Laminar Shear Stress Upregulates the Complement-Inhibitory Protein Clusterin
A Novel Potent Defense Mechanism Against Complement-Induced Endothelial Cell Activation

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Background—The complement system is implicated in the pathogenesis of atherosclerosis. Complement has been shown to activate endothelial cells (ECs) by inducing a proinflammatory response. Physiological levels of shear stress exert potent antiatherosclerotic effects. Therefore, we investigated whether shear stress antagonizes the effects of complement on ECs.

Methods and Results—Incubation of ECs with nonlytic concentrations of complement serum (CS: 0.2 U/mL for 6 hours) resulted in an upregulation of interleukin-8 (IL-8) (165±12%) and monocyte chemoattractant protein-1 (MCP-1) mRNA expression (267±6%). Preexposure of ECs for 18 hours with laminar shear stress (15 dyn/cm²) abrogated CS-induced IL-8 release to 106±10% (P<0.001) and reduced CS-induced MCP-1 expression (170±31%; P<0.05). To examine the mechanism of the protective effect of shear stress, expression of the complement-inhibitory protein clusterin was analyzed under shear exposure. Shear stress increased clusterin mRNA (225±76%, 6 hours) and protein expression (164±22%, 18 hours). Specific inhibition of clusterin by transfection with antisense oligonucleotides reversed the protective effect of shear stress on CS-induced MCP-1 and IL-8 upregulation (P<0.05 versus sense-transfected cells). Moreover, clusterin overexpression inhibited CS-induced EC activation.

Conclusions—Shear stress abrogates the complement-induced proinflammatory response of ECs by upregulation of the complement-inhibitory protein clusterin. Upregulation of clusterin may contribute to the potent antiatherosclerotic effects of shear stress by preventing endothelial activation through the complement cascade. (Circulation. 2000;101:352-355.)

Key Words: atherosclerosis ■ shear stress ■ endothelium ■ cells ■ complement

ACTIVATION OF THE COMPLEMENT SYSTEM PLAYS A KEY ROLE IN THE PATHOGENESIS OF ATHEROSCLEROSIS.1 Not only can complement complexes be detected in atherosclerotic lesions,2 but more importantly, complement deficiency prevents the formation of atherosclerotic lesions.3 Although the exact mechanisms by which complement exerts its proatherosclerotic effects are not yet clear, several studies suggest that the complement complex activates the endothelial cells (ECs), thereby inducing a proinflammatory response. Exposure of ECs to complement components triggers the induction of proinflammatory cytokines, such as interleukin (IL)-6 or IL-8.4 Moreover, monocyte chemoattractant protein-1 (MCP-1), which contributes causally to the formation of atherosclerotic lesions,5 is released by ECs in response to complement.4

A major endogenous atheroprotective signal is provided by the intraluminal flow (shear stress) acting on the endothelial monolayer, as evidenced by the preferential development of atherosclerotic lesions in areas with low or unsteady shear stress.6 Exposure of ECs to fluid shear stress modulates gene expression and thereby regulates endothelial structure and function.6-9 Therefore, we investigated the effect of shear stress on complement-induced upregulation of IL-8 and MCP-1 as indicators of EC activation.

Methods

Human umbilical vein ECs (HUVECs; CellSystems/Clonetics) were cultured (third passage) and exposed to shear stress in a cone-and-plate apparatus as previously described.10 HUVECs were incubated in endothelial basal medium complete medium containing 1% BSA. Complement serum (CS) was purchased from Sigma Chemical Co. IL-8 was detected in cell culture supernatants by Quantikine Immunoassay (R&D Systems).

Expression of mRNA

RNA was isolated, separated by agarose gel electrophoresis, and transferred to nylon membranes. The blots were hybridized with a radioactively labeled full-length human MCP-1, clusterin, or CD59 probe.
Western Blot Analysis
HUVECs (4.0x10^5 cells) were lysed and protein homogenates were prepared as described. Proteins (50 μg/lane) were loaded onto 10% SDS-polyacrylamide gels, blotted onto PVDF membranes, and incubated with clusterin antibody (Santa Cruz Biotechnology; 1:200/3% BSA) or MCP-1 antibody (R&D Systems; 1:1000/3% BSA) and detected by enhanced chemiluminescence.

Plasmid Construction and Transfection
Human full-length clusterin was cloned by polymerase chain reaction (primers: 5'-CGGGATCCGGCGCCATGTCAGAACCGGGG-3'/5'-GCGATATCGGGCTTCCTTGGAGAAGATCAG-3') into the BamHI/EcoRV sites of the pcDNA3.1-Myc-His vector. HUVECs were transiently transfected as described previously. Medium (150 μL) was mixed with 3 μg plasmid and 30 μL Superfect (Qiagen) and incubated for 10 minutes, 1 mL medium was added, and HUVECs were incubated with this mixture for 3 hours at 37°C.

Sense (5'-ATGATGAAGACTCTGCTGCTG-3'; bases 53 to 73) or antisense (5'-CAGCAGCAGAGTCTTCATCAT-3') oligonucleotides corresponding to the human clusterin sequence were preincubated with the human clusterin sequence in RPMI at 37°C.

Statistical Analysis
Data are expressed as mean±SEM. Statistical analysis was performed by t test or ANOVA for multiple comparisons.

Results
Shear Stress Inhibits Complement-Induced Upregulation of IL-8 and MCP-1
Nonlytic concentrations of CS (0.2 U/mL) stimulated HUVECs to release IL-8 into the medium (Figure 1A). Similarly, MCP-1 mRNA expression was time- and dose-dependently upregulated by complement, with a maximal response after 6 hours (Figure 1B, data not shown). Control experiments demonstrated that concentrations up to 0.5 U/mL CS did not affect EC viability (data not shown). To test the effect of shear stress, HUVECs were preexposed to laminar shear stress (15 dynes/cm²) for 6 hours and then stimulated with 0.2 U/mL CS for 6 hours under prolonged application of laminar shear stress. As shown in Figure 1A, shear stress prevented the CS-induced IL-8 release. Furthermore, CS-stimulated MCP-1 mRNA expression was significantly reduced by exposure to shear stress (Figure 1B). These data were confirmed by Western blot analysis of MCP-1 protein expression (data not shown).
Shear Stress Upregulates the Complement-Inhibitory Protein Clusterin

To elucidate the mechanism of the complement-inhibitory effect of shear stress, we examined whether shear stress upregulates the complement-inhibitory proteins clusterin and CD59. Shear stress exposure time-dependently increased the expression of clusterin mRNA (6 hours: 225 ± 76%) and clusterin protein levels (164 ± 22% increase after 18 hours; Figure 1C). In contrast, expression of the complement inhibitor CD59 was not affected by shear stress (data not shown).

To assess a causal role for shear stress–stimulated upregulation of clusterin to mediate the complement-inhibitory effect of shear stress, clusterin expression was specifically inhibited by transfection with antisense oligonucleotides (Figure 2A). Clusterin antisense oligonucleotides significantly reduced the inhibition of CS-induced MCP-1 expression by shear stress, compared with HUVECs transfected with sense or scrambled oligonucleotides (Figure 2B; data not shown). Similar data were obtained for IL-8 (data not shown). Most importantly, overexpression of clusterin significantly reduced CS-induced expression of MCP-1 and IL-8 compared with mock-transfected cells (Figure 2C and 2D, data not shown). Thus, clusterin appears to be causally involved in the suppression of complement-induced activation of ECs.

Discussion

The results of the present study demonstrate that exposure of ECs to laminar shear stress prevents complement-induced activation of ECs by upregulation of the complement-inhibitory protein clusterin. Complement components stimulate the activation of ECs, resulting in an enhanced expression of MCP-1 and other cytokines, which trigger the firm adhesion of monocytes to the endothelium, a key event in initiation of the pathogenesis of atherosclerosis. Clusterin is a heterodimeric multifunctional glycoprotein capable of interacting with complement components C7, C8, and C9, thereby preventing the formation of the membrane attack complex. The results of the present study demonstrate that overexpression of clusterin is sufficient to prevent complement-induced activation of ECs. More importantly, clusterin upregulation is necessary for the complement-inhibitory effects of shear stress.

Abrogation of the complement-induced proinflammatory response of ECs might importantly contribute to the potent antiatherosclerotic effects of shear stress. Previous studies have demonstrated that prolonged exposure to physiological levels of shear stress reduces the expression of adhesion molecules and MCP-1 in part via increased expression of the endothelial NO synthase (eNOS), which alters endothelial redox state and inhibits nuclear factor-κB–mediated gene transcription. Shear stress–induced upregulation of clusterin, however, occurs independently of enhanced NO synthesis, because NOS inhibitors did not affect shear stress–stimulated clusterin expression (data not shown). Thus, inhibition of complement-induced EC activation does not require eNOS activation. It has been reported that transforming growth factor (TGF)-β stimulates the upregulation of clusterin. Because shear stress induces gene expression of TGF-β, one may speculate that TGF-β mediates the enhanced expression of clusterin in response to shear stress.

Taken together, shear stress–induced upregulation of clusterin might interfere with endothelial activation in response to infectious agents, which activate the complement cascade and are currently receiving increasing attention in relation to atherosclerosis. Furthermore, additional biological effects of the multifunctional protein clusterin, including the transport of lipoproteins and its chaperone-like activity in combination with the promotion of cell survival, may be involved in the suppression of complement-induced activation of ECs.
in preserving the integrity and the biological functions of the endothelial monolayer.

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