Shear stress-induced shedding of soluble intercellular adhesion molecule-1 from saphenous vein endothelium

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Abstract Within 6 h, shear stress upregulated intercellular adhesion molecule-1 (ICAM-1) (two- to four-fold, $P < 0.001$) and induced matrix metalloproteinase-2 (MMP-2) in cultured human saphenous vein endothelial cells. By 8 h endothelial ICAM-1 levels returned to baseline, with concomitant increase in soluble ICAM-1 (sICAM-1) ($P < 0.001$) and MMP-9 had been induced. Inclusion of a hydroxamate metalloproteinase inhibitor partially reversed the effects on ICAM-1 and sICAM-1 at 8 h, whereas TIMP-1, -2 or -3 had no effect. MMP-9, but not MP-2, co-immunoprecipitated with ICAM-1. sICAM-1 was processed distal to Arg441, indicating that MMP-9, docking to ICAM-1, contributes to sICAM-1 shedding and attenuation of the shear stress-induced upregulation of ICAM-1.

Key words: Endothelium; Shear stress; Adhesion molecule; Metalloproteinase

1. Introduction

Endothelial cells sense shear stress at or near the cell surface. Shear stress may be transduced through interactions of activated mechanoreceptors with connections to the cytoskeleton leading to a diversity of cell responses and alterations in gene expression [1]. The upregulation of endothelial intercellular adhesion molecule-1 (ICAM-1) in response to shear stress has been well described in cultured cells and intact saphenous vein [2–4]. The transient upregulation of endothelial ICAM-1 and its influence on leukocyte recruitment and vascular remodelling are likely to be important in several clinical situations, including the fashioning of arteriovenous fistulae for dialysis access and in saphenous vein grafts, implanted to bypass arterial occlusions.

No studies have reported on mechanisms that 'switch off' or counteract the endothelial response to shear stress, but for ICAM-1 the possibility of protease-induced shedding of soluble ICAM-1 (sICAM-1) is considered here. There is controversial evidence as to the class of protease that is involved in sICAM-1 shedding from cultured cells. In U937 cells sICAM-1 shedding was effected by human leukocyte elastase (a serine protease) but not by matrix metalloproteinase-9 (MMP-9) [5]. In contrast, in astrocytes sICAM-1 shedding was blocked by hydroxamate inhibition of MMPs [6]. More recently, it has been suggested that MMP-9 binds to ICAM-1 on the surface of HL60 cells, subsequent activation of MMP-9 being associated with sICAM-1 shedding [7].

2. Materials and methods

All reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. XN908 (HONHCOCH(CH_3)CH (isobutyl)-CO-Tyr-(OMe)-NHMe) was obtained from Dupont Pharmaceuticals (Wilmington, DE, USA), tumour necrosis factor-α (TNF-α) from R&D Systems (Abingdon, UK) and α_2-macroglobulin from Research Diagnostics (Flanders, NJ, USA). Recombinant tissue inhibitors of metalloproteinases TIMP-1, TIMP-2 and TIMP-3 were prepared and tested for activity as described previously [8]. Monoclonal antibodies to MMP-2 and MMP-9, sICAM-1 and MMP enzyme-linked immunosorbent assay kits were obtained from R&D Systems. Monoclonal and polyclonal antibodies to ICAM-1 were from Dako Diagnostics (Ely, UK) and R&D Systems respectively.

Human saphenous vein endothelial cells (HSVECs) were isolated and cultured as described previously [9]. For shear stress experiments HSVECs were grown to confluence (passage 3) on chamber slides 75 mm × 25 mm (Nunc, Paisley, UK), which were subjected to shear stress for various times in a parallel plate chamber [10]. HSVECs sufficient for 20–25 slides only were obtained from each donor. Medium (M199 containing 15 μg/ml endothelial growth factor and 17 U/ml heparin) was replaced 30 min before application of shear stress, reagents added 20 min later and included in the perfusate (30 ml reservoir, gassed with 95% O_2/5% CO_2); the entire system was maintained in an enclosure at 37°C. Flow was altered to generate simulated 'venous' (7.6 ml/min, 0.08 N/m^2) or 'arterial' shear stress (76 ml/min, 0.79 N/m^2) [4]. At completion of experiments cells were fixed for 2 min with ice-cold methanol and 0.03% (v/v) H_2O_2 at room temperature and the perfusate was centrifuged (300 × *g*, 5 min) and concentrated 100-fold using Centriplus concentrator tubes (Millipore, Watford, UK). Concentrated perfusate was frozen in aliquots at −70°C. Time-matched stationary controls and TNF-α (5 ng/ml)-stimulated ICAM-1 expression (± reagent) served as negative and positive controls respectively. ICAM-1 was measured as described previously [11]. sICAM-1 was collected from the concentrated lysate by immunoprecipitation (see below), and tryptic peptides analysed by matrix-assisted laser desorption ionisation mass spectrometry.

Cell number was determined from measurement of double-stranded DNA, using a PicoGreen™ dsDNA quantitation kit (Molecular Probes, Leiden, The Netherlands). The assay, calibrated with suspended HSVECs, was linear in the range 4000–20 000 cells.

Cell lysates, cells were prepared in 50 μl of 10 mM Tris–Cl^- pH 7.4 containing 140 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium metavanadate ($NaVO_3$,), 0.8 U/ml aprotinin, 1 mM EDTA, 2.9 μM pepstatin A, 4.68 μM leupeptin, and 1% (v/v) NP40. Lysates were stored at −70°C until used for immunoprecipitation (incubation with 5 μl goat polyclonal antibodies to ICAM-1 for 2 h at 4°C, followed by incubation with 10% volume of protein G Sepharose for 2 h at 4°C) or gelatin zymography. Polyacrylamide gels (7.5%) containing 1% sodium dodecyl sulphate and 0.8 mg/ml gelatin were prepared and run in 2-amino-2-methyl-1,3-propanediol–Cl^- buffer, with 0.5 μg/ml purified MMP-2 (Matrix Biology Laboratory,

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Kennedy Institute of Rheumatology, London, UK) as a positive control. To identify the class of proteinase activity, PMSF (10 μM) or EDTA (5 mM), for inhibition of serine and metalloproteinases respectively, were added to the renaturing and developing buffers.

Statistical calculations were performed using Stata version 6 (Texas, USA).

3. Results

3.1. Effect of steady and step laminar shear stress ICAM-1 expression in HSVECs

Low shear stress ('venous', 0.08 N/m^2), for periods of up to 8 h, did not alter cellular ICAM-1 (open bars Fig. 1A). Higher shear stress ('arterial', 0.8 N/m^2) resulted in a two- to four-fold increase in cellular ICAM-1 after 6 h (solid bars in Fig. 1A), analysis of variance (ANOVA) $P = 0.002$. However, after 8 h cellular ICAM-1 was not significantly different from baseline. After 'arterial' shear stress for up to 8 h, HSVECs remained flattened with little cell detachment ($< 10\%$) (Fig. 2).

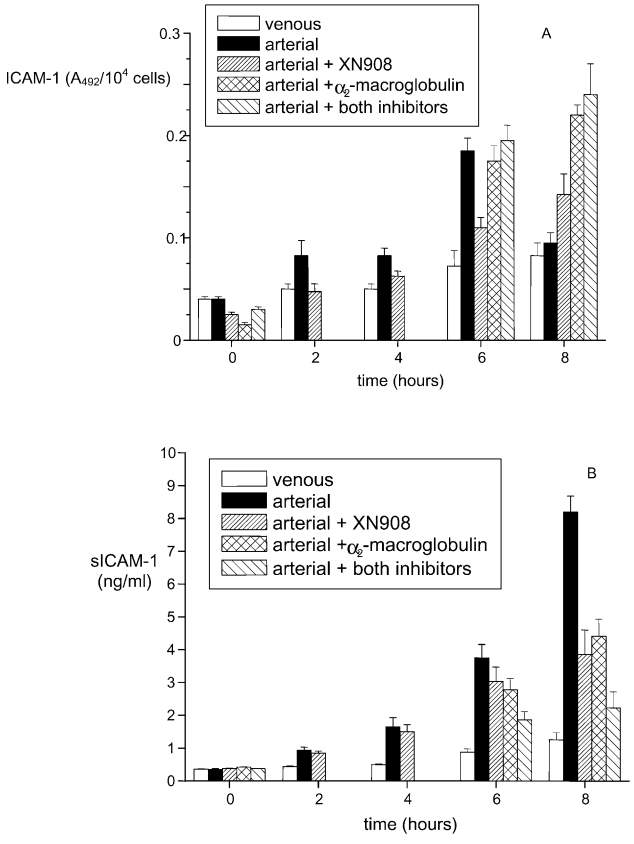

Fig. 1. Regulation of ICAM-1 in HSVECs by shear stress. All values are means ± S.E.M. from four or five separate experiments. Error bars show S.E.M. Different HSVEC donors were used for each time course, so that only statistical comparisons (ANOVA) within each bar coding are valid. A: Cellular ICAM-1. Cellular ICAM-1 expression was unchanged by 'venous' shear stress, but increased two- to four-fold after exposure to 6 h of 'arterial' shear stress under all conditions ($P < 0.01$). ICAM-1 levels increased further after 8 h in the presence of 3 μM XN908 or 0.05 μM α_2-macroglobulin ($P < 0.02$), but had reduced to near-basal levels in the absence of these protease inhibitors. B: sICAM-1 (values refer to unconcentrated perfusate). With 'arterial' shear stress, in the presence or absence of inhibitors, there was a steady increase in sICAM-1 between 0 and 6 h. In the absence of inhibitors sICAM-1 doubled between 6 and 8 h ($P < 0.01$), but this increase was abolished in the presence of inhibitors.

3.2. Effect of prolonged exposure to shear stress on sICAM-1

sICAM-1 increased steadily, from 0.35 ± 0.03 ng/ml to 3.75 ± 0.41 ng/ml, between 0 and 6 h of 'arterial' shear stress, but with a sharp increase to > 8 ng/ml between 6 and 8 h (solid bars in Fig. 1B). In contrast, any increase in sICAM-1 after the application of 'venous' shear stress was insignificant (open bars in Fig. 1B).

3.3. Effect of protease inhibitors on cellular ICAM-1

The protease inhibitors XN908 (3 μM) and α_2-macroglobulin (50 nM) did not alter the cellular ICAM-1 concentration in the absence of shear stress. In the presence of either XN908 or α_2-macroglobulin, cellular ICAM-1 expression continued to increase between 6 and 8 h of 'arterial' shear stress ($P = 0.02$ and $P = 0.023$ respectively) (Fig. 1A). The combined presence of XN908 and α_2-macroglobulin in the perfusate had a similar effect (Fig. 1A). Inclusion of 1 μM TIMP-1, -2 or -3 in the perfusate did not maintain elevated cellular ICAM-1 levels after 8 h of 'arterial' shear stress (Table 1).

3.4. Effect of protease inhibitors on sICAM-1

Inclusion of either XN908 or α_2-macroglobulin in the perfusate had no significant effect in reducing sICAM-1 levels between 0 and 6 h of 'arterial' shear stress (Fig. 1B), but the sharp increase between 6 h and 8 h was abolished ($P < 0.02$). The combination of XN908 and α_2-macroglobulin in the perfusate appeared to have increased effectiveness to reduce sICAM-1 levels (Fig. 1B). Inclusion in the perfusate of 1 μM TIMP-1, -2 or -3 did not alter the generation of sICAM-1 from HSVECs exposed to 'arterial' shear stress (Table 1).

3.5. Gelatinolytic activity in lysates of HSVECs exposed to 'arterial' shear stress

No activity was observed on gelatin zymograms in lysates of cells maintained in the absence of flow. After 6 h of 'arterial' shear stress, four gelatinolytic bands at 107 kDa, 92 kDa and doublet at 72 kDa and 64 kDa were visible (Fig. 3A). By 8 h densitometry indicated that the 92 kDa band had four-fold increased intensity ($n = 4$, $P = 0.02$), whereas the intensity of the 72/64 kDa doublet had not increased ($P = 0.15$). PMSF (10 μM) inhibited the band detected at 107 kDa only (data not shown). EDTA (5 mM) inhibited MMP-2 markers (64 kDa activated form and 45 kDa form lacking the C-terminal haemopexin domain) and bands detected at 92 kDa and doublet at 72 kDa and 64 kDa in cell lysates (Fig. 3A), suggesting their possible identity as MMP-9 and MMP-2 respectively. Western blotting confirmed the presence of proMMP-2 in 6 h and 8 h lysates (Fig. 3C), but proMMP-9 only in 8 h lysates (Fig. 3D).

3.6. Gelatinolytic activity in perfusate of HSVECs exposed to 'arterial' shear stress

In the absence of flow there was no gelatinolytic activity at < 100 kDa. Gelatinolytic activity at 72 kDa (inhibited by EDTA), compatible with proMMP-2, was present in perfusate after 6 and 8 h 'arterial' shear stress (Fig. 3B): there was no evidence of a 92 kDa band. MMP-2 concentrations in the 0, 6 and 8 h concentrated perfusates were < 0.2, 0.8 ± 0.2 and 2.2 ± 0.3 ng/ml respectively, whereas MMP-9 was not detected ($n = 4$).

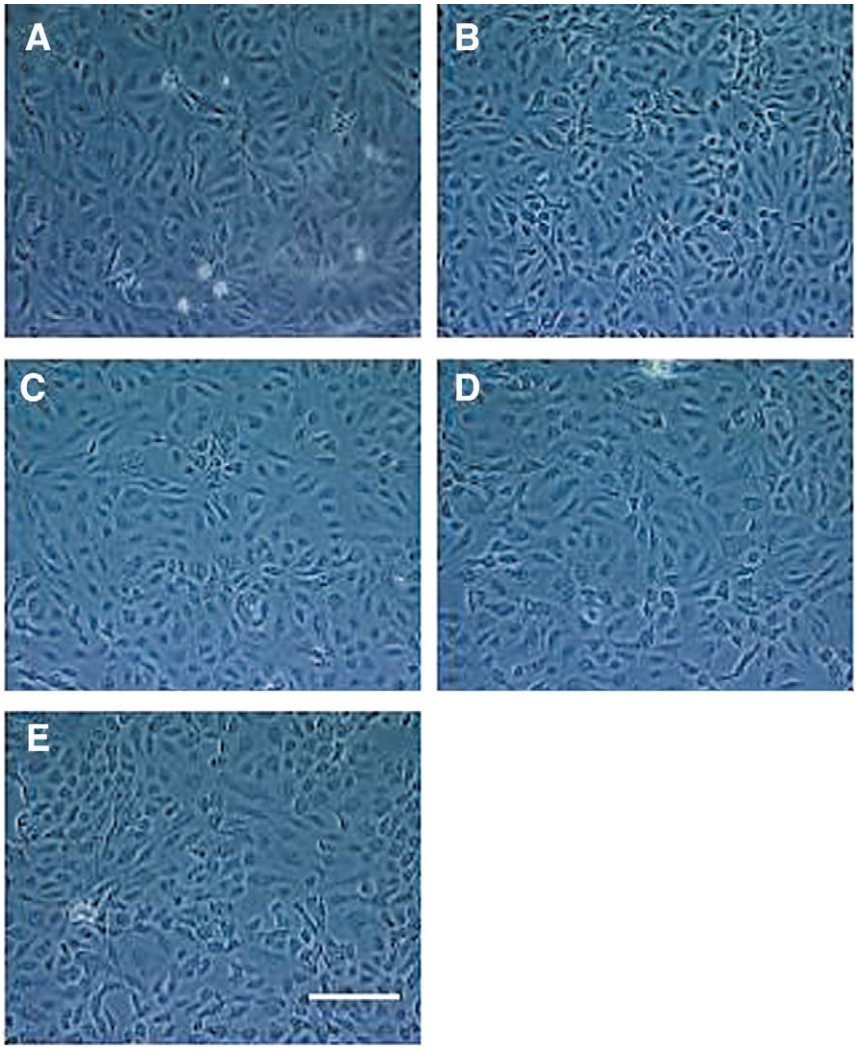

Fig. 2. Morphology of human saphenous vein endothelial cells after 0–8 h of shear stress. HSVECs cultured in the absence of flow for 8 h (A) and after exposure to 2, 4, 6 and 8 h of laminar shear stress, 0.8 N/m^2 (B–E respectively). Scale bar represents 50 μm.

3.7. Immunoprecipitation of ICAM-1 from cell lysates

Following immunoprecipitation of cell lysates (0 h and 8 h 'arterial' shear stress) with polyclonal antibodies to ICAM-1, the precipitated proteins were subject to Western blotting (Fig. 4). Probing with a monoclonal antibody to ICAM-1, the positive control for ICAM-1 was a single band (115 kDa) at 0 h, but an additional band at 95 kDa, indicative of ICAM-1 cleavage, was observed at 8 h. Probing the blots with monoclonal antibody to MMP-2 revealed no staining at 0 or 8 h, but monoclonal antibody to MMP-9 revealed a band at 92 kDa (proMMP-9) after 8 h.

3.8. Sequencing of sICAM-1 from cell perfusates

The sICAM-1 peptides identified closest to the carboxy-terminal, membrane-spanning domain were DLEGTYLCR (423–431) in the fifth immunoglobulin domain and STQGEVTR (434–441) in the hinge region (Fig. 5), to indicate that cleavage occurred between Arg441 and the transmembrane domain.

4. Discussion

This study reports the novel observation that prolonged

Table 1
The effect of TIMPs on cellular and soluble ICAM-1 from HSVECs exposed to 'arterial' shear stress

Time (h)	TIMP (1 μM)	Cellular ICAM-1 (A_{492}/10^4 cells)	sICAM-1 (ng/ml)
0	–	0.08	0.6
6	–	0.26	4.7
8	–	0.09	8.4
8	TIMP-1	0.07	7.8
8	TIMP-2	0.08	8.1
8	TIMP-3	0.08	8.2

The results show the mean values obtained from three flow chambers connected in parallel.

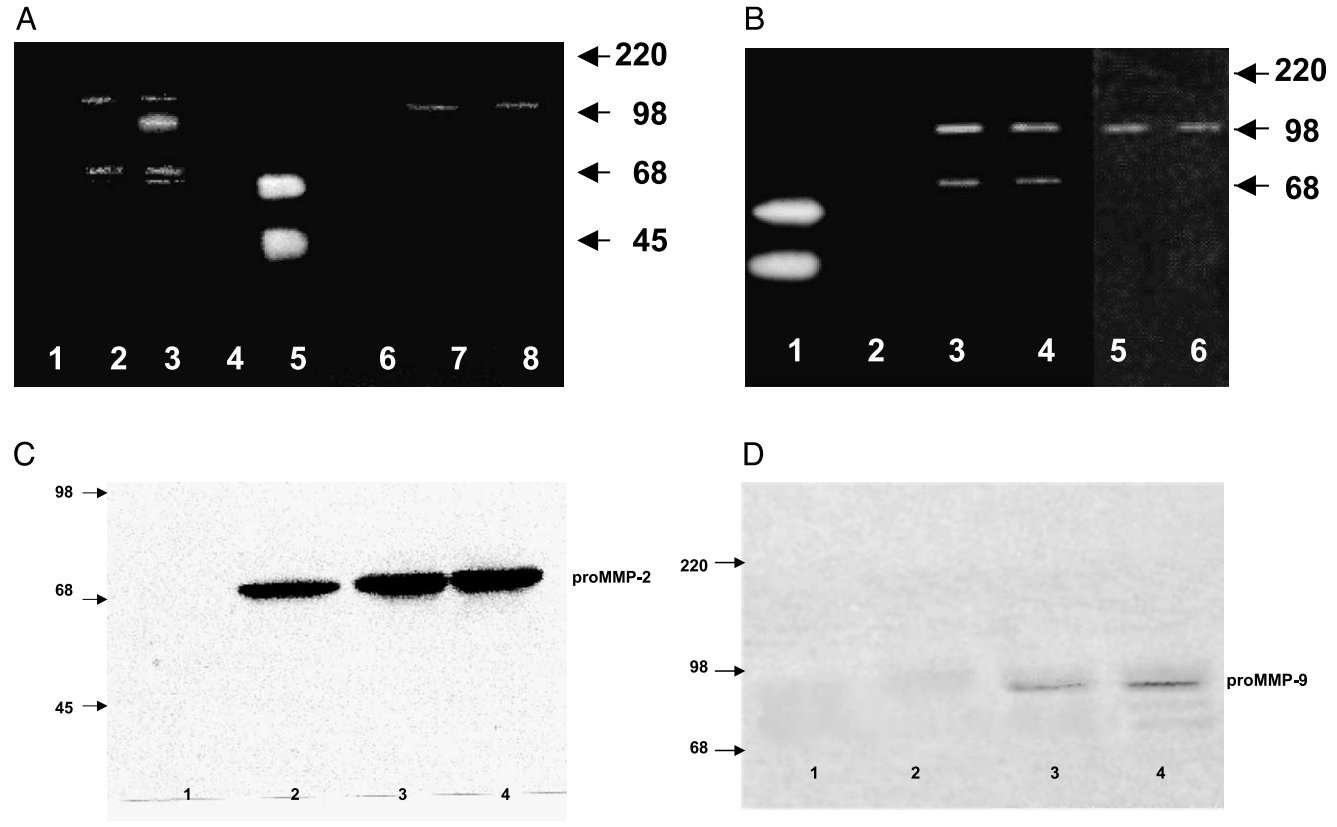

Fig. 3. The effect of shear stress on gelatin-degrading enzymes in lysates prepared from HSVECs. A: Confluent HSVECs were exposed to 4, 6 and 8 h of 'arterial' shear stress and cell lysates were analysed with gelatin zymograms. Purified active MMP-2 was the positive control (lane 5, 64 and 45 kDa bands). Lysates from cells exposed for 4, 6 and 8 h are shown in lanes 1–3 respectively, lane 4 shows the 8 h no flow control. Activity at 107 kDa, 92 kDa and a 72/68 kDa doublet is evident in lanes 2 and 3. 5 mM EDTA inhibited the MMP-2 positive control (lane 6), and all but the 107 kDa band of the 6 and 8 h 'arterial shear' lysates (lanes 7 and 8). B: Gelatin zymograms of concentrated perfusate from HSVECs exposed to 6 and 8 h of 'arterial' shear stress. Lane 1, positive control (64 and 45 kDa); lane 2, 8 h no flow; lanes 3 and 4, 6 and 8 h of 'arterial' shear stress respectively. 5 mM EDTA inhibited the lower molecular weight band from 6 and 8 h perfusates, lanes 5 and 6. C,D: Western blots of cell lysates to show the time-dependent expression of MMP-2 and MMP-9. Lysates (50 μg protein) from HSVECs cultured for 8 h with no flow, or with 6 and 8 h of 'arterial' shear stress are shown in lanes 1, 2 and 3 respectively. Lane 4, positive control, cultured human vein smooth muscle cells stimulated with 5 ng/ml TNF-α.

'arterial' shear stress leads to the metalloproteinase-dependent shedding of sICAM-1 from saphenous vein endothelium, a mechanism which could counteract the upregulation of ICAM-1 by shear stress.

After 8 h of 'venous' shear stress there was some accumulation of sICAM-1 in the medium, to indicate that shedding could be a normal physiological process. With 'arterial' shear stress, the shedding of sICAM-1 increased by an order of magnitude, the sharpest increase occurring between 6 and 8 h, with concomitant reduction in cellular ICAM-1. Sequencing of sICAM-1 identified that sICAM-1 was cleaved in the hinge region beyond the fifth immunoglobulin domain. Metalloproteinases MMP-2 and MMP-9 were induced by 'arterial' shear stress. Zymograms and immunoassays showed that MMP-2 was synthesised and secreted by 6 h, with little further increase at 8 h, with delayed induction of proMMP-9 between 6 and 8 h. All the proMMP-9 was cell-associated and MMP-9 (but not MMP-2) co-immunoprecipitated with ICAM-1. Both the hydroxamate XN908 and α_2-macroglobulin inhibited the sharp increase in sICAM-1 observed between 6 and 8 h and partially inhibited the reciprocal decline in cellular ICAM-1. The role of two sequential proteolytic steps would be concordant with both established mechanisms for metalloproteinase activation and the process for the hydroxamate-sensitive shedding of PECAM-1 from endothelial cells [12]: α_2-macroglobulin could inhibit either step. We provide circumstantial evidence for a role for MMP-9 in the shear stress-induced shedding of sICAM-1 from HSVECs. Based on the substrate

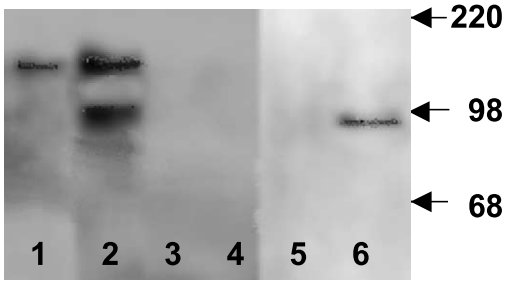

Fig. 4. Immunoprecipitation of ICAM-1 from endothelial cell lysates. Proteins were immunoprecipitated from cell lysates (50 μl lysate, 8 h of no flow or 'arterial' shear stress) with polyclonal anti-ICAM-1, subjected to Western blotting and probed with monoclonal antibodies to ICAM-1 (lanes 1 and 2), MMP-2 (lanes 3 and 4) and MMP-9 (lanes 5 and 6). After 8 h of shear stress there was evidence of the presence of a lower molecular weight form of ICAM-1, 95 kDa (lane 2), in addition to the 115 kDa form. There was staining for proMMP-9 after 8 h of 'arterial' shear stress in lane 6, but no staining for MMP-2 (lane 4).

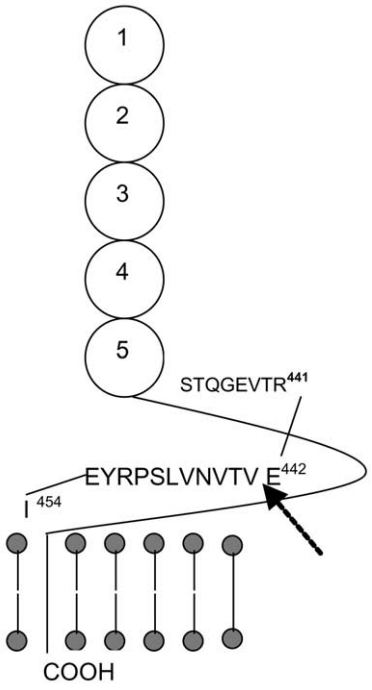

Fig. 5. Putative MMP-9 cleavage site for generation of soluble ICAM-1. Diagrammatic representation of the potential cleavage site of MMP-9 (arrow) in the hinge region of ICAM-1, between the fifth immunoglobulin domain (circle 5), sequenced peptide (434–441, bold) and the trans-membrane domain (from I^{454}).

specificity of MMP-9 [13], the most likely site cleaved, to release sICAM-1, is the Glu442–Val443 bond, but the exact site of proteolysis needs to be confirmed.

Recently it has been shown that proMMP-9 binds to ICAM-1 on the cell surface of HL60 cells and subsequent activation of MMP-9 leads to ICAM-1 shedding [7]. HL60 cells express MMP-2 and MMP-9, but only MMP-9 was co-immunoprecipitated with ICAM-1, to eliminate a similar role for MMP-2 [7]. Such a docking mechanism of MMP-9 to ICAM-1 appears to be compatible with our data for the co-immunoprecipitation of MMP-9, but not MMP-2, with induction of MMP-9 associated with the accelerated shedding of sICAM-1 from HSVECs after 6 h 'arterial' shear stress. Fiore et al. [7] hypothesised that MMP-9 bound to cell surface ICAM-1 on HL60 cells was resistant to TIMP-1 inhibition. Very recently, there has been exciting confirmation that MMP-9 on the cell surface of neutrophils is resistant to TIMP-1 and TIMP-2 inhibition, with the IC_{50} for TIMP-1 increasing >30-fold for cell-surface compared with soluble MMP-9 [14]. In contrast, the potency of a hydroxamate inhibitor was similar for both soluble and cell-bound MMP-9 [14]. This provides one explanation why TIMP-1, TIMP-2 or TIMP-3 (each at the high concentration of 1 μM) did not attenuate sICAM-1 shedding. Alternatively, the enzyme involved in sICAM-1 shedding from HSVECs could belong to the class of metalloproteinases inhibited by hydroxamate inhibitors but not by TIMPs, e.g. ADAM8 and ADAM9 [15].

The identification of the metalloproteinases responsible for ICAM-1 shedding from shear-stressed HSVECs requires further biochemical investigation, but current evidence indicates that docking of MMP-9 to ICAM-1 on the endothelial surface may have an important role.

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References

[1] Papadaki, M. and Eskin, S.G. Effects of fluid shear stress on gene regulation of vascular cells, (1997) Biotechnol. Prog. 13, 209–221.
[2] Sampath, R., Kukielka, G.L., Smith, C.W., Eskin, S.G. and McIntire, L.V. Shear-stress mediated changes in the expression of leukocyte adhesion receptors on human umbilical vein endothelial cells in vitro, (1995) Ann. Biomed. Eng. 23, 247–256.
[3] Nagel, T., Resnick, N. and Atkinson, W.J. et al. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells, (1994) J. Clin. Invest. 94, 885–891.
[4] Golledge, J., Turner, R.J., Harley, S.L., Springall, D.R. and Powell, J.T. Circumferential deformation and shear stress induce differential responses in saphenous vein endothelium exposed to arterial flow, (1997) J. Clin. Invest. 99, 2719–2726.
[5] Champagne, B., Tremblay, P., Cantin, A. and St. Pierre, Y. Proteolytic cleavage of ICAM-1 by human neutrophil elastase, (1998) J. Immunol. 161, 6398–6405.
[6] Lyons, P.D. and Benveniste, E.N. Cleavage of membrane-associated ICAM-1 from astrocytes: involvement of a metalloproteinase, (1998) Glia 22, 103–112.
[7] Fiore, E., Fusco, C., Romero, P. and Stamenkovic, I. Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell mediated cytotoxicity, (2002) Oncogene 21, 5213–5223.
[8] Kashiwagi, M., Tortorella, M., Nagase, H. and Brew, K. TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5), (2001) J. Biol. Chem. 276, 12501–12504.
[9] Gosling, M., Harley, S.L., Turner, R.J., Carey, N. and Powell, J.T. Human saphenous vein endothelial cells express a tetrodotoxin-resistant, voltage-gated sodium current, (1998) J. Biol. Chem. 273, 21084–21090.
[10] Qureshi, S.S. (2002) Mechanisms of Regulation of Endothelial ICAM-1 by Shear Stress, PhD Thesis, University of London, London.
[11] Harley, S.L., Sturge, J. and Powell, J.T. Regulation by fibrinogen and its products of intercellular adhesion molecule-1 expression in human saphenous vein endothelial cells, (2000) Arterioscler. Thromb. Vasc. Biol. 20, 652–658.
[12] Ilan, N., Mohsenin, A., Cheung, L. and Madri, J.A. PECAM-1 shedding during apoptosis generates a membrane-anchored truncated molecule with unique signaling characteristics, (2001) FASEB J. 15, 362–372.
[13] Kridel, S.J., Chen, E., Kotra, L.P., Howard, E.W., Mobashery, S. and Smith, J.W. Substrate hydrolysis by matrix metalloproteinase-9, (2001) J. Biol. Chem. 276, 20572–20578.
[14] Owen, C.A., Hu, Z., Barrick, B. and Shapiro, S.D. Inducible-expression of TIMP-resistant matrix metalloproteinase-9 on the cell surface of neutrophils, (2003) AJRCMB 29, 283–294.
[15] Amour, A., Knight, C.G., English, W.R., Webster, A., Slocombe, P.M. and Knauper, V. The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs, (2002) FEBS Lett. 524, 154–158.