Integrin-substrate interactions underlying shear-induced inhibition of the inflammatory response of endothelial cells

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Summary

Conditioning of endothelial cells by shear stress suppresses their response to inflammatory cytokines. We questioned whether signalling through different integrin-matrix interactions, previously associated with the pathogenic effects of disturbed flow, supported the anti-inflammatory action of steady shear. Primary human endothelial cells were cultured on different substrates and exposed to shear stress (2.0Pa) for varying periods before stimulation with tumour necrosis factor-α (TNF). Shear-conditioning inhibited cytokine-induced recruitment of flowing neutrophils. However, the effect was similar for culture on collagen, laminin or fibronectin, even when seeding was reduced to 2hours, and shear to 3hours before TNF treatment (to minimise deposition of endothelial matrix). Nevertheless, in short- or longer-term cultures, reduction in expression of β_1-integrin (but not β_3-integrin) using siRNA essentially ablated the effect of shear-conditioning on neutrophil recruitment. Studies of focal adhesion kinase (FAK) phosphorylation, siRNA against FAK and a FAK-inhibitor (PF573228) indicated that FAK activity was an essential component downstream of β_1-integrin. In addition, MAP-kinase p38 was phosphorylated downstream of FAK and also required for functional modification. Mechanotransduction through β_1-integrins, FAK and p38 is required for anti-inflammatory effects of steady shear stress. Separation of the pathways which underlie pathological versus protective responses of different patterns of flow is required to enable therapeutic modification or mimicry, respectively.

Keywords

Endothelial cells, shear stress, integrin, inflammation

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Financial support:
This work was supported by The British Heart Foundation (grant numbers PG/06/039/20697 and PG/06/140/21878).

Received: June 14, 2012
Accepted after major revision: November 8, 2012
Prepublished online: December 13, 2012

doi:10.1160/TH12-06-0400
ThrombHaemost 2013; 109: 298–308

Introduction

Vascular endothelial cells (EC) are exposed to fluid shear stress of a magnitude and pattern that varies throughout the vascular tree. These stresses condition the inflammatory responses of EC, particularly their ability to recruit leukocytes (1, 2). Oscillatory low-shear stress that is found in regions prone to atheroma, may directly activate inflammatory and thrombotic responses (3, 4). On the other hand, steady laminar shear stress suppresses endothelial responses to cytokines, including leukocyte adhesion and migration (5-10).

When shear stress is first applied to EC, rapid responses include activation of MAP-kinases (MAPK) and nuclear translocation and phosphorylation of the pro-inflammatory transcription factor nuclear factor (NF)κB, with later changes in gene expressions (11, 12). The responses are downstream of shear-induced activation of integrin adhesion receptors linked to engagement of sub-endothelial matrix proteins (13). More specifically, for EC cultured on fibronectin, engagement of α5β1- or αvβ3-integrin supported such pro-inflammatory responses, while α2β1-integrin engagement on collagen led to activation of the MAPK p38 which inhibited the NFκB activation (14). The situation on physiological mixed substrates was more complex, with the potential for cross-inhibition between different activated integrins, so that signals may depend on the specific content of the basement membrane (15). The responses downstream of integrins appear to require activation of focal adhesion kinase (FAK) (16), and a recent study showed that NFκB phosphorylation (but not translocation) immediately after application of shear stress was reduced in EC lacking FAK (17).

Rapid responses to application of shear appear to be pro-inflammatory and may model signals generated by exposure of EC to oscillatory shear at arterial sites prone to thrombosis (14). It is not clear if the same pathways contribute to longer-term, anti-inflammatory shear-conditioning of EC which would occur in other regions of the circulation. Investigation of functional effects of different integrin-substrate interactions is practically difficult, because cultured EC themselves deposit matrix proteins. We showed that neutrophil recruitment was reduced when EC were

conditioned by exposure to shear stress before treatment with TNF (6). The effect increased with the magnitude and duration of the shear stress, but was detectable even if shear stress and TNF were applied together for only 4 hours (h). However, the EC had been stabilised for 24 h before these assays. Thus, it is unknown whether the anti-inflammatory effects of steady shear stress depend on signals from integrin engagement, differ between substrates, or proceed via signalling pathways identified in short-term studies.

Additional complexity arises from findings that activation of integrins may occur downstream of signals from a membrane complex incorporating CD31, VE-cadherin and VEGFR2 (4). On the other hand, application of forces independently to surface integrins or CD31 can both generate signals (18). We recently showed that anti-inflammatory effects of shear on response to TNF were dependent on presence of CD31 (19). Equivalent functional experiments have not been reported for EC lacking integrins or cultured on different adhesive substrates, although it is important to ascertain whether integrin signalling can have beneficial as well as pathogenic effects. To address these questions, we examined neutrophil recruitment and gene expression by primary human EC treated with TNF after shear-conditioning on collagen, laminin or fibronectin substrates for different periods of seeding and stress. The studies were combined with use of small-interfering siRNA to reduce expression of β1- or β3-integrins, or FAK, and use of inhibitors of FAK and p38. While we did not detect substrate-specificity in anti-inflammatory effects of shear, we demonstrated for the first time that β1-integrin-mediated signalling leading to a pathway from FAK to p38 phosphorylation was required. The results suggest that dissection and selective modification of pathways specific to steady vs. oscillatory shear will be required to deliver beneficial effects in inflammatory and thrombotic disorders.

Materials and methods

Culture of endothelial cells and exposure to shear stress

Human umbilical cords were obtained from the Human Biomaterials Resource Centre (University of Birmingham) after informed consent. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in medium M199 supplemented with 20% heat-inactivated fetal calf serum, 2.5 μg/ml amphotericin B, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (all from Sigma-Aldrich, Poole, UK) and 35 μg/ml gentamycin (Gibco, Life Technologies Co., Paisley, UK) until confluent, as previously described (20). Primary HUVEC were dissociated, seeded in microslides (flattened glass capillaries with cross-section 0.3x3.0 mm; Camlab, Cambridge, UK), and cultured for chosen periods under static conditions before exposure to shear stress. The microslides were coated with either collagen type IV (20 μg/ml, from human placenta; Sigma), fibronectin (20 μg/ml, from human plasma; Sigma) or laminin (20 μg/ml, from human placenta; Chemicon, Temecula, CA, USA).

The system for culturing EC under static or flow conditions in microslides has been described in detail (21). After seeding, microslides were placed into specially constructed glass dishes, and attached to a continuous flow loop incorporating an 8-roller pump (model 502S; Watson Marlow Ltd., Falmouth, UK). The pump was set to deliver the flow rate (7.76 ml/minute [min]) to yield the desired wall shear stress of 2.0 Pa in the microslide. This stress was chosen as being in the range of values found in human arteries and providing a marked modification of the responses of EC to inflammatory cytokines (6, 9). A separate microslide in each dish was connected to a separate pump. This pumped a small amount of medium through the microslide for 30 seconds once an hour, to enable prolonged growth under 'static' conditions. Each repeated experiment used first-passage HUVEC from a different donor. HUVEC were used because of their availability as low passage primary cells. In previous studies, the effects of shear on responses to TNF were almost identical for HUVEC, human umbilical artery EC and human coronary artery EC (9) and qualitatively similar for primary murine EC (19).

Typically, microslides were cultured under static conditions for 24 h, and then exposed to shear stress for a further 24 h, with or without TNF (100 U/ml, recombinant human; Sigma) added for the last 4 h. However, in some experiments on effects of different substrate proteins, static culture was shortened to as little as 2 h to reduce the deposition of matrix proteins by the EC themselves before exposure to shear, and durations of shear exposure and cytokine treatment were also reduced (see *Results*). In all cases, seeding levels were adequate to yield confluent monolayers before exposure to shear. When seeding for 24 h before shear, trypsin/EDTA (Sigma) was used to dissociate the primary cultures, but when seeding was for 2 h we used non-enzymatic dissociation buffer (Gibco). This was because we noticed that trypsin itself induced a degree of neutrophil adhesion in briefly-seeded monolayers (not seen after 24 h seeding), which was absent with the non-enzymatic method. We concluded that trypsin induced an undesirable transient activatory response in HUVEC.

When desired, EC in microslides were treated with inhibitor of FAK (PF57328, 1 μM) or p38 (SB202190, 10 μM) for 60 min before the start of shear, and agents were included in medium throughout the following treatments.

Modification of endothelial gene expression by siRNA

Expression of β1-integrin, β3-integrin or FAK in HUVEC was reduced using small-interfering, siRNA, as described (19). Oligonucelotide pairs were purchased for β1-integrin siRNA from Thermo Scientific (Northumberland, UK) and β3-integrin or FAK siRNA from Eurogentec Ltd (Southampton, UK). Transfection of oligos specific for targets or with scrambled sequence was achieved using Lipofectamine™ RNAiMAX reagent (Invitrogen) and 20 nM siRNA as recommended by the supplier. After transfection, cells were cultured for either 48 h before seeding in microslides for 2 h, followed by exposure to shear stress and TNF for a total of 7 h, or 24 h before seeding for a further 24 h followed by exposure to shear stress and TNF for the desired period. Changes in expression at mRNA and proteins levels were assessed as described below.

Evaluation of gene expression by quantitative real-time PCR (qPCR)

RNA was extracted from EC cultured under static or flow conditions within microslides using RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) and quantified as described (19). mRNA was reverse transcribed and analysed by qPCR using Quanti-Tect™ probe RT-PCR kit according to manufacturer's instructions (Qiagen). Primers were bought as Taqman Gene Expression Assays (Applied Biosystems, Warrington, UK) for: Kruppel-like factor-2 (KLF)-2, IL-8, E-selectin, ICAM-1, VCAM-1, β1-integrin, β3-integrin, FAK, TNF-receptors 1 and 2 (p55/TNFR1 and p75/TNFR2). Samples were amplified using the 7500HT Real-Time PCR machine and analysed using the software package SDS 2.2 (Applied Biosystems, Foster City, CA, USA). Data were initially expressed as relative expression units (REU) compared to mRNA for 18S in the same samples.

Evaluation of protein expression or phosphorylation

To measure expression of integrins, HUVEC were detached with non-enzymic dissociation buffer and washed with PBA/BSA. Cells were permeabilised with 0.1% saponin and then incubated with phycoerythrin-conjugated mouse monoclonal antibody (mAb) against β1-integrin, β3-integrin (both R&D, Indianapolis, IN, USA) or mouse IgG control (DakoCytomation Ltd., Ely, UK) diluted 1: 20 in PBS containing 2% normal goat serum for 1 h on ice. Cells were then washed and fixed with 2% formaldehyde. The median fluorescence intensities were measured using a FACScan flow cytometer (Cyan, Becton Dickinson Ltd, Oxford, UK).

To quantify phosphorylation of P38 or FAK, HUVEC were lysed with Laemmli lysis buffer on ice. Whole cell lysate samples were boiled following addition of an equal volume of Laemmli reducing sample buffer. Samples were separated by SDS-PAGE on NuPAGE 4-12% Bis Tris gels (Invitrogen, Life Technologies Ltd., Paisley, UK) and transferred to polyvinylidene difluoride membrane. Phosphorylation was detected by western blotting using rabbit polyclonal against phospho-p38 (Promega, Madison, WI, USA) or mouse mAb against pY379 phospho-FAK (BD Transduction Laboratories, San Diego, CA, USA). Membranes were stripped and re-probed with rabbit polyclonal antibody against p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit monoclonal against FAK (Abcam plc, Cambridge, UK). In all cases, membranes were incubated with HRP-conjugated secondary antibodies (Amersham Biosciences, Little Chalfont, UK) and proteins were visualised by chemoluminescence (ECL; Pierce, Rockford, IL, USA) and quantified as arbitrary densitometry intensity units using Quantity One software (Biorad Laboratories, Hemel, Hempstead, UK). Values for the ratio of phospho:total protein were calculated, and results for the various treatments expressed relative to untreated controls.

Evaluation of NFκB activation

NFkB activation was measured in nuclear protein extracts (5 μg) by the TransAM-NFkB p65 activation kit (Active Motif, Carlsbad, CA, US) as described (10). This is a DNA-binding ELISA using a consensus oligonucleotide to capture members of the NFκB family of transcription factors in the extract, and an HRP-conjugated antibody against the NFκB p65 subunit to detect this specific subunit.

Analysis of adhesion and migration of neutrophils on EC

Isolation of neutrophils and adhesion assays were performed as previously described (22). Blood was collected from healthy volunteers into K_2EDTA (Sarstedt Ltd, Leicester, UK) after informed consent. Neutrophils were isolated and suspended in PBS containing 1mM Ca^{2+}, 0.5mM Mg^{2+} and 0.15% culture-tested bovine serum albumin (Sigma) (PBS/BSA) at 10^6 cells/ml. Microslides containing EC that had been cultured under static or flow conditions were viewed by phase contrast video microscopy during perfusion of a 4-min bolus of neutrophils and subsequent washout of non-adherent cells with PBS/BSA for 5 min. The flow rate was equivalent to a wall shear stress of 0.1 Pa throughout. Videomicroscopic recordings were analysed off-line using ImagePro (Media Cybernetics, Marlow, UK). Adherent cells could be characterised as slowly rolling over the surface (velocity ~5-10 μm s^{-1}), stationary adherent on the surface (phase bright) or transmigrated (phase dark and spread under the EC monolayer) (22). After washout, the total number of adherent neutrophils (rolling adherent, stationary or transmigrated) was counted, and corrected per mm^2 per 10^6 cells perfused, and the percentage that had transmigrated was also calculated.

Statistical analysis

Effects of multiple treatments were tested using analysis of variance (ANOVA), followed by post-hoc comparisons by Bonferroni test when appropriate. Samples exposed to individual treatment or inhibitors were compared to untreated controls by paired t-test. All tests were performed using the computer program Minitab (Minitab Inc, State College, PA, USA).

Results

Effects of shear stress on inflammatory responses of EC cultured on different proteins

We initially cultured EC on collagen, fibronectin or laminin for 24 h, followed by exposure to shear stress (2.0Pa) for 4 h followed by 100 U/ml TNF for a further 3 h under shear. These abbreviated periods of shear and TNF treatment were used to potentially optimise potential effects of early signalling on different substrates. Compared to cells cultured under static conditions throughout, this relatively brief exposure to shear caused slight but not signifi-

cant reduction in the number of neutrophils adhering (~25% on average), but more marked, significant inhibition of their transendothelial migration (~60%) (►Figure 1A,B). However, the effects were similar for the different substrates.

In a separate series of experiments, we proceeded to reduce periods of seeding, exposure to shear and exposure to TNF, to minimise the period in which EC could deposit their own matrix proteins. This might make it possible to detect differences between effects of shear on the different matrix protein coatings. First we reduced seeding to 2 h on each substrate. Cells were then cultured with or without shear for 5 h and then TNF for 2 h. From our previous work, 2 h treatment with TNF (but not shorter) is adequate to reliably induce adhesion of neutrophils (23). With this protocol, TNF induced high levels of neutrophil adhesion for static cultures (402 ± 66 adherent cells/mm2/106 perfused) but of the adherent neutrophils, only 1 ± 1% transmigrated (mean ± SEM from six experiments, two on each of the three different substrates). Migration was thus much less effective compared to values (40-50%) for the longer periods of culture and TNF described above. This implies that the shorter period of TNF treatment induced adhesion molecule expression but not activating agents at a level adequate to drive transmigration. Nevertheless, the brief shear conditioning was adequate to reduce adhesion induced by TNF (93 ± 7% reduction; mean ± SEM from six experiments, two on each of three different substrates). However, this reduction was similar after seeding on collagen, fibronectin or laminin (two experiments with each). We next reduced culture time even further to test if we could detect any differences between substrates. We found that for collagen or fibronectin we could obtain stable monolayers and reliable response to TNF using 2 h seeding, followed by 3 h of shear and TNF added for a further 2 h. Under these condition, again, efficient adhesion of flowing neutrophils was evident, but not transmigration. Laminin did not reliably maintain confluent monolayers under these conditions. In an extended series, experiments with these 'minimal' conditions, the level of adhesion was reduced by the brief exposure to shear, but again, the responses were not significantly different for fibronectin or collagen coatings (average reductions in adhesion 53% or 43%, respectively; ►Figure 1C).

We next used the 'minimal' incubation model (2 h seeding of EC followed by 3 h with or without shear, followed by a further period with or without TNF), to analyse NFκB activation and mRNA for adhesion molecules. In agreement with a previous report using brief seeding and shear (14), in the absence of TNF, basal NFκB activation in EC appeared higher on collagen than fibronectin, and shear tended to decrease activation for the former but increase it for the latter (►Figure 2A). However, for both surfaces, after 1 h of TNF there was marked increase in NFκB activation and the brief prior exposure to shear had little effect on this increase on either surface (►Figure 2A). After 1 h TNF, mRNA levels for E-selectin, VCAM-1, ICAM-1 and IL-8 were up regulated as expected. Data for E-selectin (the major neutrophil capture receptor) are summarised for collagen or fibronectin substrates in ►Figure 2B, which shows that shear inhibited up regulation for both surfaces. Similar trends of inhibition of upregulation by shear were seen for mRNA for VCAM-1 and IL-8, again for both collagen and fibronectin (data not shown). However, as previously reported (6), ICAM-1 upregulation by TNF was not modified by shear (data not shown). In addition, the anti-inflammatory transcription factor KLF2 was up regulated by shear as reported (24); up regulation was maintained after treatment with

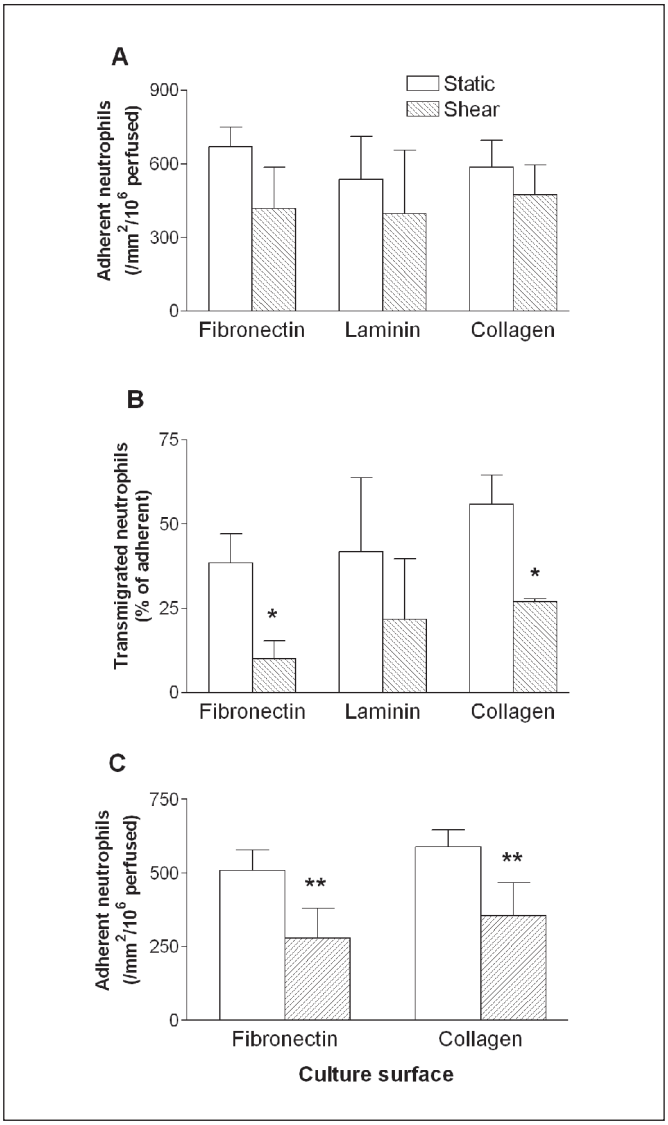

Figure 1: Effect of shear conditioning of HUVEC cultured on fibronectin, laminin or collagen on recruitment of flowing neutrophils. Neutrophil adhesion (A) and transendothelial migration (B) for HUVEC seeded for 24 h, exposed to shear stress (2.0 Pa) for 4 h and then treated with 100 U/ml TNF for a further 3 h under shear (data are mean ± SEM from three or four experiments for each substrate). C) Neutrophil adhesion for HUVEC seeded for 2 h, exposed to shear stress (2.0 Pa) for 3 h and then treated with 100 U/ml TNF for a further 2 h under shear (data are mean ± SEM from 11 experiments for each substrate). Static cultures were seeded and treated identically but not exposed to shear stress. ANOVA showed that overall, shear modified transendothelial migration (B) or adhesion (C) for all surfaces taken together ($p<0.01$). *=$p<0.05$, ** = $p<0.01$ for comparison between static and shear conditions for the individual surfaces by paired t-test.

TNF, but again in all cases expression was not dependent on the substrate (►Figure 2C).

Thus, shear-induced down regulation of response to TNF could be demonstrated in brief cultures, but little difference could be demonstrated for different protein substrates. Next, we modified integrin expression with siRNA, potentially to reveal specific effects of integrins on different substrates, or to test whether shear–induced integrin-matrix signalling was indeed functionally important.

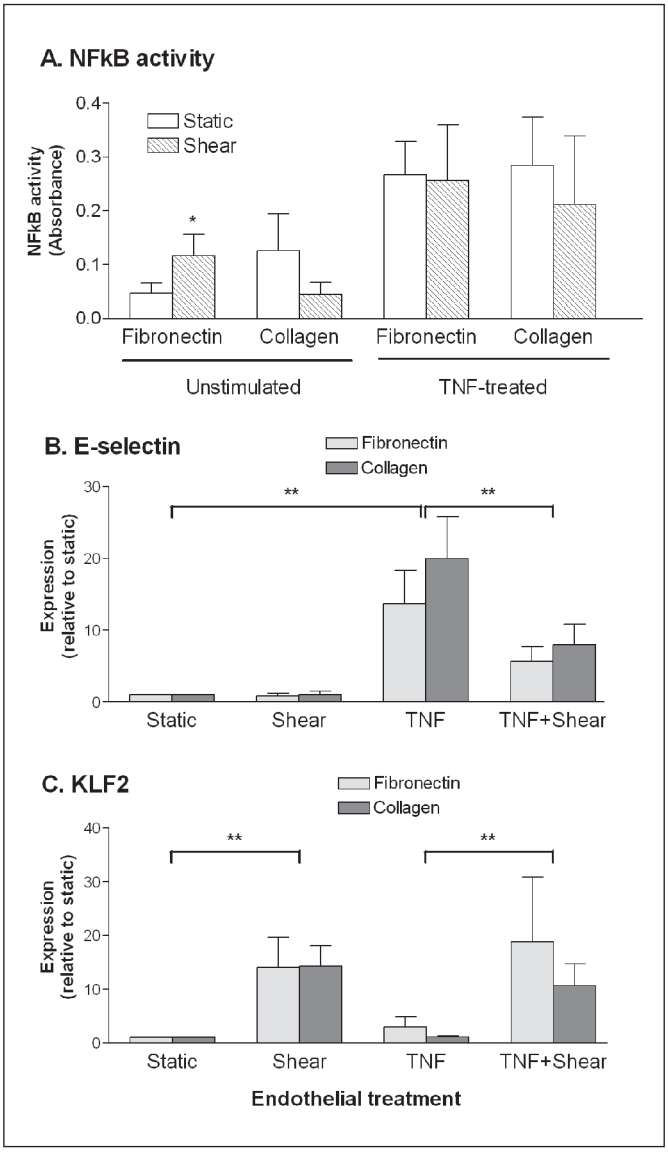

Figure 2: Effect of shear conditioning on the transcriptional activity of HUVEC cultured on fibronectin or collagen. HUVEC were seeded for 2 h, exposed to shear stress (2.0 Pa) for 2 h and then treated with 100 U/ml TNF or left untreated for a further 1 h under shear. Static cultures were seeded and treated identically but not exposed to shear stress. A) Activation of NFκB (data are mean ± SEM from four experiments for each substrate). B and C) mRNA expression for E-selectin and KLF2, respectively (data are mean ± SEM relative to values for unstimulated static HUVEC from three experiments for each substrate). In (A), ANOVA showed significant effect of TNF overall but not of substrate on NFκB activation; *=p<0.05 compared to static culture by paired t-test. For E-selectin (B) or KLF2 (C), ANOVA showed no effect of substrate, but significant effect of endothelial treatment (p<0.01 for each); *=p<0.05, ** = p<0.01 for comparison of combined data for both substrates by Bonferroni test.

Effect of reduction in expression of integrins on shear conditioning

Using siRNA against β1-integrin, we obtained marked reduction in mRNA and protein expression 48 h after transfection (88 ± 9% and 72 ± 6% reductions, respectively; means ± SEM from three and six experiments, respectively). To test whether integrin signalling might have differential effects on different matrix proteins, we used the minimal incubation model. After 48 h transfection, cells were seeded for 2 h in microslides which had been coated with collagen or fibronectin, and then exposed to shear stress of 2.0 Pa for 3 h with TNF added for a further 2 h. Transfected EC continued to form confluent monolayers capable of withstanding shear and supporting neutrophil adhesion. However, shear-induced modulation of neutrophil adhesion was essentially absent for cells lacking β1-integrin, compared to untransfected HUVEC or HUVEC treated with a non-specific siRNA (►Figure 3A,B). The requirement for expression of β1-integrin to enable shear-conditioning was evident for collagen or fibronectin coatings (►Figure 3A and B, respectively).

Following these experiments, we reverted to long-term seeding and exposure to shear stress, which was expected to show potent stress-dependent inhibition of responses (6). We aimed to test the requirement for β1-integrin signalling for responses over longer time periods. After 24 h transfection, cells were seeded on collagen without flow for 24 h and then exposed to 24 h shear, with TNF added for the last 4 h. Collagen was chosen as supporting slightly more consistent responses in the shorter experiments (►Figure 3B). As expected, adhesion was strongly down regulated by the long-term shear, but reduction of β1-integrin expression still effectively nullified the effect of shear (►Figure 3C). Thus, overall, even with short-term cultures, we could not detect any differences between responses on different matrix proteins; for short- or long-term cultures, reduction of β1-integrin expression essentially nullified the effect of shear on the functional adhesion readouts. For comparison, we tested siRNA against β3-integrin (reduction in expression at mRNA level was 89%; mean from two experiments). In this case, there was no effect of siRNA on the shear-induced reduction of adhesion (►Figure 3D). None of the targeted or non-specific control siRNAs had significant effects on the levels of adhesion for static cultures (►Figure 3A-D).

We next examined effects of siRNA against β1-integrin on NFκB activation and gene expression using long-term exposure to shear. After 24 h exposure to shear, TNF-induced activation of NFκB was reduced by shear-conditioning (in contrast to results using short-term shear); after reduction in expression of β1-integrins, NFκB activation was no longer affected by shear conditioning (►Figure 4A). Up regulation of E-selectin or VCAM-1 expression in response to TNF was inhibited by shear-conditioning; for either receptor, reduction in expression of β1-integrins tended

to lower the level of expression (although not statistically significantly), and although shear conditioning tended to reduce expression further, this did not reach statistical significance (►Figure 4B,C). As a control, expression of ICAM-1 was tested and again found not to be modified by shear stress, and this remained the case when expression of β1-integrins was reduced (►Figure 4D). Up regulation of expression of KLF2 by shear-conditioning was evident with or without reduction of expression of β1-integrin (►Figure 4D). Pre-treatment of HUVEC with non-specific siRNA left all responses essentially identical to untreated cells (►Figure 4A-D).

Signalling pathways linked to β1-integrin in shear-conditioning of neutrophil adhesion

The pathway(s) by which the effectsof β1-integrins were exerted were tested in the long-term model of 24 h seeding on collagen, followed by up to 20 h exposure to shear, with TNF added for a further 1-4 h when desired. We checked whether exposure to shear or reduction in integrin expression affected expression of the TNF receptors TNFR1 and TNFR2. Shear stress alone did not significantly modify mRNA levels of either receptor (the ratios of expression for sheared:static cultures were 0.90 ± 0.12 or 1.22 ± 0.31 for TNFR1 or TNFR2, respectively), in agreement with our previosus report on protein levels (6). Reduction in β1-integrin expression did not significantly modify the levels either (the ratios of expression for siRNA:untreated cultures were 0.99 ± 0.18 or 1.03 ± 0.13 for TNFR1 or TNFR2, respectively) and the combination of integrin reduction and shear again gave similar levels (the ratios of expression for sheared siRNA:static untreated cultures were 1.06 ± 0.36 or 0.95 ± 0.25 for TNFR1 or TNFR2, respectively; all data mean ± SEM from four experiments). We checked the effect of β3-integrin reduction (which had no functional effect in any case) on receptor expression in one experiment and found little change either (data not shown). Thus reduction in response to TNF after shear-conditioning, and loss of this effect after integrin reduction could not be attributed to changes in TNF receptor levels.

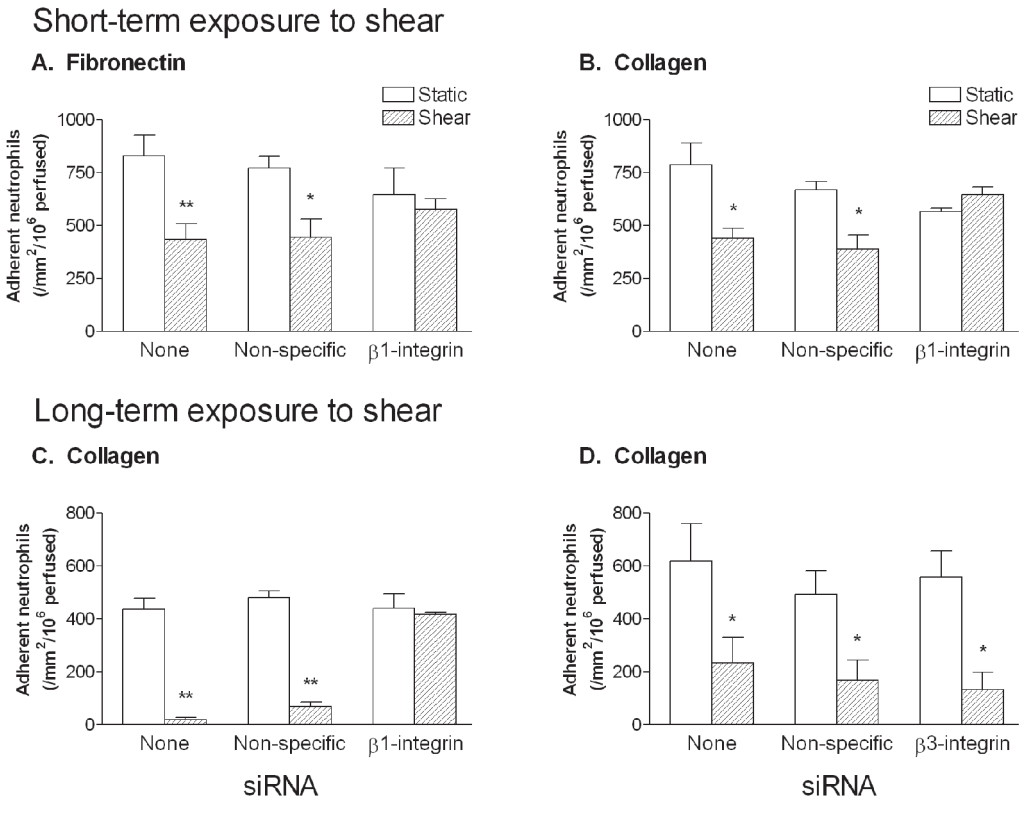

Figure 3: Effect of short or long term shear conditioning of HUVEC on recruitment of flowing neutrophils, with or without reduction of expression of β1- or β3-integrins using siRNA. A, B) Short-term exposure to shear: neutrophil adhesion to HUVEC seeded for 2 h on fibronectin or collagen, respectively, exposed to shear stress (2.0 Pa) for 3 h and then treated with 100 U/ml TNF for a further 2 h under shear. C, D) Long-term exposure to shear: neutrophil adhesion to HUVEC seeded for 24 h on collagen, exposed to shear stress (2.0 Pa) for 20 h and then treated with 100 U/ml TNF for a further 4 h under shear. A-C) HUVEC were treated with non-specific siRNA, with siRNA targeting β1-integrin or untreated (None); D) siRNA targeting β3-integrin (instead of β1-integrin) was used. Data are mean ± SEM from three experiments for each condition. ANOVA showed significant effect of shear on adhesion in all conditions ($p<0.01$). *=$p<0.05$, ** = $p<0.01$ compared to static culture by paired t-test.

We did find that phosphorylation of FAK was increased in EC 10 min after the start of exposure to shear (►Figure 5A). However, after treatment with siRNA against β1-integrin, increase in phosphorylation of FAK by shear was no longer evident (►Figure 5A). When an inhibitor of FAK activity (PF573228) was added to cultures, the level of adhesion seen in static cultures was unchanged, but the ability of shear conditioning to reduce adhesion was suppressed (►Figure 5B). We thus tested the effect of reducing expression of FAK with siRNA; mRNA was reduced by 73% judged by qPCR and protein by 55% judged by western blotting and densitometry (means from two experiments). This treatment also led to loss of shear conditioning of neutrophil adhesion, compared to untreated controls or cells treated with non-specific siRNA (►Figure 5C). Thus FAK operated downstream of β1-integrins in signalling the anti-inflammatory effects of shear stress.

We hypothesised that p38 MAP-kinase might act downstream of β1-integrin and FAK based on studies showing that its rapid phosphorylation by shear varied depending on the matrix substrate (14). Increased phosphorylation of p38 was detected 10 min after the start of exposure to shear, but increase could no longer be detected if EC were pre-treated with FAK-inhibitor, or siRNA against β1-integrin or FAK (►Figure 6A,B). In addition, after treatment of EC with an inhibitor of p38 activity (SB202190), shear-conditioning did not suppress neutrophil adhesion (►Figure 6C). Thus, modulation of the inflammatory response to TNF in EC appeared to operate through shear-induced signals from β1-integrin to FAK to p38. It may be noted that the inhibitors of FAK (PF573228) and p38 (SB202190) did not themselves influence response to TNF at the concentrations used here, judged by neutrophil adhesion to the static cultures (►Figure 5B, ►Figure 6C).

Discussion

We investigated the roles played by matrix proteins and integrin-mediated signals in shear-conditioning of the functional response of endothelial cells to TNF. We found that as little as 7 h culture after seeding (with TNF added for last 2 h), gave reproducible adhesion of flowing neutrophils which was inhibited if shear was ap-

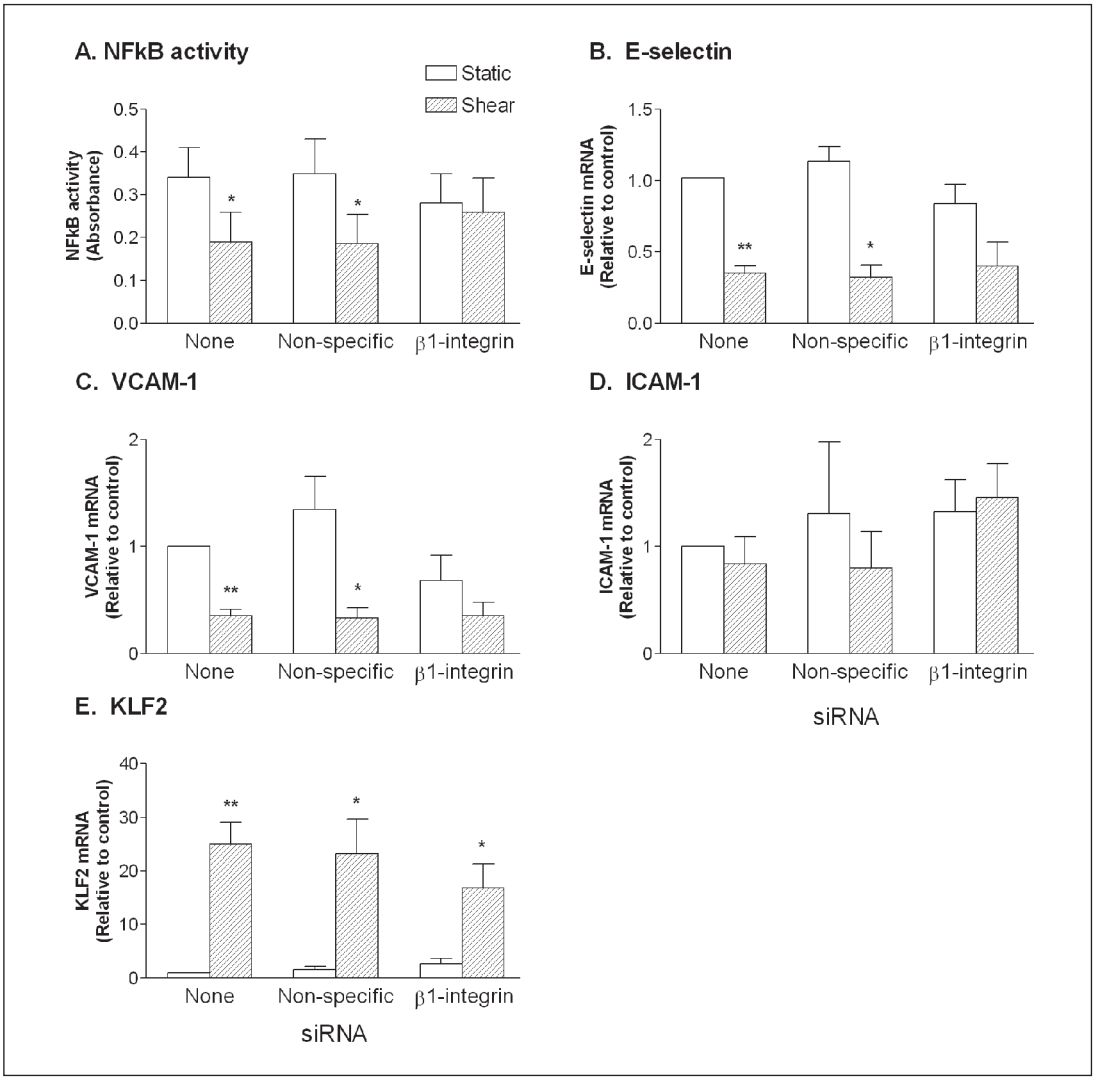

Figure 4: Effect of shear conditioning on the transcriptional activity of HUVEC with or without reduction of expression of β1-integrins using siRNA. HUVEC were seeded for 24 h on collagen, exposed to shear stress (2.0 Pa) for 20 h and then treated with 100 U/ml TNF under shear. A) Activation of NFκB 1 h after addition of TNF (data are mean ± SEM from six experiments for each substrate). B-E) mRNA expression for E-selectin, VCAM-1, ICAM-1 and KLF2, respectively, 1 h after addition of TNF (data are mean ± SEM relative to values for unstimulated static HUVEC from 3, 4 or 5 experiments, respectively). ANOVA showed significant effect of shear on all parameters ($p<0.01$) except ICAM-1 mRNA. *=$p<0.05$, ** = $p<0.01$ compared to static culture by paired t-test.

plied after the first 2 h seeding. However, under this condition which minimised matrix deposition by the EC themselves, and in longer cultures, responses were similar for different protein substrates; collagen, laminin or fibronectin. This applied to gene expression and NFκB activation as well as neutrophil adhesion. On the other hand, when β1-integrin expression was reduced by siRNA, this effectively nullified the effect of exposure to shear stress. This was true for brief or prolonged exposures to shear, but

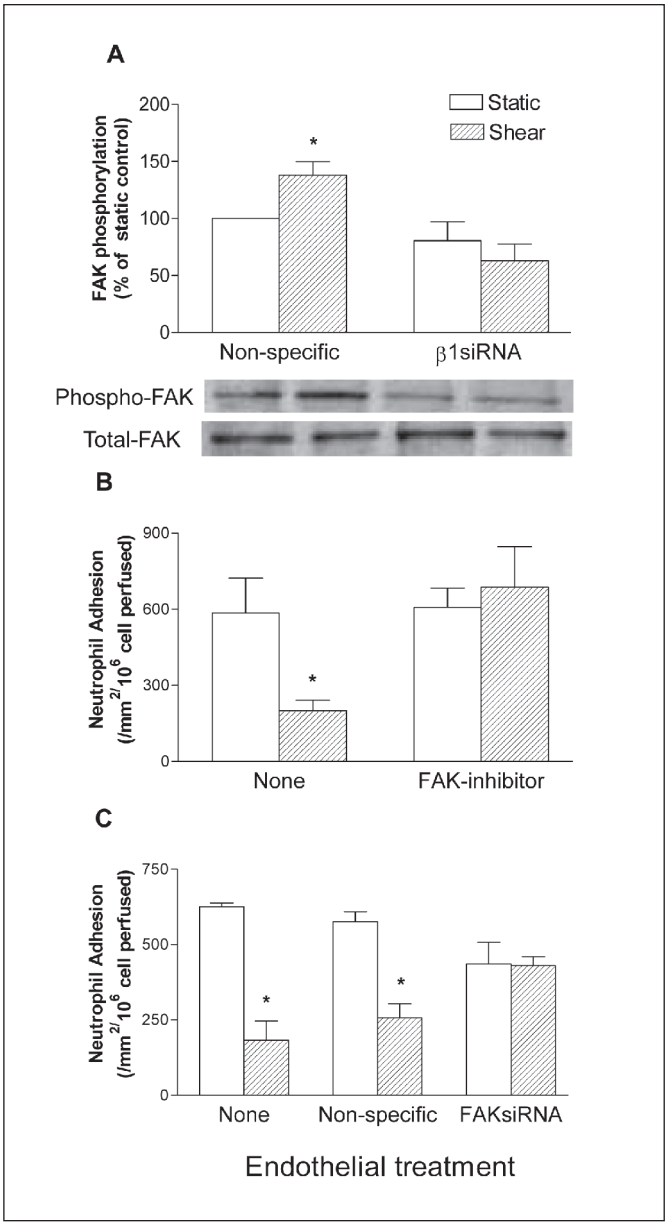

Figure 5: Role of FAK in the effects of shear conditioning on HUVEC. A) Level of phosphorylation of FAK in HUVEC which were seeded for 24 h on collagen and exposed to shear stress (2.0 Pa) for 10 min, with or without reduction of expression of β1-integrins using siRNA. Values are % of static cultures treated with non-specific siRNA. Typical western blots for phospho- and total FAK are shown below the graph. B,C) Neutrophil adhesion to HUVEC which were seeded for 24 h on collagen and exposed to shear stress (2.0 Pa) for 20 h and then treated with 100 U/ml TNF for a further 4 h; B) with or without FAK-inhibitor (PF573228, 1 μM) added 1 h before exposure to shear; C) with or without reduction of expression of FAK using siRNA. Data are mean ± SEM from three or experiments in all cases. *=p<0.05, ** = p<0.01 compared to static culture by paired t-test.

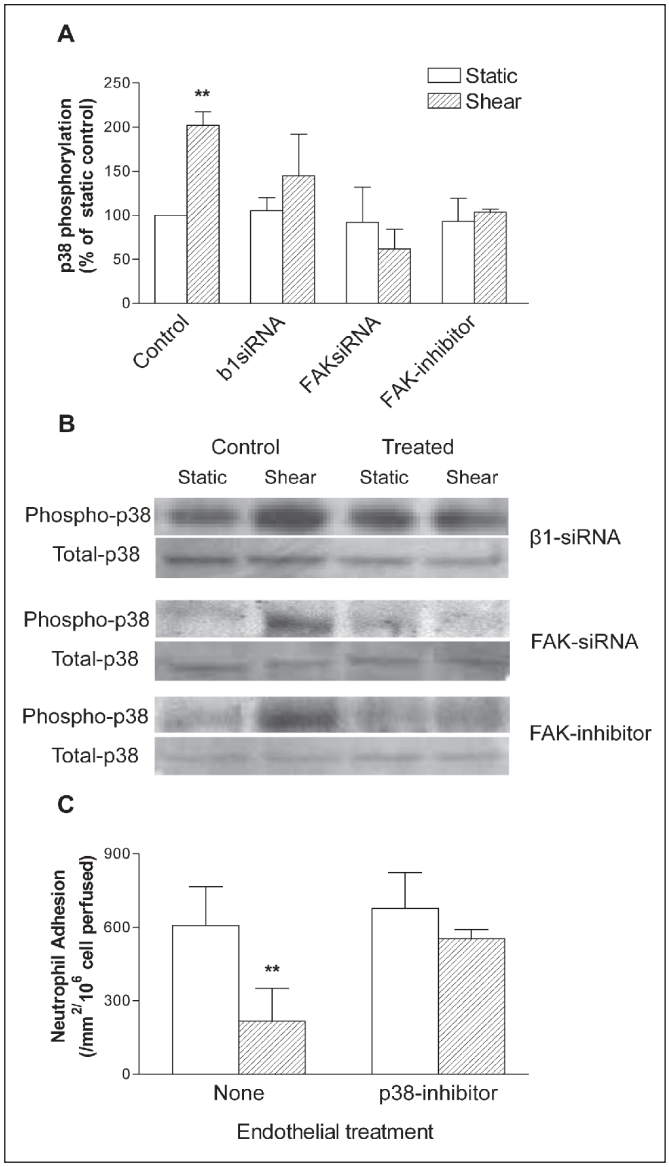

Figure 6: Role of p38 in the effects of shear conditioning on HUVEC. A) Level of phosphorylation of p38 in HUVEC which were seeded for 24 h on collagen and exposed to shear stress (2.0 Pa) for 10 min, with or without reduction of expression of β1-integrins or FAK using siRNA, or in the presence of FAK-inhibitor. Values are % of static controls. Controls were either treated with non-specific siRNA (paired with samples treated with specific siRNAs) or untreated (paired with samples treated with FAK-inhibitor) (data pooled for 11 controls in total). B) Typical western blots for phospho- or total FAK for the different treatments, from static or sheared cultures. C)Neutrophil adhesion to HUVEC which were seeded for 24 h on collagen and exposed to shear stress (2.0 Pa) for 20 h and then treated with 100 U/ml TNF for a further 4 h, with or without p38-inhibitor (SB202190, 10 μM) added before exposure to shear. Data are mean ± SEM from three or more experiments in all cases. *=p<0.05, ** = p<0.01 compared to static culture by paired t-test.

again, not specific to the substrate first deposited. Knock-down of β3-integrin did not modify shear modulation of the adhesive response. Based on studies of phosphorylation, siRNA and pharmacological inhibitors, the integrins acted upstream of a pathway leading to phosphorylation of FAK and then p38. This pathway down regulated activation of NFκB and expression of adhesion receptors, and so caused shear-induced inhibition of neutrophil recruitment in response to TNF. Thus, for the first time we have linked a pathway from integrin-signalling to protective effect of shear conditioning against inflammation.

Integrins are important transducers of shear-induced responses through their binding to extracellular matrix and formation of complexes with Src-family kinases and FAK, with downstream signalling through small GTPases and MAP-kinases leading to activation of gene transcription factors including NFκB (13). Different integrins have been shown to be capable of initiating signals for cells grown on their specific substrates, although contributions to effects on functional responses were not clearly defined. In studies of EC cultured on collagen or fibronectin for short periods, Orr et al. (14) found differences in p38 phosphorylation and NFκB activation shortly after exposure to shear. These studies suggested that the effects of shear stress on inflammatory responses of endothelial cells might be dependent on their adhesive substrate. Subsequent studies using more complex mixtures of substrate protein, showed that integrins engaged with different substrates could influence each other's activation and downstream signalling (15). This makes it hard to predict the outcome of signals from specific integrins or substrates on endogenous extracellular matrices. Since we previously found that HUVEC lay down detectable levels of collagen IV, fibronectin and laminin within 24 h (25), we first used short-term culture conditions similar to those of Orr et al. (14) to see if we could detect functional effects of differences in substrate. For unstimulated EC, we found patterns of NFκB activation similar to those published (14) (i.e. the initial level was higher for collagen and went down with shear, and lower for fibronectin but went up with shear). Under these conditions, shear-conditioning of the unstimulated endothelial cells did not cause consistent changes in neutrophil adhesion, which was very low in every case (data not shown). We thus studied how matrix and integrin signalling influenced the known ability of shear to down-regulate TNF-induced adhesion (6).

Short-term exposure to shear stress inhibited the EC response to TNF (judged by neutrophil adhesion), but the effect was similar regardless of the culture substrate. There was marked activation of NFκB by TNF which was similar on collagen or fibronectin, overriding the effects of substrate or shear on the unstimulated level. Under these conditions, short-term exposure to shear did not significantly modify the TNF-induced NFκB activation. We thus questioned whether the attenuation of the response to TNF was indeed mediated by integrin signalling, and found that reduction of expression of β1-integrin did effectively ablate shear-conditioning. This was the case on fibronectin or collagen substrates, and so we extended studies to long-term exposure to shear for EC already seeded for 24 h, where a more complex substrate would exist. These conditions, where we have previously shown profound anti-inflammatory effect of shear, might be more relevant to the physiological situation of blood vessels continually experiencing shear stress. Under these conditions, we found that expression of β1-integrin (but not β3-integrin) was critical for the protective effects of shear stress. Moreover, TNF-induced activation of NFκB was inhibited by shear-conditioning, as were TNF-induced up regulation of expression E-selectin and VCAM-1. After reduction in expression of β1-integrin, the effects of shear on NFκB, E-selectin and VCAM-1 were no longer significant, although the baseline levels of the adhesion receptors had shifted downward before shear. Previous studies indicate that inhibition of E-selectin expression is the likely mediator of reduced neutrophil adhesion after shear-conditioning (6).

To gain further insight into how shear-induced integrin signalling could translate into changes in neutrophil adhesion, we studied likely intermediary pathways. FAK was shown early on to play a role in generation of shear-dependent signals following formation of integrin-matrix bonds (16). However, little was known of functional responses downstream of FAK in this context.

What is known about this topic?

- Shear stress is an important regulator of the functions of endothelial cells. Increasing steady shear stress has progressively greater anti-inflammatory effects, but oscillatory low shear stress may predispose to thrombotic disease in arteries.
- Various mechanotransducers have been shown to initiate signals in response to changes in shear stress. Signalling through different integrin receptors can be demonstrated to depend on the matrix protein to which they bind, but these signals have not been clearly linked to changes in specific cell functions.
- Strong evidence links signals from integrins, focal adhesion kinase (FAK) and MAP-kinase p38 to pro-inflammatory signals caused by changes in shear. However, it is not known if they transduce the protective reduction in inflammatory function arising from conditioning by prolonged steady shear stress.

What does this paper add?

- Steady shear stress down-regulates the inflammatory response to tumour necrosis factor (TNF) (judged by recruitment of flowing leukocytes) but this does not depend on the matrix protein used as endothelial cell culture substrate, possibly because of deposition of endogenous matrix in hours.
- Integrin signalling thru FAK and p38 is demonstrated in response to steady shear and is shown to be essential for protective effects. On native matrix proteins, signalling from β1-integrins dominates over β3-integrins.
- Direct stimulation of this pathway might be anti-thrombotic, but it is essential to identify specificity in mechanotransduction as signals might be part of the protective or pathogenic effects of different forms of shear. Stimulation of integrin signalling might mimic protective effects of steady shear in some vessels but worsen pathology in others experiencing oscillatory flow.

Here, shear-induced FAK phosphorylation was dependent on β1-integrin expression. Reduction of FAK expression by siRNA and pharmacological inhibition both suppressed the effect of shear-conditioning on response to TNF, but did not modify the responses of static cells. Phosphorylation of p38 was also previously implicated in shear-induced integrin signalling and substrate-dependent NFκB activation or inactivation (14). Here, FAK was required for p38 phosphorylation and an inhibitor p38 activity also caused loss of shear regulation of adhesion. These results clearly identify FAK as a downstream mediator of functional effects of shear-induced signalling through β1-integrin and directly demonstrate the role of integrin-FAK-p38 in protective effects of shear against inflammation. The effects of shear-conditioning on neutrophil adhesion can thus be attributed to generation of an integrin-dependent signal acting via FAK and p38 to inhibit NFκB activity and adhesion molecule expression.

An outstanding question arises from our observation that although long-term exposure to shear inhibited TNF-induced nuclear translocation of NFκB, in short-term experiments protective effects of shear occurred without inhibition of nuclear translocation. Interestingly, a recent study showed that when a murine endothelial cell line lacking expression of FAK was exposed to shear stress, phosphorylation of NFκB was not upregulated to the same extent as control EC, although nuclear translocation of NFKB was not inhibited (17). It was suggested that shear-induced signalling via FAK modified transcriptional activity of NFκB by a novel mechanism, not linked to nuclear translocation. Although that study did not combine shear with TNF stimulation, FAK-dependent changes in phosphorylation of NFκB might be the basis of the effects of short-term exposure to shear. Over the long-term, the more powerful inhibitory effects of shear may arise from additional loss of nuclear translocation.

We also examined expression of KLF2, a transcription factor up regulated by shear stress, which may have anti-inflammatory effects (24). However, while KLF2 expression was strongly up regulated by exposure to shear stress, this remained the case when integrin expression was reduced. Similar to our previous findings (9, 19), this implies that expression of this transcription factor is not closely linked to protective effects of shear on cytokine-induced leukocyte recruitment.

Integrin-signalling is one of several mechanotransduction pathways proposed to underlie effects of shear stress on endothelial cells. In studies of acute and oscillatory shear, signalling from a complex incorporating CD31, VE-cadherin and VEGFR2 was upstream of integrin activation (4). We recently showed that ablation of CD31 could also inhibit the ability of shear-conditioning to suppress the response to TNF although we did not examine signalling downstream of that receptor (19). While it has been shown that integrins and CD31 can signal independently in response to force applied to each separately (18), our studies are consistent with their signals being at least linked in anti-inflammatory function, as loss of either nearly ablates protection. It also appears that these linked pathways play roles in both protective effects of steady shear and pathogenic pro-inflammatory effects of oscillatory shear, although the downstream signals must differ in some as yet undefined way. It will be important to understand how the pathways that underlie either type of response diverge, or possibly whether other shear-type-specific transducers feed into these pathways, if one wishes to inhibit or mimic them for therapeutic gain. It would not be beneficial, for example, to inhibit signals generated under oscillatory shear if the same signals supported down-regulation of inflammation by steady shear. Protection against atheroma and thrombosis might then occur at a cost of systemic over-responsiveness to inflammation.

Conflicts of interest

None declared.

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