Plasma-derived exosomes contributes to Endothelial-to-mesenchymal transition in moyamoya disease

Jilan Liu  
Affiliated Hospital of Jining Medical University

Chao Chen  
Jining Medical University

Xianyun Qin  
Affiliated Hospital of Jining Medical University

Yan Lu  
Affiliated Hospital of Jining Medical University

Bin Zhang  
Affiliated Hospital of Jining Medical University

Feng Jin (✉️ jinfengsdjn@163.com  )  
Affiliated Hospital of Jining Medical University  https://orcid.org/0000-0003-1307-3690

Research Article

Keywords: moyamoya disease, plasma, exosomes, Endothelial-to-mesenchymal transition, microRNA

Posted Date: January 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2399901/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License
Abstract

Moyamoya disease was a cerebrovascular disease with a high disability rate, and its pathogenesis was still unknown. Endothelium-mesenchymal transition (EndMT) was the pathological basis of many vascular diseases, however, whether EndMT played a key role in moyamoya disease has not been reported. Multiplex fluorescent immunohistochemistry staining confirmed that CD31, VE-cadherin and E-cadherin were down-regulated, α-SMA and Vimentin were significantly up-regulated in moyamoya vascular endothelial cells. Therefore, we proposed for the first time that EndMT may exist in the vessels of moyamoya disease. Plasma-derived exosomes (PDEs) can transmit information between cells and tissues and are of great value in many disease studies. PDEs significantly promoted cell proliferation and migration, and make cells slender. PDEs induced EndMT phenotype changes in cerebral vascular endothelial cells including decreased endothelial cell markers expression and increased mesenchymal cell markers expression. We demonstrate that EndMT phenotypic alterations are mediated in part by microRNA. Thus, we concluded that PDEs induce the EndMT phenotype to promote the development of moyamoya disease. This study aimed to provide a new theoretical basis for elucidating the pathogenesis of moyamoya disease.

1. Introduction

Moyamoya disease (MMD) was a chronic progressive cerebrovascular disease characterized by partial stenosis or occlusion of the distal end of the internal carotid artery and abnormal vascular network formation at the base of the brain[1]. MMD patients were mostly manifested by ischemic or hemorrhagic stroke, epileptic seizures and cognitive dysfunction, with high disability rate and even death [2]. In recent years, the incidence of MMD has been increasing year by year, and the incidence was higher in East Asia [3]. Due to the insidious onset of MMD and the lack of specificity and sensitivity indicators for early screening, the vast majority of MMD patients cannot receive timely diagnosis and treatment [3]. In view of the particularity of the pathogenesis of moyamoya disease, it was difficult to obtain clinical samples, and there are few basic studies, so it was particularly important to conduct basic research on the pathogenesis of moyamoya disease.

The pathogenesis of moyamoya disease was not fully understood, and it was currently believed to be related to genetic, inflammatory, environmental and other factors[1, 4]. Our experimental results indicated that there may be a biological process of EndMT in the blood vessels of moyamoya disease. EndMT was first discovered by Markwald et al. during atrioventricular valve development in the embryonic heart and has been observed in a variety of pathological conditions characterized by abnormal shear stress, vascular damage and chronic inflammation [5]. EndMT was a complex cellular transdifferentiation process during which endothelial cells lose endothelial properties and acquire mesenchymal characteristics, as well as enhanced migratory and proliferative capacity [6]. The molecular markers of EndMT were endothelial markers such as CD31 decreased and mesenchymal markers such as N-cadherin and Vimentin increased[7]. EndMT was the common pathological basis of various vascular diseases [8–10][8–10], and our findings suggested that EndMT plays a key role in moyamoya disease.
However, the specific role of EndMT in the occurrence and development of moyamoya disease and its upstream regulatory mechanism have not yet been reported.

Exosomes were membranous vesicles secreted into the extracellular environment by the fusion of intracellular multivesicles bodies with the cell membrane [11]. Studies have shown that PDEs in moyamoya disease significantly promote the proliferation of cerebral vascular endothelial cells compared with those in healthy samples, but have no effect on peripheral vascular endothelial cells, suggesting that PDEs in moyamoya disease specifically act on cerebrovascular endothelial cells [12]. Exosomes secreted from highly metastatic cancer cells can promote vascular permeability by downregulating the expression of VE-cadherin and ZO-1 in endothelial cells, thereby promoting tumor recurrence and metastasis [13], indicating that exosomes have the potential to regulate endothelial-mesenchymal transition. Our study showed that the PDEs in moyamoya disease significantly promoted the EndMT phenotype of cerebral vascular endothelial cells. In conclusion, we have reason to believe that the PDEs promotes the occurrence and development of moyamoya disease by regulating EndMT.

2. Materials And Methods

2.1. Patientand plasma Samples

This study was approved by the Ethics Committee of the Affiliated Hospital of Jining Medical College. Inclusion criteria for moyamoya disease: All patients were diagnosed with moyamoya disease by cerebral angiography; Age less than 60 years old; Comorbiddises such as cerebral arteriosclerosis and severe cardiopulmonary disease were excluded. People recruited in this study were from patients who visited the Affiliated Hospital of Jining Medical College from November 16, 2021 to June 4, 2022. We took the discarded vascular tissue from neurosurgery patients with moyamoya disease during surgery and the discarded vascular tissue from cardiac surgery vascular bypass surgery for tissue-level experiments. We took the plasma samples of moyamoya disease patients and the control group for exosome isolation and extraction and vitro experiments. The plasma samples of the control group were from healthy people in the Physical Examination Center of the Affiliated Hospital of Jining Medical College.

2.2. Exosomes Isolation and Identification

We used the kit from Shanghai Umibio Biotechnology Co., Ltd. to extract PDEs (UR52136). Exosome morphology were then observed by electron microscope imaging, and particle size was measured using the Nanosight LM10 system. Exosomes markers were confirmed by western blot.

2.3. Cell proliferation assays

We used cell counting Kit-8 (Dojindo, Japan) to detect the cell proliferation ability. We seeded the cells in 96-well culture plates at 5000 cells per well, and stimulated bEnd.3 for 24h and HUVEC for 72h with PDEs, respectively. After the stimulation, cells were incubated with WST-8 reagent for 2h, and the OD value was
detected by a microplate reader. Since bEnd.3 cells grew faster, they were only stimulated for 24 h for proliferation experiments.

2.4 Cell Migration assays

We used Transwell (Corining 3422) for cell migration experiments. 10,000 cells per well were seeded in the upper chamber of Transwell. The volume of the culture medium in the upper chamber was 200 ul without serum, and the volume in the lower chamber was 600 ul with 10% serum. PDEs were added to each chamber, and cultured for 24 hours and placed under a microscope. observe.

2.5 Tube Formation Assay

Tube formation assays were performed using bEnd.3 cells with treatment of PDEs for 6 hours. We added 100ul Matrigel per well to a 96-well plate and incubated at 37°C for 30 minutes. After the Matrigel solidified, 10,000 cells per well were seeded in the wells.

2.6 Immunofluorescence assays

50,000 cells per well were seeded on cell slides, and cells were stimulated with PDEs for 48 h for immunofluorescence staining. The 5-color multiplex fluorescence kit (abs50013) was used for the five-color multiplex fluorescence, and the primary antibody dilution ratio of each molecular indicator was 1:100.

2.7 Western blot

After PDEs-stimulated cells, the cell pellets were collected for proteolysis, and SDS-PAGE gel electrophoresis was performed to detect the protein expression changes of various molecular indicators. The primary antibody dilution ratio was 1:1000. The secondary antibody dilution ratio was 1:5000.

2.8 qRT-PCR

After PDEs-stimulated cells, the cells were harvested for RNA lysis and subsequently assayed for gene expression by qRT-PCR. The 2-ΔΔCq method was used for data analysis and the relative expression was normalized to β-actin.

2.9 Statistical Analysis and Date Availability

All results are presented as the mean values ± SD. The statistical significance of the results was analyzed by a 2-tailedunpaired Student t test using GraphPad Prism. The statistical significance of all the data is indicated by *P < 0.05, **P < 0.01, ***P < 0.001

3. Results

3.1. Patients Information

A total of 53 adults (including 28 MMD patients and 25 control samples) were recruited to this study from November 2021 to April 2022. We reserved 6 blood vessels for tissue level experiments. The control
Vascular samples were derived from the internal mammary artery resected by vascular bypass surgery in cardiac surgery. The MMD Vascular samples were obtained from the middle cerebral artery resected by vascular reconstruction surgery in neurosurgery. Detailed patient information is shown in the Table 1.

Table 1. Samples Information Used in Experiments

<table>
<thead>
<tr>
<th>Assay</th>
<th>group</th>
<th>Sample</th>
<th>n</th>
<th>sex</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Tissue level</td>
<td>MMD</td>
<td>artery</td>
<td>6</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>artery</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cell level</td>
<td>MMD</td>
<td>Plasma</td>
<td>22</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Plasma</td>
<td>22</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>

3.2. Expression of EndMT markers in human moyamoya disease

We used multiplex fluorescent immunohistochemistry to detect the expression of EndMT-related molecules in moyamoya disease and control vessels. The results showed decreased expression of VE-cadherin and CD31 (Figures 1A-C, F), and increased expression of Vimentin, Snail and α-SMA (Figures 1A,B,D,E,G,H), suggesting that EndMT plays an important role in moyamoya disease. Compared with the control group, the intimal layer of blood vessels is significantly thickened (Figure S1), indicating abnormal proliferation of vascular endothelial cells in moyamoya disease. The ETS (E-26 transformation-specific) transcription factor ERG (ETS-related gene) is essential for maintaining endothelial homeostasis. We detected that ERG was absent in vascular endothelial cells of moyamoya disease, suggesting that vascular endothelial cells of moyamoya disease were dysfunctional (Figure S2A).

3.3. Exosome Identification

To confirm that peripheral blood exosomes regulate EndMT, we conducted a series of cell experiments. First, we collected peripheral blood for exosome extraction and identification. Electron transmission microscopy showed that exosomes were in the shape of tea saucers (Figure 2A). Nanosight LM10 system showed that exosomes were mainly concentrated at 100nm (Figure 2B). Western blot detection showed that exosome markers TSG, CD63 and CD9 were positive expressed (Figure 2C).

3.4. Exosomes from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell proliferation and migration

To demonstrate the functional effect of exosomes on the occurrence and development of moyamoya disease, we performed in vitro cell experiments. CCK-8 assay showed that compared with control group, exosomes of moyamoya disease significantly promoted the proliferation of cerebrovascular endothelial cells, but had no effect on umbilical vein endothelial cells (Figure 3A, B). Transwell assay showed that
plasma exosomes of moyamoya disease significantly promoted the migration of cerebrovascular endothelial cells, but had no effect on umbilical vein endothelial cells compared with the control group(Figure3B). Interestingly, compared with healthy controls, exosomes of moyamoya disease had little effect on the angiogenesis ability of cerebrovascular endothelial cells, which was consistent with the results of Lian Duan et al. (Figure3C), indicating that exosomes of moyamoya disease specifically act on cerebrovascular endothelial cells, and there may be dysfunction of endothelial cells [12].

3.5. Exosomes from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell EndMT phenotype

Our results showed that cells stimulated by exosomes of moyamoya disease underwent significant morphological changes (Figure4A). Immunofluorescence was performed to detect the expression of β-tubulin, which further demonstrated that the cytoskeleton was significantly elongated (Figure4B,C). We hypothesized that exosomes regulate cell morphological changes by regulating EndMT. To further demonstrate the regulatory effect of exosomes on EndMT, we detected the expression of EndMT-related markers. Immunofluorescence and western blot showed that the expression of CD31, VE-cadherin and E-cadherin were down-regulated (Figures4D-F), and the expression of vimentin and Snail were up-regulated (Figure4F-G) after exosomes stimulated. However, there was no significant change in α-SMA. The results of RNA level showed that E-cadherin was down-regulated and Snail expression was down-regulated(Figure4H). These results suggest that exosomes promote EndMT phenotype in moyamoya disease. In addition, Western and immunofluorescence showed downregulation of ERG expression (FigureS2 B, C), indicating that endothelial homeostasis was impaired after exosome stimulation, which may be the reason for the lack of ability to promote tube formation (Figures3F, G).

3.6. Exosomal miRNAs promote endothelial cells EMT phenotype

In the previous stage, our research group carried out the sequencing analysis of miRNAs in plasma-derived exosomes, and screened the differential miRNAs. After exosome stimulation, we preliminarily performed miRNA detection and found that the miRNA151a-3p and miRNA125b-5p were up-regulated. We speculated that miRNA-151a-3p and miRNA-125b-5p may play important roles in EndMT phenotype changes. Overexpression of miRNA-125b-5p enhanced the migration ability of endothelial cells, while knockdown of miRNA-125b-5p weakened the migration ability of endothelial cells (Figure5C). Similarly, overexpression of miRNA-151a-3p enhanced the migration ability of endothelial cells, while knockdown of miRNA-151a-3p weakened the migration ability of endothelial cells (Figure5C). In order to further confirm the role of miRNA-151a-3p and miRNA-125b-5p, the expression of miRNA-151a-3p and miRNA-125b-5p was knocked down while stimulated by plasma-derived exosomes from moyamoya disease, the results showed that the cell migration ability was inhibited (Figure5E), Snail expression was also inhibited (Figure5F).

4. Discussion
Based on these results, we propose that EndMT plays a key role in the occurrence and development of moyamoya disease for the first time. PDEs of moyamoya disease promote the proliferation, migration ability and EndMT phenotype changes of cerebrovascular endothelial cells. The occurrence of EndMT in moyamoya disease is at least partly regulated by Plasma-derived exosomes.

At present, the treatment of moyamoya disease is mainly intracranial and extracranial vascular revascularization[14]. In view of the particularity of the disease site, it is difficult to generate discarded vascular tissue during surgery. Therefore, we only collected intracranial vascular tissue from 6 patients with moyamoya disease, which is a limitation of this study. Our findings suggest that EndMT plays a key role in the development of moyamoya disease. EndMT is involved in various maladaptive tissue remodeling, such as venous graft remodeling and vascular fibrosis caused by stroke[15, 16]. EndMT can also cause inflammation, and inflammatory cytokines can destroy endothelial cell barrier function, thereby causing more EndMT [5, 17]. Our previous study showed that the levels of inflammatory factors IL-1β, TNF-α and IL-12 in peripheral blood of MMD patients were significantly higher than those of normal controls, which may be related to vascular EndMT in moyamoya disease [18].

The ETS (E-26 transformation-specific) transcription factor ERG is a key regulator of endothelial cell homeostasis and contributes to maintaining vascular stability[19]. We found that PDEs in moyamoya disease promoted the proliferation and migration ability of cerebrovascular endothelial cells, but did not promote tube formation ability, which may be closely related to endothelial cell dysfunction caused by ERG deletion. We will further verify the regulatory effect of ERG on vascular endothelial cell function in vitro, and may be able to treat moyamoya disease by inducing ERG expression in the future.

At present, there are few studies on the function regulation of PDEs on cerebrovascular endothelial cells. Exosomes are encapsulation vectors for many different molecules, including proteins as well as coding and non-coding RNAs (such as microRNAs (miRNAs)), which can influence protein expression and function in recipient cells such as vascular endothelial cells[20, 21]. In this study, PDEs were isolated and extracted for in vitro experiments, which firstly demonstrated that PDEs from MMD patients significantly induced EndMT phenotype changes compared with the control group. The regulatory effect of exosomes on cerebrovascular endothelial cells needs to be further studied. Our study suggests that exosomal miRNA-151a-3p and miRNA-125b-5p may play role in inducing EndMT phenotype. However, this study still has some shortcomings. This study only proved the regulatory effect of exosomes on cerebrovascular endothelial cells, but we did not study what kind of cells secreted exosomes into peripheral blood, and whether they were secreted into plasma by cerebrovascular endothelial cells and then could interact with endothelial cells to form a feedback loop is still unclear, which is also the limitation of this study.

In the future, we will further collect tissue samples to verify the key role of EndMT in the occurrence and development of moyamoya disease, and deeply explore the specific regulatory mechanism of plasma exosomes on EndMT, so as to provide new insights into the pathogenesis, diagnosis and treatment of moyamoya disease.

**Conclusion**
In this study, we performed that EndMT plays important role in MMD progression. In addition, PDEs induce the phenotype change to promote vascular endothelial cell proliferation and migration. Exosomal miRNAs mediated this EndMT phenotype. This study provides new insights into the molecular mechanism underlying the pathogenesis of MMD.

**Declarations**

**Ethics approval**

This study was approved by Ethics Committee of Medical Science Research, Affiliated Hospital of Jining Medical University (no. 2021C107). All ethical rules and regulations were followed while conducting the study and the participants were included in the study based on informed consent. This study was conducted in accordance with the guidelines outlined in the declaration of Helsinki.

**Consent to Participate**

All participants involved in this study provided written informed consent.

**Consent for Publication**

All co-authors approved the final version of the manuscript and agreed to submit it to Molecular Neurobiology.

**Competing Interest**

The authors declare no competing interests.

**Acknowledgments**

We wish to thank the respondents for accepting to participate in the study. We also wish to thank the Management of the hospital for approval to conduct the study in the facility.

**Authors' contributions**

Jilan Liu, Chao Chen, Bin Zhang, and Feng Jin co-wrote the manuscript and designed experiments. Jilan Liu, Chao Chen, Xianyun Qin and Yan Lu performed experiments and analyzed the results. All authors have read and approved the manuscript.

**Funding**

The present study was supported by Project of Scientific Developmental Program of Shandong Provincial Administration of Traditional Chinese Medicine (grant no. Q-2022136), project of Health and Family Planning Commission of Shandong Province (grant no. 2019WS361), Teacher Support Fund of Jining Medical University (grant no. JYFC2019FKJ125).
Availability of data and materials

The results of the analyzed data presented in this study are all included herein.

References


Figures
Figure 1

Expression of EndMT-related molecular markers in arterial tissue

(A) VE-cadherin (green), Vimentin (red) and α-SMA (orange) expression in control and MMD tissues. (B) CD31, Snail (red) and N-cadherin (orange) expression in control and MMD tissues. (C) Percentage of VE-cadherin+ ECs in arterial intima (*p<0.05). (D) Percentage of α-SMA+ VE-cadherin+ ECs in arterial intima (*p<0.05). (E) Percentage of Vimentin+ VE-cadherin+ ECs in arterial intima (**p<0.001). (F) Percentage of CD31+ ECs in arterial intima (*p<0.05). (G) Percentage of N-cadherin+ CD31+ ECs in arterial intima. (H) Percentage of snail+ CD31+ ECs in arterial intima (*p<0.05).

Figure 2

Identification of plasma-derived exosomes

(A) Electron microscope image showing the intact morphology of exosomes isolated. (B) Particle size analysis confirmed that the particle size of exosomes was about 94.5nm. (C) Western blot analysis of the exosome markers TSG101, CD63 and CD9.
Figure 3

Exosomes from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell proliferation, migration but not tube formation

(A)(B) CCK-8 assay showed that exosomes (200μg/mL) from MMD patients promoted mouse brain vascular endothelial cell (bEnd.3) proliferation but had no effect on human umbilical vascular endothelial cells (HUVEC). (C) Transwell assay showed that exosomes (200μg/mL) from MMD patients

---

Page 12/16
promoted bEnd.3 migration but had no effect on HUVEC. (D, E) high power field (HPF) showed migration cells of two groups (**p<0.01). (F, G) Exosomes from MMD patients did not promote tube formation by bEnd.3 cells.

Figure 4

Exosomes from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell EndMT phenotype

(A) The morphological changes of the two groups of cells observed under the microscope. (B) Immunofluorescence assay showed the cellular localization of β-tubulin. Red represents β-tubulin and
blue represent DAPI. (C) The cell length of the control and MMD group were measured by image J tool. (D) Immunofluorescence assay showed the expression of CD31 after cells stimulated by exosomes from MMD and control group. Red represents CD31 and blue represent DAPI. (E) Western blot showed the expression of VE-cadherin, E-cadherin, CD31 and α-SMA after cells stimulated by exosomes from MMD and control group. (F, G) Immunofluorescence assay and qRT-PCR showed the expression of E-cadherin and snail after cells stimulated by exosomes from MMD and control group. Green represents E-cadherin, and red represents snail, blue represents DAPI (*p<0.05 **p<0.01). (H) Western blot showed the expression of vimentin and snail after cells stimulated by exosomes from control and MMD group.
Exosomal miRNA from Moyamoya disease (MMD) patients promote cerebrovascular endothelial cell migration

(A) The bEnd.3 cells were transfected with mimics and inhibitor, respectively for the overexpression and knockdown of miR-125b-5p. The mRNA expression of miR-125b-5p was detected by qRT-PCR normalized.
to U6 (***p<0.001). (B) The bEnd.3 cells were transfected with mimics and inhibitor, respectively for the overexpression and knockdown of miR-151a-3p. The mRNA expression of miR-151a-3p was detected by qRT-PCR normalized to U6 (***p<0.001). (C-E) Transwell assay showed the migration ability of each group. Nc, negative control. (F) The bEnd.3 cells were transfected with miRNA inhibitor and inhibitor nc, at the same time, cells were stimulated by exosome from MMD patients. The protein expression of Snail was detected by western blotting.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- figureS1.tif
- figureS2.tif