Laminar shear stress inhibits CXCR4 expression on endothelial cells: functional consequences for atherogenesis

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SPECIFIC AIMS

Laminar shear stress (LSS) represents a major atheroprotective stimulus, as it inhibits endothelial cell death and has antioxidant and antithrombotic activities. As the underlying mechanisms are still poorly characterized, we aimed at investigating the role of chemokine receptors in LSS-mediated biological activities on endothelial cells (ECs).

PRINCIPAL FINDINGS

1. Laminar shear stress strongly inhibited CXCR4 expression on human primary endothelial cells

Chemokine receptor expression in response to 15 dynes/cm^2/s^{-1} LSS was evaluated on human umbilical vein endothelial cells (HUVEC) by RNase protection assay (RPA). The CXCR4 message was strongly and rapidly down-regulated by LSS treatment at 0.5–16 h at all time points (up to 93.6%±0.5) (**Fig. 1*A, C, D***) whereas mRNA levels of other chemokine receptors expressed by ECs (i.e., CCR2 and CCR8) were not affected (Fig. 1*B*). We demonstrated that LSS-induced CXCR4 down-regulation occurred at the transcriptional but not at the post-transcriptional level (not shown). In contrast, CXCR4 message levels were unmodified at 2 and 5 h of treatment and only slightly decreased (20.7±0.6%) at 16 h in respect to static control cells (Fig. 1*E*) after exposure of ECs to 4 dynes/cm^2/s^{-1} (low) shear stress. SDF-1 mRNA levels were not modulated by 2–16 h of LSS treatment (Fig. 1*F*).

2. Laminar shear stress treatment inhibited SDF-1 mediated chemotaxis of HUVECs

We next investigated whether the CXCR4 down-regulation induced by LSS that we observed at the protein level (not shown) may be of functional significance and evaluated the responsiveness of LSS-treated HUVECs to the CXCR4 specific agonist SDF-1. HUVECs were subjected to LSS for 16 h or kept in static conditions and analyzed for their ability to migrate in response to SDF-1 in a 48-well microchamber assay. SDF-1-directed chemotaxis after LSS was totally inhibited at SDF-1 concentration of 10–500 ng/mL (not shown). HUVECs' chemotaxis in response to the CCR8-restricted chemokine I-309/CCL1, whose receptor expression in HUVECs was not regulated by LSS, was not significantly affected (not shown).

3. CXCR4 overexpression induced caspase-dependent and SDF-1-inducible ECs apoptosis. CXCR4 overexpression inhibited LSS-induced protection from apoptosis on ECs

CXC Chemokine receptor (CXCR) 4 signaling has been reported to modulate cell chemotaxis, survival, and apoptosis. As gp120 was shown to induce cellular apoptosis in ECs partly via CXCR4, we hypothesized that SDF-1-induced CXCR4 signaling in HUVECs may be proapoptotic (**Fig. 2*A***) and that LSS-triggered CXCR4 down-regulation may contribute to cell survival in response to LSS.

We found that CXCR4 overexpression significantly inhibited the antiapoptotic effect of LSS (Fig. 2*B*) and promoted a caspase-dependent (Fig. 2*C, D*) and SDF-1-inducible cellular apoptosis (Fig. 2*F*) that was dependent on CXCR4 expression levels (Fig. 2*E, F*). Such CXCR4-mediated effect was associated to the impairment of LSS-induced ERK1/2 phosphorylation, a key event in cell survival (not shown). These results suggest

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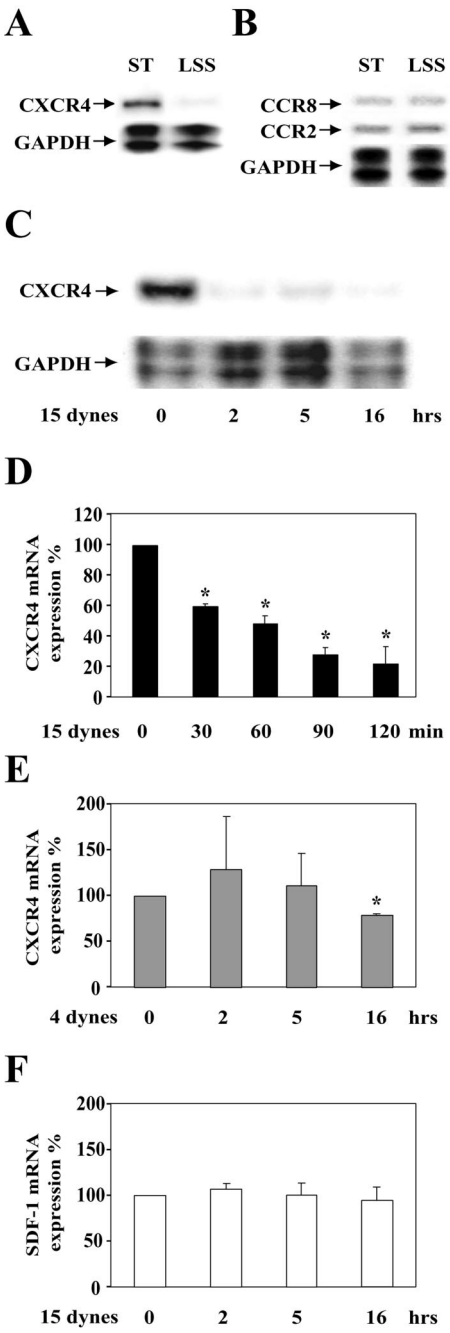

Figure 1. Laminar shear stress inhibits CXCR4 expression in HUVECs. Effect of 15 dynes/cm^2/s^{-1} laminar shear stress (LSS) on chemokine receptors expression on HUVECs as assessed by RPA. LSS down-regulated CXCR4 for 6 h ($P<0.01$) *(A)* but had no significant effect on CCR2 and CCR8 ($P>0.05$) *(B)* mRNA expression. *C)* Representative experiment of CXCR4 mRNA expression of LSS-treated HUVEC for 2, 5, and 16 h. GAPDH message was used as an internal control. *D)* Densitometric analysis of CXCR4 mRNA expression of LSS-treated vs. ST (0 h) cells. Results represent the mean ± SD of 3 independent experiments. 30 min: $P < 0.01$; 60 min: $P < 0.01$; 90 min: $P < 0.01$; 120 min: $P < 0.01$. *E)* Effect of (4 dynes/cm^2/s^{-1}) shear stress on CXCR4 expression for 2 h ($P>0.05$), 5 h ($P>0.05$), and 16 h ($P<0.05$). Results represent mean ± SD of 3 independent experiments. *F)* Densitometric analysis of SDF-1 mRNA expression of LSS-treated vs. ST (0 h) as assessed by RT-PCR. No significant differences were observed in expression levels at 2, 5, and 16 h vs. control cells ($P>0.05$). Results represent mean ± SD of 3 independent experiments. *$P < 0.05$.

that CXCR4 down-regulation may be a mediator of the antiapoptotic effect of LSS.

4. SDF-1 treatment and CXCR4 overexpression in HUVECs induce MCP-1 and IL-8 mRNA expression. CXCR4 overexpression impaired the LSS-induced inhibition of MCP-1 expression

Chemokine receptors and their ligands, such as CCR2/MCP-1 and CXCR2/IL-8, have been shown to play an important role in atherosclerotic lesion development. The inhibition of MCP-1 expression by steady laminar SS (>5 h treatment) in HUVECs has been hypothesized to contribute to the atheroprotective effect of LSS.

In our study, CXCR4-overexpressing ECs enhanced MCP-1 and IL-8 mRNA expression as measured by RNase protection assay (RPA) (not shown). Similarly, SDF-1 treatment of HUVEC for 2 and 5 h increased MCP-1 and IL-8 mRNA expression (not shown). LSS-induced inhibition of MCP-1 message was impaired in CXCR4-expressing cells (not shown).

5. CXCR4 protein was abundantly expressed by the atherosclerotic plaque endothelium of human carotid arteries while barely expressed by minimally diseased carotid arteries

Under the conditions of the present study, CXCR4 expression was maximal in static control cells, which mimic the low absent flow that can be found at sites of atherosclerotic lesion development and strongly down-regulated in ECs exposed to LSS. We therefore analyzed CXCR4 expression in human atherosclerotic carotid arteries vs. minimally diseased carotid arteries obtained from patients undergoing endoarterectomy.

CXCR4 was abundantly expressed by the plaque endothelium while its expression was low in minimally diseased endothelium (not shown).

CONCLUSIONS AND SIGNIFICANCE

The endothelial lining of the vasculature plays an important role in sensing blood flow perturbations leading to fine-tuning of gene expression, which in turn regulates blood flow and endothelial cell function. LSS inhibits endothelial cell proliferation and cellular apoptosis induced by growth factor depletion, tumor necrosis factor (TNF), or hydrogen peroxide exposure. The antiapoptotic effect of laminar shear stress is mediated, in part, by the up-regulation of superoxide dismutase and activation of nitric oxide synthase and, ultimately, by nitric oxide (NO) production leading to the inhibition of caspase activation.

Atherosclerotic lesions are preferentially found in areas with low or turbulent shear stress while areas exposed to steady LSS are protected by plaque formation. A large body of evidence suggests that endothelial apoptosis contributes to the development of atherosclerotic lesions in areas of low or turbulent flow with a prevalent occurrence of apoptosis in the downstream part of the plaque.

Endothelial apoptosis is proadhesive and promotes smooth cell migration from the media to the intima, an important event in intimal thickening. Further, it stimulates plaque erosion leading to thrombosis and to the establishment of acute coronary syndromes.

Our findings suggest that CXCR4-mediated signals may trigger apoptosis in primary endothelial cells provided sufficient levels of CXCR4 are present. Our data suggest that LSS keeps CXCR4 expression on the endothelium at lower levels than in endothelial cells subjected to low/absent shear stress. This may help regulate endothelial cell survival and integrity, thus contributing to protection toward the development of atherosclerotic lesions.

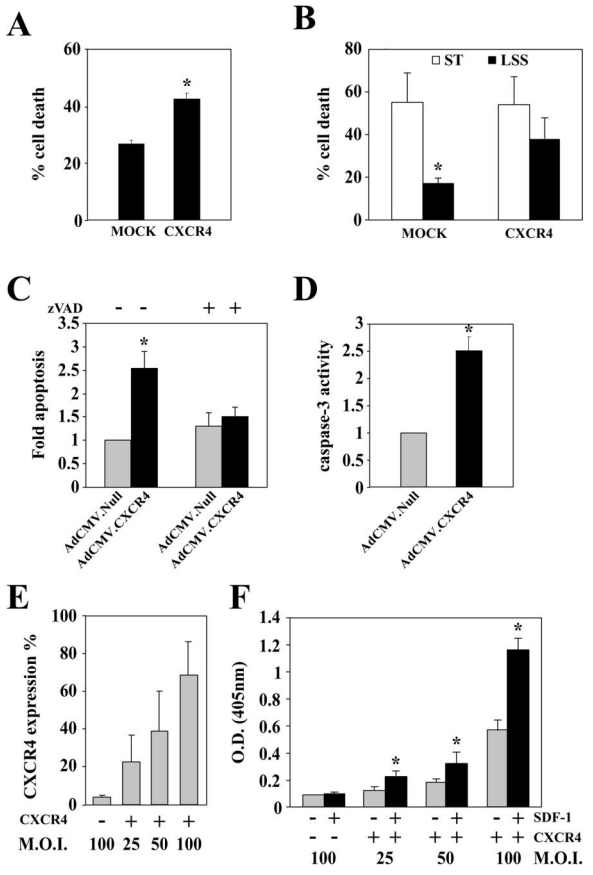

Figure 2. CXCR4 overexpression inhibits LSS antiapoptotic activity. CXCR4 overexpression induces a caspase-dependent, CXCR4 expression level-dependent and SDF-1 inducible cell apoptosis. *A)* HUVECs were transiently transfected in complete medium with a CXCR4-expressing plasmid (CXCR4) or with an empty control vector (MOCK). Cells were harvested after 48 h from transfection and the percentage of cell death was determined by PI staining of GFP-positive cells by FACS analysis. Mean cell death ± SD of 5 experiments is shown. *B)* Serum-deprived transfected cells were exposed to LSS for 16 h or kept in ST. % of cell death was analyzed as above. *C)* Cells were infected with a CXCR4-expressing adenovirus vector (AdCMV.CXCR4) or with a control vector (AdCMV.Null), cultured in the absence (–) or presence (+) of the caspase inhibitor zVADfmK (100 μM) for 48 h, and the percent of cell death analyzed by PI uptake. Results are expressed as fold induction of apoptosis of AdCMV.null-infected cells. *D)* CXCR4 overexpression increased caspase 3 activity by 2.5-fold ($P<0.01$). Experiments performed in triplicate. *E)* Dose-dependent surface CXCR4 expression in cells infected with 25–100 MOI AdCMV.CXCR4. The level of CXCR4 expression on infected cells was detected with 12G5 anti-CXCR4 antibodies by FACS analysis. *F)* Cells were infected with 25, 50, 100, MOI of AdCMV.CXCR4 or with 100 MOI of AdCMV.Null, cultured in the absence (–) or presence (+) of 100 ng/mL SDF-1 for 48 h, and apoptosis measured by nucleosome fragmentation determination. O.D., optical density. Results represent the average of 3 experiments and are expressed as corrected mean value of absorbance at 405 nm. The dose-dependent CXCR4-induced apoptosis (AdCMV.CXCR4-infected cells vs. AdCMV. Null-infected cells) was further increased by SDF-1 treatment (AdCMV.CXCR4-infected HUVECs, black bars vs. gray bars) ($P < 0.05$). *$P < 0.05$.

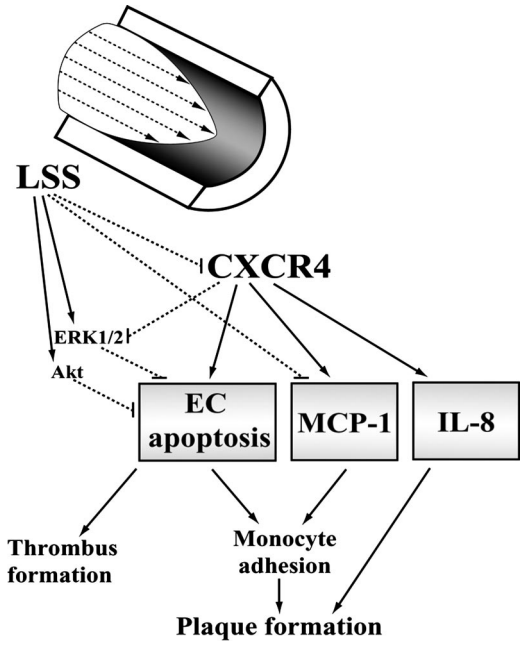

Figure 3. Schematic diagram. LSS-induced inhibition of CXCR4 expression and function may contribute to the antiatherogenic effect of laminar flow by affecting endothelial cell survival and proatherogenic chemokine production.

MCP-1 down-regulation exerted by constant levels of laminar shear stress is hypothesized to play a role in maintaining atherosclerotic lesion-free areas under steady flow. CXCR4 signaling induced an up-regulation of MCP-1 and IL-8 mRNA levels in endothelial cells and impaired LSS-induced inhibition of MCP-1 message.

In agreement with our hypothesis that CXCR4 expression may play a role in atherosclerotic plaque development, we found high levels of CXCR4 expression in endothelial cells from human carotid artery atherosclerotic lesions. In contrast, CXCR4 expression was low in minimally diseased carotid artery endothelium.

The results of the present study suggest that the antiatherogenic effect of laminar shear stress may be mediated, in part, through the down-regulation of CXCR4 (**Fig. 3**), thereby affecting the expression of proatherogenic chemokines and the integrity of the endothelial barrier. FJ