Original Article

MiR-145 facilitates proliferation and migration of endothelial progenitor cells and recanalization of arterial thrombosis in cerebral infarction mice via JNK signal pathway

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Abstract: Arterial thrombosis in cerebral infarction severely affects patients' lives. Classical treatment including surgery and medication both had significantly adverse effects, making it necessary to find novel strategy. Endothelial progenitor cells (EPCs) have been shown to enhance the recanalization of thrombosis, while leaving its molecular mechanism unclear. EPCs were separated from peripheral blood, and were transfected by microRNA (miR)-145. The growth, proliferation and migration abilities were quantified by MTT, clone formation and Transwell assays, respectively. Cell apoptosis was evaluated by flow cytometry. The activation of JNK signaling pathway was measured by Western blotting, followed by JNK inhibitor SP600125. In a mouse cerebral infarction model, miR-145 transfected EPCs were injected to observe the condition of arterial thrombosis. MiR-145 transfection enhanced growth, migration and proliferation of EPCs without induction of apoptosis. MiR-145 exerts its effects via JNK signaling pathway, as the blocking inhibited cell migration/proliferation.

In vivo injection of miR-145 transfected EPCs also potentiated cell proliferation and migration, in addition to the recanalization of arterial thrombosis. MiR-145 facilitates proliferation and migration of EPCs and recanalization of arterial thrombosis in cerebral infarction mice via JNK signal pathway. This study provided new insights regarding infarction treatment.

Keywords: MicroRNA-145, JNK signal pathway, endothelial progenitor cells, cerebral infarction

Introduction

The incidence of cardio-cerebrovascular disease especially the arterial thrombosis has been increasing due to the lifestyle transition [1, 2] and severely affects people’s lives and public health [3]. Classical treatment strategies including surgical recanalization and medical thrombolysis have saved millions of lives [4] but also own amounts of adverse effects [5]. Therefore the novel treatment method with higher efficacy and less adverse effects has drawn lots of research interests [6].

Previous study has revealed the role of endothelial progenitor cells (EPCs) in facilitating recanalization of blood vessel and thrombosis [7], but leaving the molecular mechanism unsolved. Recent study has suggested the critical role of EPCs in the thrombolysis and blood vessel recanalization [8]. Further data showed the time-dependent decrease of EPCs in peripheral blood within 60 hours of arterial thrombosis formation, in both animal models and clinical observation [9]. The number of EPCs resurfaced after thrombolytic drugs [10], suggesting critical function of EPCs in formation and degradation of arterial thrombosis. Therefore, EPC-targeting treatment may own satisfactory effects on arterial thrombosis.

MicroRNA (miR) is a kind of small RNA molecule with 20~23 nucleotides and has been found in almost all living creatures from virus to higher animals [10]. Although not coding proteins, miR owns pluripotent biological functions [9], including signaling transduction [11], cell growth [12, 13], proliferation [14, 15], cell cycle modulation...
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However, the role of miR in EPCs still requires further elaborations [20]. Certain study has suggested the implication of miR-145 in the recanalization of cerebral infarction [21]. The modulatory role of miR-145, especially in the occurrence and progression of cerebral infarction, requires further illustration. This study thus utilized both in vitro primary cell culture and in vivo mouse models of cerebral infarction, and investigated the molecular mechanism of miR-145 in regulating EPCs in recanalization of arterial thrombosis, in an attempt to provide valuable information for thrombolysis.

Materials and methods

EPCs culture and transfection

Mouse EPCs (Procell, China) were thawed and centrifuged to remove cell preserving buffer. After re-suspension in high-glucose DMEM medium (Gibco, US) containing 10% fetal bovine serum (FBS), cells were cultured in a humidified 37°C chamber with 5% CO₂. Based on previous reports of miR-145 in other tumor cell lines [1] and preliminary study, 10 μM miR-145 was chosen to transfect cells. In brief, one day before transfection, cells were seeded into 96-well plate (10⁵ cells each well) to reaching 85% density. MiR-145 was mixed with Lipo200 reagent (Invitrogen, US) diluted in DMEM medium (1:500). After 15-min incubation, transfection mixture was added into each well, followed by continuous incubation at 37°C.

MTT assay

Proliferation of EPCs was quantified by MTT assay kit (Dingguo, China) following the manual instruction. In brief, cells were seeded into 96-well plate (N=6). 30 μL MTT reagents (40 mg/mL) were added to each well for 37°C incubation for 8 hours. The reaction was quenched by PBS washing for 3 times. 0.15 mL DMSO was then added to each well for 15-min development. A microplate reader was then used to quantify absorbance value at 490 nm.

Cell apoptosis

EPCs after transfection were examined for cell apoptosis using phosphatidyl serine method. In brief, cells were re-suspended (10⁶ per mL) and mixed with Annexin V and Annexin V-EGFP (Beyotime, China) solutions (150:50:1). After 30-min dark incubation, cells were loaded for flow cytometry assay to detect the percentage of phosphatidyl serine flipping cells.

Western blotting

Total proteins were extracted from transfected and controlled EPCs. Western blotting was deployed to quantify JNK and p-JNK levels as previous reported [21].

Clone formation assay

EPCs were examined under clone formation assay according to previous documentation [22]. In brief, cells in all groups were firstly counted and diluted serially. Cells were then inoculated into agarose gel and cultured in 37°C chamber for 2 weeks. Culture medium was discarded until clone formation. Cells were fixed in method at room temperature for 15 min, and were stained in Giemsa dye for 30 min. The number of clones was counted under an inverted microscope.

Transwell assay

Cells from all groups were tested for the migration ability using Transwell assay as previously documented [22]. In brief, the upper surface of Transwell plate was pre-coated with solid medium. The bottom of plate was filled with culture medium containing chemotactic factors. EPCs after transfection were seeded on the top layer. After 48-hour incubation, the number of cells in the lower phase was counted.

Mouse infarction model

The generation of mouse cerebral infarction model and intravenous injection of EPCs were performed as previously described [23].
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Statistical analysis

SPSS 14.0 software package was used to process all collected data, which were presented as mean ± standard deviation (SD). The comparison across groups was performed by analysis of variance (ANOVA). A statistical significance was defined when P<0.05.

Results

MiR-145 facilitated EPCs growth

MTT assay showed the facilitation of cell growth after miR-145 transfection when compared to controlled cells (P<0.05, Figure 1). As no difference existed between blank control cells and those cells with nonsense microRNA transfection (MiR control), blank control group was omitted in the following study.

MiR-145 enhanced EPCs proliferation

As shown in Figure 2, EPCs transfected with miR-145 had relatively stronger ability in clone formation, when compared to microRNA controlled group (P<0.05, Figure 2).

Elevated migration ability of EPCs after miR145 transfection

Transwell assay was used to detect the effect of miR-145 on the migration ability of EPCs. Results showed significantly facilitated migration ability of EPCs after miR-145 transfection, as compared to microRNA control group (P<0.05, Figure 3).

MiR-145 did not cause EPCs apoptosis

As shown by flow cytometry, miR-145 transfection did not cause the flipping of phosphatidyl serine, in contrast to those cell transfected with tamoxifen, which worked as the positive control (P<0.05, Figure 4).

MiR-145 activated JNK pathway in EPCs

Western blotting showed the activation of JNK signaling pathway after miR-145 transfection (P<0.05, Figure 5). This model received further support as the JKN inhibitor SP600125 inhibited the JNK activation induced by miR-145 (Figure 6).

JNK signaling pathway inhibition suppressed miR-145-induced cell migration and proliferation

As shown in Figure 7, JNK signaling pathway inhibitor SP600125 significantly suppressed miR-145-induced migration and proliferation of EPCs (P<0.05, Figure 7). These results support-
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ed the ERK-dependent enhancement of EPC proliferation and migration by miR-145.

Recanalization of arterial thrombosis in cerebral infarction mice after injecting miR-145-transfected EPCs

As shown in Figure 8, the intravenous injection of EPCs transfected with miR-145 caused the recanalization of arterial thrombosis in cerebral infarction mice.

Discussion

Cerebral infarction severely affects patients’ life quality and brings heavy burdens to the family and the society as a whole [15]. Therefore the comprehensive study about recanalization of cerebral infarction thrombosis is of critical importance [17]. As one important miR molecule [18], miR-145 still has unclear molecular mechanisms in EPCs. This study thus investigated the modulatory role of miR-145 using cultured EPCs. Our results showed that the transfection of miR-145 enhanced EPC growth, migration and proliferation but without cell apoptosis. JNK pathway was activated by miR-145 transfection in EPCs. The inhibitor of JNK pathway suppressed miR-145-induced migration and proliferation but without cell apoptosis. Further in vivo study showed the intravenous injection of miR-145-transfected EPCs facilitated recanalization of arterial thrombosis. This study provided new insights regarding the treatment of cerebral infarction related arterial thrombosis, as consistent with previous study [19]. Our study for the first

Figure 3. EPCs migration ability. A. Representative images of migrated cells in Transwell chamber; B. Relative area occupying by migrating cells. *, P<0.05 compared to microRNA control group.

Figure 4. EPCs apoptosis. A. Flow cytometry data; B. Percentage of cells with phosphatidyl serine flipping. *, P<0.05 compared to microRNA control group.
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Figure 5. JNK signaling pathway in EPCs. A. Western blotting bands showing p-JNK and JNK protein levels; B. Relative JNK activity. *, P<0.05 compared to microRNA control group.

Figure 6. JNK inhibitor in miR-145 transfected EPCs. A. Western blotting bands showing p-JNK and JNK levels in EPCs with or without SP600125 treatment; B. Relative JNK activity. *, P<0.05 compared to microRNA control group.

Figure 7. EPCs migration (A) and clone formation (B) abilities after JNK inhibition. *, P<0.05 compared to the control group.

Figure 8. Recanalization rate of arterial thrombosis. *, P<0.05 compared to the control group.

Time demonstrated the potency of miR-145 as the novel molecular marker and target for treating cerebral infarction and facilitating recanalization.

Our results had certain differences compared to previous studies in which over-expression of miR actually facilitated cell apoptosis [21]. No apoptosis, however, has been discovered in our study. In contrast, miR-145 actually facilitated cell growth and proliferation. Such inconsistency may be attributed to the difference of cell types and miR molecules [22]. As certain miR molecules exert pro-apoptotic roles [23] while others have anti-apoptotic functions [24]. Moreover, differential expressional level of miR across different tissues [25] may also cause the various biological functions of miR.

Certain limitations also existed in this study, as only cell model was used to demonstrate the biological function of miR-145 in regulating EPC growth, proliferation and migration, but without clinical samples from cerebral infarction patients [26] as supporting evidence to show the relationship between EPCs growth and miR-145 [27]. Moreover, although miR-145 has been found to activate JNK signal pathway in EPCs, the relationship between miR-145 level and severity of cerebral infarction has not been...
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discussed [28]. Future studies can be pursued using small interference RNA (siRNA) approach to further elucidate the molecular mechanism of miR-145 in EPCs.

In summary, this study showed the enhancement of EPC proliferation and migration and recanalization of cerebral infarction by miR-145 via JNK signaling pathway. Our results provided new insights regarding the potency of EPCs in treating arterial thrombosis of cerebral infarction.

Disclosure of conflict of interest

None.

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