Original Article

Activation of JNK and induction of VSMC apoptosis via macrophage secreted TNFα in rupture of intracranial aneurysm

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Abstract: Elevated apoptosis of vascular smooth muscle cells (VSMCs) is correlated with formation and rupture of intracranial aneurysm (IA). A previous study showed the role of TNFα in activating JNK and cell apoptosis. Elevated JNK activity has been found in the vascular wall of IA. We thus investigated whether macrophage-secreted TNFα plays a role in activating JNK, VSMC apoptosis, and aneurysm rupture. IA patients with or without rupture were collected for vascular samples, in parallel with normal intracranial artery. Flow cytometry was used to test infiltration and apoptosis mediated by macrophages. TNFα expression, JNK signal pathway, and Bax expression were tested. An in vitro model of VSMC-macrophage co-culture system was prepared in control, TNFα blocking antibody, JNK inhibitor SP600125, and TNFα + SP600125 groups. Western blot was used to test the JNK signal pathway activity and Bim/Bax protein expression. Activity of caspase-9 and caspase-3 was observed, followed by apoptosis assay in flow cytometry. Compared to control or IA without rupture group, the IA rupture group had increased infiltration of macrophages, TNFα expression, cell apoptosis, JNK activity, and Bax expression. The blockade of TNFα and/or JNK effectively inhibited JNK activity, decreased Bim/Bax protein expression, inhibited caspase-9 and caspase-3 activity, and lowered VSMC cell apoptosis. Macrophage infiltration and TNFα expression were significantly elevated in IA artery wall. TNFα secreted by macrophages can elevate expression of apoptotic proteins Bim and Bax, and initiate endogenous apoptosis of VSMCs, via activating the JNK signal pathway, thus possibly playing a role in IA formation and rupture.

Keywords: Intracranial aneurysm, macrophage, TNFα, JNK, vascular smooth muscle cells, apoptosis

Introduction

Intracranial aneurysm (IA) is one tumor-like protrusion of artery wall caused by focal dilation of the intracranial artery under pathological conditions. It often occurs in a major artery branch of Wills ring. As the third common cerebrovascular incidence, only next to cerebral thrombosis and hypertensive cerebral hemorrhage, rupture of IA severely affects patient life [1]. IA is the primary reason causing subarachnoid hemorrhage, occupying about 80% of all cases [2]. IA has relatively high mortality and morbidity, with the death rate of first rupture at 30%~40%, and over 70% for recurrent rupture. For those case where the patient survives, certain neurological dysfunction can exist, thus severely affecting patient’s life and health [3]. Recent studies reported that the occurrence and progression of IA are related with infiltration of inflammatory cells in focal artery wall [4] and release of inflammatory mediators [5, 6]. Aoki et al. and Kanematsu et al. observed abundant infiltration of macrophage in the IA vascular wall tissues [7, 8], suggesting an important role of macrophage migration and aggregation in IA pathogenesis. Tumor necrosis factor α (TNFα), as one inflammatory factor predominantly produced from macrophages, has also been demonstrated to play a critical role in IA occurrence [9, 10]. Studies show the important step of vascular remodeling dysfunction, as featured with decreased proliferation of vascular smooth muscle cells (VSMCs) and over-apoptosis for imbalance of proliferation-apoptosis ratio, in aneurysm occurrence and rupture [11]. c-Jun
N-terminal kinase (JNK) signal protein plays an important role in inducing cell apoptosis [12]. A recent study has shown significantly elevated JNK signal pathway activity inside IA vascular wall tissues [13]. Other studies have shown an important role of TNFα in activating the JNK signal pathway [14] and inducing apoptosis [15]. This study thus investigated whether macrophage-secreted TNFα plays a role in activating JNK, inducing VSMC apoptosis, and inducing aneurysm rupture.

Materials and methods

Major reagents and materials

DMEM, HEPES, α-MEM, FBS, and streptomycin-penicillin were purchased from Gibco (US). Human recombinant M-CSF was purchased from Peprotech (US). Total RNA extraction kit was purchased from Toyobo (Japan). Reverse transcription kit PrimerScript RT was purchased from Takara (US). SYBR Green fluorescent dye was purchased from Toyobo (Japan). PCR primer was designed by Gimma (China). Mouse anti-human CD68 was purchased from BD Pharmingen. Flow cytometry antibody for TNFα and ELISA kit were purchased from eBioscience (US). Mouse anti-human CD68 antibody was purchased from BD Pharmingen (US). Mouse anti-human p-MKK4, rabbit anti-human p-JNK antibody was purchased from Abcam (US). Anti-TNFα blocking antibody was purchased from B&D system. Rabbit anti-human Bim, Bax, and p-c-Jun antibody were purchased from Santa Cruz (US). Horseradish peroxidase (HRP)-conjugated goat anti-rat and goat anti-rabbit secondary antibody was purchased from Jackson (US). Annexin V/PI apoptosis, caspase-3, and caspase-9 activity kits were purchased from Beyotime (China). JNK inhibitor SP600125 was purchased MedChemExpress (US).

Collection of clinical samples

A total of 39 IA patients who received surgery in Laiwu City People’s Hospital from June 2015 to March 2016 were recruited. All patients were diagnosed by DSA. There were 9, 10, 8, and 12 cases of anterior communicating artery aneurysm, posterior communicating artery aneurysm, middle cerebral artery aneurysm, and internal carotid artery aneurysm, respectively. Twenty-eight patients had aneurysm rupture while 11 others did not. There were 20 males and 19 females in patient cohort, with an average age of 39.4 ± 10.8 years old. Another 15 patients were recruited as the control group having clearance of intracranial hematoma or decompression surgery for collecting normal cerebral cortical artery vessels. There were 8 males and 7 females in the control group, with an average age of 41.6 ± 12.5 years old. Sample collection was done following informed consent from patients, and was approved by the Ethical Committee of Laiwu City People’s Hospital.

Assay for macrophage

Freshly prepared vascular wall tissues were placed in digestive enzymes including 0.2% type II collagenase and 0.1% hyaluronidase for 1.5 h incubation to completely separate cells. After rinsing twice in PBS, 2% FBS was used to re-suspend cells. PE-labelled CD68 antibody was used to incubate cells for 60 min at 4°C. PBS containing 2% FBS was used to rinse cells, followed by flow cytometry assay.

VSMC separation and culture

Normal intracranial cortical vessels were placed into DPBS buffer to detach the outer membrane. The artery was opened to remove the inner membrane with a blade. Tissues were incised into 2–3 mm³ cubes and were placed into culture dish. After incubation under 37°C with 5% CO₂ for 2 h, DMEM medium containing 20% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin were applied. After 8–10 days, cells emerged from tissue cube edge. Culture medium was changed every 3 days. Further experiments were performed when cell growth density reached 70%–80%.

Separation and induction culture of human bone marrow macrophage

Ten mL bone marrow samples separate from iliac bone explant patients without vascular disease were diluted in equal volume of PBS. Mononuclear cells were separate by density gradient centrifugation, and were cultured in MEM medium containing 10% FBS, 30 ng/mL M-CSF, 100 U/mL penicillin and 100 μg/mL streptomycin with medium changing every 72 hours. After 1 week, differentiation of macrophage was observed.
Establishment of HA-VSMC and macrophage co-culture system and grouping

Macrophage cells were inoculated into the upper chamber of Transwell with 8 μm pore size, while VSMC was placed into the lower chamber. Cells were divided into four groups: control group, TNFα blocking group with 0.1 μg/ml anti-TNFα blocking antibody, JNK inhibitor group with 5 μM specific inhibitor SP600125, and combined blocking group with both TNFα antibody and SP600125 treatment.

ELISA TNF-α and flow cytometry

ELISA was performed following manual instruction. In brief, microplate was pre-coated with 100 μL antibody dilution, and was washed in buffer. Two hundred μL blocking buffer was added for 60 min room temperature incubation, followed by washing buffer. 100 μL test samples or standard dilutions were added for 2 hour incubation at room temperature. After washing, 100 μL test antibody was added and incubated for 60 minute at room temperature. One hundred μL avidin-HRP was added for 30 minute room temperature incubation. After washing, 100 μL TMB was added for 15 minute room temperature incubation, and the reaction was stopped in 50 μL quenching buffer. The absorbance value at 450 nm was measured.

Flow cytometry was performed as follows: 1% Monesin solution was added 12 hours before testing. VSMC and macrophage cells were separated collected via trypsin digestion. Cells were fixed in 4% paraformaldehyde for 30 minutes, and were treated in 0.1% Triton X-100 to rupture the membrane. Five μL of APC-labelled TNF-α antibody was added for 30 minute incubation in the dark, followed by 1000 rpm centrifugation for 5 minutes. With one more washing by centrifugation in PBS containing 0.5% BSA, cells were re-suspended in PBS for online test.

qRT-PCR for gene expression assay

Total RNA kit (OMEGA) was used to extract total RNA. In brief, 350 μL TRK buffer was used to lyse cells for homogenization. Three hundred and fifty μL 70% ethanol was added, and the mixture was loaded onto the filtration column, which was centrifuged at 10000 g for 1 minute. Five hundred μL wash buffer I was added twice followed by centrifugation in wash buffer II. Ethanol was air dried, and the membrane was incubated with 30 μL DEPC water for 2 min. Soluble RNA was centrifuged for 1 min and was collected into RNAase-free tubes. cDNA was synthesized by applying PrimeScript RT reagent kit under reverse transcription. Using cDNA as the template, PCR amplification was performed using primer sequences: TNF-αP_F: 5’-CCTCT CTCTA ATCAG CCCTC TG-3’; TNF-αP_R: 5’-GAGGA CCTGG GAGTA GATGA G-3’; BimP_F: 5’-TAAAG TCTGA GTGTG ACCGA GA-3’; BimP_R: 5’-GCCCA GAGGT CTTTT TCCGA G-3’; BaxP_F: 5’-CCAGC CCATG ATGCT TCTGA T; BaxP_R: 5’-TAAGC GCCCA C-3’; β-actinP_F: 5’-TGTCA CGCAC GATTT CC-3’. In a 10 μL PCR system, 4.5 μL 2X SYBR Green mixture was added, along with 0.5 μL of forward/reverse primer (2.0 μM), 1 μL cDNA, and 3.5 μL ddH2O. PCR was performed in 40 cycles under the following conditions: 95°C for 15 seconds, and 60°C for 60 seconds, on ABI-7500 fluorescent quantitative PCR cycler.

Western blotting

Cells were lysed in SDS and boiled for 5 minutes. The protein concentration was determined by BCA kit. Sixty μg sample was loaded, separated by SDS-PAGE, and was transferred onto PVDF membrane, which was blocked by 5% defatted milk powder for 1 hour. After 4°C overnight incubation in primary antibody (MKK4 at 1:400, p-JNK at 1:200, p-c-Jun at 1:100, Bim at 1:300, Bax at 1:300, and β-actin at 1:1000), PBST was added for three times of rinsing (5 min each), followed by HR conjugated secondary antibody incubation for 60 minutes (1:10000 dilution for both anti-mouse and anti-rabbit). After PBST rinsing (5 minutes, 3 times), ECL chromogenic substrate was added for dark room exposure, development and visualization. The film was scanned and saved. Gray value of bands was analyzed in Quantity One software.

Activity assay of caspase-3 and caspase-9

Standard dilution buffer was used to prepare 0, 10, 20, 50, 100, and 200 μM pNA standards. Absorbance values at 405 nm wavelength were measured by a microplate reader to prepare a standard curve with pNA concentration against A405 value. Attached cells were digested in trypsin, and were collected into culture medium for 4°C centrifugation for 5 minutes at 600 g. Supernatant was carefully removed and wash-
ed out by PBS. 100 μL lysis buffer was added for every $2 \times 10^6$ cells. Cells were lysed at 4°C for 15 minutes, and were centrifuged at 18000 g with 4°C for 10 minutes. Supernatants were saved for further use. Caspase-3 activity was tested in the following step: 2 mM Ac-DEVD-pNA was placed on ice, mixed with buffer and test samples, with 10 μL Ac-DEVD-pNA. The mixture was incubated at 37°C for 2 hours. A $A_{405}$ was measured when the color changed significantly. Caspase-9 activity was measured as follows: 2 mM Ac-DEVD-pNA was placed on ice. Test buffer was firstly added followed by test samples. Ten μL Ac-DEVD-pNA was then mixed for 37°C incubation for 2 hours. $A_{405}$ was measured when the color changed significantly.

Flow cytometry for cell apoptosis

Cells were digested by trypsin and collected. After that cells were washed in PBS, and centrifuged in 2000 rpm for 5 minutes. The supernatant was discarded and re-suspended in 195 μL binding buffer. Five μL Annexin V-FITC dye was added for gentle mixture. Ten μL PI was then added for 20 minute incubation in the dark at room temperature. The mixture was immediately placed on ice for measurement.

Statistical analysis

SPSS18.0 software was used for data analysis. Measurement data are presented as mean ± standard deviation (SD). One-way ANOVA with Newman-Keuls multiple comparison post-hoc analysis has been performed for multiple groups' comparison. A statistical significance was defined when $P < 0.05$.

Results

Significant elevation of macrophage and TNFα in vascular walls of IA patients

A total of 28 samples of ruptured IA vascular tissue, 11 samples of non-ruptured IA tissues, and 15 samples of normal cerebral cortical vessels. Flow cytometry showed significantly
Cell apoptosis in aneurysm

Figure 1. Elevated macrophage infiltration in vascular wall tissues of IA patients. A. Protein expression by Western blotting. The expression of Bax was quantified as a ratio to β-actin and the phosphorylation level of JNK and MKK4 was quantified as a ratio to the total level. B. Flow cytometry for cell apoptosis. *P < 0.05.

Enhanced JNK activity and cell apoptosis in IA vascular wall

Western blotting results showed the highest phosphorylation level of MKK4, JNK and Bax proteins in IA vascular wall tissues with aneurysm rupture, next by IA patients without rupture, and the control population (see Figure 2A for representative results). Flow cytometry revealed significantly more cell apoptosis in aneurysm with rupture compared to those IA patients without rupture, which had higher apoptosis level than the control group (see Figure 2B for representative results).

Macrophage secreted TNFα and facilitated VSMC cell apoptosis

Flow cytometry results showed about 98% expression of CD68, which is macrophage specific marker, after 7 days of M-CSF induction, indicating successful induction of macrophage differentiation for further experiments (Figure 3A). ELISA results showed no TNFα expression in medium of VSMC culture (Figure 3B). The basal apoptotic rate of VSMC was relatively low, at only 3.5% (Figure 3C). After co-culture with macrophage, the apoptosis of VSMC significantly increased (Figure 3C), along with remarkably increased TNFα content (Figure 3B). To investigate the source of TNFα in co-culture system, flow cytometry was performed to test TNFα expression in macrophage and VSMC. TNFα expression in macrophage was significantly higher than that in VSMC (Figure 3D and 3E), indicating that TNFα in the culture medium came from macrophage.

TNFα activated JNK signal pathway and facilitated VSMC cell apoptosis

Tests of clinical samples showed significantly elevated macrophage infiltration and TNFα content in the vascular wall of IA patients, accompanied with enhanced JNK activity and cell apoptosis. In the co-culture system, macrophage had effects of secreting TNFα and facilitating VSMC apoptosis. This study utilized an in vitro culture system, in which whether macrophage activated JNK signal pathway and facilitated VSMC apoptosis via secreting TNFα were investigated. After blocking TNFα using an inhibitor, phosphorylation of MKK4, JNK, and c-Jun was significantly weakened (Figure 4A), accompanied with decreased mRNA and protein expression of pro-apoptotic factor Bim and Bax (Figure 4A, 4B), and decreased caspase-9 and caspase-3 activity (Figure 4C), and lower VSMC cell apoptotic rate (Figure 4D), suggest-
ing that TNFα could activate JNK signal pathway inside VSMC cells, up-regulate apoptotic protein Bim and Bax expression, and facilitate cell apoptosis. Application of JNK specific inhibitor SP600125 blocked phosphorylation of JNK kinase on downstream c-Jun protein, and decreased apoptotic protein Bim and Bax expression inside VSMC, weakened caspase-9 and caspase-3 activity, and decreased cell apoptosis remarkably. The combined treatment using TNFα blocking antibody and SP600125 further down-regulated phosphorylation of c-Jun protein, expression of Bim and Bax, caspase-3 and caspase-9 activity, and cell apoptosis. These results further demonstrate that macrophage could initiate endogenous apoptosis of VSMC cells via secreting TNFα, activating JNK signal pathway, and up-regulating Bim and Bax expression.

Discussion

IA is one common cerebrovascular disease in clinics, with an incidence of about 3.2% in the population. Among those around 1% of all patients may have aneurysm rupture within one year, causing subarachnoid hemorrhage, whose mortality was over 30% [16]. Due to insidious onset of IA, current diagnosis technique has limitation on IA patients without aneurysm rupture. Therefore the investigation of pathogenesis, progression and rupture mechanism of IA, is of critical importance for preventing aneurysm rupture, improving treatment efficacy and prognosis. IA is a vascular disease caused by focal thinning for cerebral vascular wall and dilation, and is featured with the absence of inner elastic lamina, thinning of smooth muscle layer, degeneration of extracel-
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Various studies assigned inflammation induced pathological reconstruction of vascular tissues as one of important pathogenesis mechanisms governing IA generation and rupture, with critical role of mononuclear induced inflammatory response [8]. Aoki et al found significantly elevated macrophage infiltration in IA vascular wall tissues [18]. MMP-2 and MMP-9 secreted by macrophage could accelerate enzymatic rearrangement of vascular wall matrix including elastin and collagen, thus inducing aneurysm formation and rupture. TNFα is one inflammatory factor secreted by macrophage, and has been shown to be related with IA occurrence. Jayaraman et al. found significantly elevated expression of TNFα in ruptured IA walls, as it can initiate inflammatory response, damage vascular endothelial cells and inner plastic membrane, accelerating structural and functional degeneration of vascular wall, thus playing a direct role in IA pathogenesis and rupture [9]. Starke et al. also showed correlation between elevated TNFα expression and formation or rupture of IA [19]. Results from this study showed significantly more macrophage infiltration in IA patient’s artery walls, accompanied with potentiated TNFα expression. In those patients with aneurysm rupture, both macrophage and TNFα expression were all higher than those without rupture. Results from Aoki et al. [18] and Starke et al. [19] agreed with each other, further demonstrating the role of macrophage and TNFα in formation and rupture of IA.

Middle membrane of normal intracranial artery mainly consists of smooth muscles cells, which

Figure 4. Activation of JNK signal pathway by TNFα and facilitation of HA-VASMC cell apoptosis. A. Western blot for protein expression; B. qRT-PCR for mRNA expression; C. Spectrometry for caspase-9 and caspase-3 activity; D. Flow cytometry for cell apoptosis. Compared with blocking TNFα or blocking TNFα + SP600125, *P < 0.05.
can synthesize multiple extracellular matrix such as collagen, elastin, and proteoglycan in addition to the normal contraction function. Normal number of vascular smooth muscle cells is critical for maintaining structural stability and functional integrity of vascular wall [20]. In addition to infiltration of inflammatory cells, pathological changes of IA also include destruction of vascular wall structure featured as degradation of collagen fiber, rupture of inner elastin fiber, and decreased number or even disappearance of vascular smooth muscle cells [21]. Decreased number of smooth muscle cells and consequently suppressed extracellular matrix synthesis and renew are important pathological processes in degenerative disease of IA [22]. Recently, the role of cell apoptosis mechanisms in the decrease of smooth muscle cells of intracranial artery have drawn much research interest [23]. JNK is one key protein at the most downstream of MAPK protein kinase cascade reaction, and is important signal molecule connecting membrane surface receptor and regulation of nuclear downstream target gene expression. Located in the branch of multiple signal pathways, it participates in the regulation of various biological processes including cell proliferation, apoptosis, and differentiation [24]. Guo et al. showed significantly elevated phosphorylated JNK protein in rat IA vascular wall tissues [25]. Laaksamo et al. found significant up-regulation of p-JNK in IA tissues, and correlation with tumor size. These studies all indicate possible involvement of the JNK signal pathway abnormality and IA pathogenesis. Okuno et al. showed that JNK signal protein could phosphorylate c-Jun, induce Bax protein expression, initiate cell apoptosis [26], suggesting the role of JNK in modulating cell apoptosis. Takagi et al. found significant enhancement of auto-phosphorylation level of JNK upstream proteins such as MKK4 and JNK in vascular wall tissues from ruptured aneurysms, plus elevated expression of downstream regulator pro-apoptotic protein Bax, indicating the potential role of enhanced JNK signal pathway activity in facilitating cell apoptosis. Co-culture with macrophage significantly facilitated apoptosis of HA-VSMC cells, with elevated TNFα contents in culture medium. Addition of TNFα blocking agent in such co-culture system significantly depressed phosphorylation of downstream proteins MKK4, JNK, and c-Jun, and apoptotic proteins Bim and Bax, plus the suppression of enzymatic activity of caspase-9 and caspase-3, and decreased mitochondria-dependent cell apoptosis. Young et al. performed a study and found that specific inhibition of TNFα could decrease IA incidence [32], suggesting that TNFα up-regulation might accelerate IA pathogenesis, and the antagonism of TNFα could decrease IA risk. This study was consistent with
our observation that TNFα could reduce VSMC cell apoptosis. The addition of SP600125 directly inhibited phosphorylation of JNK on c-Jun and expression of downstream apoptotic proteins Bim and Bax, with significantly reduced cell apoptosis. Results show that macrophage could initiate endogenous apoptosis pathway of VSMC cells via secreting TNFα, activating JNK signal pathway, and up-regulating expression of apoptotic proteins Bim and Bax. Takagi et al. showed high level of co-expression of c-Jun and apoptotic marker ssDNA in VSMC [27]. This study further illustrated the role of inflammatory mediatory in activating JNK signal pathway and inducing apoptosis of vascular smooth muscle cells, which have not been described before. Yamanouchi et al. revealed the role of inflammatory response and macrophage in inducing apoptosis of vascular smooth muscle cells and abdominal aorta formation [33]. This study further replenished and expanded such opinions.

Conclusion

Both macrophage infiltration and TNFα expression were significantly elevated in artery wall tissues of IA patients. TNFα secreted by macrophage could initiate endogenous apoptosis pathway in VSMC cells via activating JNK signal pathway and up-regulating expression of apoptotic proteins Bim and Bax. This may play a role in inducing IA formation and rupture.

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Disclosure of conflict of interest

None.

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