# Low shear stress upregulates the expression of fractalkine through the activation of mitogen-activated protein kinases in endothelial cells

Amanguli Ruze[a,b,*], Yiwei Zhao[a,*], Hui Li[c], Xiayimaidan Gulireba[d], Jing Li[a], Dongyu Lei[a], Hongyan Dai[a], Jiang Wu[e], Xin Zhao[f] and Yongmei Nie[a]

**Fractalkine (FKN) is a cytokine which plays an important role in atherosclerosis and other inflammatory diseases. Studies have shown that FKN induces integrin-independent leukocyte adhesion to primary endothelial cells under physiological flow conditions. Further, increased expression of FKN has been demonstrated in atherosclerotic lesions induced by low shear stress. However, the signal transduction mechanisms involved in low shear stress-induced FKN upregulation are not well characterized. In this study, EA.hy926 cells were subjected to varying intensity of fluid shear stress for different time durations. Further, mRNA and protein expressions of FKN were assessed by quantitative real-time PCR and Western blotting, respectively. Upregulation of FKN expression, which was induced via activation of mitogen-activated protein kinases signaling pathway under conditions of low shear stress, was studied both in the presence and absence of inhibitors. Low shear stress (~4.58 dyne/cm$^2$) for more than 1 h promoted FKN expression and activated the extracellular signal-regulated kinase (ERK)1/2, p38, and Jun N-terminal kinase (JNK) mitogen-activated protein kinases signaling pathways by their phosphorylation. Inhibitors of ERK1/2, p38, and JNK pathways downregulated the FKN expression. In this study, fluid shear stress affected FKN expression in endothelial cells via activation of ERK1/2, p38, and JNK in a time-dependent manner. Our findings serve to advance the theoretical basis for prevention and treatment of atherosclerosis. *Blood Coagul Fibrinolysis* 29:361–368**


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[a]Department of Physiology, School of Medicine, Xinjiang Medical University, [b]Department of Cardiology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, [c]Institute of Biology and Medicine, Wuhan University of Science and Technology, Wuhan University, Wuhan, [d]Department of Cardiac Function, Second Affiliated Hospital, Xinjiang Medical University, Urumqi, [e]Institute of Biomedical Engineering, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, Chengdu and [f]Department of Physics, East China Normal University, Shanghai, PR China

Correspondence to Yongmei Nie, PhD, Department of Physiology, School of Medicine, Xinjiang Medical University, Urumqi, PR China
E-mail: nieyongmei@126.com

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## Introduction

Atherosclerosis is a common underlying disorder in several cardiovascular disorders, which accounts for a large proportion of overall morbidity and mortality burden. Inflammation plays a role in all phases of the atherosclerosis [1–4]. Chemokines and adhesive molecules are the main proinflammatory factors that mediate vascular inflammation. Fractalkine (FKN), an identified chemokine with both chemoattractant and adhesive functions, participates in the atherosclerotic pathological process by mediating the activation of inflammatory cells and their aggregation or adhesion to the vascular walls [5–8]. Normal vascular endothelial cells (VECs) have a negligible or nil expression of FKN, as against that in atherosclerotic lesions, in which a high expression of FKN has been reported [9–11]. Knockout of the FKN gene in CCR2$^{-/-}$ mice resulted in a phenotype manifesting reduced atherosclerosis [12]. Endothelial injury is the initial step in the pathogenesis of atherosclerosis [13–15]. VECs form an interface between the blood and vessel wall, the VECs and are subjected to constant blood-flow-induced shear stress. A shear stress of 5–12 dyne/cm$^2$ is essential to maintain the structural and functional integrity of VECs [16]. However, fluid shear stress outside this range injures the VECs, which initiates the atherosclerosis [17]. Atheromas typically occur at bends and bifurcations in which shear stress is low and uneven. Low shear stress and oscillatory shear stress have been shown to promote atherosclerosis in animal models [18]. The alteration in hemodynamics and its effects on cell rheology may be important in the pathogenesis of atherosclerosis [19].

* Amanguli Ruze and Yiwei Zhao contributed equally to the article.

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Mitogen-activated protein kinase (MAPK) signaling pathway is ubiquitous in mammals and is widely involved in several physiological and pathological processes [20]. There are four subfamilies of MAPKs; the most studied members are extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 proteins [21]. Many extracellular stimuli such as physical stress, inflammatory cytokines, growth factors, and bacterial complexes can activate MAPK signaling pathway [22]. Activated MAPK regulates cell proliferation, differentiation, and apoptosis through phosphorylation of nuclear factor (NF)-κB family proteins [23]. In-vitro studies have shown that MAPK signaling mediates low shear-stress-induced oxidative damage, which is crucial in promoting atherosclerosis in human VECs [24]. Furthermore, FKN is an efficient cell-adhesion protein that adheres to endothelial cells under physiological flow conditions and can be upregulated in atherosclerotic lesions induced by low shear stress [25–27]. However, not much is known about the signal transduction mechanisms underlying the low shear-stress-induced FKN upregulation.

In this study, EA.hy926 cells were cultured and exposed to a certain shear stress for varying durations. Expression levels of FKN mRNA and protein under shear stress were measured. Further, we also assessed the effect of specific inhibitors of ERK1/2, p38, and JNK pathways on the expression of FKN under low shear stress conditions.

## Methods

### Cell culture

EA.hy926 cell line was purchased from Shanghai Cell Bank (Shanghai, China) and was cultured in HyClone Medium 199 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), including 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml); the medium was changed every 48 h until the cells reached confluence. Then the cells were detached with 0.25% trypsin-ethylenediamine tetraacetic acid (Gibco-BRL, Grand Island, New York, USA), and the cell suspension was transferred onto Fibronectin (BD, Franklin Lakes, New Jersey, USA)-coated sterile glass slides and incubated in 37 °C humidifed incubator until the cells adhered to the wall. Subsequently, cells were serum starved for 8 h before every single experiment. For cell signaling pathway experiments, cells were pretreated with the MAPK pathway inhibitors PD98059, SB203580, and SP600125 (Sigma, Ronkonkoma, New York, USA) for 30 min before exerting the shear stress.

### Exposure of EA.hy926 cells to shear stress

The parallel plate flow chamber was designed on the basis of the model by Stephen-G [28]. Briefly, varying intensities of shear stresses (0, 2.62, 4.58, 6.54, 10.47, 15.71, and 19.64 dyne/cm$^2$) were applied to EA.hy926 cells grown on glass slides for fixed time (2 h). In separate experiments, a fixed intensity of shear stress (4.58 dyne/cm$^2$) was applied for different time durations (0, 0.5, 1, 2, 3, 5, 7, 9, and 11 h). Cells and perfusate were collected for further analysis.

### Quantitative real-time PCR analysis

Total RNA was extracted from EA.hy926 cells using Trizol reagent (Invitrogen, Waltham, Massachusetts, USA), and its quality was confirmed by Nanodrop 2000 (Thermo Scientific, USA). Template complementary DNA (cDNA) was synthesized from 1 μg of RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Two microliters of the template cDNA was added to PCR reaction mixture (25 μl) containing 2 × SYBR Green PCR master mix (12.5 μl) (QIAGEN, Germantown Maryland, USA) and the primers (10 μmol/l) for PCR. The following primers were used (Shanghai Biological Engineering Company, Shanghai, China): FKN,

Forward, 5′-GACCCCTAAGGCTGAGGAAC-3′

Reverse, 5′-CTCTCCTGCCATCTTTCGAG-3′; and

β-actin,

Forward, 5′-TGGCACCCAGCACAATGAA-3′,

Reverse, 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′.

The PCR conditions were as follows: one cycle of 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Experiments were performed as triplicates, including non-Reverse Transcriptase and nontemplate controls. Dissociation analysis confirmed the specificity of the reaction.

### ELISA

After each experiment, the perfusate (in serum-free HyClone Medium 199) was harvested, kept at −80 °C for 48 h, and placed in a vacuum freeze-drier to reduce its volume. The whole-dried solid powder was weighed, placed in a tube, and dissolved in 1 ml distilled water and a diluted solution (200 ng/ml) of which was prepared before detection. FKN concentration was determined using a human chemotactic factor CX3CL1 (FKN) ELISA kit (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions. The microplate reader was used at an optical density of 450 nm to measure the absorbance (Thermo Scientific Multiskan Spectrum, Vantaa, Finland).

### Western blot analysis

Cells were washed in ice-cold PBS and lysed in RIPA buffer (Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, USA). The lysates were centrifuged at 12 000 × *g* for 10 min at 4 °C, and supernatants were collected. The protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA)

according to the manufacturer's instructions. In brief, 30 μg of protein was separated using 12% SDS–PAGE gel (Solabri, Beijing, China) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% BSA (Boshide, Wuhan, China) in Tris Buffered Saline Tween (TBST) at room temperature (RT) for 1 h, then incubated overnight at 4 °C with the following primary antibodies: ERK1/2 (1 : 1000), phospho-ERK1/2 (1 : 1000), P38 (1 : 1000), phospho-p38 (1 : 1000), JNK (1 : 1000), phospho-JNK (1 : 1000), β-actin (1 : 1000), glyceraldehyde phosphate dehydrogenase (1 : 1000) (all from Cell Signaling Technology, Danvers, Massachusetts, USA). The membranes were washed three times in TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1 : 5000; Zhongshan Golden Bridge Biotechnology, China) for 2 h at RT. Immunoreactive bands were visualized by enhanced chemiluminescence substrate (Thermo Fisher Scientific, USA), and the band intensities were analyzed using Image J software (National Institute of Health, Bethesda, Maryland, USA).

### Statistical analysis

All results were expressed as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA), least significant difference test, or by multiple ANOVA using SPSS software version 17.0 (SPSS Inc., Chicago, Illinois, USA). A value of $P$ less than 0.05 was considered as statistically significant.

## Results

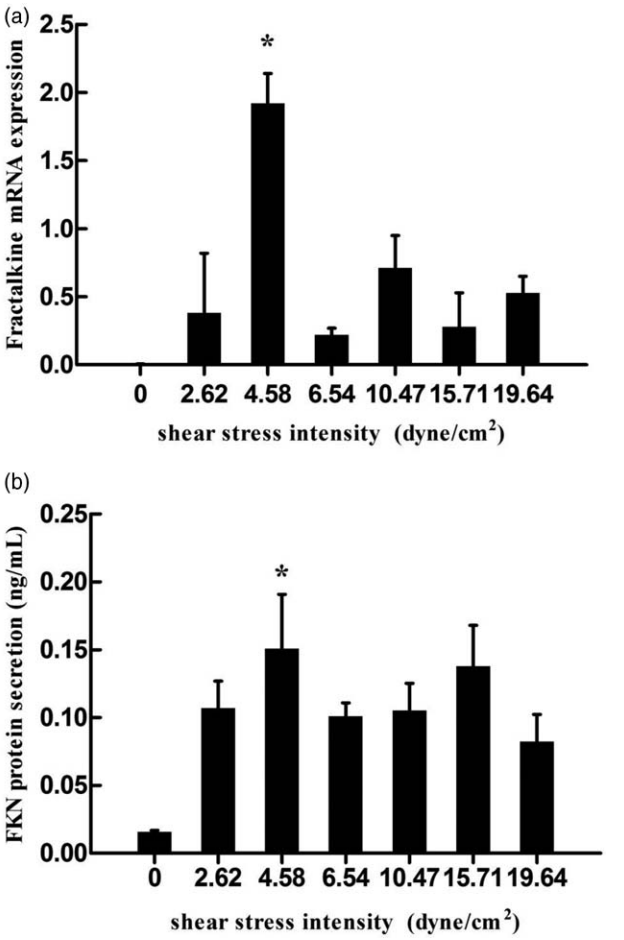
### Identification of the EA.hy926 cell line: supplementary data

To confirm if EA.hy926 cells showed characteristic of endothelium, EA.hy926 cell is used for two to six passages following which the morphology, ultrastructure/Weibel–Palade bodies, and stained factor-VIII-related antigen were examined using optical microscopy (OLYMPUS-CKX41, Tokyo, Japan) (Sup Fig. 1A, http://links.lww.com/BCF/A48), transmission electron microscopy (LEICA-DMI4000B, Wetzlar, Germany) (Sup Fig. 1B, http://links.lww.com/BCF/A48) and immunohistochemistry and hematoxylin (Sup Fig. 1C–D, http://links.lww.com/BCF/A48), respectively. All tests showed that the factor-VIII-related antigen was strongly expressed in cells which confirmed that the EA.hy926 cells had the characteristics of endothelium.

### Effect of shear stress intensity on fractalkine expression

To assess the effect of shear stress intensity on FKN gene expression, a flow rate of 0–19.64 dyne/cm$^2$ was used for 2 h. Each slide was exposed to shear stress separately, and each experiment was repeated by using five separate slides. After each experiment, fluid was collected to measure the concentration of FKN in the perfusate. The cells were harvested and total RNA was extracted for quantitative real-time PCR (qRT-PCR). FKN mRNA

Fig. 1



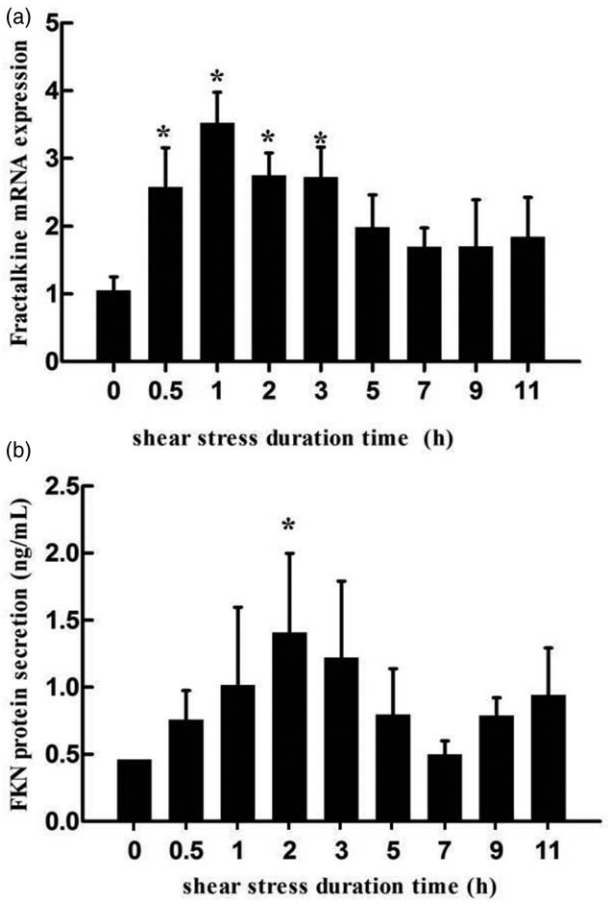

Effect of shear stress intensity on fractalkine expression. (a) mRNA expression of fractalkine in EA.hy926 cells subjected to shear stress of different intensities (0, 2.62, 4.58, 6.54, 10.47, 15.71, and 19.64 dyne/cm$^2$) for a fixed time (2 h). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (panel a1). (b) Results of ELISA showing fractalkine expression levels (ng/ml) in the perfusate collected from EA.hy926 cells subjected to shear stress of different intensities (0, 2.62, 4.58, 6.54, 10.47, 15.71, and 19.64 dyne/cm$^2$) for 2 h (panel b1). (*$P < 0.05$; $n = 3$ in each group). FKN, fractalkine.

expression (Fig. 1a) was significantly higher at 4.58 dyne/cm$^2$ than that at other intensities of shear stress ($P < 0.05$, $n = 3$) and FKN protein expression (Fig. 1b) was significantly higher at 4.58 dyne/cm$^2$ than that at other intensities except 15.71 dyne/cm$^2$. These results indicated that FKN gene was more sensitive to a shear stress of 4.58 dyne/cm$^2$.

### The effect of shear stress duration on fractalkine expression

To assess the effect of the duration of exposure to shear stress on FKN expression, we exposed the cells to a fixed shear stress of 4.58 dyne/cm$^2$ for a range of time durations (0, 0.5, 1, 2, 3, 5, 7, 9, and 11 h). Each experiment was repeated using five separate slides. After each

**Fig. 2**




Effect of the duration of exposure to shear stress on fractalkine expression. (a) mRNA expression of fractalkine in EA.hy926 cells subjected to a fixed intensity of shear stress (4.58 dyne/cm$^2$) applied for different time durations (0, 0.5, 1, 2, 3, 5, 7, 9, and 11 h). (b) Concentration of fractalkine in the perfusate after exposure to shear stress of 4.58 dyne/cm$^2$ for different durations of time (0, 0.5, 1, 2, 3, 5, 7, 9, and 11 h) (*$P < 0.05$; $n = 3$ in each group). FKN, fractalkine.

experiment, fluid was collected to measure the concentration of FKN in the perfusate, and mRNA was extracted from cells for qRT-PCR. At a shear stress of 4.58 dyne/cm$^2$, the FKN mRNA expression in the cells increased and reached a peak at 1 h of application of the shear stress (Fig. 2a). The FKN secreted in the perfusate increased over time, and the FKN protein level peaked at 2 h (Fig. 2b). The results of one-way ANOVA were significant ($P < 0.05$, $n = 3$), which indicated that expression of FKN gene was sensitive to a shear stress of 4.58 dyne/cm$^2$ and that the effect also depends on the duration of exposure to shear stress.

### Low shear stress activates mitogen-activated protein kinases signaling

To determine the relationship between low shear-stress-induced FKN expression and MAPK signal transduction pathways, levels of phosphorylated ERK1/2, p38, and JNK were measured in the cells exposed to low shear stress for different time intervals (0, 5, 10, 30, and 60 min). As shown in Figs. 3–5, low shear stress promoted ERK1/2, p38, and JNK phosphorylation in a time-dependent manner. Phosphorylation of ERK1/2 reached a peak level at 5 min and began to decline thereafter (Fig. 3a). When pretreated with PD98059 before low shear stress (4.58 dyne/cm$^2$) intervention, ERK1/2 activation was inhibited and decreased significantly when compared with control group (Fig. 3b). Highest phosphorylation of p38 was observed at 30 min (Fig. 4a) under low shear stress (4.58 dyne/cm$^2$), and this p38 activation process was inhibited when pretreated with SB203580 (Fig. 4b). JNK phosphorylation was enhanced with the extending of low shear stress exposure time and reached a peak level at 60 min (Fig. 5a); this progression was suppressed by JNK-specific inhibitor SP600125 (Fig. 5b).

### Mitogen-activated protein kinase inhibition deregulated low shear-stress-induced fractalkine expression

Different MAPK inhibitors were used before exposure to shear stress to assess their effect on the mRNA expressions of FKN induced by low shear stress. Cellular RNA was extracted for qRT-PCR analysis, respectively. qRT-PCR results showed that PD98059, SB203580, and SP600125 downregulated the activation of ERK1/2, p38, and JNK in EA.hy926 cells subjected to shear stress of 4.58 dyne/cm$^2$. In addition, downregulation of FKN expression was seen in stress-exposed cells as compared with that observed in the control without shear stress (Fig. 6a–c). In other words, FKN expression in the PD98059, SB203580, and SP600125 groups was considerably lower than that in the low shear stress group. The change in FKN mRNA expressions after treatment with different MAPK inhibitors indicated the involvement of MAPK signaling pathways in low shear-stress-induced FKN upregulation.

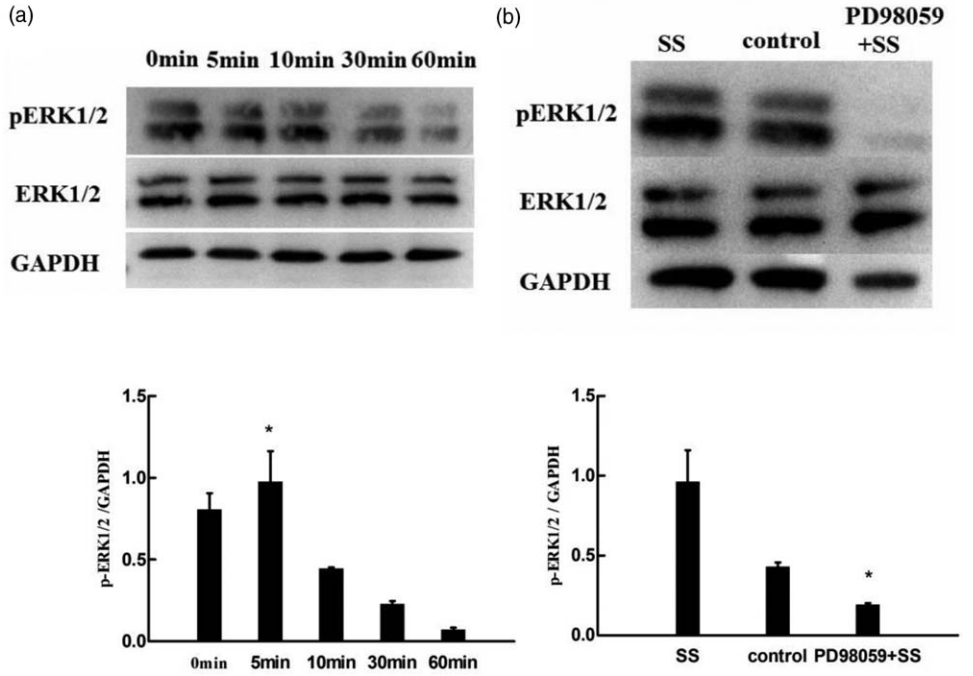
## Discussion

Endothelial cell dysfunction was shown to be caused by disordered shear stress. Shear stress refers to the frictional force exerted by the flowing blood onto the vascular wall, which is thought to play a critical role in angiogenesis, vascular remodeling [29], and atherosclerosis [30].

In the current study, low shear stress (4.58 dyne/cm$^2$) was shown to affect FKN expression in endothelial cells; further, low shear stress increased ERK1/2, p38, and JNK activation in a time-dependent manner. Furthermore, this activation was inhibited by specific inhibitors, which resulted in a downregulation of the FKN expression induced by low shear stress.

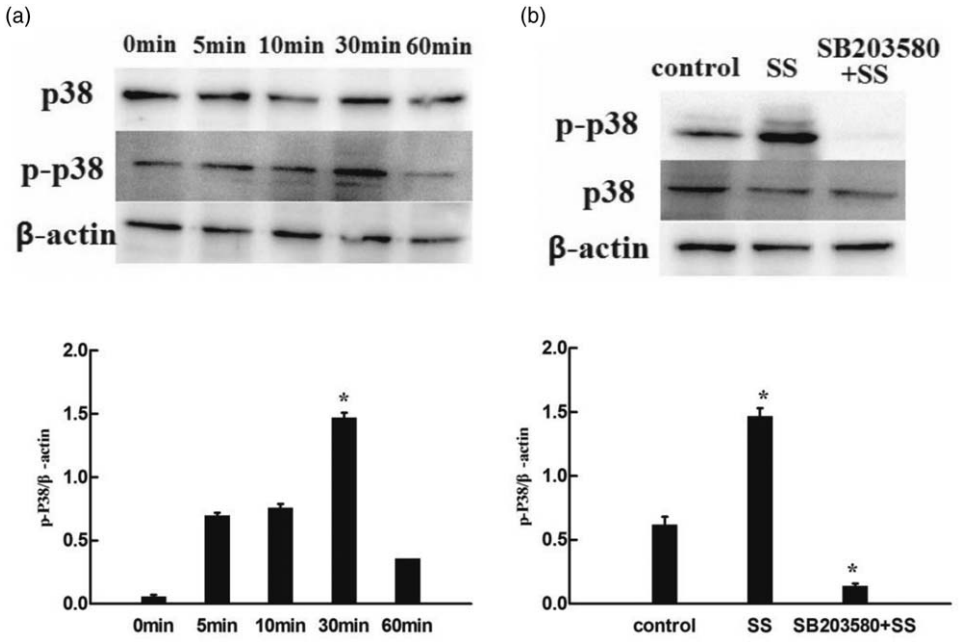
Higher shear stress protects endothelial cell function [31–33] which may reduce the process of atherosclerosis. On the contrary, low shear stress may cause endothelial cell damage and atherosclerosis by promoting secretion of vasoactive and inflammatory mediators such as ET-1,

**Fig. 3**



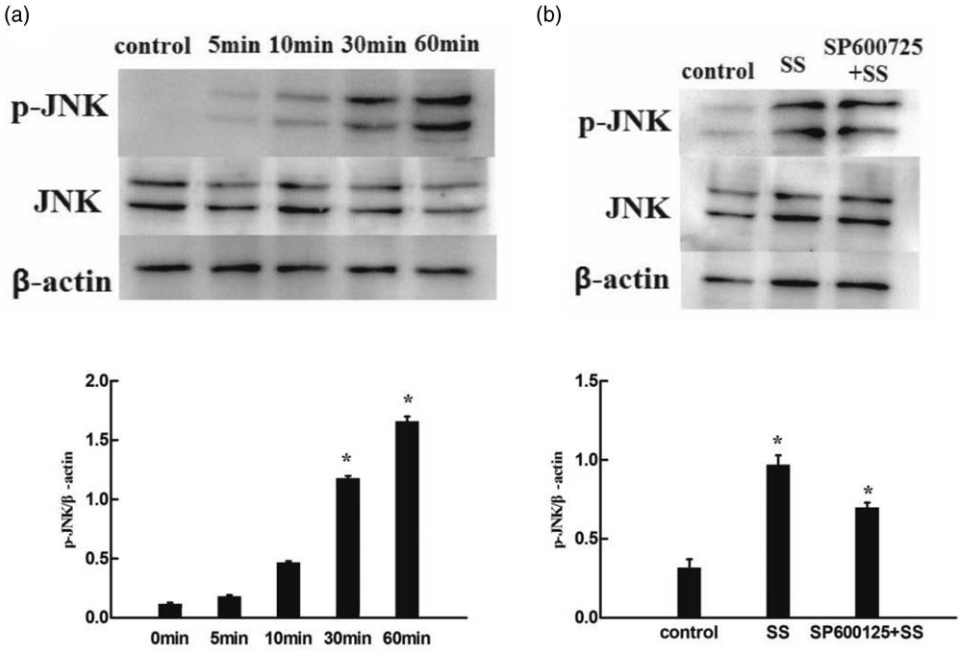

Low shear stress activates extracellular signal-regulated kinase 1/2 in human umbilical vein endothelial cells. (a) Results of Western-blot showing expression levels of extracellular signal-regulated kinase 1/2 and phosphorylated extracellular signal-regulated kinase 1/2 after different durations (0, 5 10, 30, 60 min) of exposure to low shear stress (4.58 dyne/cm$^2$); (b) Inhibition of p-extracellular signal-regulated kinase 1/2 after PD98059 treatment when exposed to 4.58 dyne/cm$^2$ of shear stress for 5 min ($*P < 0.05$; $n = 3$ in each group). ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase1/2; HUVECs, human umbilical vein endothelial cells; p-ERK1/2, phospho-extracellular signal-regulated kinase1/2.

**Fig. 4**




Low shear stress activates p38 in human umbilical vein endothelial cells. (a) Expressions of p38 and phosphorylated p38 after exposure to 4.58 dyne/cm$^2$ of low shear stress for different durations (0, 5 10, 30, 60 min); (b) Inhibition of p38 after SB203580 treatment when exposed to 4.58 dyne/cm$^2$ of shear stress for 30 min ($*P < 0.05$; $n = 3$ in each group). HUVECSs, human umbilical vein endothelial cells.

**Fig. 5**




Low shear stress activates Jun N-terminal kinase in human umbilical vein endothelial cells. (a) Results of Western-blot showing expression levels of Jun N-terminal kinase and phosphorylated Jun N-terminal kinase after exposure to 4.58 dyne/cm$^2$ of low shear stress for different durations (0, 5 10, 30, 60 min); (b) Inhibition of p-Jun N-terminal kinase after SP600125 treatment when exposed to 4.58 dyne/cm$^2$ of shear stress for 60 min (*$P < 0.05$, $n = 3$ in each group). HUVECSs, human umbilical vein endothelial cells; JNK, Jun N-terminal kinase; p-JNK, phospho-Jun N-terminal kinase.

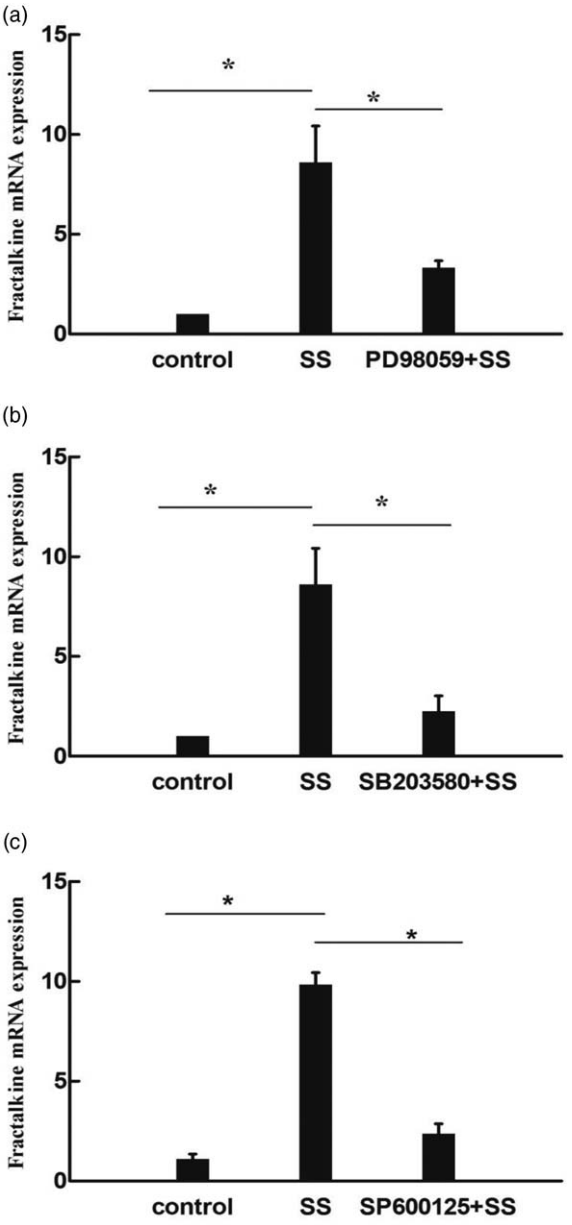
VEGF, IL-8, MCP-1, and lipocalin-type prostaglandin D2. Low shear stress may also increase oxidative stress and induce subendothelial accumulation of LDL [34–36]. Recent studies have shown that FKN, a unique chemotactic factor, has both chemoattractant and adhesive functions. It is upregulated in low shear-stress-induced atherosclerotic lesions [25]. However, not much is known about the underlying molecular mechanisms.

FKN is the sole member of the CX3C subfamily of chemokines which exists in both membrane-bound and soluble forms [8,37]. It has been shown to be associated with a variety of inflammatory diseases [38,39]. In a recent study, Nie and Chen [40] demonstrated increased expression of FKN and its receptor CX3CR1 in the aortic-arch atherosclerotic plaques in a rabbit model of atherosclerosis. Inhibition of expression of FKN and CX3CR1 was shown to alleviate the severity of atherosclerosis [12,38,41]. These findings indicate that FKN/CX3CR1 pair is closely involved in the pathogenesis of atherosclerosis.

We used EA.hy926 cells as an in-vitro model and examined the effect of shear stress (range, 0–19.64 dyne/cm$^2$) on FKN expression. FKN mRNA expression and protein secretion significantly increased at a shear stress level of 4.58 dyne/cm$^2$. FKN protein expression in the perfusate was consistent with FKN gene expression level. FKN expression began to increase within 30 min of loading and peaked at 1 h at shear stress level of 4.58 dyne/cm$^2$. In parallel with the increase in FKN expression, endothelial cells released FKN into the perfusate. In a recent study, expression of ICAM-1, an important mediator of adhesion responses, was shown to be regulated by low shear stress in a time-dependent manner. In the current study, expression of FKN, an adhesion molecule like ICAM-1, showed a similar response to low shear stress. Our results show that low shear stress may induce inflammatory response during the development of atherosclerosis. Subsequently, we investigated the mechanism by which low shear stress induced expression of FKN.

Studies have shown that low shear stress increased expression of inflammatory cytokines through activation of JNK and NF-κB. We investigated the mediatory role of the MAPK pathway in low shear-stress (4.58 dyne/cm$^2$)-induced FKN upregulation using relative signal pathway inhibitors and the results obtained by qRT-PCR analysis. Low shear stress promoted ERK1/2, p38, and JNK phosphorylation in a time-dependent manner, and this activation was inhibited by their corresponding inhibitors. This indicates that low shear stress may stimulate MAPK signaling pathways in endothelial cells. Of note, all inhibitors could downregulate the expression of FKN. This result too demonstrated the involvement of MAPK signaling

**Fig. 6**




Effect of PD98059, SB203580, and SP600125 on fractalkine mRNA expression induced by low shear stress. (a)–(c) Results of real-time PCR showing changes in fractalkine expression after treatment with mitogen-activated protein kinases inhibitors (PD98059, SB203580, and SP600125) when exposed to 4.58 dyne/cm$^2$ of shear stress for 5, 30, 60 min, respectively. ($*P < 0.05$; $n = 3$ in each group). FKN, fractalkine; MAPK, mitogen-activated protein kinases; RT-PCR, real-time PCR.

pathways in mediating the increase in the expression of FKN induced by low shear stress, and that ERK1/2, p38, and JNK pathways were associated with endothelial dysfunction in cells exposed to shear stress.

It could be inferred that the low shear-stress-induced FKN expression promoted atherosclerosis through the above-mentioned mechanism, at sites subjected to 4.58 dyne/cm$^2$ of shear stress (e.g., at vascular bifurcation). However, as FKN expression was reduced under high shear stress, it may contribute to antiatherosclerotic processes. Indeed, it is conceivable that MAPK inhibitors may be used as potential antiatherosclerotic therapeutic targets. However, cellular signaling pathways typically involve a cascade of subpathways such as NF-κB and $Ca^{2+}$ signaling pathways, which increases the complexity of their interlinkages; it is very difficult to exclude factors other than the MAPK pathways. To further characterize low shear stress-induced FKN expression, other molecular mechanisms such as those involving the cytoskeleton, integrin, and ion channels should also be investigated.

In this study, exposure to low shear stress (of the order of 4.58 dyne/cm$^2$) induced a time-dependent increase in FKN synthesis and secretion by endothelial cells. Low shear stress appeared to activate ERK1/2-related, p38-related, and JNK-related MAPK signaling pathways, which in turn induced upregulation of FKN expression. Inhibitors of ERK1/2, p38, and JNK pathways down-regulated FKN expression induced by low shear stress. Our findings serve to advance the theoretical basis for prevention and treatment of atherosclerosis.

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Expression of FKN in HUVECs under low shear stress.

### Conflicts of interest

There are no conflicts of interest.

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