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Tumor Necrosis Factor- α Regulates p27^{kip} Expression and Apoptosis in Smooth Muscle Cells of Human Carotid Plaques via Forkhead Transcription Factor O1

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Abstract

Apoptosis of vascular smooth muscle cells (SMCs) is controlled by a balance between the effect of growth factors and cytokines, and is involved in plaque instability in advanced atherosclerotic lesions. Recently, we reported high levels of atheroma-associated cytokines, including tumor necrosis factor- α (TNF- α), in carotid plaques of symptomatic patients. These cytokines induce apoptosis of vascular SMCs, and thus could be responsible for plaque rupture, a clinically devastating event. In this study, we examined the effect of TNF- α on the cell cycle inhibitor p27^{kip} and apoptosis of SMCs in human carotid plaques, and the underlying mechanism. Both Forkhead box subclass o1 (FoxO1) and p27^{kip} were more strongly expressed in symptomatic than asymptomatic atherosclerotic plaques. TNF- α significantly induced the expression of FoxO1 in asymptomatic plaque SMCs in a dose- and time-dependent manner via JNK signaling pathway. TNF- α also induced phosphorylation of FoxO1, resulting in its cytoplasmic translocation/nuclear exclusion of transcription factors. The effect of TNF- α was blocked by the PI3K inhibitor, LY294002. Meanwhile, TNF- α not only induced the p27^{kip} expression and cell cycle arrest in G₀–G₁ phase, but also enhanced caspase-3 activity and induced apoptosis in SMCs of asymptomatic plaques. However, the potential effect of TNF- α on the cell cycle inhibitor p27^{kip} and apoptosis of SMCs was inhibited by siRNA against FoxO1 in asymptomatic patients. These data suggest the involvement of FoxO1 transcription factor in TNF- α -induced expression of a cell cycle regulatory protein and apoptosis of SMCs, thus regulating the stability of atherosclerotic plaques with carotid stenosis.

Keywords

Apoptosis; Atherosclerosis; Forkhead Transcription Factor; Plaque Stability; Vascular Smooth Muscle Cell

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Introduction

Atherosclerosis is the hardening of the arteries that leads to stroke or heart attack, and is characterized by many complex changes to the arteries. In the United States alone, atherosclerosis affects about 70 million people, causing approximately 42% of all deaths (LaBelle and Tulenko, 2004). Morbidity and mortality from atherosclerosis are associated with complicated atherosclerotic lesions due to vascular plaque rupture, which is regulated by a balance between proliferation and death of vascular smooth muscle cells (SMCs) (Moran and Agrawal, 2007). Therefore, how this balance is maintained remains of utmost importance in understanding the mechanisms underlying plaque stability.

Stable plaques have a thick fibrous cap composed mainly of VSMCs and extracellular matrix enveloping a necrotic lipid core. In the unstable plaques, however, part or the entire fibrous cap is thin and contains few VSMCs and many macrophages (DeGaba, 2004). Our previous study showed that insulin-like growth factor-1 (IGF-1) is more potent in inducing the survival of VSMCs from the endarterectomy specimens of asymptomatic patients as compared to that of symptomatic subjects, and that cytokines associated with atheromatous lesions decrease the activity of IGF-1-induced survival in the vascular SMCs of asymptomatic plaques (Jia et al., 2006a). Thus, inflammation is now thought to be a key event in the initiation and progression of atherosclerosis, and there appears to be a good correlation between inflammation and plaque instability. Tumor necrosis factor- α (TNF- α) is a pluripotent mediator of inflammation, is believed to play a pathophysiological role in vascular atherosclerosis, and can be expressed by vascular SMCs in atheromatous plaques (Prins et al., 1998). By signaling through cell membrane receptors, TNF- α can induce mitogenic and pro-apoptotic effects, activating monocytes, endothelial cells, and macrophages (Rakesh and Agrawal, 2005). Thus, TNF- α may play a significant role in the formation and development of atherosclerosis.

Forkhead box subclass O (FoxO) factors include FoxO1/FKHR, FoxO3a/FKHRL-1, and FoxO4/AFX (Potente et al., 2005). FOXO members can bind to the consensus DNA sequence of TTGTTTAC through their function as transcription factors and regulate the expression of cell cycle regulators such as the cyclin-dependent kinase inhibitor, p27^{kip} (Abid et al., 2005; Paik et al., 2007), as well as apoptosis (Jia et al., 2007b). However, it is not known if and how the transcription regulation occurs in the atherosclerotic plaques and how inflammatory cytokines affect plaque stability. In this study, we assessed the effect of tumor necrosis factor- α on the expression of p27^{kip} and apoptosis in SMCs of atherosclerotic plaques from asymptomatic and symptomatic patients with carotid stenosis.

Methods

Carotid Endarterectomy Specimen Collection and culture of VSMC

Carotid endarterectomy specimens were processed by a method previously reported from our laboratory (Jia et al., 2007b). Carotid endarterectomy specimens were excised after longitudinal arteriotomy, rinsed in saline to remove surface blood and the specimen placed in the University of Wisconsin solution and transported to the laboratory within 2–3 hours. Briefly, after gentle removal of the endothelial cells and adventitia, the specimen was minced and digested with digestion media (containing elastase, collagenase, M199SF medium, glucose, HEPES, and FBS). The isolated cells were removed by centrifugation at 900g for 10 min at 4°C, suspended in M199SF and incubated at 37°C in a humidified 5% CO₂ atmosphere for 10–14 days and passaged. The cultured VSMCs were used between passages 2–3. The confluent cells showed the characteristic hill-and-valley pattern associated with spindle-shaped VSMCs. The purity of isolated VSMCs was examined with

positive immunostaining to smooth muscle α -actin and caldesmon. Prior to stimulation experiments, cells were serum-starved for 24 hours in DMEM.

According to patients' history and clinical examination, carotid endarterectomy specimens were categorized as asymptomatic (mean patient age 72.5 ± 3.7 yrs) and symptomatic (mean patient age 70.6 ± 2.4 yrs). The time of collection of the specimen from the patients with symptoms was within two weeks of developing symptoms that included hemispheric transient ischemic attacks, amaurosis fugax, or stroke. The Institutional Review Board of Creighton University approved the research protocol.

Immunostaining

The carotid plaques were transversely sectioned at 2–3 mm intervals. Each tissue block was embedded separately into OCT (optimum cutting temperature- a mixture of glycols) and frozen in liquid nitrogen. Thin sections ($6\mu\text{m}$) were cut using the Cryocut 1800 (Reichert – Jung) and cryostat sections were mounted on electrostatically coated slides and stored at -70°C before staining. Sections were treated with 3% peroxide in methanol for 15 minute followed by an overnight incubation with the primary antibody at 4°C . Rabbit primary monoclonal antibody directed against FoxO1 and p27^{kip} (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was used in these experiments. After rinsing with PBS, the staining in the sections was detected by the rat ABC Staining System (Vector Laboratories, Burlingame, CA). The immunopositivity in plaque sections was microscopically quantified with NIH Scion Image analysis by randomly selecting four different areas of 1-mm length under the light microscope. Then the area of positive immunostaining per millimeter squared was calculated.

SMCs were plated onto 4-well chamber slides at a density of 50,000 cells/well. The cells were fixed in 3.7% paraformaldehyde for 20 min at room temperature, washed with phosphate-buffered saline, and incubated in blocking buffer for 10 min. The cells were incubated with anti-p-FoxO1 for 1 h at room temperature. After washing, FITC-conjugated goat anti-rabbit IgG 1/200 was applied at room temperature for 1hr, followed by thorough washing, then staining with 4',6'-diamidino-2-phenylindole (300 nM). Staining was visualized using fluorescence microscopy.

Small Interfering RNA and Cell Transfection

We used small interfering RNA (siRNA), an effective tool to downregulate the expression of target genes in cultured mammalian cells. The small interfering RNAs (siRNA) with following sense and antisense sequences were for FoxO1: sense, 5'-GGAAAGUGAUGUAUAGUUAtt-3'; and antisense, 5'-UAACUAUACAUCACUUUCtg-3'; for control (nonsilencing): sense, 5'-UUCUCCGAACGUGUCACGUAAdTdT-3'; and antisense, 5'-ACGUGACACGUUCGGAGAAAdTdT-3'. All siRNAs were purchased from Ambion (Austin, USA). siRNAs were diluted in RNase-free water to a final concentration of $20\mu\text{M}$ ($20\text{ pmol}/\mu\text{l}$). Forty-eight hours after transfection, gene silencing was monitored.

Transfection of VSMCs was performed using the nucleofection device and solutions (Amaza Biosystems, Amaza Inc, USA). The Nucleofection device is an apparatus which delivers unique electrical stimuli to the target cells. The resulting transfection efficiency was 70–78% in vascular smooth muscle cells.

Western blot analysis

Nuclear and cytosolic extracts kit (Active Motif, Calsbad, CA, USA) were prepared by the standard procedure (Sekine et al., 2007). Proteins were resolved by SDS-PAGE and

transferred to Immobilon P membrane. Non-specific proteins were blocked by incubation in blocking buffer (5% milk powder/TRIS-buffered saline, pH 7.8/0.1% Tween 20) and the membranes were incubated overnight at 4°C with blocking buffer containing antibodies to FoxO1, p-FoxO1 and p27^{kip} respectively. Membranes were washed and incubated with HRP-conjugated secondary antibody and detected using an ECL kit (Pierce). As a loading control, cytosolic protein GAPDH or nuclear protein Lamin B was probed and visualized in all the blots.

Cell Cycle Distribution

In each experiment, 10,000 cells were counted, and these events are thought to represent at least 90% to 95% of the total cell population. The area versus the width of the fluorescent signal was analyzed to gate out cellular multiples. Histograms of DNA content were analyzed with Modfit Lt V1.01 (Verity Software House, Topsham, ME, USA) to determine fractions of the population in each phase of the cell cycle (G₀, G₁, S, and G₂/M).

Annexin V Labeling and Caspase-3 Assay

An annexin V-FITC kit (BD Biosciences, Pharmingen, San Jose, USA) was used to measure phosphatidylserine externalization. The cells were incubated in the presence of annexin V-FITC and propidium iodide for 15 minutes at 37°C. Labeled cells were analyzed with flow cytometry with FACS Calibur (BD Biosciences, Pharmingen, San Jose, USA) and Lysis II software (Verity Software House, Topsham, ME, USA). In each experimental group 10,000 cells were counted, and these events were thought to represent at least 90% to 95% of the total cell population. Apoptosis was also quantified by measuring activity of caspase-3 using the CPP32 colorimetric assay kit (MBL, Woburn, MA USA). Five milliliters of 1 mmol/L caspase-3 substrate and 50 µL of 2xreaction buffer/dithiothreitol mix were added, and the samples were incubated at 37°C for 1 hour. Protease activity was quantified by spectrophotometer (Bio-Rad3550; Bio-Rad, Hercules, CA, USA) at 405 nm.

Statistical analysis

Data are presented mean ± SEM. Statistical analysis was performed using Student's *t* test or ANOVA when appropriate to analyze differences between groups. *p* < 0.05 was considered significant.

Results

FoxO1 and p27^{kip} expression in human carotid plaques

Our studies have previously shown that symptomatic plaques show a higher expression of cytokines, interleukin-12 and interferon-γ, compared with asymptomatic plaques (Jia et al., 2006a). However, cytokines can induce the activation of the Forkhead family of transcription factors which regulate cell cycle and apoptosis gene expression. The immunopositivity to FoxO1 antibody was greater in the symptomatic plaques compared to the asymptomatic plaques (Fig. 1A). The immunopositive areas in the shoulder region of carotid plaques were captured, and the mean area of immunoreactivity was $19.3 \pm 1.53 \text{ mm}^2$ in symptomatic plaques and $9.7 \pm 1.1 \text{ mm}^2$ in asymptomatic plaques (Fig. 1A). Previous studies in non-VSMCs have demonstrated a link between forkhead activity and expression of cell cycle inhibitor, p27^{kip}. Here, p27^{kip} was strongly expressed in symptomatic atherosclerotic plaques ($8.33 \pm 1.15 \text{ mm}^2$) as compared to asymptomatic plaques ($5.32 \pm 0.45 \text{ mm}^2$) (Fig. 1B). To confirm these findings, we again measured the expression of FoxO1 nuclear protein and p27^{kip} proteins using western-blot in patient plaques. The expression of FoxO1 nuclear protein and p27^{kip} proteins was significantly higher in symptomatic than in asymptomatic plaques (Fig. 1C).

TNF- α induced expression of FoxO1 nuclear protein via JNK signaling pathway in the SMCs of asymptomatic plaques

The expression of FoxO1 nuclear protein in the SMCs of asymptomatic plaques was evaluated in a time- and dose-dependent manner. FoxO1 expression was temporarily increased significantly at 0.5 h and 3 h in response to TNF- α (20 ng/ml) (Fig. 2A). Also, TNF- α (20, 50, and 100 ng/ml) induced a significant maximal response in the SMCs of asymptomatic plaques at 0.5 h in a dose-dependent manner (Fig. 2B). However, JNK inhibitor inhibited the effect of TNF- α on the expression of FoxO1 (Fig. 2C). These suggest that TNF- α induced expression of FoxO1 nuclear protein via JNK signaling pathway in the SMCs of asymptomatic plaques.

TNF- α induced phosphorylation of FoxO1 nuclear proteins and nuclear exclusion in SMC of asymptomatic plaques

To determine whether TNF- α modulates the phosphorylation of forkhead proteins in VSMC, Western blot analyses were performed in SMCs of asymptomatic plaques treated with TNF- α . As shown in Fig. 3A, incubation of SMCs with TNF- α (20 ng/ml for 30 min) resulted in phosphorylation of FoxO1 nuclear protein. However, TNF- α -mediated induction of FoxO1 phosphorylation was blocked by pretreatment with the PI3K inhibitor, LY294002. These data indicate that TNF- α induced phosphorylation of FoxO1 in SMCs of asymptomatic plaques through PI3K-dependent signaling pathways.

To determine the effect of TNF- α on nuclear localization of p-FoxO1, SMCs were cultured in 4-well chamber slides, serum-starved for 24 h, and then treated with TNF- α (20 ng/ml). Subcellular distribution of p-FoxO1 was assayed by immunofluorescence and Western-blot. Treatment with TNF- α resulted in nuclear export of p-FoxO1 and a steady increase of cytosolic p-FoxO1 protein at 30 minutes, 1 hr, and 3 hrs in SMCs of asymptomatic plaques (Fig. 3B). These results suggest that TNF- α -mediated phosphorylation of FoxO1 results in cytoplasmic translocation/nuclear exclusion of the transcription factors in SMC of asymptomatic plaques.

Involvement of activation of transcription factor FoxO1 in the TNF- α -induced p27^{kip} expression and cell cycle arrest in G₀-G₁ phase in SMCs of asymptomatic plaques

In order to confirm the involvement and activation of the transcription factor FoxO1 in TNF- α -induced p27^{kip} expression and cell cycle arrest in SMCs of asymptomatic plaques, FoxO1 expression was knocked down using siRNA. Initially, SMCs were transfected with siRNA against FoxO1 and non-silencing siRNA, and FoxO1 was determined by Western blot analysis. A marked reduction in FoxO1 protein expression was observed 48h and 72h following siRNA treatment (Fig. 4A). SMCs were then treated with siRNA against FoxO1 and subsequently exposed to TNF- α (20 ng/ml) for 24 hrs. TNF- α significantly induced the p27^{kip} expression and cell cycle arrest in G₀-G₁ phase of SMCs in asymptomatic plaques. However, the effect of TNF- α on the p27^{kip} expression and cell cycle arrest of SMCs were significantly blocked by FoxO1-siRNA (Fig. 4B, C). These results provide strong evidence as to the involvement of FoxO1 in the TNF- α -induced p27^{kip} expression and cell cycle arrest in G₀-G₁ phase in plaque VSMCs from asymptomatic patients.

Involvement of activation of transcription factor FoxO1 in the TNF- α -induced caspase-3 activity and apoptosis in SMCs of asymptomatic plaques

We further examined the effect of TNF- α on caspase-3 activity and apoptosis of SMCs isolated from asymptomatic patients with carotid disease *via* FoxO1, and performed an apoptosis assay by annex V labeling. SMCs were treated with siRNA against FoxO1 and subsequently exposed to TNF- α (20 ng/ml) for 24 hrs. TNF- α significantly enhanced

caspase-3 activity and induced apoptosis in SMCs of asymptomatic plaques (Fig. 5A, B). However, the effect of TNF- α on the caspase-3 activity and apoptosis of SMCs was blocked by FoxO1-siRNA (Fig. 5B, C).

Discussion

In atherosclerotic lesions, inflammatory processes change the structural integrity of the artery plaques, and activated macrophages are known to produce cytokines, proteolytic enzymes, and growth factors that alter growth and survival of VSMCs (Rakesh and Agrawal, 2005). Our studies have previously shown that the area of the macrophage infiltration, as defined by positive immunoreactivity to CD68, in the base of the lesion and fibrous caps of symptomatic plaques was significantly greater than that in asymptomatic plaques (Dhume et al., 2003). Because macrophages can release several cytokines, including TNF- α , which can induce VSMCs to undergo apoptosis which, in turn, may destabilize the lesion (Gerdes et al., 2002), this could be a reason that symptomatic plaque cells are more susceptible to apoptosis than asymptomatic plaques. However, we still do not know the transcription regulatory mechanisms of inflammatory cytokines in the apoptosis of SMCs. In this study, we provide new evidence that increased expression and activity of FoxO1 transcription factor with an increased level of cell cycle inhibitor p27^{kip} and apoptosis may significantly contribute towards TNF- α -induced destabilization of the atherosclerotic plaques in human carotid arteries.

FoxO1 recently has been implicated in cell growth, cell cycle, and differentiation of neointimal hyperplasia in a rat balloon carotid arterial injury model (Park et al., 2005). Our results suggest that TNF- α significantly enhances the expression of FoxO1 in a time- and dose-dependent manner in asymptomatic plaque SMCs. Because inflammatory cytokines were primarily released by monocytes and macrophages, which are present in larger quantities in the plaques of symptomatic subjects as compared to asymptomatic subjects (Dhume et al., 2003), cytokines may have already increased the expression of FoxO1. This could be the reason that the expression of FoxO1 was higher in symptomatic plaques than in asymptomatic plaques. The mechanism by which TNF- α increases FoxO1 nuclear protein remains to be determined. In our study, we found that TNF- α induced expression of FoxO1 nuclear protein via JNK signaling pathway in the SMCs of asymptomatic plaques. These results are consistent with previous reports that TNF- α increased FoxO protein in the nucleus, whereas IGF-1 resulted in a higher protein level of FoxO in the cytoplasm of rat aortic vascular SMCs (Li et al., 2007). Further, they found that TNF- α upregulated nuclear FoxO expression through the ROS-JNK/ROS-MST1-activated stress signaling pathway after vascular injury (Li et al., 2007). None-the-less, further studies are warranted to elucidate and dissect cellular and molecular mechanisms underlying TNF- α -induced expression of FoxO1 in vascular VSMCs.

Data from other laboratories demonstrated that one major upstream target of FoxO is the PI3K-Akt signaling pathway (Marchetti et al., 2006). We found that TNF- α could induce phosphorylation of FoxO through PI3K-dependent signaling pathways in SMCs of atherosclerotic plaques. These results are consistent with the previous report that TNF- α stimulated phosphorylation of FoxO1 in human coronary artery smooth muscle cells via MEK1/2 and/or phosphatidylinositol 3-kinase-dependent signaling pathways under *in vitro* conditions (Abid et al., 2005). Interestingly, we further found that TNF- α -mediated phosphorylation of FoxO results in cytoplasmic translocation/nuclear exclusion of the transcription factors. Since TNF- α could affect the inactivation of Akt through the Ras- and PI3K-dependent pathways (Li et al., 2008), this could in turn result in hyperphosphorylation of FoxO transcription factor, and Akt-mediated phosphorylation allows FoxO proteins to bind to the chaperone proteins 14-3-3 and be exported into the cytoplasm, where they are

unable to regulate gene expression (Fig. 6), thus inactivating FoxO1 protein in a CRM1-dependent manner (Huang et al., 2006; Huang and Tindall, 2007). Therefore, this may explain our results that the FoxO1 protein was decreased in the nucleus after 3 h by TNF- α stimulation in SMCs of asymptomatic plaques.

A link between forkhead activity and expression of the cell cycle inhibitor-p27^{kip} has been demonstrated (Charvet et al., 2006). The cell cycle machinery is regulated by oscillatory and locally coordinated expression of complexes, consisting of cyclins and their associated cyclin-dependent kinases(CDK), and cell cycle transit is regulated at a number of checkpoints, the best characterized of which is the G₁-S transition (von Harsdorf et al., 1999). p27^{kip} is one of the cyclin-dependent kinase (CDK)2 interacting protein (CIP)/CDK inhibitory proteins (Fiaschi-Taesch et al., 2006). In endothelial cells and VSMCs, TNF- α and IFN- γ can induce a G₀-G₁ arrest and apoptosis associated with reduced levels of cyclin D1 and cdk2, and increased expression of the cdk inhibitors p16, p21 and p27^{kip} expression (Dormond et al., 2002; Jia et al., 2006a). In order to confirm the involvement and activation of FoxO1 in TNF- α -induced p27^{kip} expression, we knocked down FoxO1 expression using siRNA. We found that TNF- α induced the expression of FoxO1 nuclear protein, increased expression of p27^{kip} protein, and arrested the cells of asymptomatic plaques in the G₀-G₁ phase. Conversely, silencing of FoxO1 expression was able to inhibit the effect induced by TNF- α . These results are consistent with previous reports that FoxO1 overexpression increased p27 expression, leading to G₁/S phase cell cycle arrest in neointimal hyperplasia (Park et al., 2005). However, the phosphorylation of FoxO1 decreased expression of p27 in vascular SMCs (Park et al., 2005). These data suggested a key role of the forkhead family transcription factors in the cell cycle regulation.

Apoptosis in atherosclerotic lesions is triggered by inflammatory processes, both via cell-cell contact and by cytokines and oxidized lipids (Jia et al., 2006b). We and other investigators have reported that inflammatory cytokines could induce the apoptosis of vascular SMCs (Anwar et al., 2002; Jia et al., 2006b). However, TNF- α induces proliferation or apoptosis in human vascular SMCs depending on the phenotype. Wang et al (Wang et al., 2005) have showed that TNF- α can only induce the apoptosis of rhomboid VSMCs, which is related with the pathogenesis of atherosclerosis. Therefore, cytokines released by activated T-lymphocytes and macrophages lead to apoptosis and result in the instability of atherosclerotic plaques from symptomatic patients (Dhume et al., 2003). The downregulation of IGF-1 receptor by inflammatory cytokines is critical for the apoptosis of SMCs (Jia et al., 2007a). One of the important findings in our study was involvement of activation of the transcription factor foxO1 in the TNF- α -induced caspase-3 activity and apoptosis in SMCs of asymptomatic plaques. Indeed, FoxO1 overexpression increased apoptosis of SMCs in neointimal hyperplasia (Park et al., 2005). In human fibroblasts, TNF- α increased cell survival, growth, and development *via* NF-KB, however, TNF- α switched to control apoptosis through activation of FoxO1. Further, silencing Foxo1 decreased the TNF- α -induced apoptosis by 76% and caspase-3, -8, and -9 activities (Alikhani et al., 2005). Thus, TNF- α controlled the survival of SMCs through upregulation of FoxO1 expression. This could be a potential mechanism underlying instability of atheromatous plaques in symptomatic patients.

In summary, data from this study supported our hypothesis that there is increased expression and activity of the FoxO family of transcription factors, together with an increased level of the cell cycle inhibitor p27, which could induce cell cycle arrest and apoptosis after stimulation with TNF- α in the SMCs of atherosclerotic plaques. This could be a potential mechanism underlying instability of atherosclerotic plaques and a novel therapeutic target in fibroproliferative vasculopathies.

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Fig. 1.

A: Histological serial adjacent sections taken from shoulder region of carotid plaque. The immunohistochemistry demonstrates that FoxO1 are expressed differently in symptomatic and asymptomatic carotid plaques. Arrows indicate FoxO1 positive cells in the medial layer as smooth muscle α -actin positive cells. B: Histological serial adjacent sections taken from shoulder region of carotid plaque. The immunohistochemistry demonstrates that p27^{kip} proteins are expressed differently in symptomatic and asymptomatic carotid plaques. Arrows indicate p27^{kip} positive cells in the medial layer as smooth muscle α -actin positive cells. Image analysis indicated significantly increased immunoreactivity to FoxO1 or p27^{kip} in the shoulder region of the plaques from symptomatic that in asymptomatic patients (as the area

of the staining per mm² in 10 high power fields). C: FoxO1 nuclear protein and p27^{kip} protein expression in symptomatic and asymptomatic plaques. The relative amount of FoxO1 and p27^{kip} expression was measured by Western blot. Data are expressed as mean $\pm$ SE in the plaques of 3 asymptomatic and 3 symptomatic patients. # $p < 0.01$ compared with the symptomatic group.

Fig. 2.

TNF- α induced transcription factor FoxO1 nuclear protein expression in vascular smooth muscle cells of asymptomatic plaques. A: TNF- α (20 ng/mL) induced FoxO1 nuclear protein expression in SMCs of asymptomatic plaques in a time-dependent manner. B: TNF- α induced FoxO1 nuclear protein expression of SMCs from asymptomatic plaques at 0.5 h in a dose-dependent manner. C: TNF- α (20 ng/mL) induced FoxO1 nuclear protein expression via JNK signaling pathway in SMCs of asymptomatic plaques. Cultured SMCs were exposed to JNK inhibitor II (1 μ g/ml) for 30 min before the addition of TNF- α (20 ng/mL) for 0.5 h. The relative amount of FoxO1 protein expression was measured by Western blot. Each bar represents the ratio of FoxO1/Lamin B (mean $\pm$ SE) from three independent experiments. [#] p <0.01 compared with the control group. ^{*} p <0.01 compared with the TNF- α group.

Fig. 3.

A: TNF- α induced phosphorylation of FoxO1 proteins in SMC of asymptomatic plaques. SMCs of asymptomatic plaques were treated with TNF- α (20 ng/ml for 30 min) where indicated, and serum-starved cells were pretreated with LY294002 (LY, 10 μ mol/L) for 30 min. The blots shown are representative of three independent experiments. [#] P <0.01 compared with control group. * P <0.01 compared with TNF- α group. B: TNF- α induced nuclear exclusion of p-FoxO1 in asymptomatic plaque SMC. SMCs of asymptomatic plaques were treated with TNF- α (20 ng/ml) for 30 min, 1 h and 3 hrs, respectively. The relative amount of cytosolic p-FoxO1 expression was measured by Western blot. The blots shown are representative of three independent experiments. [#] p <0.01 compared with control group.

Fig. 4.

Involvement of activation of transcription factor FoxO1 in TNF- α induced p27^{kip} expression and cell cycle distribution in G₀-G₁ phase in SMCs from asymptomatic plaques. A: SMCs of asymptomatic plaques were transfected with siRNA against FoxO1 and non-silencing siRNA, and protein expression of FoxO1 was determined by western blot analysis after 48h and 72h. B: p27^{kip} expression was induced by TNF- α (20 ng/mL) *via* FoxO1 in SMCs of asymptomatic plaques. C: The SMCs of asymptomatic plaques were arrested in the G₀-G₁ phase by TNF- α (20 ng/mL) *via* FoxO1. Data are mean $\pm$ SE of triplicate observations. In A and B: [#] $p < 0.01$ compared with control group. ^{*} $p < 0.01$ compared with TNF- α group. In C: [#] $p < 0.05$ compared with control group. ^{*} $p < 0.05$ compared with TNF- α group.

Fig. 5. Effect of TNF- α on caspase-3 activity and apoptosis of SMCs in asymptomatic plaques. A: TNF- α (20 ng/ml) enhanced caspase-3 activity *via* FoxO1 in SMCs of asymptomatic plaques. B: TNF- α (20 ng/ml) induced apoptosis *via* FoxO1 in SMCs of asymptomatic plaques. Data are mean $\pm$ SE of triplicate observations. [#] p < 0.01 compared with control group. ^{*} p < 0.01 compared with TNF- α group.

Fig. 6.

Proposed schema which depicts our hypothesis for tumor necrosis factor- α -induced increase in the expression and activity of p27^{kip} and caspase-3 via JNK-FoxO1 to induce cell cycle arrest and apoptosis, respectively, in smooth muscle cells of human carotid plaques. However, FoxO1 may also undergo phosphorylation through PI3K-Akt pathway, and p-FoxO1 is then sequestered by chaperone protein, 14-3-3, and exported to the cytoplasm in SMCs. Thus, the latter signal would act as a negative feedback signal and protect smooth muscle cells and thus increase plaque stability.