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MIF Promoted Cardiovascular Angiogenesis *via* Erk/Mapk Pathway

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Abstract

As the pivotal part of cardiovascular angiogenesis, endothelial cells dysfunction is the leading cause of cardiovascular diseases. Macrophage migration inhibitory factor (MIF) is a tumor growth factor with important roles in cervical tumor formation, invasion, progression and metastasis. However, there was no report on effect of MIF on endothelial cells is unclear, and it is still unknown whether MIF is associated with angiogenesis of endothelial cells. Our study was focused on the effect of MIF and PD98059 on endothelial cells HUVEC cell line, so as to investigate the influence of MIF on expression of vascular endothelial growth factor (VEGF). We also explored whether MIF will influence angiogenesis of endothelial cells *via* ERK/MAPK pathways. Endothelial cells HUVEC cells were conventionally cultured, and Western blot were used to detect the expression of MIF, ERK1 and VEGF proteins after HUVEC cells were intervened by MTT and PD98059. Inter-group difference was statistically assessed. Positive expressions of MIF, ERK1 and VEGF were observed in HUVEC cells. Proliferation activity in MIF group gradually increased after 24 h, 48 h or 72 h treatment ($P<0.05$). Expressions of ERK1 and VEGF were increased after MIF 48 h treatment ($P<0.05$). Proliferation activity in PD98059 group gradually decreased after 24 h, 48 h or 72 h treatment ($P<0.05$). Expressions of ERK1 and VEGF were decreased after MIF 48 h treatment ($P<0.05$). Compared with control group, expressions of ERK1, IL-2 and VEGF were significantly decreased in both MIF pre-stimulation +PD98059 inhibition group and PD98059 pre-exposure+MIF stimulation group ($P<0.05$), while no difference was observed between MIF pre-stimulation+PD98059 inhibition group and PD98059 pre-exposure+MIF stimulation group ($P>0.05$). Expressions of ERK1 and VEGF are involved in the process of endothelial cells HUVEC cell line. MIF is correlated with increased cell proliferation and promoted cardiovascular angiogenesis via ERK/MAPK pathway

Keywords: MIF; Endothelial cells; HUVEC cells; Cell number/activity; ERK1; VEGF

Introduction

Endothelial cells is the most part of cardiovascular angiogenesis, however, there is no curative therapy for endothelial cells repairing in cardiovascular diseases [1]. At present, multiple clinical trials have indicated that endothelial cells dysfunction aggravate development of restenosis post-PCI, moreover, endothelial injury itself is the cause of many cardiovascular diseases, including myocardial infarction and atherosclerosis [2]. Promoting angiogenesis is the basic aim for endothelial repair. The occurrence and development of endothelial injury is a multi-factor, multi-step and multi-gene interactive process [3,4]. Sufficient blood vessels are essential for blood flow. Moreover, angiogenesis increases viability of ischemic myocardium *via* multiple mechanisms, while VEGF plays an important role in angiogenesis of cardiovascular diseases [5]. Macrophage migration inhibitory factor (MIF) is a very important cytokines within the organism that can promote the occurrence and development of tumors. Studies have found that the change of MIF level were associated with tumor metastasis and malignant potential [6,7], while there was no report on its cardiovascular angiogenesis effect. ERK/MAPK pathways were associated with angiogenesis, and PD98059 was a reported specific inhibitor of cell permeability and selectivity, which was involved in ERK1/2 pathways [8]. Thus, we determined to explore whether the effect of MIF on endothelial cells was associated with ERK/MAPK pathways, and endothelial cells HUVEC cell line was used in our study for MIF or PD98059 treatment. In summary, we aimed to examine expressions of ERK1 and VEGF so as to explore the effect of MIF on endothelial cells and its relationship with ERK/MAPK pathways.

Material and Methods

Experimental cell

Endothelial cells HUVEC cell were kindly provided by Shangdong University.

Reagents and antibodies

PD98059 were purchased from the Beyotime Biotechnology (Jiangsu, China); DMEM medium, penicillin/streptomycin, fetal bovine serum, MTT reagent, Propidium iodide (PI) and were purchased from Sigma-Aldrich (St. Louis, MO, USA). MIR, ERK1, VEGF antibody were purchased from Bioworld (Bioworld company); glyceraldehyde 3-phospahte dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Bechtop (Formal205, USA); Inverted Microscope was purchased from Olympus[Olympus, Japan]; Incubator was purchased from Thermo (Thermo, USA); carbon dioxide incubator and -80°C refrigerator were purchased from SANYO (SANYO, USA); Low temperature high-speed desk centrifuge was purchased from Beckman (Beckman, USA).

Experimental method

Cell culture: Cells culture cells were cultured in RPMI1640 medium, it was incubated at 37°C in an atmosphere of 5% CO_2/95% air with saturated humidity.

Protein analysis for MIF, ERK1, pERK1 and VEGF: ELISA was preliminarily performed with routine protocol to assess the concentration of VEGF, and showed the concentration was far below 10-3/ml, suggesting this index did not influence result. Lysate proteins (40 ug per lane) were separated by 8% SDS polyacrylamide gel-electrophoresis were diluted in DMEM and incubated for 1 h at room temperature. Then discard confining liquid, adding first antibody (l: 200 serial dilutions, β-action 1:500) incubated for 24 h at 4°C refrigerator, after 24 h of plating, added second antibody (1:200 serial dilutions) and these were incubated for 1 h. Combined developer solution A, B (2 mL of each one), and perform chemiluminescence photographic detection, after scan the developed stripe, Quantity One was used to measure the optical density.

MIF intervene HUVEC cells: Logarithmic phase HUVEC cells were transfected into cell cultured plate with 2% fetal bovine serum, regular culture, making them grow adhering to the wall, cultured for 24 h. Cells (80% confluent) were harvested using 10% DMEM medium, added 250 ng/ml of MIF for 48 h. Control group use normal ervical cancer HUVEC cells.

Cell counting for proliferation of HUVEC cells after MIF treatment: HUVEC cells were treated with MIF (250 ng/ml) for 48 h. Cell counting was performed with routine protocols to assess proliferation of HUVEC cells after 24 h, 48 h or 72 h treatment.

Western blot analysis of ERK1, pERK1 and VEGF protein expression: Levels of ERK1, VEGF were determined by Western blotting, 250 ng/ml of MIF was added for 48 h.

PD98059 intervene HUVEC cells: Logarithmic phase HUVEC cells were transfected into cell cultured plate with 2% fetal bovine serum, regular culture, making them grow adhering to the wall, cultured for 24 h. Cells in PD98059 group were harvested using 10% DMEM medium. PD98059 was dissolved in DMSO. Add PD98059 (20 μmol/L) into PD98059 group for 48 h. In control group, endothelial cells HUVEC cells were cultured in the same DMEM medium with the same amount of DMSO.

Cell counting for the proliferative activity after intervene by PD98059: After using 100 μmol/L of PD98059 for 48 h, cell counting was performed to detect the proliferative activity after inhibition in 24 h, 48 h, and 72 h. See 1.3.4 for detailed procedure.

Western blot analysis of ERK1, VEGF protein expression after inhibit by PD98059: Logarithmic phase HUVEC cells were filled with 100 μmol/L of PD98059 for 48 h.

Western blot analysis of ERK1, VEGF of different variables groups: Two groups used logarithmic phase HUVEC cells, one used 250 ng/ml of MIF for stimulation then 100 μmol/L of PD98059 for inhibit; the other used 100 μmol/L of PD98059 for inhibit and 250 ng/ml of MIF for stimulation.

Statistical analysis

Invasion and motility results are expressed as mean ± SE. Multiple comparisons were performed using SPSS17.0 with P value<0.05 considered statistically significant.

Results

Expression of MIF, ERK1, pERK1 and VEGF protein before and after intervene by MIF

Logarithmic phase HUVEC cells were conventionally cultured. Expression of MIF, ERK1, VEGF protein were detected by Western blotting analysis. The result shows that these protein have positive staining, the expression increased after intervene by MIF, the difference was statistically significant (P<0.05, Figures 1).

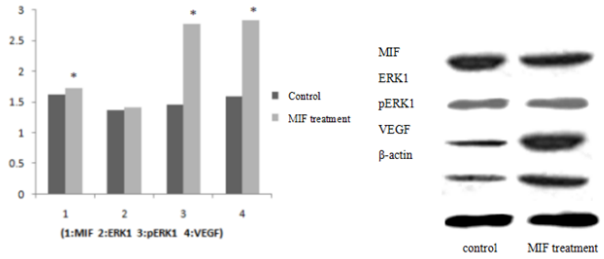

Figure 1: Expression of ERK1, pERK1 and VEGF after MIF treatment. *Compared with control, P0.05

Proliferative activity of HUVEC cell after intervened by MIF

MIF has been reported as being able to intervene proliferative activity of HUVEC cell, the result shows that proliferative activity increased significant in 24, 48, and 72 h compared with control group, the difference is statistical significant (P<0.05). Table 1 shows that as time went on, proliferative activity increased (P<0.05).

Group	24 h	48 h	72 h
Control	38.2 ± 5.2	46.5 ± 4.7 2	52.3 ± 2.8 23
MIF	50.2 ± 6.5 1	59.4 ± 5.3 12	62.9 ± 4.2 123

Table 1: Proliferation of HUVEC cell after MIF treatment Compared with control, P0.05, Compared with 24 h, P0.05, Compared with 48 h, P0.05.

Proliferative activity of HUVEC cell after intervened by PD98059

After PD98059 inhibit endothelial cells Siha, cell proliferation HUVEC cells detected by MTT assay, we found that proliferative activity increased significantly in 24 h, 48 h, and 72 h, compared with control group, the difference is statistical significant (P<0.05).

Group	24 h	48 h	72 h
Control	38.2 ± 5.2	46.5 ± 4.7 2	52.3 ± 2.8 23
PD98059	25.1 ± 2.7 1	19.6 ± 3.3 12	10.5 ± 1.2 123

Table 2: Proliferation of HUVEC cell after PD98059 treatment Compared with control, P0.05, Compared with 24 h, P0.05, Compared with 48 h, P0.05.

As time become longer, proliferative activity of cell suffers a corresponding decrease (P0.05, Table 2).

Expression of ERK1pERK1 and VEGF protein in Siha HUVEC cell after intervened by PD98059

Western blotting was performed to assess ERK1, pERK1 and VEGF protein after PD98059 inhibition endothelial cells Siha, which showed a decrease in ERK1, VEGF protein. Compared with the control group, the difference is statistical significant (P<0.05) (Figure 2, Table 3).

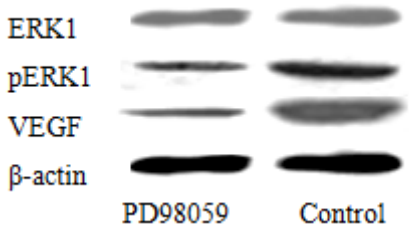

Figure 2: Expression of ERK1, pERK1 and VEGF after PD98059 treatment.

Group	ERK1	pERK1	VEGF
Control	1.15 ± 1.17	1.23 ± 1.01	1.08 ± 0.87
PD98059	1.12 ± 1.22	0.46 ± 0.32*	0.61 ± 0.22*

Table 3: Expression of ERK1, pERK1 and VEGF after PD98059 treatment.

Expression of ERK1, pERK1 and VEGF protein in Siha HUVEC cell of MIF inhibition PD98059 stimulation group, PD98059 inhibition MIF stimulation group

The HUVEC cell lines was investigated by examining the signaling pathways of ERK1, pERK1 and VEGF, stimulated both ERK1 and VEGF by MIF, then inhibit by PD98059. Protein expression levels of ERK1 and VEGFMIF decreased significantly in both groups (MIF pretreatment and PD98059 subsequent treatment, PD98059 pretreatment and MIF subsequent treatment), with no difference between them (P>0.05), (Figure 3 and Table 4).

Discussion

Endothelial cells is a not only a common part of vessels, but also a natural cover for defense against adverse factors of cardiovascular diseases. However, the patient population with endothelial injury becomes much younger recently and the disease rate is rising. The treatment options for patients including surgical repairing, PCI or drug therapy [9,10].

With the development of molecular biology techniques, further studies were performed on tumor-related factor in molecular level. The occurrence and development of endothelial injury is a multi-factor, multi-step and multi-gene regulation process. MIF is a type of cytokine which can promote tumor genesis and produced by activated T cells. It can also inhibit the migration of mononuclear and macrophage, influence the cell divides and induce malignant transformation [9].

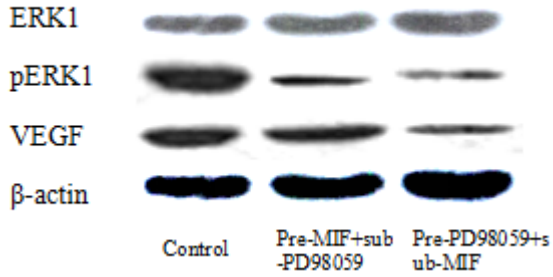

Figure 3: Expression of ERK1, pERK1 and VEGF after MIF and PD98059 cotreatment

Group	ERK1	pERK1	VEGF
Control	1.14 ± 0.26	1.23 ± 1.01	1.58 ± 0.87
MIF pretreatment and PD98059 subsequent treatment	1.13 ± 0.21	0.67 ± 0.41*	0.62 ± 0.23*
PD98059 pretreatment and MIF subsequent treatment	1.19 ± 0.32	0.63 ± 0.35*	0.65 ± 0.18*

Table 4: Expression of ERK1, pERK1 and VEGF after MIF and PD98059 cotreatment.

MIF is not only limited to generate angiogenesis in tumor, but also expressed in ECM and basement membrane; rather, it is also profoundly involved in the secretion of vascular endothelial growth factor (VEGF), and even plays a critical part in carcinogenesis [10]. Previous studies showed that MIF was at low expression level under normal condition, but overexpressed in multiple malignant tumors, including prostate cancer, liver cancer, lung cancer, breast cancer and esophageal cancer. The main effect of MIF was induction of tumor angiogenesis and degradation of ECM or basement membrane, but mechanisms are still unclear. Accordingly, we explored detailed mechanisms of MIF on HUVEC cells.

In this study, HUVEC cells were conventional culture. Western blotting analysis of HUVEC cells lines showed higher MIF, ERK1, VEGF protein expression than in normal cholangiocytes. As described above, previous studies showed that MIF was overexpressed in multiple tumors, moreover, MIF was associated with blood-supply of cancer [11,12]. In addition, Fujiwaki et al. found that expression of VEGF progressively increased in the progress of normal cervical epithelium→carcinoma in situ→invasive carcinoma [13]. Angiogenesis growth factors, including VEGF, were overexpressed in the progress of cancer. What's more, angiogenesis of cancer was under regulation of VEGF, which was secreted by interstitial cells and cancer cells. MIF was reported to regulate secretion of Angiogenesis growth factors in tumor lesion, which was proved in our study.

Our study used MIF to intervene and stimulate endothelial cells HUVEC cells, proliferation of tumor cell was detect by MMT, the result shows that MIF increased the proliferation of tumor cell in 24 h, 48 h, 72 h, when incubation time was prolonged, the activity of tumor cell increased correspondingly. This indicates that MIF increased the

proliferation of endothelial cells HUVEC cells and reduced the expression of VEGF, ERK1 protein. The study of Na et al. shows that MIF is involved in tumor cell proliferation and differentiation, promotes tumor invasion and metastasis and is capable of increasing the susceptibility to diseases [14]. The expression of MIF plays an important role in carcinogenesis and tumor progression, the high expression of MIFin cells can hypertrophy induced EK1/2 tyrosine kinase activation, the replace the fuction of Ras in keeping the activation of EK1/2 tyrosine kinase [15,16]. Our findings indicated that MIF promoted phosphorylation of ERK and maintained activation of ERK1/2 kinases. Furthermore, MIF regulated proliferation of HUVEC cells *via* ERK1/2 pathways, and influenced angiogenesis *via* regulating VEGF, which was consistent with previous studies.

In further studies, we PD98059 to inhibited endothelial cells HUVEC cells, proliferation of tumor cell was detect by MMT, the result shows that PD98059 inhibit proliferation of tumor cell in 24, 48, 72 h, when incubation time was prolonged, the activity of tumor cell decline correspondingly. This indicates that PD98059 inhibited the proliferation of endothelial cells HUVEC cells and reduced the expression of VEGF, ERK1 protein. ERK1/2 activation is known to regulate a variety of cellular functions, such as proliferation, PD98059 have the ability to interdict the activation of ERK1/2 in a time-and a dose-dependent manners [17]. PD98059 sestrain the growth and the proliferation of cancer cell by operating the phosphorylation of ERK1/2, it can exert functions in transcriptional regulation, thus inhibit tumor cells proliferation, migration, and invasion which is also related with ERK1/2 [18-20]. Our study showed that ERK pathways played an important role to promote proliferation of HUVEC cells, and VEGF might be a target of ERK pathways. In addition, as reported previously, PD98059 decreased phosphorylation of ERK and inhibited activities of ERK1/2 pathways so as to inhibit proliferation of HUVEC cells and expression of VEGF.

MIF was used to stimulate HUVEC cells, and added PD98059 to inhibit, on the other hand, the other group were used PD98059 inhibit HUVEC cells, and further underwent the MIF stimulation. The expression of VEGF, ERK1 protein was assessed by Western blot, and both of them reduced the expression of VEGF, ERK1 protein. Thus, it can be concluded that both MIF stimulation and PD98059 inhibition can lower the expression of VEGF, ERK1 protein.

ERK1, VEGF were highly expressed in in endothelial cells HUVEC cells, MIF can increase cell proliferation of HUVEC cells, and improve the protein expression level of ERK1, VEGF, while PD98059 significantly blunted cell proliferation. Our study demonstrates that both the stimulation of MIF and the treatment of PD98059 can regulate the expression of ERK1, VEGF, decreasing the protein expression of them. MIF can affect the expression of angiogenic growth factors, such as VEGF, by regulating the ERK/MAPK pathways, to promote the angiogenesis. MIF regulated VEGF to enhance both angiogenesis and proliferation of HUVEC cells *via* promoting phosphorylation of ERK and maintained activation of ERK1/2 kinases, but more basal researches and studies are need to be done.

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