# Original Article Chemerin promotes the viability and migration of human placental microvascular endothelial cells and activates MAPK/AKT signaling

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Received January 31, 2017; Accepted December 13, 2017; Epub February 15, 2018; Published February 28, 2018

**Abstract:** In preeclampsia, maternal serum chemerin level is significantly increased and associated with dyslipidemia. However, the mechanism by which chemerin contributes to the pathogenesis of preeclampsia remains elusive. This study aimed to investigate the effect of chemerin on placental microvascular generation using human placental microvascular endothelial cells (HPMECs) as the experimental model. HPMECs were isolated and cultured and then treated with chemerin at different concentrations (0.1, 0.3, 1.0, 3.0 and 10.0 ng/ml). The viability, migration and tube formation of HPMECs were evaluated. The expression of chemerin receptor ChemR23 and the activation of MAPK/AKT pathway were analyzed by Western blot analysis. HPMECs isolated were positive for vWf and CD31 staining. Chemerin enhanced the viability, migration and tube formation of HPMECs, and significantly increased the expression of ChemR23. In addition, chemerin activated ERK1/2, p38MAPK and Akt pathways. In conclusion, chemerin promotes the formation of blood vessels in human placenta and activates Akt and MAPKs pathways, which may be involved in the occurrence and progression of preeclampsia.

Keywords: Preeclampsia, chemerin, HPEMCs, ChemR23, angiogenesis

#### Introduction

Preeclampsia is the most common medical disorders during pregnancy, and is a leading cause of maternal and perinatal morbidity and mortality [1]. Preeclampsia can lead to perinatal deaths, preterm birth and intrauterine growth restriction [2]. The etiology of preeclampsia is still unclear. The only effective cure of preeclampsia is delivery of the placenta. The placenta is the key to the development of preeclampsia, with shallow placentation and maternal endothelial dysfunction being strongly associated with the pathogenesis of preeclampsia [3]. Placental vascular endothelial cells play an important role in the formation of new blood vessel [4]. Human placenta is a rich source of angiogenic factors, which is vital to vessel formation. Pro-angiogenic and antiangiogenic factors are important in the regulation of placental development [5]. However, the placental vessel network is abnormal in preeclampsia. The angiogenic imbalance impairs local cellular function and leads to systemic endothelial cell dysfunction [6].

Chemerin is a novel adipokine mainly expressed in placenta, liver, adipocytes and ovary [7]. ChemR23, the receptor of chemerin, is expressed in the placenta and vascular endothelial cells [8]. Chemerin has been implicated in obesity, insulin resistance and inflammation [9, 10], which are high-risk factors of preeclampsia. Chemerin stimulated angiogenesis, and chemR23 up-regulated endothelial angiogenesis via the activation of PI3K/AKT and MAPKs pathways [11, 12].

Our previous study demonstrated that serum concentration of maternal chemerin was significantly increased in preeclampsia and strongly associated with markers of dyslipidemia [13]. We hypothesized that chemerin may play an important role in the pathogenesis of preeclampsia. In present study, human placental microvascular endothelial cells (HPMECs) were used as the experimental model to elucidate the mechanism by which chemerin contributes to the pathogenesis of preeclampsia.

#### Materials and methods

### Isolation and culture of HPMECs

The placentas (approximately 40 g) from preeclampsia pregnancies were collected aseptically during cesarean sections. The study was approved by the local Hospital's Ethics Committee, and all the women signed the consent form before the initiation of the study. After removal of the deciduas, fetal membranes and the chorionic plate under aseptic conditions, placentas were washed twice with chilled antiseptic solution containing 100 U/ml of penicillin and 5 µg/ml of amphotericin, and then immediately minced into small fragments. The minced tissues were digested for 2 h in a shaking incubator at 37°C in PBS containing 0.28% (W/V) collagenase II (Invitrogen, USA), 0.25% dispase II (Invitrogen, USA) and 2.5 mg DNasel (Roche, USA). After incubation, cell suspension was collected and filtered using 10 µm mesh, and then centrifuged at 1,000 g for 10 min at 4°C. The pellets were gently resuspended in 3 ml of DMEM (Gibco, USA) and layered onto the surface of a gradient and centrifuged for 20 min at 400 g. The gradient was harvested by using mixture consisting of 9 portions of pure Percoll and one portion of 8.5% NaCl, diluted with 0.85% NaCl to yield three concentrations of Percoll (25%, 30% and 35%). A white band was observed after centrifugation between 30% and 35% (2-4 cm from the top of the Percoll gradient). The band was collected and washed twice with DMEM to eliminate Percoll. The cells were resuspended in DMEM containing 20% FCS and incubated at 37°C in an incubator with 5% CO<sub>2</sub>. The medium was replaced after 24 h and every 48 h thereafter [14-16].

# Immunofluorescence

Cells grown on the coverslips were washed twice in PBS, and fixed in 4% paraformaldehyde for 15 min at room temperature, The cells were permeabilized with 0.1% Triton for 10 min, washed 3 times in PBS and incubated with 7% BSA at 37°C for 30 min. The cells were then incubated overnight at 4°C with antibody against the von Willebrand (vW) factor or CD31 (Santa Cruz, USA). The cells were washed 3 times with PBS and incubated for 1 h at room temperature with goat anti-rabbit IgG coupled with fluorescence.

### MTT assay

Cells were seeded into 96-well plate at a concentration of  $5 \times 10^4$  cells/well. After incubation with 0.1, 0.3, 1.0, 3.0 and 10.0 ng/ml chemerin for 48 h, the cells were incubated with 5 mg/ml MTT (Gibco, USA) for 4 h at 37°C. Following the incubation, the insoluble blue formazan products were dissolved in Dimethyl sulfide (DMSO) (GIBCO, USA) and quantified by measuring the absorbance at 570 nm.

# Migration assay

HPEMCs suspension of  $1.0 \times 10^6$  cells/ml was prepared and 100 µl was put into the upper chamber of Trans well. Then 600 µl of serum free medium containing 0.1, 0.3, 1.0, 3.0 and 10.0 ng/ml chemerin was added to the lower chamber. After incubation for 6 h at 37°C, HPEMCs were fixed in 4% formaldehyde and stained with crystal violet. The migrated cells were quantified by counting five randomly selected fields.

# Tube formation assay

Growth factor-reduced Matrigel (300  $\mu$ l) was pipetted onto 24-well culture plates and polymerized for 30 min at 37°C. HPMECs were seeded onto the surface of the Matrigel, and 0.1, 0.3, 1.0, 3.0 and 10.0 ng/ml chemerin was added individually. The plates were incubated for 8 h at 37°C. The morphological changes of the cells were observed under a microscope.

# Western blot analysis

Serum-starved HPMECs were treated with or without chemerin. The protocol for protein lysate preparation and Western blot analysis had been previously described [12, 17]. Briefly, cells were lysed with Laemmli buffer and centrifuged at 5,000 rpm for 2 min.

Each sample was separated on a 10% sodium dodecyl sulfate polyacrylamide gel, and transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was then incubated with 5% bovine serum album in Tris buffered solution plus 0.05% Tween 20 (TBST, pH 7.4) for 1 h at room temperature, and then incubated overnight at 4°C with primary antibodies for phospho-p38



MAPK, p38 MAPK, phospho-Akt, Akt, phospho-ERK1/2, ERK1/2, CMKLR1 and  $\beta$ -actin (Affinity, USA). The membrane was washed with TBST and then incubated with horseradish peroxidase conjugated secondary antibody (Dako Ltd, Cambridge, UK) for 1 h at room temperature. The bands were visualized using chemiluminescence (GE Healthcare, Little Chalfont, UK) and band densities were analyzed using a scanning densitometer.

#### Statistical analysis

The data were expressed as mean  $\pm$  SE. Comparisons among groups were made by ANOVA followed by a post hoc LSD multiplecomparison test. P < 0.05 was considered significant difference.

#### Results

#### Characterization of HPMECs

Increasing evidences suggest that shallow placentation and maternal endothelial dysfunction are responsible for the development of preeclampsia [3]. *In vitro* model for the development of preeclampsia is from the umbilical vein or aorta endothelial cells [18-20]. But these endothelial cells are highly heterogeneous [21]. Endothelial cells have different morphological, phenotype and physiological function in different tissues and organs, even within same organ but in different position [22-25]. To understand the mechanism of preeclampsia, human placental microvascular endothelial cells (HPMECs) were isolated from the placenta and cultured for 7 days, and uniformed dendritic cells were observed, which was represented of typical morphology of endothelial cells (Figure 1A). These cells were immunostained by von Willebrand factor (vWF) and showed a positive staining (Figure 1B). To confirm these cells as HPMECs, another endothelial marker, PECAM-1 (CD31) antibody was used to stain these cells. These isolated cells showed positive CD31 staining (Figure 1C). Taken together, these isolated cells from placenta were confirmed as endothelial cells.

# Chemerin enhanced the viability, migration and tube formation of HPMECs

MTT assay showed that the optical density of HPMECs treated with 0.1, 0.3, 1.0, and 3.0 ng/



Figure 2. Chemerin promoted the viability and migration of HPMECs. Serum starved HPMECs were treated with 0-10 ng/ml chemerin for 48 h. The viability (A) and migration (B) of HPMECs were measured. \*P < 0.05 vs. basal, \*\*P > 0.01 vs. basal.

ml chemerin was significantly higher compared to control (0.505  $\pm$  0.005, 0.594  $\pm$  0.006, 0.573  $\pm$  0.004, 0.446  $\pm$  0.005 VS. 0.396  $\pm$ 0.005, **Figure 2A**, P < 0.05). These results indicated that the viability of HPMECs significantly increased after treatment with chemerin.

Migration assay showed that the numbers of migrated cells in 0.1, 0.3, 1.0, and 3.0 ng/ml chemerin treatment groups were  $25.6 \pm 2.60$ ,  $42.4 \pm 3.20$ ,  $33.4 \pm 2.70$ ,  $24.4 \pm 2.88$ , respectively, significantly higher than in control group (19.2 ± 3.19, P < 0.05, Figure 2B).

Tube formation assay showed that chemerin enhanced HPMECs tube formation in a dosedependent manner. The numbers of tube in 0.1, 0.3, 1.0, and 3.0 ng/ml chemerin treatment groups were  $1.20 \pm 0.83$ ,  $2.80 \pm 0.83$ ,  $3.40 \pm 1.14$ ,  $3.80 \pm 0.83$ ,  $5.60 \pm 1.14$ , respectively, significantly higher than in control group (control  $0 \pm 0$ , **Figure 3**, P < 0.05).

# Chemerin induced the activation of MAPK/Akt pathway in HPMECs

p38 signal, Akt and ERK1/2 signal pathways have been involved in endothelial proliferation and angiogenesis [12, 26, 27]. To better understand the mechanism of chemerin-mediated HPMECs proliferation and angiogenesis, we investigated whether p38, ERK or Akt are involved in this process.

Chemerin increased p38 phosphorylation in a dose-dependent manner in HPMECs and reached to maximum at 0.3 ng/mL (Figure 4A). Similar activation induced by chemerin was observed on Akt phosphorylation in HPMECs

(Figure 4B). In contrast, chemerin-mediated ERK1/2 phosphorylation reached the maximum at 1.0 ng/mL and then decreased to basic level at 3.0 ng/mL, and higher concentration of chemerin at 10.0 ng/mL caused ERK1/2 phosphorylation (Figure 4C). In addition, chemerin induced a dose-dependent chemR23 expression in HPMECs and reached the maximum at 0.3 ng/mL (Figure 4D).

#### Discussion

In this study, we demonstrated that the proliferation, migration, tube formation of placental microvascular endothelial cells could be promoted by chemerin, indicating that chemerin is a placental vascular growth factor. Additionally, we found that chemR23 expression in HPMECs was upregulated by chemerin. Furthermore, we reported that chemerin activated MAPK/AKT signaling pathways in placenta.

It has been reported that chemerin mRNA and protein expression levels in placenta were significantly higher in preeclampsia, and correlated with high serum chemerin level [28]. Our previous study demonstrated that serum chemerin level was significantly increased in preeclampsia and independently associated with markers of dyslipidemia and the severity of preeclampsia [13]. In addition, Carlino et al. reported that chemerin was upregulated in decidua and played a role in NK cell infiltration and vascular remodeling during early pregnancy [29]. In our present study, we isolated and cultured HPMECs and found that chemerin significantly increased the proliferation and migration of HPMECs. The strongest effects of chemerin to promote the proliferation and migration of HPMECs were observed at the dose of 0.3 ng/ml. Interestingly, higher dose of chemerin (10 ng/ml) failed to induce any significant changes in the viability and migration of HPMECs. In addition, chemerin dose-dependently induced tube formation of HPMECs. Chemerin significantly enhanced the viability, migration and tube formation of HPMECs, indicating that chemerin is a strong promoter of placental vascular development. Vascular branch formation and vascular extension are



**Figure 3.** Chemerin promoted tube formation of HPMECs. (A-F) Typical images of tube formation of HPMECs treated with chemerin at 0 ng/ml (A), 0.1 ng/ml (B), 0.3 ng/ml (C), 1.0 ng/ml (D), 3.0 ng/ml (E) and 10.0 ng/ml (F). (G) Quantitative analysis of the number of tube formation (n = 5). \*P < 0.05 vs. basal.



**Figure 4.** Chemerin induced the activation of p38 MAPK, ERK1/2 and Akt signaling and the expression of ChemR23. Serum starved HPMECs were treated with 0-10 ng/ml chemerin for 10 min. The phosphorylation of p38 MAPK (A), Akt (B) and ERK1/2 (C), and the expression of ChemR23 (D) were measured by Western blot analysis. Results were mean  $\pm$  SEM (n = 5). \*P < 0.01, \*\*P > 0.05 vs. basal.

important parts of placental blood vessel development [30]. We suggest that 0.3 ng/ml chemerin exerts better effect on vascular extension that is conducive to material exchange. Future studies are required to elucidate precise effects mediated by different concentrations of chemerin.

The mechanism of placental vascularization is complex. MAPKs and Akt pathways play important role in angiogenesis and cell survival [31, 32]. In present study, we demonstrated that chemerin induced the activation of ERK1/2 in biphasic pattern, peaking at 1.0 ng/ml, followed by a rapid decline at 3.0 ng/ml, and then a subsequent increase at 10 ng/ml. Furthermore, we observed significant upregulation of chemokine receptor ChemR23 expression by chemerin in HPMECs, and 1.0 ng/ml chemerin induced the strongest expression of chemR23. The pattern of chemR23 expression corresponded with the activation of ERK1/2. On the other hand, we found that chemerin dose-dependently activated p38 MAPK and Akt pathways. The proliferation and migration of HPMECs seemed to follow a dose-dependent response to p38 MAPK and Akt activation.

In conclusion, we present the first evidence that chemokine receptor ChemR23 was expressed on HPMECs and upregulated by chemerin. Chemerin could promote the proliferation, migration, and tube formation of HPMECs. Chemerin activates MAPK/AKT signaling pathways, which are the key survival and angiogenic pathways. Our results suggest that chemerin plays a role in placental vascularization and could be a therapeutic target for the treatment of preeclampsia.

#### Acknowledgements

This work was supported by National Natural Science Foundation of China (81771611), Shenzhen Science and Technology Innovation Committee Special Funding for Future Industry (JCYJ20170412140326739), Sanming Project of Medicine in Shenzhen (SZSM201612035 and SZSM201512023), and Science and Technology Planning Project of Guangzhou city (2014J4100043).

# Disclosure of conflict of interest

#### None.

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