KLF-2-DEPENDENT, SHEAR STRESS-INDUCED EXPRESSION OF CD59: A NOVEL CYTOPROTECTIVE MECHANISM AGAINST COMPLEMENT-MEDIATED INJURY IN THE VASCULATURE

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Summary

Complement activation may predispose to vascular injury and atherogenesis. The atheroprotective actions of unidirectional laminar shear stress led us to explore its influence on endothelial cell expression of complement inhibitory proteins CD59 and decay-accelerating factor. Human umbilical vein and aortic endothelial cells were exposed to laminar shear stress (12 dynes/cm^2), or disturbed flow (+/-5 dynes/cm^2 at 1Hz), in a parallel-plate flow chamber. Laminar shear induced a flow rate-dependent increase in steady-state CD59 mRNA, reaching 4-fold at 12 dynes/cm^2. Following 24-48 hours of laminar shear stress, cell surface expression of CD59 was upregulated by 100%, while decay-accelerating factor expression was unchanged. The increase in CD59 following laminar shear was functionally significant, reducing C9 deposition and complement-mediated lysis of flow-conditioned endothelial cells by 50%. While CD59 induction was independent of PI-3K, ERK1/2 and nitric oxide, an RNA interference approach demonstrated dependence upon an ERK5/KLF2 signalling pathway. In contrast to laminar shear stress, disturbed flow failed to induce endothelial cell CD59 protein expression. Likewise, CD59 expression on vascular endothelium was significantly higher in atheroresistant regions of the murine aorta exposed to unidirectional laminar shear stress, when compared to atheroprone areas exposed to disturbed flow. We propose that upregulation of CD59 via ERK5/KLF2 activation leads to endothelial resistance to complement-mediated injury, and protects from atherogenesis in regions of laminar shear stress.

Introduction

The complement cascade provides an essential defence against bacterial infection and a bridge between innate and adaptive immunity (1). However, by the nature of its cytolytic activity, complement has the potential to inflict injury on bystander host tissues including vascular endothelium. C1q, C3a, C5a and the C5b-9 membrane attack complex (MAC)[2] have the capacity to exert pro-inflammatory effects on vascular endothelial cells (EC) including induction of cellular adhesion molecules and cytokine secretion, increased leukocyte adhesion and generation of a pro-thrombotic endothelial surface (2-5). Mechanisms implicated in complement deposition on the surface of EC include activation of the classical pathway by immune complexes, anti-phospholipid and anti-endothelial cell Abs (6), and through recognition of apoptotic cell blebs by the globular head of C1q (7). Alternatively, activation of the lectin pathway may follow exposure to hypoxia-reoxygenation (8).

Complement activation on the surface of human EC is regulated by membrane-bound inhibitory proteins: decay-accelerating factor (DAF) (CD55), membrane cofactor protein (MCP, CD46) and CD59. The genes encoding DAF and MCP are clustered on the long arm of chromosome 1, while that for CD59 is located on chromosome 11. These proteins use distinct mechanisms for complement regulation. DAF prevents the formation and accelerates the decay of C3 and C5 convertases (9), while MCP accelerates the degradation of C3b and C4b by Factor I (10). CD59 inhibits the terminal pathway of complement activation, preventing incorporation of C9 into the MAC (11,12).

Complement activation may be an early pre-lesional event in atherogenesis, as revealed by colocalisation of C5b-9 with lipid deposits in the tunica intima prior to monocyte recruitment (13). Experimental models suggest that vascular endothelial injury is the earliest detectable event in atherogenesis. Apoptosis of EC occurs

preferentially at bifurcations and curvatures, where denudation of vascular endothelium enhances the risk of plaque development and local thrombosis. Moreover, aging and exposure to oxidized low-density lipoprotein (ox-LDL), or reactive oxygen species, increases EC apoptosis, consistent with a role in the initiation of atherogenesis (14). In addition to local lipid-related activation of complement, exposure of the underlying extracellular matrix, resulting from apoptosis of EC, may further reinforce complement activation (13,15).

The susceptibility of branch points and curvatures to atherosclerosis is in large part related to exposure to disturbed blood flow (DF), with low shear reversing or oscillatory flow patterns. In contrast, the arterial tree exposed to unidirectional laminar shear stress (LSS) >10 dynes/cm^2 tends to be protected (16). This is reflected in the phenotype of EC exposed to LSS, typically characterized by enhanced nitric oxide (NO) biosynthesis, prolonged cell survival and an anticoagulant, anti-adhesive cell surface (17-19). In contrast, endothelium exposed to DF exhibits reduced levels of eNOS, increased apoptosis, generation of reactive oxygen species, permeability to LDL and leukocyte adhesion (reviewed in (16)).

The importance of unidirectional LSS for vascular endothelial cytoprotection, together with the observation that LSS protects against complement-induced EC activation and chemokine synthesis (20), led us to explore whether LSS may be protective against complement-mediated vascular injury through suppression of the C5b-9 membrane attack complex. We show for the first time that CD59, the predominant membrane-bound regulator of the MAC, is preferentially induced by unidirectional atheroprotective LSS, providing enhanced protection against complement activation. This response was independent of NO and dependent upon an ERK5, Kruppel-like Factor2 (KLF2) signalling pathway. In contrast, an atheroprone disturbed flow waveform failed to increase CD59 protein expression. These observations were confirmed *in vivo*, where CD59 expression was significantly higher on arterial EC in atherosclerosis-resistant areas of the murine aorta. Thus, upregulation of CD59 may represent an important component of endothelial cytoprotection in regions of LSS, preserving vascular integrity and minimizing both complement activation and atherogenesis.

Experimental procedures

Materials

Anti-human CD59 mAb (IgG1) Bric 229 was purchased from the International Blood Group Reference Laboratory (Bristol, UK). The anti-murine CD59 mAb (MEL-4) was a gift from BP Morgan (University of Wales School of Medicine). Anti-DAF monoclonal antibody (mAb) 1H4 (IgG1) and anti-MCP mAb TRA-2-10 (IgG1) were gifts from D. Lublin and J. Atkinson respectively (Washington University School of Medicine, St Louis, Mo). UO126 and N^G-monomethyl-L-arginine (L-NMMA) were from BIOMOL (Plymouth Meeting, PA) and LY290042 from Merck Biosciences (Nottingham, UK). Other products were from Sigma-Aldrich (Poole, UK).

Endothelial cell exposure to flow

Human umbilical vein ECs (HUVEC) and human aortic ECs (HAEC; purchased from Promocell, Heidelberg, Germany), were cultured as described (21). The use of human EC was approved by Hammersmith Hospitals Research Ethics Committee (ref no. 06/Q0406/21). Confluent HUVEC or HAEC cultures were exposed to control static conditions, high shear unidirectional laminar flow (up to 20 dynes/cm^2), or disturbed flow with directional changes of flow at 1Hz (+/- 5 dynes/cm^2), for up to 48h using a parallel-plate flow chamber (Cytodyne, La Jolla, CA, USA) as described previously (22,23). Cell viability was assessed by examination of EC monolayers using phase contrast microscopy, cell counting and estimation of trypan blue exclusion.

RNAi design and transfection

The short interfering RNA (siRNA) duplexes were from Dharmacon Inc. (Lafayette, CO) or Ambion (Austin, TX). siRNA sequences for KLF2: (Sense: 5'-GCCCUACCACUGCAACUGGUU-3'), (Antisense: 5'-CCAGUUGCAGUGGUAGGGCUU-3'), siRNA sequences for ERK5: (Sense: 5'-GGCUCGGCUUGGUUAAUUCtt-3'), (Antisense:5'-GAAUAAUCCAAGCCGAGCCtc-3'). Corresponding negative control sequences were commercially synthesized. For siRNA delivery, HUVECs were plated at 3 x 10^5 cells per well on fibronectin-coated glass slides in Endothelial Cell Basal Media (EBM2) (Cambrex

BioScience Wokingham, UK) to obtain 50% confluency. siRNA targeting KLF2, ERK5 or scrambled control siRNA (all at 10-50nM) was transfected into EC using oligofectamine-based transfection in EBM2 media. EC were cultured for 48h in EBM2 and analysed for target gene expression by qRT-PCR or immunoblotting. The specificity of siRNA targeting was confirmed using a second set of sequences.

Quantitative real-time (RT)-PCR

Quantitative RT-PCR was performed using an iCycler (BioRad, Hercules, CA) as described (24,25). β-actin, GAPDH and HPRT were used as housekeeping genes, with data calculated in relation to β-actin and verifed with GAPDH and HPRT. DNase-1-digested total RNA (1 μg) was reverse transcribed using 1μM oligo-dT and Superscript reverse transcriptase (Invitrogen, Paisley, UK), according to the manufacturers instructions. cDNA was amplified in a 25 μl reaction containing 5 μl cDNA template, 12.5 μl iSYBR supermix (BioRad), and 0.5pM sense and antisense gene-specific primers and ddH_2O. Primer sequences used were: β-actin forward 5'-GAGCTACGAGCTGCCTGACG-3', β-actin reverse 5'-GTAGTTTCGTGGATGCCACAGGACT-3'; KLF2 forward 5'-CTTTCGCCAGCCCGTGCCGCG-3', KLF2 reverse 5'- AAGTCCAGCACGCTGTTGAGG-3'; CD59 forward 5'-ATGCGTGTCTCATTAC-3', CD59 reverse 5'-TTCTCTGATAAGGATGTC-3'; ERK5 forward 5'-AGTACGAGATCATCGAGACC-3'; ERK5 reverse 5'-CTCCCTGAGGGTCCGCTTGG-3'.

Northern blotting

RNA was extracted from HUVEC using the RNeasy kit Qiagen Ltd (Crawley, UK). Total RNA was separated on a 1% agarose/formaldehyde gel, transferred overnight to Hybond-N nylon membranes (Amersham Biosciences) and analysed by specific hybridization to a radiolabeled cDNA probe for human CD59 (gift from H Waldmann, University of Oxford, UK) as previously described (26). Integrated density values for each band were obtained with an Alpha Innotech ChemiImager 5500 Alpha Innotech (San Leandro, CA), normalised with respect to the 28S band on ethidium bromide-stained rRNA loading patterns and expressed as percent change from control.

Flow cytometry

Flow-cytometry was performed as previously described using a Beckman-Coulter flow cytometer (Luton, UK) (26). The results are expressed as the relative fluorescent intensity (RFI), representing mean fluorescent intensity (MFI) with test mAb divided by the MFI using an isotype-matched irrelevant mAb.

Complement deposition assays

Cell surface C9 deposition and complement-mediated lysis was assessed by flow-cytometry as described previously (24). EC were exposed to LSS at 12 dynes/cm^2 for 24h, harvested and suspended in veronal buffered saline containing 0.1% gelatin (VBSG). The non-complement fixing, inhibitory CD59 mAb BRIC 229 (27) was used at 20 μg/ml. For analysis of C9 deposition, EC were exposed to normal human serum (NHS) or heat-inactivated NHS (HIHS) for up to 3h at 37°C. C9 binding was detected with mouse anti-human C5b-C9 Technoclone (Vienna, Austria) and FITC-rabbit-anti-mouse Ig. Complement-mediated cell lysis was quantified by assessing the percentage of cells permeable to propidium iodide (PI), by flow-cytometry, following exposure to NHS or HIHS for 3h.

Confocal microscopy

Confocal immunostaining and microscopy was by modification of an established method (28). C57BL/6 mice (8 weeks) were killed by CO_2 inhalation, and the vasculature perfused-fixed with 1% paraformaldehyde in PBS. Thoracic organs were removed *en bloc* and equilibrated in OCT for 18 hours. The aortic arch was dissected and a transverse slice cut, which encompassed the circumference of the aorta at the origin of the brachiocephalic trunk, the brachiocephalic trunk itself and the proximal portions of its branches (right common carotid and right subclavian). The block was frozen in isopentane and serial cryostat sections cut and incubated with 10% normal goat serum (Dako, Ely, UK), followed by rat anti-mouse CD59 mAb MEL-4 for 30 mins. Sections were washed and incubated with 1:200 goat-anti-rat-AlexaFluor 568 (Molecular Probes, Invitrogen, Paisley, UK). Sections used for quantitation were stained only for CD59. Adjacent sections were immunolabelled with anti-CD59 as before, and then incubated with biotinylated *Griffonia simplicifolia* isolectin B4 (Vector Laboratories, Burlingame, CA), followed by steptavidin-

AlexaFluor-488 (Invitrogen). Other serial sections were immunolabelled with anti-CD59, followed by AlexaFluor 488, then incubated with Cy3-labelled mouse monoclonal anti-smooth muscle actin (clone α-1A4, Sigma). After the second label, sections were washed in PBS and incubated for 10 min with TOPRO-3 (Molecular Probes) and mounted in PBS/glycerol.

Sections were examined with a Zeiss LSM 510 Meta inverted confocal microscope (Thornwood, NY). Scan and photomultiplier (PMT) settings were set to optimize signal/noise ratio for each emission wavelength. Using these PMT settings, there was no detectable crossover between channels. However, to eliminate any possibility of data skew by signal contamination, quantitation was performed on CD59-only stained sections. Processing was with Zeiss LSM Image Browser and quantitation by export of the images into Image J. The regions of interest were selected and the histogram function used to calculate the distribution of pixel intensities on the red channel (corresponding to AlexaFluor 568).

Animals
C57BL/6 mice were purchased from Harlan Olac (Bicester, Oxford, UK) and housed under controlled climactic conditions in microisolator cages with autoclaved bedding. Irradiated food and drinking water were readily available. All animals were housed and studied according to UK Home Office guidelines. Sentinel mice were housed alongside test animals and regularly screened for a standard panel of murine pathogens.

Statistics
Data are expressed as the mean of individual experiments ± standard error of the mean (SEM). Data were grouped according to treatment and analyzed using GraphPad Prism software (San Diego, CA) and the analysis of variance with Bonferroni correction or an unpaired Students *t*-test. Kolmogorov-Smirnov analysis was used to compare immunofluorescence intensity in different regions of the murine aorta using an Excel plug-in Stat-Plus Professional. Differences were considered significant at P values of <0.05.

Results

CD59 expression is induced by unidirectional LSS
To assess the influence of shear stress on the surface expression of the complement inhibitory proteins MCP, DAF and CD59, HUVEC in a parallel-plate flow chamber were exposed to unidirectional LSS (12 dynes/cm^2) for up to 48h and analyzed by flow cytometry. No change in the cell surface expression of either MCP or DAF was seen (Figure 1). In contrast, a significant increase in CD59 expression of up to 2.5-fold was seen following exposure to unidirectional LSS for 24h and this was sustained at 48h (Figure 1).

Northern analysis detected alternatively spliced variants of CD59 (as described previously (24)) and demonstrated that induction by LSS was associated with an increase in mRNA, first detectable 2h post the onset of flow and continuing to rise over 16h. This pattern was confirmed by qRT-PCR, which revealed a sustained increase of up to 6-fold, 24-48h post-initiation of LSS (Figure 2). To determine whether LSS-induced upregulation was dependent upon the magnitude of shear force, HUVEC were exposed to increasing LSS for 24h and changes in CD59 mRNA analyzed by qRT-PCR. CD59 mRNA levels rose progressively as LSS increased to 12 dynes/cm^2 (Fig 2C).

Experiments performed on HAEC, to represent EC derived from a vascular bed affected by atherosclerosis, also showed a significant upregulation of CD59 mRNA and cell surface protein in response to LSS (Figure 3). The extracellular matrix upon which EC are cultured may also influence their responsiveness to shear stress (29). To address this, HUVEC were cultured on fibronectin, gelatin and collagen type I and exposed to LSS for 24h. However, the nature of the underlying matrix did not alter the ability of LSS to induce CD59, with equivalent induction seen under all conditions (data not shown).

LSS-induced CD59 is cytoprotective
To address the functional significance of CD59 upregulation, the effect of LSS on cell surface deposition of C9 was measured. HUVEC were cultured under static conditions or in the presence of LSS (12 dynes/cm^2) for 24h prior to exposure to 20% normal human serum (NHS) and flow-cytometric analysis, using an antibody against a neo-epitope on C9 that is revealed upon C5b-9 complex formation. In addition, complement-mediated EC lysis was quantified using propidium iodide uptake (24). Exposure to NHS resulted in measurable C9 deposition on the surface of

HUVEC cultured under static conditions. A significant reduction in C9 deposition was seen on EC exposed to LSS ($p<0.05$), with levels comparable to that seen on EC exposed to serum that had been heat-inactivated to prevent complement activation (Figure 4A). Likewise, LSS conferred protection against complement-mediated lysis, when compared to EC cultured under static conditions ($p<0.05$) (Figure 4B). The inhibitory, non-complement fixing CD59 mAb BRIC 229 (27) was used to confirm the role of CD59 in protection against lysis. BRIC 229 reversed the protective effect of LSS and its presence resulted in lysis of >75% of EC (Figure 4B).

Mechanosensitive signalling pathways regulating expression of CD59

Activation of signaling pathways such as those regulated by PI-3K/Akt and ERK1/2, and enhanced eNOS-dependent NO biosynthesis, may contribute to the vasculoprotective effects of LSS (30). Thus, we initially adopted a pharmacological approach to explore the role of these pathways in the induction of CD59 by LSS. HUVEC were pre-treated with PI-3K antagonist LY290042, or MEK-1 inhibitor UO126, at concentrations we have shown to inhibit phosphorylation of Akt and ERK1/2 respectively (31,32). Inclusion of LY290042 had no significant inhibitory effect on the induction of CD59 mRNA in response to LSS (Figure 5A). Although UO126 reduced expression, this did not reach significance (Figure 5B). Likewise, the presence of nitric oxide synthase inhibitor L-NMMA, at concentrations capable of inhibiting the induction of CD59 by atorvastatin under hypoxic conditions (24), did not inhibit LSS-induced CD59 protein (Figure 5C) or mRNA expression (not shown).

In contrast, inclusion of the protein synthesis inhibitor cycloheximide abrogated the LSS-mediated increase in CD59 mRNA (Figure 5D), suggesting dependence upon *de novo* synthesis of an inducible intermediary protein in response to LSS. Furthermore, it has been reported that in addition to MEK-1, UO126 may inhibit ERK5 (33), hence the decrease in flow-induced CD59 mRNA observed in the presence of UO126 (Figure 5B) may in fact reflect a reduction in ERK5 activation. These data, combined with (i) the identification of ERK5 (BMK-1) as a shear-inducible cytoprotective member of the MAPK family (34), (ii) identification of the LSS-inducible transcription factor KLF2 as a downstream target of ERK5 (35,36), and (iii) microarray data suggesting over-expression of KLF2 may increase CD59 mRNA in EC (36), led us to investigate the role of ERK5/KLF2 further. The KLFs are a subclass of the zinc-finger transcription factors, within which KLF2 has emerged as an important factor in the maintenance of endothelial homeostasis (37,38). We adopted an siRNA approach, which reduced the expression of ERK5 transcripts in EC by 80% (Supplementary Figure 1A). Further analysis demonstrated that the induction of both CD59 mRNA (Supplementary Figure 2A) and surface protein by LSS was significantly attenuated in HUVEC treated with ERK5 siRNA, when compared to scrambled siRNA controls ($p<0.01$) (Figure 6A).

LSS significantly induced expression of KLF2 mRNA, a response that was reversed by the presence of specific siRNA oligonucleotides targeting KLF2 (Supplementary Figure 1B). In addition, knockdown of ERK5 significantly inhibited induction of KLF2 by LSS (Supplementary Figure 1C). Analysis of CD59 mRNA in HUVEC pretreated with KLF2-specific siRNA, demonstrated that LSS induction of CD59 was inhibited, an effect that was not seen with scrambled control siRNA (Supplementary Figure 2B). This was further confirmed by flow-cytometric analysis, which showed that cell surface expression of CD59 on EC treated with KLF2 siRNA and exposed to LSS was equivalent to that on static cells (Figure 6B).

CD59 expression is differentially regulated by LSS and DF

Atheroprotected and atheroprone regions of the aorta are exposed to unidirectional LSS and a low velocity reversing flow pattern respectively. To compare the effect of these different flow patterns on CD59 expression, EC were exposed to LSS (12 dynes/cm^2), or an oscillatory flow pattern (±5 dynes/cm^2 at 1 Hz) to model DF. Changes in endothelial cell morphology and CD59 expression were compared with cells cultured under static conditions. Preliminary experiments performed to validate the model, demonstrated characteristic morphological changes in response to LSS (Figure 7A) and induction of intercellular adhesion molecule-1 (ICAM-1) by LSS and vascular cell adhesion molecule-1 (VCAM-1) by DF respectively (22) (Supplementary Figure 3).

Furthermore, while LSS induced KLF2 mRNA expression, no such response was seen in EC exposed to DF (Supplementary Figure 3C). In subsequent experiments, a 4.3-fold increase in CD59 mRNA above static levels was seen following 24h LSS. In contrast, CD59 mRNA induction was reduced to 2-fold in EC exposed to DF, significantly lower than LSS ($p<0.05$) (not shown). Likewise, LSS induced a significant increase in EC surface CD59 expression when compared to static cultured EC ($p<0.01$), while no change was seen in response to DF (Figure 7B).

CD59 expression is differentially expressed in the murine aorta
A murine model was used to explore further the *in vitro* observation of differential regulation of CD59 by unidirectional LSS and DF. Regions of murine aorta exposed to LSS and DF can be determined by geometry, with straight portions exposed to LSS and the inner curve of the aortic arch and the aortobrachiocephalic junction exposed to DF (39). Murine aortae were perfused-fixed, sectioned longitudinally and analyzed by immunohistochemistry for CD59 expression. As seen in Figure 8 A-C, CD59 expression was greater on vascular endothelium in areas of the aorta and brachiocephalic artery (not shown) predicted to be exposed to atheroprotective LSS, when compared to the inner curve of the aorta and the aortobrachiocephalic branch point, exposed to DF. To confirm the presence of intact endothelium at the DF sites, sections were stained with *Griffonia simplicifolia* isolectin B4 (Figure 8C). Similar analysis demonstrated intact EC at LSS sites (not shown). Quantification of endothelial CD59 expression at multiple sites within LSS and DF exposed areas of the aorta confirmed a significant difference (Figure 8D). These results demonstrate differential expression of CD59, the most potent regulator of the MAC, with specific reduction on endothelium in atheroprone sites of the aorta.

Discussion
The data presented herein suggest that LSS enhances vascular endothelial resistance to complement-mediated injury through induction of CD59 expression. Vascular wall injury contributes to the initiation of atherogenesis, a complex multifactorial inflammatory disease process propagated by both local and systemic factors. Activation of the classical or alternative complement pathways (40-42) may be involved in the pathogenesis of atherosclerosis from the pre-lesional stage (13), through early arterial wall lesions (43) and intermediate and advanced plaques (44), via generation of C3a, C5a and the C5b-9 MAC. LSS is an essential component of vascular endothelial homeostasis, contributing to resistance against apoptosis and the maintenance of an anti-proliferative, anti-oxidant, anti-thrombotic, anti-adhesive endothelial barrier (reviewed in (45)). Although LSS-dependent induction of eNOS and NO biosynthesis is an important regulator of many of these mechanisms (17), the cytoprotective effects of LSS remain to be fully elucidated.

CD59 is a glycosylphosphatidylinositol-anchored, 18-25 kDa molecule, belonging to the Ly-6 superfamily of cell surface proteins (11,12). Through its ability to bind to the α-γ-subunit of C8, CD59 prevents the incorporation of C9 into C5b-9 (46). Thus CD59, which is constitutively expressed on the vascular endothelial surface, is the predominant membrane-bound regulator of the MAC. In light of the role of complement activation in atherogenesis and of LSS in atheroprotection, we explored the effect of shear stress on endothelial cell CD59 expression. We have shown that 24-48h of atheroprotective LSS enhances CD59 protein expression in HUVEC and HAEC and that this response is attenuated in EC exposed to an oscillatory flow pattern (±5 dynes/cm^2 at 1 Hz), modelling disturbed flow characteristic of atheroprone regions of the vasculature. Comparison of the level of CD59 expression at sites of the murine aorta exposed to LSS and DF supported the relevance of these observations to the *in vivo* situation. Moreover, the enhanced expression of CD59 in response to LSS resulted in increased endothelial resistance to C5b-9 deposition and complement-mediated injury.

Urbich and colleagues demonstrated an increase in EC clusterin in response to LSS and, as we show for CD59, this response was independent of enhanced NO synthesis (20). Of note, they did not detect a change in CD59 protein expression, which may reflect differences in experimental conditions including the time courses used. Urbich analyzed CD59 protein expression following 18h of LSS, whereas we first saw a significant increase in protein expression at 24h. Clusterin, a multifunctional cytoprotective molecule, acts to

inhibit complement-mediated cytolysis by binding to C5b-6 and preventing formation of the MAC. Thus, increased expression of both soluble (clusterin) and membrane-bound (CD59) inhibitors of the MAC may contribute significantly to the atheroprotective actions of LSS through reduction of complement-mediated EC activation and injury. Of these, CD59 is considered to be the most potent endothelial regulator of the MAC. In contrast DF, in addition to reducing protection against complement, may in fact increase complement activation by enhancing alternative pathway activity through induction of endothelial properdin synthesis. EC synthesis of properdin, a stabilizer of C3 and C5 convertases, is a significant contributor to plasma levels and is markedly enhanced following exposure of EC to DF (47).

KLF2 is one of 17 KLFs, a subclass of the zinc-finger transcription factors. KLF2 is flow inducible and differentially expressed in areas of the aorta exposed to LSS and DF (48). KLF2 activity has emerged as an important regulator of endothelial cytoprotective genes including eNOS, thrombomodulin and heme oxygenase-1, which exert anti-inflammatory, anti-thrombotic and anti-oxidant effects (25,36-38). An ERK5/myocyte enhancing factor 2 pathway regulating KLF2 transcription has been identified (35,36). Thus, KLF2 represents an important transcriptional effector in the cytoprotective actions of LSS (38). We have now added induction of CD59 and protection against complement-mediated injury to the atheroprotective profile of shear stress, a response which mirrors the effect of LSS and DF on KLF2 expression. Using siRNA we have demonstrated that CD59 induction by LSS is dependent-upon ERK5 and KLF2 activity. In support of a role for KLF2, microarray analysis revealed a 2.65-fold increase in CD59 mRNA following adenoviral over-expression of KLF2 in HUVEC for 24h (36). In contrast, lentiviral over-expression of KLF2 for 7 days failed to detect a change in CD59 mRNA, while showing a 1.98-fold induction of DAF (38). Thus, further studies are required to delineate the regulation and outcomes of KLF2 pathway activity, including its relationship to upstream mechanotransducers and signaling mediators and with downstream target gene promoters. Analysis of the 5kb region upstream of the CD59 gene transcription start site revealed potential KLF2 5'-CACCC-3' binding sites. Detailed examination of the CD59 promoter and definition of the precise relationship with KLF2 are beyond the scope of the current manuscript, and will be addressed in future studies.

Complement activation is tightly controlled so as to avoid host injury. While lysis of nucleated cells is rare, sub-lytic C5b-9 may induce tissue factor expression and release of soluble factors from EC and VSMC including platelet-derived growth factor, IL-1, IL-6 and MCP-1 (49-51). The resultant proliferation of EC and VSMC, combined with induction of cellular adhesion molecules, monocyte chemotaxis, EC apoptosis and thrombosis may contribute to atherogenesis. Hence, maintenance of cell surface CD59 to limit C5b-9 deposition is essential. We propose that in areas of the vasculature where this is insufficient, such as sites exposed to DF, a threshold is exceeded whereby levels of C5b-9 deposition are reached that induce EC injury with pro-inflammatory, pro-atherogenic sequelae. A concept supported by our recent report of increased atherosclerosis in CD59/LDL receptor-deficient mice (S Yun, VWY Leung, M Botto, JJ Boyle, DO Haskard: submitted for publication). Moreover, our observation that the induction of cell-surface CD59 expression by LSS is significantly reduced by the immunosuppressive drug cyclosporine A (CsA) (data not shown), suggests this may be a contributory factor in CsA-mediated vasculopathy.

A further indication of the importance of CD59 in vasculoprotection comes from the study of diabetes mellitus (DM). EC protection mediated by CD59 may be compromised in DM by two mechanisms; glycation of CD59 leading to loss of function (52) and hyperglycemic-induced shedding of cell surface protein (53). Dysfunctional glycated CD59 is detectable in the urine of patients with DM, and colocalises with the increased MAC deposited on the vascular endothelium of target tissues (54). The consequent release of growth factors and the pro-inflammatory, pro-thrombotic sequelae in the vasculature may be a significant factor in the accelerated atherogenesis associated with DM.

In conclusion, our data reveal induction of the complement-inhibitory protein CD59 to be a novel cytoprotective outcome of LSS-induced activation of the ERK5/KLF2 signaling pathway. The demonstration of differential regulation of CD59 by LSS and DF suggests CD59 expression may be

a contributory factor in the protection afforded by LSS against atherogenesis. Through the inhibition of the terminal MAC, CD59 has the potential to exert anti-inflammatory and vasculoprotective effects. Moreover, data demonstrating that HMG-CoA reductase antagonists activate KLF2 (25,55,56) and enhance CD59 expression (24), suggest that modulation of KLF2 represents an important component of the vasculoprotective profile of statins and emphasizes the therapeutic potential of KLF2-related signalling pathways.

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Footnotes

[1]ARK and FA contributed equally to this study.

[2]The abbreviations used are: MAC, membrane attack complex; EC, endothelial cells; DAF, decay-accelerating factor; ERK, extracellular signal-regulated kinase; KLF2, Kruppel-like Factor2; Ox-LDL, oxidized low-density lipoprotein; LSS, laminar shear stress; DF, disturbed flow; NO, nitric oxide; C; PI-3K, phosphoinositide-3 kinase; L-NMMA, N^G-Monomethyl-L-arginine; HUVEC, human umbilical vein endothelial cells; HAEC, human aortic endothelial cells; Quantitative real-time-polymerase chain reaction, qRT-PCR; RFI, relative fluorescent intensity; MFI, mean fluorescent intensity; FBS, fetal bovine serum; VBSG; veronal buffered saline containing 0.1% gelatin; NHS, normal human serum; HIHS, heat-inactivated NHS; PI, propidium iodide; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; SEM, standard error of the mean.

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Figure legends

Figure 1. CD59 expression is upregulated by prolonged laminar shear stress. HUVEC were exposed to unidirectional laminar shear stress (LSS) 12 dynes/cm^2 or cultured under static conditions for up to 48h after which DAF, MCP and CD59 expression were determined by flow-cytometry. Data are expressed as mean relative fluorescence intensity (RFI) ± SEM from three experiments.*$p<0.05$.

Figure 2 CD59 induction is dependent upon the magnitude of shear force
A and B. HUVEC were exposed to LSS for up to 48h and CD59 mRNA quantified by **A**. northern blotting (with fold change calculated by densitometric quantification of 3 separate experiments) and **B**. real-time quantitative PCR (qRT-PCR). **C**. HUVEC were exposed to varying LSS (0-12 dynes/cm^2) for 24h and CD59 mRNA quantified by qRT-PCR. Data expressed as mean ± SEM from 2-5 experiments.*$p<0.05$ **$p<0.01$, ***$p<0.001$.

Figure 3. HAEC CD59 expression is upregulated by prolonged LSS. HAEC were exposed to LSS (12 dynes/cm^2) or cultured under static conditions for 24h prior to quantification of **A**. CD59 mRNA by qRT-PCR and **B**. CD59 surface protein by flow-cytometry. Data expressed as mean ± SEM from three experiments. *$p<0.05$.

Figure 4. LSS enhances EC resistance to complement-mediated lysis. HUVEC were exposed to LSS (12 dynes/cm^2) (grey bars) or cultured under static conditions for 24h (black bars). EC were then left untreated (UT) or exposed to 20% normal human serum (NHS) or heat-inactivated serum (HIHS) for up to 3h. **A**. C9 deposition was measured by flow-cytometry using an antibody against a neo-epitope on C9 that is revealed upon C5b-9 complex formation. **B**. Percentage EC lysis was calculated as the number of propidium iodide positive cells expressed as a percentage of total cells. CD59 activity was inhibited by pre-treatment with mAb Bric 229. Data expressed as mean RFI ± SEM from 3 experiments. *$p<0.05$.

Figure 5. **Induction of CD59 by LSS is independent of PI-3K, ERK1/2 and NO**. HUVEC were exposed to LSS (12 dynes/cm^2) or cultured under static conditions for 24h in the presence of **A**. LY290042 (20μM) **B**. U0126 (5μM), **C**. L-NMMA (100μM) and **D**. Cycloheximide (3μg/ml) or vehicle control. CD59 mRNA was quantified by qRT-PCR and EC surface CD59 expression by flow-cytometry. Values are normalized and shown as the increase in CD59 expression above constitutive levels on untreated EC cultured under static conditions. Data expressed as mean ± SEM from three experiments. *$p<0.05$ **$p<0.01$.

Figure 6. **Induction of CD59 by LSS is dependent upon ERK5 and KLF2**. HUVEC were left untransfected (UT) or transfected with scrambled control siRNA (Scr) or ERK5-specific siRNA (**A**), or KLF2 siRNA (**B**), prior to exposure to LSS (12 dynes/cm^2) or culture under static conditions for 24h. Cell surface CD59 expression was analysed by flow-cytometry. Data expressed as mean ± SEM from 3 experiments. *$p<0.05$.

Figure 7. CD59 is differentially regulated by laminar and disturbed flow. HUVEC were exposed to static culture, LSS (12 dynes/cm^2) or disturbed flow (DF) (1Hz, +/- 5 dynes/cm^2) for 24h. **A**. Phase-contrast photomicrographs demonstrating EC monolayer morphology, the arrow shows the direction of LSS. **B**. EC surface CD59 expression was quantified by flow-cytometry. Data expressed as mean ± SEM from three experiments. *$p<0.05$ **$p<0.01$.

Figure 8. Analysis of CD59 expression in the murine aorta. Murine aortae were analyzed by immunohistochemistry and laser-scanning confocal microscopy. Endothelial CD59 expression (red) indicated by arrows in regions of the aorta exposed to **A**. laminar flow and **B**. low shear disturbed flow at the inner arch of the aorta. **C**. inner arch of the aorta stained with *Griffonia simplicifolia* (EC marker, red), CD59 (bright green), elastin (dull green), Topro (nuclear dye, blue). **D**. Image analysis quantification demonstrating maximal CD59 expression on EC in areas of the aorta exposed to LSS and reduced CD59

on EC at the inner curve of the aorta (predicted disturbed flow), n = 3 mice (*p<0.001, Kolmogorov-Smirnov analysis). Scale bars = 50 μm.