaved caspase3 in HG group increased compared with that in control group, while there were no significant differences between NG group and control group (Figure 5). HG could enhance the expression of MMP9 (Figure 4), implying that MMP9 might induce cell apoptosis via enhancing the activity of these apoptotic proteins.

Discussion

The present study demonstrated the role of MMP9 in the development of DR and elucidated potential regulatory mechanism by which MMP9 contributed to DR progression. The results showed that increased expression of MMP9 decreased cell viability and induced apoptosis in RMCs. Moreover, MMP9 was a downstream target of Ang2 and increased Ang2 expression promoted the expression of MMP9. Besides, the expression of MMP9 significantly increased under HG condition and consequently promoted the expression of several apoptotic proteins, such as Bax2, Bcl2, cleaved PARP and cleaved caspase3, thus leading to induce cell apoptosis.

MMP-9 as the largest member of the MMP family MMPs is shown to be elevated in the retina and always associated with many diabetes complications, including DR [13]. Kowluru et al. also demonstrated that MMP9 can trigger apoptosis of retinal capillary cells via modulated by a small molecular weight G protein, H-Ras [14]. Moreover, mitochondrial dysfunction is postulated to play a crucial role in the apoptosis of retinal capillary cells in the development of DR [7]. Active MMP in cardiac myocytes is considered to degrade mitochondrial membrane potential and impair mitochondrial func-
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Besides, Madsen-Bouterse et al. demonstrated that inhibition of MMP-9 could prevent the continuation of the vicious cycle of mitochondrial damage and ultimately inhibit the of DR [16]. In our study, the results of MTT assay showed MMP9 could decrease cell viability. Moreover, flow cytometry analysis revealed that MMP9 could induce cell apoptosis. These results are consistent with previous findings and imply the apoptotic effect of MMP9 in the development of DR.

To further verify the apoptotic effect of MMP9 in the development of DR, we further explored the interaction of MMP9 with Ang2. Ang2, a member of the angiopoietins, is thought to play an important role in retinal neovascularization and angiogenesis in retinal development [17]. Increased Ang-2 can lead to persistent disruption of the cellular cross-talk between pericytes and endothelial cells in early DR [18]. Moreover, Ang2 is reported to promote cell apoptosis [19, 20]. Ang2 is also proved to have proapoptotic activity and its increased expression may be associated with endothelial apoptosis [21]. Besides, high concentrations of Ang2 are considered as an apoptosis survival factor for endothelial cells via activation of the phosphatidylinositol 3'-kinase/Akt (PI3K/Akt) signal transduction pathway [22]. PI3K/Akt pathway is widely involved in high glucose-induced apoptosis in human vascular endothelial cells [23]. Inhibition of the PI3K/Akt pathway may have the potential therapeutic roles in pathophysiology of DR [24]. In combination, we speculate that Ang2 be a key apoptosis factor to induce cell apoptosis in the development of DR. In our study, MMP9 expression was significantly regulated by Ang2 whereas no significant changes occurred in the expression of Ang2 when MMP9 expression changed, indicting MMP9 was a downstream target of Ang2. It can therefore be hypothesized that MMP9 may induce cell apoptosis in the development of DR via regulating by Ang2.

In addition, the expression of apoptotic proteins, such as Bax2, Bcl2, cleaved PARP and cleaved caspase3, in HG group was determined compared with that in NG group. Bax/Bcl-2 is regarded as a key mediator known to play a central role in cell apoptosis. Risso et al. revealed that Bax/Bcl-2 was involved in apoptosis enhanced by intermittent HG in human umbilical vein endothelial cells [25]. Moreover, PARP activation is considered a downstream effector of oxidative-nitrosative stress in the pathogenesis of diabetes complications [26]. PARP activation contributes to the inhibition of PI3K/Akt pathway and consequently inhibits cell apoptosis [27], thus increased expression of cleaved PARR may promote cell apoptosis. PARP activation is also thought to be a fundamental step in the pathogenesis of DR via regulation of nuclear factor-kB (NF-kB) in the retinal cells [28]. Soufi et al. confirmed that reduced NF-kB activity by resveratrol could finally decrease apoptosis rates in the retinas of diabetic rats in the development of DR [29]. Besides, caspase-3 is the executioner caspase involved in the proteolytic cascade during apoptosis [30]. El-Asrar et al. demonstrated that the executioner caspase-3 was observed in ganglion cells in diabetic retinas [31]. Kowluru et al. also confirmed that diabetes could induce activation of caspase-3 in retina, thus inhibiting apoptosis in the development of DR [32], suggesting increased expression of cleaved caspase-3 may promote the development of DR via inducing cell apoptosis. In our study, the expression of these apoptotic proteins in HG group increased compared with that in control group. Considering that HG could enhance the expression of MMP9, we speculate that MMP9 could induce cell apoptosis in the development of DR via regulating the activity of these apoptotic proteins.

In conclusion, our findings indicate that MMP9 may play an important role in the development of DR via inducing cell apoptosis. MMP may induce cell apoptosis via regulating by Ang2 or targeting apoptotic proteins, such as Bax2, Bcl2, cleaved PARP and cleaved caspase3. Understanding the apoptotic mechanism of MMP9 in the development of DR may help identify novel molecular targets for future pharmacological interventions of this disease.

Disclosure of conflict of interest

None.

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References


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