European Review for Medical and Pharmacological Sciences 2018; 22: 2044-2051

MST1 down-regulation in decreasing apoptosis of aortic dissection smooth muscle cell apoptosis

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Abstract. – OBJECTIVE: Elevated apoptosis of vascular smooth muscle cell (VSMC) is correlated with the occurrence of aortic dissection (AD). Mammalian ste20-like protein kinase 1 (MST1) is one important component of Hippo-YAP signal pathway for activation and cell apoptosis facilitation. Whether MST1 plays a role in AD pathogenesis is unclear yet. This study established an AD rat model to investigate the role of MST1 in regulating VSMC apoptosis and AD pathogenesis.

MATERIALS AND METHODS: Cell apoptosis was compared between AD vascular tissues and normal rats, in addition to Caspase-3 activity, and expression of MST1, p-LATS1, p-YAP1, YAP1. In vitro cultured VSMCs from AD rats were treated with siRNA-MST1 to test apoptotic rate and Caspase-3 activity. AD model rats were treated with pGLVU6/GFP-MST1 for comparing MST1, p-LATS1, p-YAP1, and YAP1 expression, along with Caspase-3 activity, cell apoptosis, AD formation rate, diameter, and length.

RESULTS: Compared to control group, AD rats had elevated vascular cell apoptosis, Caspase-3 activity, expressions of MST1, p-LATS1, and p-YAP1, plus lower YAP1 expression. siRNA interference of MST1 significantly inhibited apoptosis of in vitro cultured VSMC. shRNA lentivirus targeting MST1 pGLVU6/GFP-MST1 remarkably decreased expression of MST1, p-LATS1, and p-YAP1 in AD rat vascular tissues, increased YAP1 expression, decreased VSMC apoptosis, AD formation rate, AD diameter/length.

CONCLUSIONS: MST1 up-regulation plays a role in facilitating VSMC apoptosis and AD pathogenesis. Down-regulation of MST1 decreased VSMC apoptosis and AD formation.

Key Words:
MST1, Hippo-YAP, VSMC, Aortic dissection, Apoptosis.

Introduction

Aortic dissection (AD) is featured as atrophy of aorta wall middle layer with ruptured vascular endo-membrane, through which blood flows into aorta middle layer to rupture it and form a hematoma or pseudo-cavity, eventually leading to the dual cavity of the aorta or even aorta complete rupture[1]. AD is the most severe pathology of aorta disease, with rapid progression and high mortality. Currently, no effective drug treatment is available for AD. Various risk factors including hypertension, drinking, smoking, obesity, mental stress are involved in AD pathogenesis. Various studies[2,3] showed that apoptosis of vascular smooth muscle cell (VSMC) plays a critical role in AD pathogenesis. Hippo-YAP signal transduction pathway is one kinase cascade reaction consisting of a series of protein kinase and transcriptional co-activators, and can regulate normal growth and size of tissue/organ via modulating dynamic balance of cell survival, proliferation, and cell apoptosis. Abnormality of Hippo-YAP signal pathway transduction is closely correlated with organ hypertrophy[4,5], cell decrease or organ atrophy, and tumor occurrence[6,7]. Hippo is one serine/threonine regulatory kinase firstly discovered in Drosophila. Mammalian ste20-like protein kinase (MST) is Hippo homolog in mammals, and plays critical roles in Hippo-YAP signal pathway transduction[8]. MST1 is the basic gene for regulating cell apoptosis in MST protein family, and can effectively activate Hippo-YAP signal pathway to facilitate cell apoptosis and to inhibit cell proliferation via transduction role of downstream signal molecules. Researches showed that abnormally

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elevated expression of MST1 accelerated cell apoptosis, leading to tissue cell decrease and organ atrophy, whilst abnormally decreased MST1 expression suppressed cell apoptosis, causing organ hypertrophy[9-11] or even tumor occurrence[12,13]. Various studies[10,14,15] showed the participation of MST1 kinase in mediating cardiovascular cell apoptosis and structural re-modeling process during cardiovascular apoptosis. However, the role of MST1 in AD occurrence has not been illustrated. This investigation thus generated a rat AD model, on which possible regulatory roles of MST1 in VSMC apoptosis and AD pathogenesis were investigated.

Materials and Methods

Major Reagent and Materials

DMEM, FBS, dual antibiotics, type II collagenase, and trypsin were purchased from Gibco (Rockville, MD, USA). RNA extraction kit Rneasy MiNi Kit, fluorescent quantitative kit QuantiTech SYBR Green RT-PCR Kit were purchased from Qiagen (Hilden, Germany). Rabbit anti-p-LATS1 and mouse anti-β-actin were purchased from Bio-Rad (Hercules, CA, USA). Beta-amino propionitrile (BAPN) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Lentivirus interference vector pGLVU6/GFP-MST1 and negative control virus pGLVU6/GFP-NC were purchased from Gimma Pharm (Shanghai, China). MST1 siRNA-MST1 and negative control siRNA-NC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TdT-mediated dUTP Nick-End Labeling (TUNEL) apoptosis assay kit was purchased from Sangon (Shanghai, China). Annexin V/PI apoptosis kit and Caspase-3 activity assay kit were purchased from Beyotime (Suzhou, China).

Generation of AD Rat Model and MST1 Interference

A total of 30 male Sprague-Dawley (SD) rats (3 weeks age, body weight 55 ± 1.3 g) were purchased from Shandong University Medical Laboratory Animal Center. Animals were randomly divided into two groups. The control group (N = 14) received normal diet feeding for 6 weeks. AD group (N = 16) were fed with the same diet containing 0.25% BAPN for 6 weeks. Both groups of rats were kept in a facility with normal light, 40-70% relative humidity, and 20-22°C temperature with food and water *ad libitum*. All animal protocols were approved by the Ethical Committee of our hospital and followed relevant guidelines. During 6-week experiment, AD formation rate was observed between two groups to record AD diameter. At the end of the experiment, rats were anesthetized by tail vein injection of pentobarbital. For those rats with AD formation in model group, aorta middle layer tissues were collected at the rupturing site of the aorta. For the control group, middle layer tissues of the aorta at initial descending segment were collected. All samples of aorta middle layer tissues were tested for cell apoptosis and Caspase-3 activity, and for RNA extraction and protein extraction. In MST1 interference study, rat AD model was generated as above-mentioned, and animals were further divided into three groups (N = 20 each). One group received a normal diet containing 0.25% BAPN for AD model preparation. One group received tail vein injection of 100 μL pGLVU6/GFP-NC (viral titer = 1×10^9TU/mL) for 6 consecutive weeks with 0.25% BPAN diet feeding. A third group received tail vein injection of 100 μL pGLVU6/GFP-MST1 (viral titer=1×10^9TU/mL) for 6 consecutive weeks with 0.25% BPAN diet feeding.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the affiliated Yantai Yuhuangding Hospital of Qingdao University.

TUNEL for Cardiomyocytes Apoptosis

Rat aorta middle layer tissues were prepared for paraffin sections, and were tested for cell apoptosis following the manual instruction of TUNEL kit. Five fields were randomly selected from each slide. The number of apoptotic cells in 100 cells was counted in each field to calculate cell apoptotic rate.

Caspase-3 Activity Assay in Aorta Middle Layer Tissues

Following the manual instruction of Caspase-3 activity assay kit, pNA standard samples were prepared for measuring A405 to plot standard curves. Aorta middle layer membrane tissues were lysed on ice in 100 μL lysis buffer for every 5 mg tissues. The supernatant was transferred to pre-cold tubes for further use. Assay buffer, testing samples, Ac-DEVD-pNA were sequentially added into 96-well plate and incubated at 37°C for 2 h. A405 values were measured immediately when the color changed significantly to reflect Caspase-3 activity of testing samples.

Rat Aorta VSMC Separation, Culture, and Grouping

AD model rats were anesthetized by tail vein injection of pentobarbital. AD tissues were separated to remove outer and inner vascular membranes but leaving middle membrane intact. The middle membrane tissue was lysed in type II collagenase and 0.05% trypsin. The digestion was quenched and the mixture was filtered to remove supernatant. Cell precipitation was saved and cultured in Dulbecco's Modified Eagle (DMEM) medium containing 20% fetal bovine serum (FBS), 1% streptomycin-penicillin and incubated at 37°C with 5% CO_2. Cells were passed at 1:4 ratio. Cells at 5-6 generation were obtained for further experiments.

Rat aorta VSMC at 5th generation was divided into three groups: control group, siRNA-NC transfection group, siRNA-MST1 transfection group. Cells after 72 h culture were collected for assays.

qRT-PCR for Gene Expression

QuantiTest SYBR Green RT-PCR Kit was used to test gene expression by one-step qRT-PCR, using RNA extracted by Rneasy MiNi Kit as the template. In a 20 μL qRT-PCR system, there were 10 μL 2XQuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL of forward and reverse primer (0.5 μm/L), 2 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH_2O. Reverse transcription conditions were: 50°C for 30 min. PCR conditions were: 95°C pre-denature for 15 min, followed by 40 cycles each containing 94°C denature for 15 s, 60°C annealing for 30 s, and 72°C elongation for 30 s. Gene expression was examined on Bio-Rad CFX96/CFX connect Real-time fluorescent quantitative PCR cycler.

Western Blot for Protein Expression

SDS lysis buffer was used to lyse cells by 5 min boiling. After protein quantification by BCA method, 50 μg samples were separated in SDS-PAGE and were then transferred to polyvinylidene difluoride (PVDF) membrane, which was blocked in 5% defatted milk powder at room temperature incubation. Primary antibody (MST1 at 1:3000, p-LATS1 at 1:1000, p-YAP1 at 1:2000, YAP1 at 1:5000, β-actin at 1:8000) was added for 4°C overnight incubation. After phosphate-buffered saline Tween (PBST) rinsing for three times, horseradish peroxidase (HRP) conjugated secondary antibody (1:10000 dilution) was added for 60 min incubation. The membrane was rinsed in PBST and incubated using electrochemiluminescence (ECL) method. After dark exposure and development, the film was scanned and analyzed.

Flow Cytometry for Cell Apoptosis

Cells were digested with trypsin and collected. After re-suspending in 500 μL 1X Binding Buffer, 5 μL Annexin V-FITC and 5 μL PI dyes were sequentially added. Gallios flow cytometry (Beckman Coulter, Brea, CA, USA) was used for measuring cell apoptosis.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Enumeration data were presented as percentage and were tested by chi-square test. The Student *t*-test was used to compare measurement data between groups. A statistical significance was defined when $p < 0.05$.

Results

Significant AD Formation, and Elevated MST1 Expression and Cell Apoptosis in Model Rats

Control group rats had normal general conditions, with no AD formation during experimental process. In model group, 11 rats had AD formation (68.7%). Two of them died at 4th week of feeding, 3 rats died at 5th weeks, and 6 rats survived until experimental endpoint. Model group rats had AD diameter of 5.92 ± 1.12 mm and 8.67 ± 2.25 mm length. TUNEL assay showed significantly elevated apoptosis in AD middle membrane layer in model group compared to control group (Figure 1A). Spectrometry results showed remarkably elevated Caspase-3 activity in AD vascular middle membrane in model group (Figure 1B). Western blot results showed significantly elevated expression of MST1, p-LATS1, and p-YAP1 in AD middle membrane in model group compared to control group, whilst YAP1 protein expression was significantly decreased (Figure 1C). These results indicated that activation of Hippo-YAP signal pathway might be correlated with elevated cell apoptosis and AD occurrence.

siRNA Interference of MST1 Significantly Inhibited VSMC Apoptosis in AD Model Rats

qRT-PCR results showed that, compared to control group or siRNA-NC group, siRNA-MST1

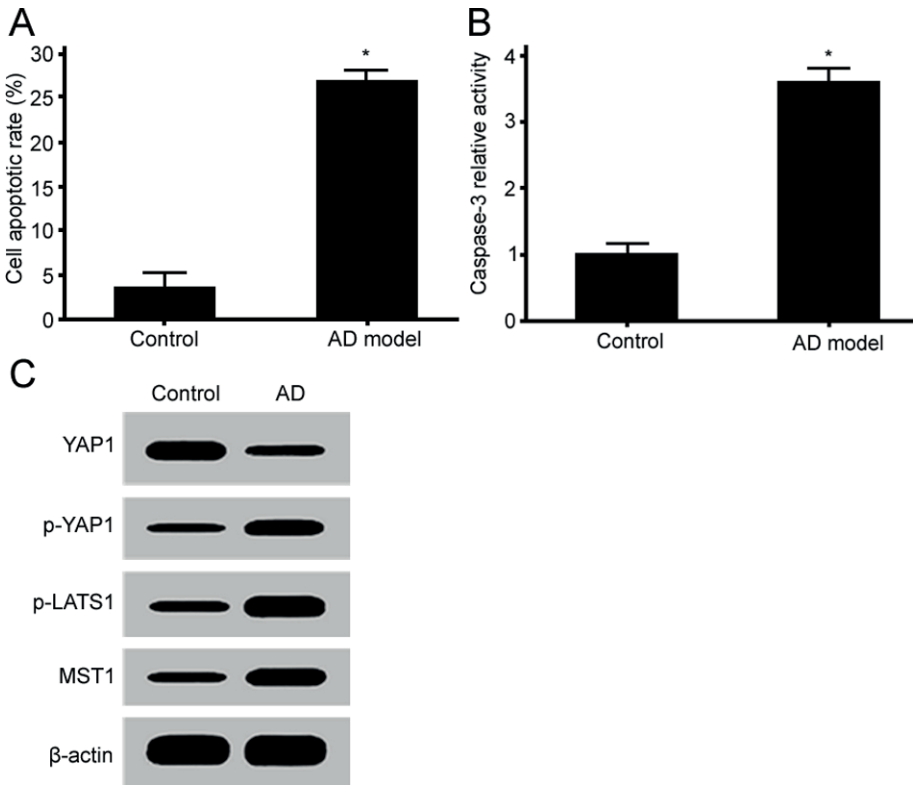

Figure 1. Significant AD formation, and elevated MST1 expression and cell apoptosis in model rats. (***A)*** TUNEL assay for vascular middle membrane cell apoptosis; ***(B)*** Spectrometry for Caspase-3 activity in the vascular middle membrane; ***(C)*** Western blot for protein expression in the vascular middle membrane. *, $p<0.05$ compared to control group.

transfection group had significantly lower MST1 mRNA expression in VSMC, indicating relatively higher interference efficiency (Figure 2A). Western blot results showed that, compared to control group or siRNA-NC group, siRNA-MST1 transfection group had significantly decreased expression of MST1, p-LATS1, and p-YAP1 proteins in VSMC cells, whilst YAP-1 expression was elevated, indicating that MST1 interference significantly suppressed the activity of Hippo-YAP signal pathway (Figure 2B). Spectrometry results showed that siRNA-MST1 transfection group had lower Caspase-3 activity than control or siRNA-NC transfection group (Figure 2C). Flow cytometry results showed that the apoptotic rate of VSMC cells in siRNA-MST1 transfection group was significantly lower than control or siRNA-NC transfection group (Figure 2D).

shRNA Interference Virus Injection Significantly Suppressed Cell Apoptosis and Decreased AD Formation Rate

Compared to AD model group or pGLVU6/GFP-NC group, shRNA interference virus pGLVU6/GFP-MST1 injection significantly decreased protein expressions of MST1, p-LATS1, and p-YAP1 in AD middle membrane tissues, whilst YAP-1 expression was significantly elevated (Figure 3A). pGLVU6/GFP-MST1 injection group had significantly lower Caspase-3 activity (Figure 3B) and cell apoptotic rate (Figure 3C) in AD middle membrane tissues compared to AD model or pGLVU6/GFP-NC group. pGLVU6/GFP-MST1 injection also remarkably decreased rat AD formation rate, and decreased AD diameter and length (Table I).

Discussion

Hippo-YAP is one newly discovered intracellular signal transduction pathway in recent years, and can modulate various biological processes including cell number, organ volume, cell adhesion, mechanic force transduction, and contact-dependent inhibition between cells via regulating cell proliferation, migration, and apoptotic balance. Hippo-YAP signal transduction abnor-

Table I. Comparison of AD formation rate, AD diameter and length of rats.

Group	AD formation rate (%)	AD diameter (mm)	AD length (mm)
AD model	67.6	6.13±1.06	8.89±1.96
pGLVU6/GFP-NC	69.3	6.08±1.13	9.06±2.02
pGLVU6/GFP-MST1	48.2*#	4.11±0.56*#	6.36±1.37*#

Note: *, p <0.05 compared to AD model group; #, p <0.05 compared to pGLVU6/GFP-NC group.

mality is closely correlated with organ hypertrophy[4,5], cell number decrease or organ atrophy, and tumor occurrence[6,7]. Hippo is one serine/threonine regulatory kinase initially discovered in Drosophila. The mammalian homolog of Hippo, mammalian ste20-like protein kinase (MST) was later identified[8]. Hippo-YAP signal pathway consists of MST and its co-factor Salvador (Sav), large tumor suppressor (Lats) and regulatory protein Mps One Binder 1 (Mobl), effector protein Yes-associated protein (YAP) or it homolog TAZ[16]. When Hippo-YAP signal pathway is activated, MST kinase can bind with Sav protein to form a complex for phosphorylation and activation of Lats kinase, which can phosphorylate S127 site of YAP. Phosphorylated YAP protein can be translocated from nucleus to cytoplasm, where it is degraded in proteasome, thus impeding nuclear gene expression. When Hippo-YAP signal pathway is inactivated, dephosphorylated YAP protein expression is significantly elevated and was translocated into the nucleus, where it functions as one co-transcriptional factor to bind with other factors such as TEADs, RUNx, ErbB4, PEB2α, p73, to facilitate transcription and expression of target gene such as cmyc or survivin, thus exerting critical regulatory roles in facilitating cell proliferation and decreasing cell apoptosis[17,18]. Previous studies showed the close correlation between Hippo-YAP signal pathway occurrence of multiple diseases including myocardial hypertrophy[9-11], liver cancer[19], breast cancer[20] and prostate cancer[21]. Various researches[10,14,15] showed that MST1 kinase could

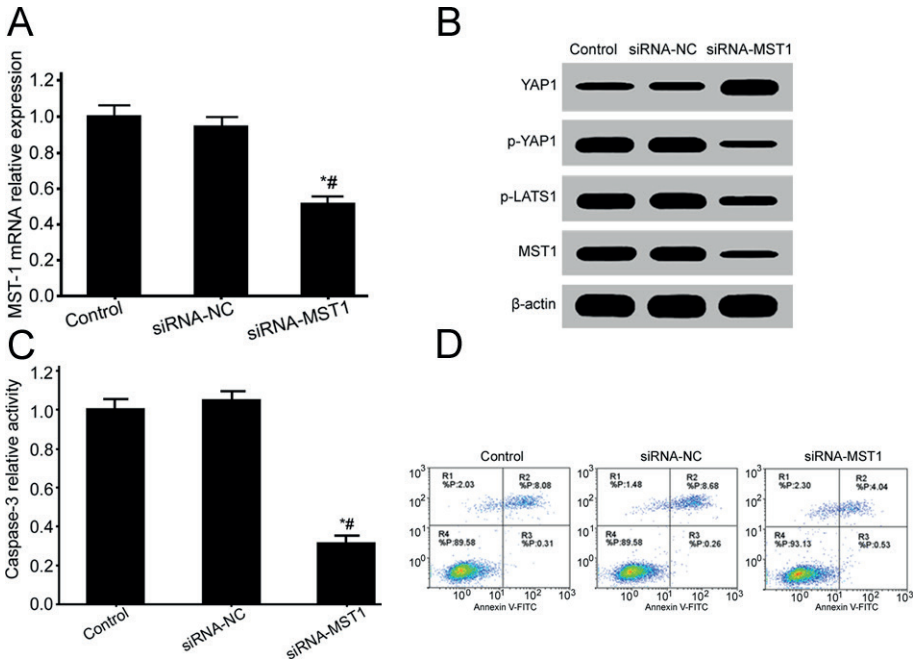

Figure 2. siRNA interference of MST1 significantly inhibited VSMC apoptosis in AD model rats. ***(A)*** qRT-PCR for MST1 mRNA expression in VSMC cells; ***(B)*** Western blot for protein expression in VSMC cells; ***(C)*** Spectrometry for Caspase-3 activity in VSMC cells; ***(D)*** Flow cytometry for VSMC cell apoptosis. *, p <0.05 compared to control group; #, p <0.05 compared to siRNA-NC group.

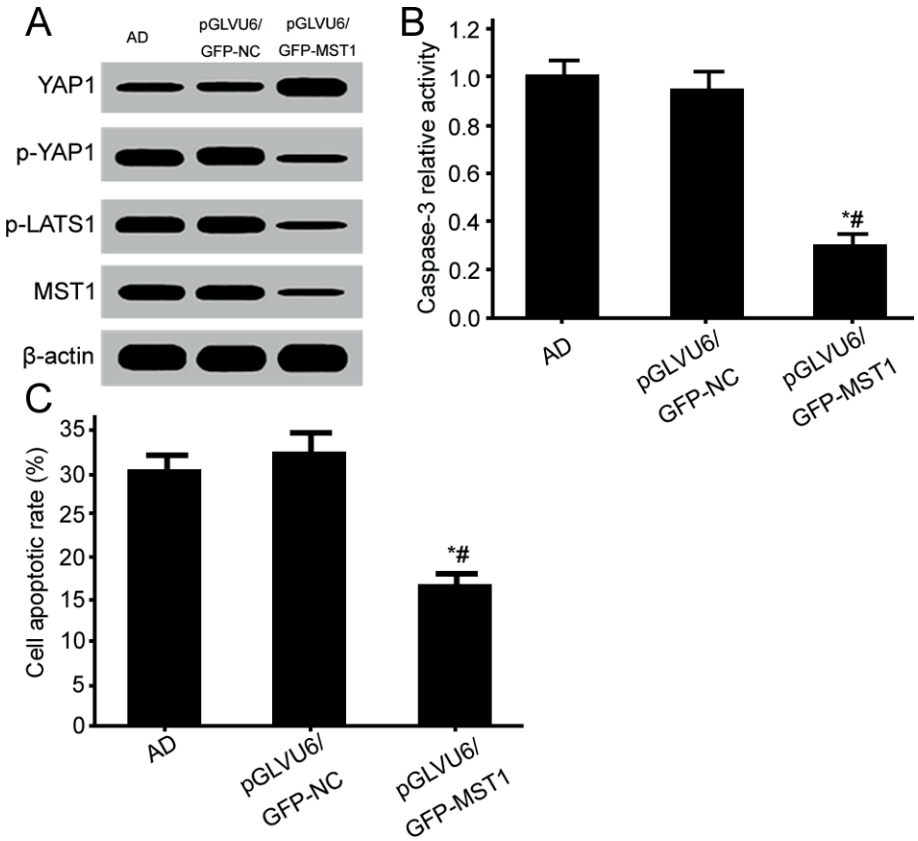

Figure 3. shRNA interference virus injection significantly suppressed cell apoptosis and decreased AD formation rate. ***(A)*** Western blot for protein expression in the vascular middle membrane; ***(B)*** Spectrometry for Caspase-3 activity in the vascular middle membrane; ***(C)*** TUNEL assay for cell apoptosis in the vascular middle membrane. *, p <0.05 compared to AD model group; #, p<0.05 compared to pGLVU6/GFP-NC group.

regulate apoptosis of cardiomyocytes, endothelial cells, and VSMCs, in addition to the structural remodeling of cardiovascular structures. Abnormality of MST1 expression and activity facilitates occurrence and progression of various cardiovascular diseases. However, its role in AD pathogenesis has not been illustrated. This study established a rat AD model, on which the role of MST1 in aorta VSMCs apoptosis and AD pathogenesis was investigated.

Our results showed no AD formation in vascular tissues of control rats, whilst AD group had 68.7% AD formation rate, plus significantly higher Caspase-3 activity and cell apoptotic rate in AD middle membrane layer tissues. These results showed critical roles of elevated cell apoptosis in AD pathogenesis. Yuan et al[2] showed significantly elevated VSMC cell apoptotic rate in AD vascular tissues. Durdu et al[3] revealed significantly elevated Bax/Bcl2 ratio and VSMC cell apoptotic rate in AD vascular tissues. In this study, model rats had significantly higher cell apoptotic rate in AD middle membrane tissues compared to control group, indicating the involvement of cell apoptosis abnormality in AD pathogenesis, as consistent with Yuan et al[2] and Durdu et al[3]. Compared to control group, model rats had a significantly elevated expression of MST1, p-LATS1, and p-YAP1 in AD middle membrane, whilst YAP1 protein expression was decreased. These results indicated the correlation between MST1 up-regulation induced Hippo-YAP signal pathway activity and higher cell apoptosis or AD pathogenesis. Ono et al[15] showed significant aorta VSMC cell apoptosis and MST1 up-regulation in bulb blockade of carotid artery rat model. They also showed a higher activity of MST1 in the process of staurosporine-induced rat carotid artery VSMC apoptosis[15]. Li et al[22] showed the involvement of MST1 down-regulation in decreased VSMC apoptosis and hypoxia-induced pulmonary hypertension. We showed a possible role of MST1 up-regulation in facilitating VSMC apoptosis and AD

occurrence, as similar with Ono et al[15] and Li et al[22] who reported the role of MST1 in mediating VSMC cell apoptosis. *In vitro* study showed that MST1 siRNA transfection significantly inhibited VSMC cell apoptosis in AD rats. Compared to AD model group of pGLVU6/GFP-NC group, shRNA interference virus pGLVU6/GFP-MST1 injection decreased Caspase-3 activity or cell apoptotic rate in AD middle membrane tissues, with decreased AD formation rate, diameter, and length. Jia et al[23] revealed that CHOP gene knockout significantly decreased mechanic induced cell apoptosis in mouse VSMC, and decreased AD formation. Liu et al[24] showed that treatment using ursodeoxycholic acid (UDCA) significantly decreased angiotensin II (Ang II) induced VSMC apoptosis, and decreased AD incidence. In this study, the inhibition of aorta VSMC apoptosis could also suppress AD pathogenesis, similar to Jia et al[23] and Liu et al[24]. Odashima et al[14] found significantly elevated cardiomyocyte apoptosis in acute myocardial infarction rats, accompanied by decreased cardiac function and potentiated MST1 activation. The inhibition of MST1 expression also decreased cardiomyocyte apoptosis and improved cardiac function post-myocardial infarction. In this work, interference of MST1 expression also plays a role in decreasing cell apoptosis and AD, as similar with Odashima et al[14] showing the role of MST1 down-regulation in decreasing cell apoptosis and improving cardiovascular functions. This study also revealed the role of MST1 up-regulation in AD pathogenesis, whilst MST1 down-regulation decreased VSMC cell apoptosis and AD formation, both of which have not been reported before.

Conclusions

MST1 up-regulation plays a role in facilitating VSMC cell apoptosis and AD pathogenesis. Down-regulation of MST1 expression decreases VSMC cell apoptosis and AD formation.

Acknowledgments

This work was supported by Natural Science Foundation of Shandong Province (General Program, No. 2013GGB14098).

Conflict of Interest

The Authors declare that they have no conflict of interest.

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