YKL40 potentially via activating the P38 pathway in decreasing atherosclerotic plaque stability

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To the Editor: Atherosclerosis is the main pathological feature of ischemic stroke, which remains a major cause of disability and death worldwide.[1] Vulnerable plaque, was characterized by ulceration, platelet activation, and thrombosis, potentially leading to emboli detachment and stroke.[2] Vascular smooth muscle cells (VSMCs), a major cellular component within lesions, can display pleiotropic phenotypes, including osteogenic or inflammatory phenotypes, which may be harmful in maintaining advanced atherosclerotic plaque stability.[3] The modulation of the function of VSMCs, including proliferation, migration, and synthesizing the extracellular matrix (ECM) and matrix metalloproteinase (MMP), is thought to markedly affect plaque formation and vulnerability.[4] Therefore, it is of considerable significance to drive harmful VSMCs toward VSMCs having beneficial properties to strengthen plaque stability and retard plaque development.

YKL40 is associated with atherosclerosis and serves as a potential biomarker predicting the occurrence and clinical outcome of carotid atherosclerosis. YKL40 messenger RNA (mRNA) expression has been found to be significantly elevated in human stable carotid plaques compared with nondiseased arterial tissues and is even higher in ruptured plaque.[5] Also, YKL40 knockdown can decrease plaque burden in partially ligated carotid artery of mouse model, which indicates that YKL40 contributes to the exacerbation of atherosclerosis.[6] However, the exact regulatory role of YKL40 and its downstream mechanisms in VSMCs and atherosclerosis are largely unknown. Herein, we explore the regulatory role of YKL40 in regulating the function of VSMCs and atherosclerotic lesion stability.

In this study, primary human aortic vascular smooth muscle cell (HAVSMC) was treated by adenovirus vector overexpressing the *YKL-40* gene or YKL40 recombinant protein. The capacity of proliferation, migration, and synthesis of ECM and MMP was thereafter detected. On the other hand, low density lipoprotein-deficient (Ldlr–/–) mice were fed with a high cholesterol diet and received the tandem stenosis surgery, after which recombinant YKL40, YKL40 neutralizing antibody, or immunoglobulin G (IgG) was intravenously administered. Finally, carotid and aortic atherosclerotic plaques were collected for further analysis. The detailed experimental procedures can be found in the Supplementary materials and methods, http://links.lww.com/CM9/B921.

First, we evaluated the exogenous role of YKL40 in functional changes in VSMCs. The cell counting kit-8 (CCK-8), 5-ethynyl-2′-deoxyuridine (EdU) assays were used to analyze cell proliferation. CCK-8 results showed that human aortic smooth muscle cell (HASMC) viability is significantly promoted after YKL40 treatment over time (24 h P <0.05, t = 2.457; 48 h P <0.01, t = 3.946; 72 h P <0.05, t = 2.471; Supplementary Figure 1A, http://links.lww.com/CM9/B921). Moreover, the percentage of EdU-positive cells was significantly increased in the YKL40 group compared with control, which indicates that YKL40-induces proliferation of HASMC (P <0.05, t = 3.149; Supplementary Figure 1B, http://links.lww.com/CM9/B921). In addition, the cell scratch assay and transwell assay were performed to evaluate the effects of YKL40 on HASMC migration. Results showed that the migration of HASMC was significantly induced by YKL40 treatment compared with control (scratch assay, P <0.05, t = 3.605; transwell assay, P <0.05, t = 4.457, Supplementary Figure 1C,D, http://links.lww.com/CM9/B921). Additionally, after YKL40 treatment, the expression

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of MMP-1, 2, 3 was not significantly altered compared with control, while MMP-9 expression was significantly induced by YKL40 in HASMC (mRNA, P <0.05, t = 4.057; protein, P <0.05, t = 3.679; Supplementary Figure 1E,F, http://links.lww.com/CM9/B921). We further explored the effects of YKL40 on the expression of contractile and synthetic markers. The results demonstrated that the expression of contractile markers including α-SMA (α-smooth muscle actin), smooth muscle 22-α (SM22α), calponin, smoothlin, and myosin heavy chain 11 were not markedly altered in HASMC treated with YKL40 compared with control [Supplementary Figure 2, http://links.lww.com/CM9/B921]. The above results indicate that YKL40, as an exogenous stimulus, could induce proliferation, migration, and MMP-9 expression of VSMC, but without switching its phenotype.

We further explored the endogenous role of YKL40 on VSMC function using adenovirus-YKL-40 or ad-negative control (NC). The infection efficacy has been evidenced by reverse transcription quantitative real-time PCR and Western Blotting results, which demonstrated an approximately 1.2 × 10-fold[6] and 1.0-fold elevation in mRNA and protein expression of YKL40, respectively, in VSMCs (mRNA, P <0.001, t = 10.79; protein, P <0.01, t = 6.843, Supplementary Figure 3, http://links.lww.com/CM9/B921). In accordance with VSMCs exogenously treated by recombinant YKL40, YKL-40–overexpressed VSMCs also exhibited notably increased proliferation, migration, and elevated MMP-9 expression without altering the expression of phenotypic markers [Supplementary Figures 4,5, http://links.lww.com/CM9/B921]. The results indicated that YKL40 not only produces an external effect on VSMCs function, but also acts as an internal factor regulating the capacity of proliferation, migration, and matrix breakdown in VSMCs.

We further elucidate the mechanism of YKL40-mediated functional changes in VSMC. RNA sequence was performed on HASMC infected with Ad-YKL-40 or Ad-NC to explore the potential downstream mediators. The results of RNA sequence are demonstrated in Supplementary Figure 6, http://links.lww.com/CM9/B921. The mitogen-activated protein kinase (MAPK) signaling pathway is observed to be markedly altered and has been previously demonstrated to be related to VSMC functional changes. We further verified the bioinformatic results and demonstrated that the phosphorylation level of p38 in HASMC was significantly increased in the Ad-YKL-40 group compared with the Ad-NC group (P-P38, P <0.05, t = 4.201; Supplementary Figure 7A, http://links.lww.com/CM9/B921). The protein level of p38, p53, p-p53, JNK, and p-JNK was not markedly changed upon YKL40 overexpression. It has been demonstrated that the MAPK pathways plays a significant role in regulating the VSMC functional changes. The above results indicated that YKL40 might promote the proliferation, migration, and MMP-9 expression in VSMC via activating p38 MAPK-mediated actions.

Finally, to validate the role of YKL40 in atherosclerosis, Ldlr–/– mice were subjected to tandem stenosis surgery on carotid artery and injected with recombinant YKL40, YKL40 neutralizing antibody, and IgG as control. The administration and feeding panels are summarized in Supplementary Figure 7B, http://links.lww.com/CM9/B921. Mice receiving recombinant YKL40 developed larger necrotic core and decreased fibrous cap content than those receiving IgG in carotid artery, whereas mice neutralized with YKL40 antibody presented an opposing effect compared with control [Figure 1]. Similarly, the recombinant YKL40 group had a notable decrease in

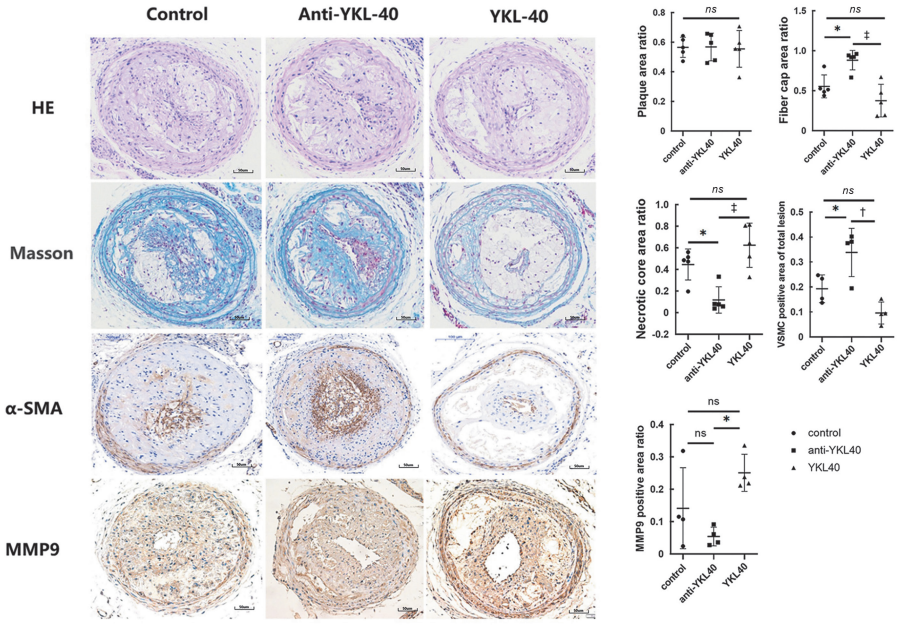

Figure 1: Verification of the *in vivo* effects of YKL40 modulation in carotid atherosclerotic plaque stability. Histological analysis, α-SMA and MMP-9 immunostaining of carotid atherosclerotic plaque in the YKL40-treated, YKL40-neutralized, and control IgG Ldlr–/–mice. Scale bar = 50 μm, n = 5/group, *P <0.05, †P <0.01, ‡P <0.001. α-SMA: α-smooth muscle actin; HE: Hematoxylin and Eosin; IgG: Immunoglobulin G; MMP-9: Matrix metalloproteinase-9; Ldlr–/–: Low density lipoprotein-deficient; *ns*: Not significant; VSMC: Vascular smooth muscle cell.

fibrous content in carotid atherosclerotic plaque while the YKL40 neutralizing group showed a significant increase in the fibrous content compared with the IgG group [Figure 1]. However, there was no difference in total plaque area between the groups. The results indicated that YKL40 modulation could pose a notable effect upon plaque stability without intervening in the progression of atherosclerosis. Furthermore, staining of α-SMA demonstrated significantly more VSMCs in the carotid plaques of mice injected with YKL40 neutralizing antibody compared with control mice. YKL40-treated mice had a nonsignificant decrease in VSMC content compared with control IgG; however, the difference is remarkable between the recombinant YKL40 group and the neutralizing group (P <0.01, q = 7.003; Figure 1). MMP-9 levels were also examined in plaques and the results showed that YKL40-treated mice had a tendency of a larger MMP-9-positive area compared with control IgG but the difference is not significant. Conversely, YKL40-neutralized mice had a nonsignificant opposing effect. Notably, YKL40-treated mice had significantly higher MMP-9 content compared with YKL40-neutralized mice (P <0.05, q = 4.845; Supplementary Figure 7C, http://links.lww.com/CM9/B921), indicating that YKL40 could promote MMP-9 expression *in vivo*. Overall, the results indicated that neutralizing YKL40 might be a viable and potent therapeutic panel for enhancing plaque stability through affecting the function of VSMCs.

Overall, our study found that YKL40 could promote the proliferation, migration, and MMP-9 expression in VSMC potentially via activating P38 MAPK signaling pathway, thus decreasing atherosclerotic plaque stability. Neutralizing YKL40 may present a viable and potent therapeutic strategy in enhancing atherosclerotic plaque stability.

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