Osteopontin in the Pathogenesis of Aortic Dissection by the Enhancement of MMP Expressions

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Summary

The pathogenesis of aortic dissection (AD) is unclear. The aim of this study was to explore the relationship between osteopontin (OPN) and AD. Fifty AD patients were enrolled; 29 had hypertension with AD (H-AD) and 21 no hypertension with HD (NH-AD). Twenty-five healthy controls (NH-C) and 14 patients with hypertension (H-C) were also enrolled. Serum and aortic wall OPN levels were determined. Human vascular muscle cells (HVSMC) were stimulated by both low (1 μg/mL) and high (5 μg/mL) concentrations of OPN and cell proliferation as well as apoptosis was measured. Transforming growth factor-β (TGF-β), matrix metalloproteinase 1 (MMP-1), MMP-2, MMP-9, TIMP-1, and TIMP-2 gene expressions by HVSMC were measured and Akt, IκB, Smad1/5/8 and Erk1/2 signaling pathways were detected. Our results showed that AD patients demonstrated significantly higher levels of serum and local OPN expressions compared to healthy controls. In those with hypertension, the serum concentrations of OPN were increased compared to those without hypertension. In in vitro culture, a high dose of OPN stimulation promoted the proliferation of HVSMC but did not affect cell apoptosis. Both concentrations of OPN enhanced MMP-2 gene expression and its activity in HVSMC. Moreover, Akt and IκB signaling pathways were significantly activated after OPN stimulation while the Smad1/5/8 and Erk1/2 signaling pathways were not changed. The addition of an IκB inhibitor significantly abrogated MMP-2 gene expression. Our data show that OPN may participate in the pathogenesis of AD by the enhancement of MMP-2 expression.

Key words: Matrix metalloproteinase, Hypertension

Aortic dissection (AD) is a life-threatening medical emergency associated with high rates of morbidity and mortality. More than 50% of patients who cannot receive urgent surgical intervention will die within 48 hours after onset.1 In fact, the population of AD patients was much greater than previously believed. An analysis by the International Registry of Acute Aortic Dissections (IRAD) reported an incidence of 16 per 100,000 in men and 7.9 per 100,000 in women,2 figures which are much higher than that of hospital studies because the data might not include deaths before hospital admission. Further etiological studies on AD can provide information about the mechanisms and may improve the clinical outcomes.

Several common risk factors for AD, such as systemic hypertension, atherosclerosis, genetic diseases (for example, Marfan syndrome), and autoimmune disorders are known.3 Inflammatory responses and signaling pathways exist in the pathophysiology of these diseases. In the past decade, it has been found that several important proteins are involved in the pathogenesis of AD as inflammatory mediators.4,5 Osteopontin (OPN) is a bone-specific sialoprotein, expressed in several cells including endothelial cells and smooth muscle cells, which has been studied as a multifunctional protein that is upregulated in fibrosis, autoimmune disease, and atherosclerosis.6,7 OPN is generally regarded as a proinflammatory and proatherogenic molecule,8 but its role in the pathogenesis of AD is unclear. Recently, Yuan, et al. reported comparable serum levels of OPN in AD patients as well as in patients with aortic aneurysm and coronary artery disease, but markedly higher OPN expressions in the aortic tissues of AD patients compared to those in patients with coronary artery disease.9,10 However, little is known about the exact mechanism by which OPN participates in the formation of AD. In the present study, we examined the changes in OPN in AD patients and the role of OPN in human vascular muscle cell (HVSMC) activity, which may suggest its potential role in the pathogenesis of AD.

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Methods

Patient selection: This study was approved by the Ethics Committee of Drum Tower Clinical Medical College of Nanjing Medical University, and all patients gave research authorization. The clinical data was collected for patients with Stanford type A aortic dissection from October 2013 to December 2014. To minimize confounding bias, we excluded patients with coronary artery diseases, degenerative valvular heart diseases, and/or malperfusion syndrome (end-organ ischemia in the setting of an aortic dissection). We also excluded patients with connective tissue diseases and autoimmune diseases. Finally, 50 AD patients were eligible for the study, 29 of whom had hypertension combined with AD (H-AD) and 21 no hypertension with AD (NH-AD). Twenty-five healthy controls (NH-C) and 14 patients with hypertension (H-C) were also enrolled. Procoagulant peripheral blood was collected on an empty stomach in the morning before the operation for each patient. The blood was left for one hour and then spin down at 2000 rpm for 20 minutes. Serum samples were collected and stored at -80 °C before use.

Enzyme-linked immunosorbent assay (ELISA): Serum levels of OPN were tested using a Human Osteoprotegerin Instant ELISA kit from eBioscience. Serum transforming growth factor β (TGF-β) levels were analyzed by ELISA (BioLegend, USA). MMP-2 and TIMP-2 levels were determined by ELISA (R&D, USA) according to the instructions. For MMP activity assay, an Amplite Universal Determined by ELISA (R&D, USA) according to the instructions. For MMP activity assay, an Amplite Universal Fluorimetric MMP Activity Assay Kit from AAT Bioquest was used and the assay was performed according to manufacturer’s protocol.

Immunohistochemistry: Aortic wall tissues from 5 patients with AD and 3 heart transplant donors were collected to investigate the distribution of cells expressing OPN. Tissue fractions were deparaffinized and fixed with different concentrations of alcohol, and then antigen retrieval was achieved by pressure-cooking for 5 minutes in 10 mM sodium citrate buffer (pH 6.0). All sections were retrieved to investigate the distribution of cells expressing OPN. Tissue fractions were deparaffinized and fixed with different concentrations of alcohol, and then antigen retrieval was achieved by pressure-cooking for 5 minutes in 10 mM sodium citrate buffer (pH 6.0). All sections were soaked in 3% H2O2 for 5 minutes to inhibit endogenous peroxidase, rinsed, and then incubated with normal rabbit peroxidase, rinsed, and then incubated with normal rabbit serum for 10 minutes at ambient temperature to block nonspecific binding. Rabbit derived polyclonal OPN antibody (Proteintech, 22952-1-AP) was diluted to 1:50. The primary antibody was applied to the specimens in a wet chamber at 4°C overnight and an Ultrasonic sensitive TM S-P kit (anti-rabbit, Maxim, China) was used as secondary antibodies, following which 3,3′-diaminobenzidine (DAB) (Sigma, USA) was applied as a substrate for coloration and hematoxylin as the counter stain. The stained specimens were covered with a cover slip for observation under a light microscope.

HVSMC cultivation and stimulation: HVSMC from passage 1 were purchased from GuangZhou Jennio Biotech Co., Ltd. (Guangzhou, China). The cells were then recovered and resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS). The cells were incubated at 37°C in a humidified atmosphere with 5% CO2. When well-developed colonies of fibroblast-like cells appeared after 10 days, the cultures were trypsinized and passaged into a new flask for further expansion.

Cells from passage 3 were resuspended in RPMI 1640 medium, and then distributed to 24-well plates (Corning, Tewksbury, MA, USA) at 5 × 104 per well. Cells incubated at 37°C in a humidified atmosphere with 5% CO2 were treated as follows: (1) in RPMI-1640 medium containing 10% FCS alone (no stimulation group), (2) low dose of human OPN (1 μg/mL) added, or (3) high dose of human OPN (5 μg/mL, PeproTech, Rocky Hill, NJ) added. Experiments were carried out in duplicate and cell numbers were equal among the 3 groups. After 48 hours, the cell supernatant was collected for MMP activity assay and HVSMC were collected for real-time PCR analysis. In some experiments, an NF-κB inhibitor (Parthenolide, PTL, Sigma, final concentration 10 μM) was used to inhibit the activation of IκB signaling.

Cell proliferation: The HVSMC cells were cultured in 96-well plates at a density of 1 × 104 cells. Each group was designed to have 4 duplicated wells. After 24 hours culture, the cell proliferation was examined with a Cell Counting Kit-8 (CCK-8, Thermo, USA) at 24, 72 and 120 hours, respectively. The procedures were as follows: After aspiration of the original medium, CCK-8 solution was added to the cells. The medium was detected at 450 nm absorbance after 2 hours culture. The experiments were repeated 3 times, and the average values were obtained.

Flow cytometer for cell apoptosis: Annexin V-FITC and PI (Abcam Co., Cambridge, MA, USA) were used for analysis of the cell apoptosis rate. The HVSMC cells were washed twice with PBS and 0.25% pancreatin was added for digestion. After adjusting the density to 107/mL, the cells were incubated with fluorescent antibody at room temperature for 30 minutes. The cells were precipitated and suspended after 200 ×g centrifugation. The samples were analyzed using a flow cytometer (BD Bioscience, San Jose, CA, USA). The experiments were repeated 3 times, and the average values were obtained.

Real-time quantitative PCR: Complementary DNA (cDNA) was synthesized from TRIzol-isolated total RNA using a SuperScript III First Strand Synthesis SuperMix for quantitative reverse transcription-PCR (Takara). For real-time PCR experiments, reactions containing SYBR Premix EX Taq (Takara), ROX Reference Dye (50 ×; Takara), cDNA, and gene primers were run on a StepOne Plus real-time PCR system and analyzed using StepOne Software, version 2.1 (Applied Biosystems). Gene primers were listed in the Table. The relative gene quantification was conducted using the 2-ΔΔCt method following normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the un-stimulated group. Primers for real-time PCR are presented in the Table.

Western blot analysis: We used antibodies recognizing human Akt, IκB, Smad1/5/8, Erk1/2, and their phosphorylation forms, and GAPDH (1:1,000; Cell Signaling Technology) to examine the concentrations of proteins in HVSMC lysates.

Statistical analysis: Categorical data are presented as frequencies and percentages and continuous data as the mean ± standard deviation. We used a t-test to conduct a statistical analysis of continuous data for normal distributions and the Mann-Whitney U test for non-normal distributions. The Mann-Whitney test was used for analysis of
Table. Primers for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>TGF-β1</td>
<td>5'-CCCAGCATCTGCAAAAGCTC-3'</td>
<td>5'-GTCAATGTACAGCTGCAGGGA-3'</td>
</tr>
<tr>
<td>MMP-1</td>
<td>5'-CTGGCCCAACTGCAAAAATG-3'</td>
<td>5'-CTGTCCCCAGAACAGCCCCATCTAA-3'</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5'-TCTCCTGACATGACCTTTGGC-3'</td>
<td>5'-CAAGTGCTGCTGAGTAGATC-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-TTGCACAGCGACAGGAGTGG-3'</td>
<td>5'-GCCATTCAGCAGGCTTTTAT-3'</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>5'-TCTGCAATTCGCCACCTTCATCA-3'</td>
<td>5'-AAGGTTGCTGTTGACTGTTGAT-3'</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>5'-CTGGCCACGACTTGGGCTGTC-3'</td>
<td>5'-CGAGAAACTCCGGCTGTTGGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGTTGAAGAGGCCAGTGGGA-3'</td>
<td>5'-TGTTGAAGAGGCCAGTGGGA-3'</td>
</tr>
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Figure 1. Serum OPN increased in AD patients. Serum levels of OPN significantly increased in AD patients compared to healthy controls (A). AD patients with hypertension showed significantly higher levels of serum OPN compared to those without hypertension (B). However, there was no significant change of serum OPN before and after the operations (C). Immunohistochemistry staining of OPN expression on aortic wall from AD patients (n = 5) and heart transplant donors (n = 3, D). AD indicates aortic dissection; CON, control; H-AD, aortic dissection with hypertension; NH-AD, aortic dissection without hypertension; H-CON, control with hypertension; NH-CON, control without hypertension; and OPN, osteopontin; *P < 0.05; **P < 0.001.

Elevated serum OPN in AD patients: First we found that serum levels of OPN were significantly increased in AD patients compared to healthy controls (237.2 ± 20.8 pg/mL versus 25.2 ± 4.6 pg/mL, P < 0.001, Figure 1A). Because of the strong association of hypertension with AD formation,11) the expression of serum OPN in AD patients with or without hypertension was analyzed. We found that although there were comparable levels of serum OPN between hypertension patients and healthy controls (24.6 ± 5.9 pg/mL versus 24.7 ± 4.5 pg/mL, P > 0.05), AD patients with hypertension showed significantly higher levels of serum OPN compared to those without hypertension (272.4 ± 30.6 versus 176.3 ± 21.2 pg/mL, P < 0.05, Figure 1B). Moreover, we observed serum OPN levels in 14 AD patients before and 7 days after surgery and found there was no significant change in serum OPN before and after surgery (232.6 ± 32.2 pg/mL versus 225.2 ± 28.4 pg/mL, n = 14, P > 0.05, Figure 1C). In addition to serum, we also determined aortic wall OPN expressions and found that local OPN was expressed much more in AD patients than in controls (Figure 1D).

Serum OPN was positively correlated with MMP-2 in AD patients: We found that serum TGF-β (39.5 ± 3.3 pg/
In the heart

Figure 2. Serum OPN was positively correlated with MMP-2 in AD patients. Serum TGF-β (A) and MMP-2 (B) were significantly increased in AD patients compared to healthy controls, while serum TIMP-2 was significantly decreased (C). Serum OPN was positively correlated with serum MMP-2 expression (D), but there was no correlation between serum OPN and TGF-β (E), TIMP-2 (F) and CRP expressions (G). CRP indicates C reactive protein; OPN, osteopontin; TGF-β, transforming growth factor β; MMP, matrix metalloproteinase; and TIMP, tissue inhibitor of metalloproteinases. *** P < 0.001.

mL versus 21.0 ± 2.6 pg/mL) and MMP-2 (69.2 ± 9.2 ng/mL versus 20.0 ± 4.2 ng/mL) levels were significantly increased in AD patients compared to healthy controls (both P < 0.001, Figure 2A and B), while serum TIMP-2 was significantly decreased (91.9 ± 4.5 pg/mL versus 141.2 ± 8.4 pg/mL, P < 0.001, Figure 2C). We also observed correlations between serum OPN concentration and MMP-2, TIMP, TGF-β, and C reactive protein (CRP) levels in AD patients, which showed that serum OPN was positively correlated with serum MMP-2 concentration (P = 0.001, Figure 2D). However, no relationship was observed between serum OPN and TGF-β, or TIMP-2 and CRP levels (both P > 0.05, Figure 2E-G).

High dose OPN stimulated HVSMC proliferation and MMP-1 and MMP-2 expressions: In order to determine the relationship between OPN and HVSMC cell proliferation in vitro, we used low dose (LD, 1 μg/mL) and high dose (HD, 5 μg/mL) OPN to stimulate HVSMC for 1, 3, and 5 days, respectively. We observed that high dose OPN significantly enhanced HVSMC proliferation in 5 days, while low dose OPN did not (Figure 3A). After 24 hours, both low and high dose OPN did not affect cell apoptosis by Annexin V and PI staining (P > 0.05, Figure 3B). Moreover, recombinant OPN dose-dependently stimulated MMP-2 expression in HVSMC but only high dose OPN could enhance MMP-1 expression (Figure 3C). Neither low dose nor high dose OPN stimulations had any effect on TGF-β1, MMP-9, TIMP-1, or TIMP-2 expression by real-time PCR analysis. However, for the detection of MMP activity in cell culture supernatant, we found OPN dose-dependently enhanced MMP-2 (Figure 3D) activity.

Signaling pathways involved in MMP expression by OPN: We found that in the presence of both 1 ng/mL and 5 ng/mL OPN, significantly higher levels of Akt and IκB phosphorylation existed (Figure 4A), while Erk1/2 and Smad1/5/8 signaling pathways were not activated (Figure 4B), which suggested that OPN may increase MMP expression in HVSMC by Akt and IκB signaling pathways. To further confirm the involvement of IκB signaling, we added an NF-κB inhibitor to the culture system and found that its addition significantly abrogated the induction of MMP-2 by OPN stimulation (Figure 4C), and also partially reduced MMP-2 activity (Figure 4D).

Discussion

Aortic dissection (AD) is one of the most dangerous cardiovascular disorders with high mortality, although many patients undergo an operation. Further exploration of the pathogenesis of AD may provide information crucial to improve the clinical outcomes. It has been established that the abnormal expressions of TGF-β, MMPs, and TIMPs are associated with the development of AD. In the present study, we found that there were significantly higher levels of serum OPN in the patients with AD compared to healthy controls, and OPN might participate in the formation of AD by regulating the expression of MMPs and TIMPs.

Osteopontin (OPN), a bone-specific sialoprotein, was first cloned from rat sarcoma cells. Encoded on chromosome 4 in the human genome, OPN is a member of...
the small integrin-binding N-linked glycoprotein family of proteins, which is known as an active player in the progression of vascular remodeling diseases. Previous studies have demonstrated that the concentration of OPN was increased in human atherosclerotic plaques as well as in neointima after experimental angioplasty. Moreover, OPN is known as a biomarker of acute atherothrombotic ischemic stroke and a potential therapeutic target for ischemic stroke. Among patients who underwent elective coronary artery bypass grafting (CABG) surgery, serum OPN levels were much higher in those with a prior acute myocardial infarction than those without. However, the expression of OPN was significantly increased in the aortic tissues of AD patients as opposed to that of patients with coronary artery disease. Thus, we postulated that OPN might be involved in the generation of AD, although the underlying mechanisms remain to be explored.

In this study, we found that serum OPN was significantly increased in AD patients, especially in those with hypertension, while there was no difference between healthy controls and hypertension patients, indicating that other factors may also participate in the promotion of AD pathogenesis. However, we did not find any difference in serum OPN in patients before and after surgery, suggesting that the elevated serum OPN in AD patients was stable and would not change during surgical intervention.

OPN played an important role in vascular disease and in the response to vascular injury by stimulating VSMC migration, proliferation, and ECM synthesis, demonstrating a major role in hypertension-induced vascular remodeling. Mechanical stretch-induced MMP-2 production was attenuated in cells treated with OPN siRNA or anti-OPN antibody as well as in OPN-deficient VSMC cultured from aorta of OPN-deficient mice, further indicating a crucial role of OPN in vascular remodeling. Vascular remodeling involves enhanced collagen decomposition and extracellular matrix reorganization, and these processes are regulated by matrix metalloproteinases (MMP) within vascular walls. The MMP family is a group of enzymes that degrade extracellular matrix and participate in the migration and proliferation of VSMC, which is one of the most important mechanisms of AD formation. It is difficult to say whether serum or aortic wall OPN is more important for disease pathogenesis. We believe local OPN expression is more important. However, for disease diagnosis, it is more convenient to detect serum OPN than local expression, and we seek to find a serum biomarker for AD patients. To further explore the direct evidence, we plan to generate OPN knock out mice and see whether OPN knock out mice are less prone to be AD, have less destruction of the aortic wall, and a higher survival rate.

Our study shows that after stimulation by OPN, there were significantly higher levels of Akt and IκB phosphorylation in HVSMC, although Erk1/2 and Smad1/5/8 signaling pathways were not activated. OPN may be associated with the increase of MMP expression in HVSMC by Akt and IκB signaling pathway activation, and this needs to be explored further in the future.

Conclusions

The expression of serum OPN was significantly...
higher in AD patients than in healthy controls. In in vitro culture, OPN stimulation could promote the proliferation of HVSMC and enhance MMP-2 gene expression as well as its activity. OPN may participate in the formation of AD by activating the Akt and IκB signaling pathways. However, the exact mechanisms need to be explored further and the relations between OPN and AD should be clarified.

Disclosures

Conflicts of interest: The authors have declared that no competing interests exist.

References