Original Article
The role of autophagy in chemerin-induced angiogenesis of RF/6A cells

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Abstract: Chemerin is a novel adipokine associated with obesity and metabolic syndrome and previous studies indicate that chemerin may also function as a stimulator of angiogenesis. However, the underlying mechanism of its regulatory role in angiogenesis remains unclear. In this study, we determined the role of autophagy in chemerin-induced angiogenesis of RF/6A cells. Proliferation, migration and angiogenesis of RF/6A cells can be promoted by treating with different concentrations of chemerin. The expression of LC3II was increased and the autophagy-related gene beclin-1 was upregulated by chemerin. Treatment with 3-MA, a common autophagy inhibitor, can significantly inhibit chemerin-induced proliferation, migration and tube formation by inhibiting the autophagy level of RF/6A cells. These studies show that autophagy play an important role in chemerin-induced angiogenesis and that targeting at autophagy may provide a new tool for treating retinal neovascularization.

Keywords: Chemerin, diabetic retinopathy, angiogenesis, autophagy

Introduction
Diabetic retinopathy (DR) is a serious microvascular complication of Diabetes mellitus (DM), and it is one of the leading causes of blindness in working-age population [1, 2]. DR can be divided into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). And one of the characteristics of PDR is the formation of retinal neovascularization (RNV). It results in subsequent intravitreal hemorrhage and tractional retinal detachment, which lead to blindness.

Adipose tissues produce several hormones and cytokines termed ‘adipokines’, which have widespread metabolic effects in T2DM patients. Chemerin, one of the newly discovered adipokines, is mainly expressed in liver, kidney and adipose tissue and it has been shown to be involved in glucose and lipid homeostasis [3]. In addition, chemerin was suggested to be correlated with diabetic peripheral vascular disease in T2DM patients [4]. We have previously shown that serum levels of chemerin were significantly increased in the PDR patients [5], but it is not clear whether chemerin is functionally important to the formation of RNV in PDR patients. Recent studies revealed that chemerin are involved frequently in the formation of neovascularization [6, 7]. Chemerin could stimulate the proliferation, migration and vascular tube formation of endothelial cells [8]. Study also found that chemerin mediated angiogenesis to a similar extent as VEGF in human endothelial cells [6].

Recent studies found that autophagy is of great importance to the formation of neovascularization and is involved in the process of vascular endothelial cells proliferation, migration and angiogenesis [9, 10]. Study also found that autophagy can be activated by chemerin in endothelial cell [11]. However, whether autophagy participates in the formation of RNV induced by chemerin and whether regulating
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Autophagy can inhibit RNV remain to be ascertained.

We take this study in order to investigate whether chemerin is involved in the formation of RNV in vitro and whether autophagy play a key role in the formation of angiogenesis, which may open the new way for the treatment of PDR.

**Materials and methods**

*RF/6A* cell line was purchased from Type Culture Collection of Chinese Academy of Sciences. Human recombinant chemerin was purchased from R&D Systems (Minneapolis, MN, USA). The anti-Beclin-1, β-actin antibodies were purchased from Bioword technology, MN, USA. And the anti-LC3 from Santa Cruz Biotech, CA, USA. 3-MA was purchased from Sigma (St. Louis, MO, USA). MTT and matrigel were purchased from BD Biosciences. The other cell culture reagents were purchased from Invitrogen.

**Cell culture**

The RF/6A cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humidified 95% room air with 5% CO₂. Culture medium was replaced 24 h after seeding and chemerin of different concentrations was added into the medium. RF/6A cells were randomly divided into four groups: control group, chemerin group (10 ng/ml) and chemerin with 3-MA group (10 ng/ml chemerin and 3-MA pretreated). Western blotting was used to detect Beclin-1 and LC3B (LC3 I/II ratio) in cell culture after 24 and 48 h treatment. Corresponding method was used to detect the cell proliferation, migration and angiogenesis. To inhibit autophagy, cells were pretreated with the autophagic inhibitor 3-MA (Sigma, St. Louis, MO) (final concentration, 5 mmol/L) for 1.5 h prior to the treatment with chemerin. Cells cultured without chemerin and 3-MA served as normal controls. All experiments were performed in triplicate.

**Detection autophagy related proteins by Western blotting**

Autophagy activation was monitored by expression of autophagy related proteins Beclin-1 and LC3. RF/6A cells were lysed and processed for determination of LC3-II/LC3-I ratio and Beclin-1. After treatment, Cell lysate (30 μg protein) was loaded onto and separated by 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes, which were then blocked with Tris-buffered saline (TBS)-T buffer containing 5% non fat milk, incubated with primary antibodies overnight directed against β-actin (1:500, Bioword technology, MN, USA), LC3 (1:500, Santa Cruz Biotech, CA, USA) and Beclin-1 (1:500, Bioword technology, MN, USA) and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotech, CA, USA) for 1 h at room temperature. The labeled bands were visualized and quantified using a chemiluminescence imaging system (ClinX, Shanghai, China).

**Cellular proliferation analysis**

Cell viability was measured using MTT assay, which reflects the dehydrogenase activity of mitochondria. RF/6A cells were seeded onto 24-well plates. The medium was replaced with MTT-containing medium and the cells were incubated for 4 h at 37°C after treatment. The cells were lysed with dimethyl sulfoxide solution and the absorbance was measured at 540 nm with a 630-nm reference absorbance. Cell proliferation was determined by using the BrdU labeling kit (Calbiochem) to show cells at various stages of DNA synthesis. 10 μM BrdU was added to the cells and it was incorporated into freshly synthesized DNA for 2 h. A peroxidase-labeled antibody to BrdU was added after cell fixation. In the final step, the peroxidase substrate was added the peroxidase enzyme, which catalyzed the cleavage of the substrate and then appeared colored complex. The absorbance of the sample was determined at 405 nm with the reference wavelength set at 490 nm. At least three independent experiments were conducted for each group of cells.

**Cellular migration assay**

RF/6A cells of a 5×10⁵/ml density were seeded in 6-well plates (2 ml in each well). After the RF/6A cells grew to approximately 90% confluency, the cells were wounded with pipette tips and washed for four times with PBS to remove the dissociate cells. Then the cell-free “bare areas” from the bottom side of plates were formed. DMEM containing various concentrations of chemerin was added into the wells.
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Every group was designed to set three parallel holes. Images of the closing wound were acquired by microscope at 0 and 24 hrs of incubation. The migration area (representing the number of cell migration) was detected and analyzed by using the Photoshop image analysis software [12]. Three independent experiments were performed.

**Tube formation assay**

The tube formation assay was performed using matrigel according to the protocol. Briefly, the matrigel was pipetted into 96-well plates (100 µl matrigel per well) and polymerized for 30 minutes at 37°C. RF/6A cells were placed onto the layer of matrigel (20,000 cells per well) in DMEM medium containing various concentrations of chemerin. After 24 hrs of incubation, the cells were photographed using an inverted microscope (magnification ×200). The perimeter of RF/6A formational tubes were measured and analyzed. Every group was designed to set three parallel holes.

**Statistical analysis**

Statistical analysis was performed by using the SPSS 13.0 software program. All quantitative data are representative of at least three independent experiments. The Kolmogorov-Smirnov test was used to determine if continuous variables were normally distributed. The data are presented as the means ± SEM. Between-group differences were tested by analysis of variance (ANOVA). The least significant difference procedure was used for pairwise comparisons. A two-tailed p-value of P<0.05 was considered significant.

**Results**

**Chemerin promoted autophagy and 3-MA suppressed the expression of chemerin-induced autophagy-related proteins in RF/6A cells**

To determine whether treatment with chemerin triggered autophagy in RF/6A cells, RF/6A cells were treated with 10 ng/ml chemerin for 24 to 48 h. The level of LC3 and Becline-1 were determined by western blotting as an indicator of autophagic activity. As shown in **Figure 1**, chemerin significantly increased the expression of Becline-1 and LC3II/I. 3-MA reduced Becline-1 and LC3II/I expression in RF/6A cell treated with chemerin of 10 ng/ml. These results suggested that chemerin can promote cellular autophagic processes in RF/6A cells. 3-MA significantly suppressed the expression of chemerin-induced autophagy-related proteins in RF/6A cells.
Chemerin increased RF/6A cells proliferation and 3-MA inhibited chemerin-induced proliferation

Proliferation assay show that the relative proliferation rates of chemerin group were 1.31 ± 0.012 and 1.22 ± 0.024 at 24 h and 48 h respectively. While the relative proliferation rates of chemerin+3-MA group were 1.13 ± 0.014 and 1.08 ± 0.089 at 24 h and 48 h respectively. Data analysis discovered a significant increase in proliferation of RF/6A cells treated with 10 ng/ml chemerin at 24 h and 48 h. Pretreatment with 3-MA significantly reduced chemerin-induced proliferation of RF/6A cells (Figure 2).

Chemerin induced migration of RF/6A cells and 3-MA inhibited chemerin-induced migration

We used scratch method to detect cell migration. The relative migration distance of RF/6A cells in chemerin group was significantly increased than control group (P<0.05 at 24 h, P<0.01 at 48 h). Pretreatment with 3-MA significantly reduced chemerin-induced cell migration of RF/6A cells (Figure 3).

Chemerin induced tube formation of RF/6A cells and 3-MA inhibit chemerin-induced tube formation

We performed Matrigel based tube formation assay. Treatment with chemerin (10 ng/ml) promoted tube formation at 24 h. Quantitative analyses revealed a significant increase in tube formation induced by chemerin. The numbers of tube formation in the three groups were 5.4 ± 1.14, 13.2 ± 1.30, 7.8 ± 0.84, respectively (Figure 4). These results showed that chemerin significantly promoted the tube formation of RF/6A cells. Pretreatment with 3-MA significantly reduced the chemerin-induced tube formation of RF/6A cells.

Discussion

DR is the leading cause of vision loss in diabetic patients. The formation of RNV is one of the important pathological changes of PDR. However, the pathogenesis of RNV is not completely clear. Furthermore, chemerin can activate the autophagy in endothelial cells [11]. Some other research also found that autophagy dysfunction is a key factor for causing endothelial cell homeostasis of diabetic vascular injury and the formation of new blood vessels, and the angiogenesis of artery endothelial cells.
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Recent studies revealed that chemerin are involved frequently in the formation of neovascularization [6, 7]. Chemerin has been implicated in mediating cellular migration under inflammatory conditions [13]. Evidences showed that chemerin mediated the formation of new blood vessels and functional angiogenesis to a similar extent as VEGF in human endothelial cells [6]. In a similar study, chemerin also induced functional angiogenesis in human endothelial cells by promoting migration, tube formation, activation of endothelial gelatinase (MMP-2/-9) and activation of PI3K/Akt and MAPKs pathways, which is a key mechanism for angiogenesis [8].

Chemerin was shown to correlate with inflammation [14-16], and was strongly correlated with markers of inflammation, such as TNF-α and IL-6 [14, 17]. In addition, CMKLR1 is expressed in vascular endothelial cells and its expression level is regulated by inflammatory cytokines [18]. These results indicate that chemerin and CMKLR1 system may be key factors in the inflammatory state of vascular endothelial cells, which is one of the promoting factors for the formation of new blood vessels. Furthermore, chemerin can increase the generation of mitochondrial reactive oxygen species (ROS) in human endothelial cells [11]. Other research also found that chemerin was correlated with the process of oxidative stress and it can enhance the response to oxidative stress [19, 20]. ROS function as key signaling molecules to mediate angiogenesis. Thus, chemerin may be also involved in the development of RNV through promoting oxidative stress.

However, chemerin may participate in the angiogenesis through various channels. Our study confirmed that chemerin can activate autophagy in RF/6A cells. And inhibition of autophagy by 3-MA can significantly inhibit chemerin-induced RF/6A cells proliferation, migration and tube formation. Autophagy was unveiled by Ashford and Porter in 1962 [21]. Autophagy has been found to be related to individual development, oxidative damage protection, malignant proliferation of tumor cells and neurodegenerative diseases [22-27]. Specific gene expression and protein level of autophagy related detection can reflect the level of autophagy. 3-MA is a commonly used autophagy inhibitor, which can definitely inhibit cell autophagy and was widely used in autophagy research [28]. It was reported that using 3-MA to pretreat cells can effectively inhibit autophagy [29, 30]. Therefore, we used 3-MA in our research. Our results confirmed that chemerin significantly activated autophagy and the level of autophagy can be effectively inhibited by 3-MA. Furthermore, 3-MA significantly inhibited RF/6A cells proliferation, migration and tube formation. Studies have shown that autophagy plays an important role in the formation of neovascularization [9, 10]. It was also found that inhibiting autophagy can reduce angiogenesis of bovine aortic endothelial cells, and induction of autophagy can promote cell tube formation and cell migration [9]. Study also showed an inhibition of VEGF-induced angiogenesis by autophagy depression [9]. Beclin-1, a mammalian ortholog of yeast Atg6 and a core component of the autophagy

Figure 4. Representative images of the tube formation of RF/6A cells in the presence of chemerin or chemerin+3-MA for 24 hours (A), and the number of tube formation of the cells incubated with different drugs (B). Quantitative analyses revealed a significant increase in tube number induced by chemerin at 24 h and 3-MA significantly reduced the tube number induced by chemerin. **P<0.01 vs Control; *P<0.01 vs chemerin.
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machinery, plays a central role in the regulation of autophagy [31]. The expression of Beclin-1 is positively associated with autophagy. Previous study demonstrated that knockdown of Beclin-1 expression also decreased chemerin-induced angiogenesis and cell proliferation [11]. Other study also found that Beclin-1 shRNA blocked VEGF-induced tube formation and migration [9]. These studies indicate that autophagy is involved in the process of angiogenesis.

Furthermore, angiogenesis inhibitors can induce autophagy of endothelial cell which is independent of nutritional deprivation, hypoxic stress and vascular endothelial growth factor (VEGF) expression [32-34]. This may be one of the mechanisms of angiogenesis recurrence in angiogenesis inhibitors treatment.

Our present data indicated that autophagy may play a critical role in chemerin-induced angiogenesis. Our study provides the first evidence that chemerin contributes to RF/6A angiogenesis through the upregulation of autophagic activity. In addition, inhibiting autophagy can significantly inhibit the RF/6A angiogenesis induced by chemerin in vitro.

However, the role of autophagy in angiogenesis maybe complex and some evidence suggests that autophagy may have an antiangiogenic role [35]. Thus, the mechanisms of autophagy in RNV in vivo require further investigation. Furthermore, in spite of the extensive application of RF/6A cells in studies on retinal angiogenesis, it may be different from human retinal vascular endothelial cells. Therefore, further studies using human retinal vascular endothelial cells as well as in vivo models are required to clarify the role of chemerin and autophagy in angiogenesis.

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Disclosure of conflict of interest

None.

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