TNF-α regulates apoptosis of human vascular smooth muscle cells through gap junctions

MEI TANG[1] and JUN FANG[2]

[1]Infusion Preparation Center of Pharmacy Department; [2]Division of Nephrology, Department of Internal Medicine, Xianning Central Hospital & The First Clinical Hospital of Hubei University of Science and Technology, Xianning, Hubei 437100, P.R. China

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Abstract. Inflammatory cytokines are released by immune cells and are able to induce vascular smooth muscle cells (VSMCs) to undergo apoptosis, causing atherosclerotic plaque rupture. Changes in the expression levels of connexins (Cxs) have been demonstrated in VSMCs to be involved in the pathogenesis of atherosclerotic progression. The present study examined the effect of tumor necrosis factor-α (TNF-α) on Cx43 expression levels and apoptosis in human VSMCs. Overexpression of Cx43 plasmids notably stimulated VSMC proliferation. TNF-α directly inhibited Cx43 expression levels in a dose- and time-dependent manner in VSMCs, however this was blocked by c-Jun N-terminal kinase inhibitor. TNF-α also increased caspase-3 activity and apoptosis of VSMCs through the inhibition of Cx43. These data suggested that TNF-α induced the apoptosis of VMSCs and prompted the destabilization of atherosclerotic plaques by downregulating Cx43.

Introduction

Atherosclerotic occlusion affects the arteries in the heart, brain and legs, and is an important cause of morbidity and mortality worldwide (1). Atherosclerotic plaque stability is partly determined by a balance between the apoptosis and the survival of vascular smooth muscle cells (VSMCs) in the fibrous cap. Stable plaques possess a thick fibrous cap that includes an abundance of VSMCs and extracellular matrix (2). Unstable plaques possess thin fibrous caps that contain a smaller number of VSMCs and more inflammatory cells, including macrophages (3). Atherosclerosis is a chronic inflammatory disease of the arterial vessels and pro-inflammatory immune responses regulates the structural integrity of the atherosclerotic plaques. Inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-12 and IL-1β, alter the growth and survival of VSMCs and are implicated in this pathophysiological process (4).

Gap junctions are a form of cell-to-cell connection that involve direct intercellular communication (5) and mediate the direct exchange of ions, small metabolites and other secondary messenger molecules between adjacent cells (6). Three connexins (Cxs), Cx37, Cx40 and Cx43, are reported to express in the vascular wall. Cx43 is predominantly expressed in the VSMCs of the vessel wall (7,8). Gap junctions have been described in cardiac rhythm (9) and a previous study identified that gap junctions are involved in cell growth and differentiation, and in intimal hyperplasia (10). However, little is known about the interaction of cytokines and Cx43 in VSMC apoptosis. The present study examined the effect of inflammatory cytokines, including TNF-α, on C43 expression and the apoptosis of VSMCs and its signaling pathways.

Materials and methods

Culture of VSMCs. Human VSMCs were purchased from Clonetics (San Diego, CA, USA) and cultured in a cell incubator at 37˚C and a humidified 5% CO_2 atmosphere. The VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich China, Inc., Shanghai, China) containing L-glutamine (Sigma-Aldrich China, Inc.), 10% fetal bovine serum (FBS; Sigma-Aldrich China, Inc.), 10,000 U/ml penicillin, 10,000 μg/ml streptomycin and 250 μg/ml amphotericin B (Sigma-Aldrich China, Inc.), and subcultured strains of the VSMCs were used between passages 3 and 6. Prior to experimentation, the cultured VSMCs were serum-starved for 24 h in DMEM without FBS.

pcDNA3-1-Cx43 expression vector construction and transfection. The total RNA was isolated from the cultured VSMCs using RNAzol (Sigma-Aldrich China, Inc.). RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed in a Veriti Thermal Cycler (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as a hot-start PCR. Primer sequences were as follows: Cx43,

Correspondence to: Dr Jun Fang, Division of Nephrology, Department of Internal Medicine, Xianning Central Hospital & The First Clinical Hospital of Hubei University of Science and Technology, 228 Jingui Road, Xianning, Hubei 437100, P.R. China
E-mail: junfang.xn@gmail.com

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forward: 5'-AGAGGAAGAACTCAAGGTTGCCCA-3' and reverse: 5'-AGGACACCACCAGCATGAAGATGA-3'. The primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and the Genbank Accession Code (NM000165) for cx43. Following successfully amplification of cDNA from human VSMCs, a Cx43 gene segment was cloned into pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The transfection of VSMCs was performed in a 6-well plate using Lipofectamine® 2000, according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). VSMCs were collected after 48 h of transfection.

Proliferation assay. A bromodeoxyuridine (BrdU) incorporation assay was performed using a cell proliferation ELISA BrdU kit (Roche Diagnostics GmbH, Mannheim, Germany), as described previously (11). Briefly, the cells were labeled with 10 μM BrdU solution and denatured using FixDenat solution. A mouse anti-BrdU monoclonal antibody (cat. no. B8434; 1:200 dilution; Sigma-Aldrich China, Inc.) conjugated with peroxidase and tetramethylbenzidine was added, and the cells were incubated for 30 min at room temperature. Finally, the absorbance of the samples was measured using a microplate reader at 370 nm.

Western blot analysis. VSMCs were collected and lysed with radioimmunoprecipitation acid lysis buffer (Sigma-Aldrich China, Inc.) and the protein concentration of the lysate was determined by Bio-Rad protein assay [Bio-Rad Laboratories (Shanghai) Ltd., Shanghai, China]. The proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes (Sigma-Aldrich China, Inc.). Non-specific proteins were blocked by incubation in blocking buffer containing Tris-buffered saline with Tween-20 (TBST) and 5% non-fat dried milk (Sigma-Aldrich China, Inc.) at room temperature for 1 h and membranes were incubated overnight at 4˚C with blocking solution containing antibodies targeting Cx43 (cat. no. 3512; 1:1,000 dilution; Cell Signaling Technology, Danvers, MA). The membranes were washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. A9542; 1:5,000 dilution; Sigma-Aldrich China, Inc.) at room temperature for 1 h. After membranes were washed with TBST, the HRP activity was detected using an enhanced chemiluminescence kit [Bio-Rad Laboratories (Shanghai) Ltd.]. As a loading control, glyceraldehyde 3-phosphate dehydrogenase was run in parallel.

Caspase-3 assay. The activity of caspase-3 in VSMC apoptosis was quantified using a CPP32 colorimetric assay kit (MBL International Co., Woburn, MA, USA). Briefly, 5 ml of 1 mM/l caspase-3 substrate and 50 μl of 2X reaction buffer/dithiothreitol mix were added, and protease activity was quantified using a spectrophotometer (Bio-Rad3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 405 nm.

Statistical analysis. The data are reported as the mean ± standard error of the mean. Statistical analysis was performed using Student's t-test or analysis of variance using Sigma Plot software version 12 (Systat Software Inc., San Jose, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

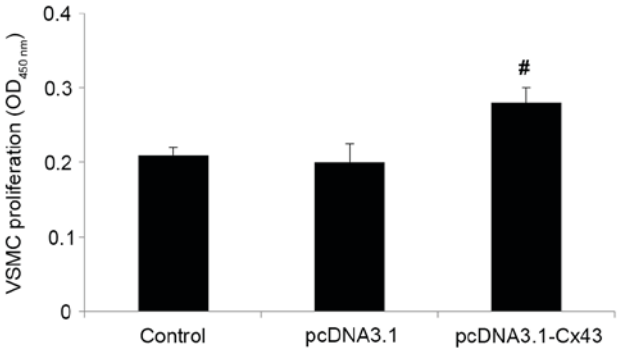

Figure 1. Effect of pcDNA3.1-Cx43 transfection on the proliferation of vascular smooth muscle cells. Each bar represents bromodeoxyuridine incorporation (mean ± standard deviation) for five independent experiments. #P<0.05 vs. pcDNA3.1 empty vector group. Cx43, connexin 43; VSMCs, vascular smooth muscle cells.

Results

Effect of pcDNA3.1-Cx43 transfection on the proliferation of VSMCs. To determine whether the cells were actively proliferating or quiescent, the proliferation of VSMC in the presence of the pcDNA3.1 empty vector or pcDNA3.1-Cx43 at 48 h was examined by measuring the incorporation of BrdU. The proliferation of VSMCs was significantly increased in pcDNA3.1-Cx43 compared with that in the pcDNA3.1 empty vector (Fig. 1).

Effect of TNF-α on the expression of Cx43 on VSMCs. TNF-α treatment (100 ng/ml) resulted in a rapid reduction in Cx43 protein expression levels, detectable at 1 h and reaching a decrease of 80% at 24 h (Fig. 2A). Exposure of VSMCs to different doses of TNF-α for 24 h demonstrated a significant reduction at 50 ng/ml and near-maximal reduction with 100 ng/ml (Fig. 2B).

TNF-α inhibited Cx43 expression in VSMCs via the c-Jun N-terminal kinase (JNK) pathway. To investigate the possible signaling pathway that mediates TNF-α inhibition of Cx43 on human VSMCs, the VSMCs were incubated with TNF-α for 24 h in the absence or presence of mitogen-activated protein kinase (MAPK) inhibitors. As shown in Fig. 3, TNF-α inhibited Cx43 expression, which was completely blocked after the addition of JNK inhibitor II (1 μg/l) for 30 min prior to TNF-α treatment.4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl] pyridine (an inhibitor of p38 MAPK; 10 μmol/l) and 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (an inhibitor of extracellular signal-regulated kinases (ERK) 1/2; 40 μM/l) failed to noticeably block the TNF-α inhibition of Cx43 expression. Dimethyl sulfoxide alone as a vehicle did not affect the Cx43 expression. These findings implied that JNK pathways mediate the reduction of Cx43 on human VSMCs.

Effect of IFN-γ, IL-12 and IL-1β on the expression of Cx43. IFN-γ (100 ng/ml), IL-12 (100 ng/ml) and IL-1β (10 ng/ml) were enough to cause certain effects in the VSMCs in a previous study (4). As presented in Fig. 4, IFN-γ and IL-12 did not affect Cx43 expression levels, but IL-1β did inhibit the Cx43 expression levels in VSMCs.

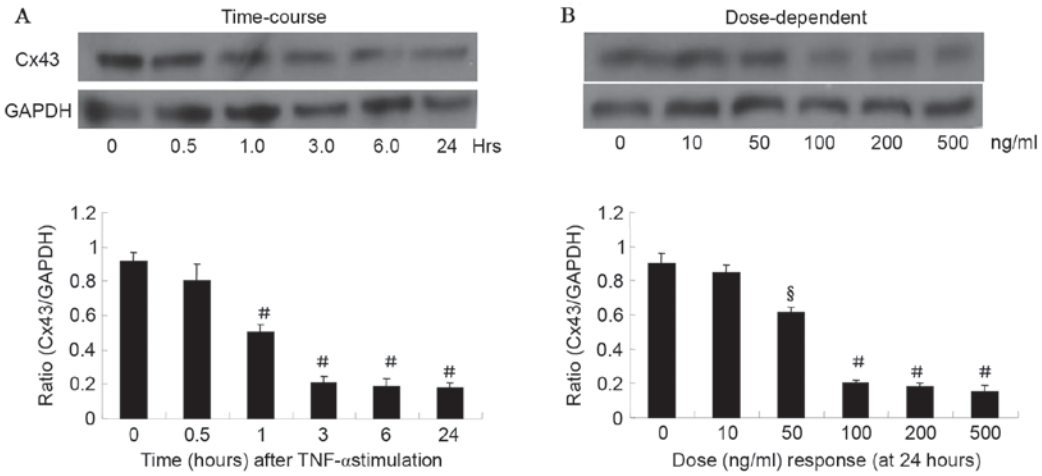

Figure 2. Effect of TNF-α on the expression of Cx43 in VSMCs. (A) TNF-α (100 ng/ml) inhibited Cx43 protein expression in a time-dependent manner in human VSMCs. (B) TNF-α inhibited Cx43 protein expression at 24 h in a dose-dependent manner in human VSMCs. Each bar represents the ratio of Cx43/GAPDH (mean ± standard error of the mean) from four independent experiments. #P<0.01, §P<0.05 vs. control group. TNF-α, tumor necrosis factor-α; Cx43, connexin 43; VSMCs, vascular smooth muscle cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

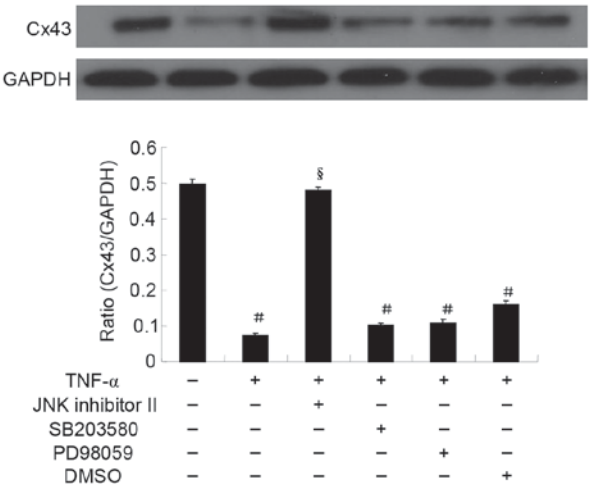

Figure 3. Effect of MAPK inhibitors on TNF-α-inhibited expression of Cx43 in human VSMCs. The MAPK inhibitors include JNK inhibitor II (an inhibitor of JNK; 1 μg/l), SB203580 (an inhibitor of p38 MAPK, 10 μmol/l), and PD98059 (an inhibitor of ERK1/2 kinase, 40 μM/l). Cultured VSMCs were exposed to either solvent or an inhibitor for 30 min prior the addition of TNF-α for 24 h. Proteins were quantified and each well was loaded with 20 μg protein. Each bar represents the ratio of Cx43/GAPDH (mean ± standard error of the mean) from four independent experiments. #P<0.01 vs. control group. §P<0.01 vs. TNF-α group. MAPK; TNF-α, tumor necrosis factor-α; Cx43, connexin 43; VSMCs, vascular smooth muscle cells; JNK, c-Jun N-terminal kinase; SB203580, 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl] pyridine; PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; ERK, extracellular signal-regulated kinases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide.

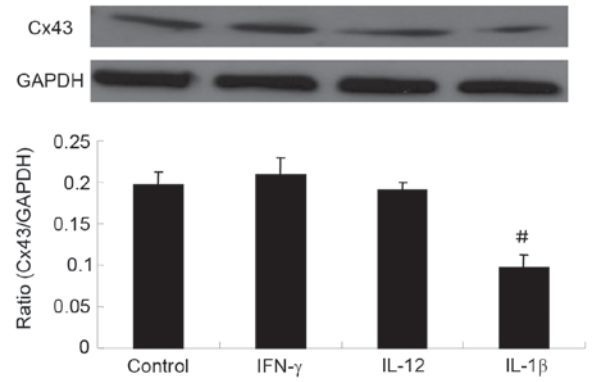

Figure 4. Effect of IFN-γ, IL-12 and IL-1β on the expression levels of Cx43 in VSMCs. Quiescent VSMCs were treated with IFN-γ (100 ng/ml), IL-12 (100 ng/ml) and IL-1β (10 ng/ml) for 24 h respectively. Proteins were quantified and each well was loaded with 20 μg protein. Each bar represents the ratio of Cx43/GAPDH (mean ± standard error of the mean) from four independent experiments. #P<0.01 vs. control group. IFN, interferon; IL, interleukin; Cx43, connexin 43; VSMCs, vascular smooth muscle cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Involvement of Cx43 in the TNF-α-increased caspase-3 activity. Human VSMCs were exposed to pcDNA3.1-Cx43 or natriuretic peptide B (NPPB, an inhibitor of Cx43; 100 μM) and subsequently exposed to TNF-α (100 ng/ml) for 24 h. TNF-α significantly enhanced VSMC caspase-3 activity and apoptosis. However, the effect of TNF-α on caspase-3 activity was inhibited by overexpression of pcDNA3.1-Cx43 (Fig. 5). Furthermore, inhibiting Cx43 with NPPB recovered the role of TNF-α in VSMC apoptosis, suggesting that TNF-α induced VSMC caspase-3 activity and apoptosis, at least partly, via the downregulation of Cx43.

Discussion

Atherosclerotic plaque rupture is considered to serve a key role in thrombus formation and thereby results in the symptoms observed in acute coronary syndrome; stroke and limb ischemia (12). One of the important factors is that the interaction of VSMC and inflammatory mediators, including TNF-α, may alter the survival rate of VSMCs and modify the extracellular matrix, thus modifying the integrity of the atherosclerotic plaque (13). The present study provided new evidence that Cx43 is associated with VSMCs proliferation and that TNF-α prompted VSMC apoptosis through the downregulation of Cx43, suggesting that the interaction of inflammatory cytokines and gap junctions regulated the atherosclerotic plaque stability.

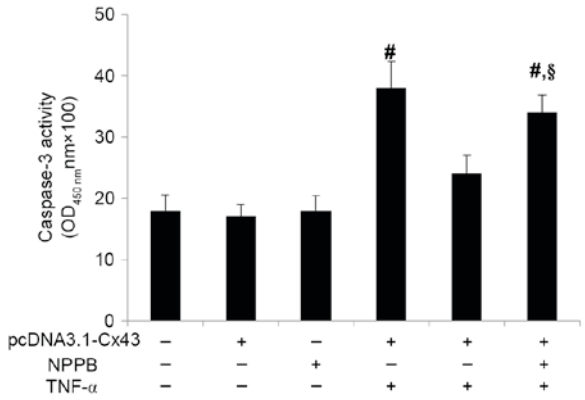

Figure 5. Effect of Cx43 on TNF-α-induced caspase-3 activity in cultured human VSMCs. Cultured VSMCs were exposed to pcDNA3.1-Cx43 or NPPB (100 μM) prior to the addition of TNF-α for 24 h. #P<0.01 vs. control group. §P<0.05 vs. TNF-α + pcDNA3.1-Cx43 group. Cx43, connexin 43; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells; NPPB, natriuretic peptide B.

Gap junctions provide direct signal communication and drive numerous important biological processes, including the rapid transmission of electronic signals in cardiac and smooth muscle (14,15). Growing evidence suggests that Cx43 is involved in VSMC growth, differentiation and the development of intimal hyperplasia (10). In early atheromas, VSMC Cx43 and macrophage Cx37 were observed in the neointima (8,16). In particular, Cx43 demonstrated a higher expression level in asymptomatic compared with in symptomatic plaques (16). Previous studies (17,18) have demonstrated that reducing Cx43 limited neointima formation following acute vascular injury in hypercholesterolemic mice *in vivo*. The present study further confirmed that Cx43 induces the proliferation of VSMCs *in vitro*, and suggested a role for Cx43 in the maintenance of plaque stability.

Atheromatous lesion and plaque stability are partly determined by the balance between the apoptosis and survival of VSMCs in the fibrous cap (19). For example, there is a higher expression of apoptotic genes in the symptomatic compared with the asymptomatic plaques and this change is involved in caspase-3 activation (20). Previous studies (21,22) have demonstrated that the inflammatory cytokines TNF-α, IFN-γ and IL-12 may be responsible for the apoptosis of VSMCs and the destabilization of atherosclerotic plaques. However, the interaction of inflammatory cytokines and Cx43 on the apoptosis of VSMCs and the regulation of plaque stability remains to be elucidated. The current study demonstrated that TNF-α inhibited Cx43 in a dose- and time-dependent manner in VSMCs and prompted caspases-3 activity, resulting in VSMC apoptosis. To the best of our knowledge, this is the first report on the regulation of Cx43 and VSMC apoptosis induced by TNF-α in VSMCs.

The important finding of the present study was that TNF-α can selectively modulate the expression of Cx43 on the VSMCs through JNK pathways. JNK and p38MAPK kinase have been reported to be important intracellular signaling pathways that regulate Cx43 (23,24). A previous study (25) investigated the effects of amphetamine on the Cx43 gene expression through the JNK-AP-1 pathway in cultured neonatal rat cardiomyocytes. However, the role of JNK in the regulation of VSMC Cx43 is has yet to be elucidated (25). The data from the present study implied that the JNK pathway, but not the ERK1/2 or p38 MAPK pathways, is the major pathway involved in the downregulation of Cx43 by TNF-α on VSMCs. Another study (26) also demonstrated that TNF-α decreased Cx43 expression through inhibition of the Cx43 promoter activity in rat hearts. Although the precise mechanism remains to be elucidated, an AP-1 site in its promoter is likely involved in the cytokine regulation of Cx43 (26). Other cytokines including IFN-γ, IL-12 and IL-1β are also implicated in the induction of VSMC loss and plaque instability (27). Although IL-1β has been suggested to regulate Cx43 expression on cultured VSMCs, neither IFN-γ nor IL-12 regulate Cx43. A possible explanation is that the VSMCs are in different conditions *in vivo* and *in vitro*. A previous study (28) also demonstrated that TNF-α signaling initiated the activation of the tyrosine (Tyr) kinase c-Src in airway epithelial cells, which physically associates with Cx43 by the phosphorylation of Tyr-247 and 265. Furthermore, mutation of Tyr-247 and Tyr-65 completely prevented Cx43 channel closure by Src, suggesting that phosphorylation of both Tyr residues is involved in the regulation of gap junctions (28). Further investigation is warranted to examine the regulatory mechanisms underlying the effects of these cytokines.

In conclusion, the present study demonstrated that TNF-α potently inhibited Cx43 expression and prompted VSMC apoptosis; these findings may explain the stabilization of atheromatous plaques. A further understanding of the interaction of inflammatory cytokines and Cx43 on human VSMCs may possess considerable biological and clinical impact, and provide a potential therapeutic strategy in maintaining the stability of atherosclerotic plaques.

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