Cytokine stimulated vascular cell adhesion molecule-1 (VCAM-1) ectodomain release is regulated by TIMP-3

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Abstract

Objectives: Vascular cell adhesion molecule-1 (VCAM-1) is a cell surface adhesion molecule involved in the recruitment of leukocytes to endothelial cells on arterial walls during the pathogenesis of atherosclerosis. The soluble ectodomain of VCAM-1 (sVCAM-1) is proteolytically released from the cell surface into the circulation, a process which is up-regulated in patients with cardiovascular or inflammatory disease. Here we investigate mechanisms involved in sVCAM-1 generation in response to cytokine stimulation.

Methods: VCAM-1 ectodomain release into the conditioned media of MCEC-1 murine endothelial cells and cells grown from primary aortic explants from timp3/C0/C0 mice and wild-type littermates was measured by sandwich ELISA and Western blot after stimulation with tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), or the phorbol ester PMA. Protease expression was inhibited (knocked down) with siRNA and validated using real-time PCR.

Results: Proinflammatory cytokines IL-1β and TNFα up-regulated VCAM-1 ectodomain release from the MCEC-1 cells, and this was dependant on p38 and mitogen-activated protein kinases (MAP kinases) and inhibited by the matrix metalloproteinase (MMP) inhibitor BB94 and tissue inhibitor of metalloproteinase (TIMP)-3, but not TIMP-1 or TIMP-2. Timp-3−/− cells exhibited greater VCAM-1 ectodomain release following cytokine stimulation than TIMP-3-expressing cells. Additionally, cytokine stimulation of MCEC-1 cells was shown to cause down-regulation of TIMP-3 expression. Knockdown of the metalloproteinase ADAM17, but not ADAM10 or ADAM12, gene expression reduced cytokine-stimulated VCAM-1 shedding.

Conclusions: TIMP-3 regulates the release of sVCAM-1 from cytokine-stimulated endothelial cells, which is mediated by ADAM17.

Keywords: Cytokines; Endothelial function; Matrix metalloproteinases

1. Introduction

Vascular adhesion molecule-1 (VCAM-1), a member of the immunoglobulin family of cell–cell adhesion receptors, is expressed principally on endothelial cells, where it is cytokine-inducible and facilitates leukocyte adhesion. VCAM-1 up-regulation has been shown to be important in inflammatory diseases including atherosclerosis, where
induction precedes leukocyte adhesion and transmigration across the vascular endothelium [1–3]. Elevated serum levels of the extracellular domains (ectodomains) of several cell surface adhesion molecules including VCAM-1 are found in patients with atherosclerosis and there is some evidence to suggest that changes in VCAM may correlate with destabilization of atherosclerotic plaques [4,5]. Furthermore, proinflammatory cytokine levels are increased concurrently with elevated circulating VCAM-1 in atherosclerosis [5], and in a manner similar to inflammatory disorders such as rheumatoid arthritis [6]. These findings are substantiated by in vitro data which indicate a close association with increased ectodomain shedding and the inflammatory response [7,8].

Proteolytic release of the ectodomain of VCAM-1 and other transmembrane proteins, or ectodomain shedding, provides a post-translational mechanism by which cell surface proteins can be rapidly down-regulated and a means by which any downstream signaling of the released domain can be regulated. This ectodomain release can occur in response to a number of exogenous triggers, including calcium ionophores, cytokines and growth factors [9–11]. The protein kinase C activating phorbol ester, phorbol 12-myristate-13-acetate (PMA), has been demonstrated to be a potent agonist of shedding for many proteins and is extensively used for in vitro studies [12], but little information is available regarding the roles of cytokine in shedding events.

Matrix metalloproteinases (MMPs) and ADAMs (A Disintegrin and Metalloproteinase) have been implicated in many instances of ectodomain release [12,13]. There are 33 known ADAM family proteins, of which the proteolytically active members contain a zinc binding catalytic domain. ADAM17 (TACE) has been studied most extensively in terms of ectodomain release, and is responsible for the release of several transmembrane proteins including neuregulins 1 and 2, TNFα, TGFα and fractalkine [14–16].

In order to address the important question concerning the mechanism of release of sVCAM-1 during inflammation we have characterized VCAM-1 ectodomain release from the murine cardiac endothelial cell line MCEC-1, derived from H-2Kb-tsA58 mice [17], in response to the cytokines TNFα and IL-1β. The effect of exogenous TIMPs on VCAM-1 shedding was determined and then substantiated using cells isolated from timp-3 null mice [20] and wild-type (WT) littermates were killed by anaesthetic overdose followed by cervical dislocation. Aortas were collected and sliced into 2–3 mm² fragments which were washed in sterile PBS and then plated a gelatin coated well in a drop of DMEM (supplemented as for the MCEC-1 cells). After 5 h, a further 1 ml of medium was added to each explant. The medium was changed every 2 days. Cell outgrowth was observed at 2–3 days and experiments were performed after 10–14 days. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No.85–23, revised 1996).

2.2. Culture of MCEC-1 cell line

MCEC-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), (Invitrogen), 10% fetal calf serum (FCS; Globepharm), 30 μg/ml endothelial cell growth supplement (ECGS), 10 U/ml heparin (Invitrogen) and 2 mM L-glutamine (Invitrogen), as described in [17]. Cells were expanded at 33 °C then plated at 38 °C 24–48 h prior to experimentation, to arrest SV40 T antigen dependant immortalization. TNFα (10 ng/ml), IL-1β (10 ng/ml) (R&D Systems) and PMA (20 ng/ml) were added in serum-free DMEM. Conditioned medium and cell lysates were collected at the times shown. Inhibitors [BB94, SB202190, UO126 (Calbiochem) and TIMPs] were incubated with cells, at the concentrations indicated, for 20 min prior to addition of other treatments. TIMPs were expressed and purified as described [18,19].

2.3. Primary culture of mouse aortic explants

The timp-3 null mice [20] and wild-type (WT) littermates were killed by anaesthetic overdose followed by cervical dislocation. Aortas were collected and sliced into 2–3 mm² fragments which were washed in sterile PBS and then plated a gelatin coated well in a drop of DMEM (supplemented as for the MCEC-1 cells). After 5 h, a further 1 ml of medium was added to each explant. The medium was changed every 2 days. Cell outgrowth was observed at 2–3 days and experiments were performed after 10–14 days. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No.85–23, revised 1996).

2.4. Western immunoblotting and dot blots

Conditioned media were concentrated 10-fold using Centricron concentrators (Millipore). Cell lysates were standardized for total protein concentration. Samples were subjected to SDS-PAGE (10%) and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) containing 5% milk for 1 h, and incubated with 1:400 anti-VCAM-1 (Santa Cruz Biotechnology) overnight at 4 °C. Following 45 min incubation with anti-rabbit horseradish peroxidase (Jackson ImmunoResearch; 1:5000), protein was detected using ECL reagent (Amersham Biosciences). For dot blots, cell lysates were standardized for total protein concentration and pipetted onto the nitrocellulose membrane in equal volumes, then processed as described above using anti-ADAM17 (Abcam) at 1:1000.

2.5. ELISA

A sandwich ELISA was performed using matched anti-mouse VCAM-1 antibodies (R&D Systems) according to
the manufacturer’s instructions. Conditioned media were diluted between 1:30 and 1:70 and lysates (standardized according to protein concentration) 1:100 before assay.

2.6. Quantitative real-time PCR

RNA was isolated using the SV Total kit (Promega) and reverse transcribed using Superscript II (Invitrogen). Gene expression was measured with quantitative real-time PCR using an ABI Prism 7700 (Applied Biosystems) as described previously [21,22] and normalised to 18S rRNA (primers and probes from Applied Biosystems). TIMP and ADAM primers and probes sequences are described previously [22].

2.7. RNA interference

Transfection was performed either using Lipofectamine 2000 (Invitrogen) or using an AMAXA Nucleofector (Amaxa Inc) in accordance with the manufacturer’s instructions. MCEC-1 cells were plated in serum free DMEM with a mixture of Lipofectamine 2000 and siRNAs previously incubated at room temperature for 20 min. Two siRNA duplexes targeting two regions of the murine ADAM10 (Sense: 5’-ATGGGACACATGGCCACGCTA-3’ and 5’-ACAGTTCACTACGAATGAA-3’), ADAM12 (Sense: 5’-CAGGAACCTTGAAGTATTAA-3’ and 5’-CCGAGTTTCTAAAGTGTTTA-3’; Qiagen) and ADAM17 gene (Sense: 5’-AAGTCTGATCGATACGGAT-3’ and 5’-AAGCTGACGAGCAGAATGG-3’; Dharmacon Inc) were used in combination and are designated as ADAM10, ADAM12 and ADAM17 siRNAs respectively. A non-targeting scrambled RNA was used as a negative control (SCR; Qiagen). FCS (final concentration 10%) was added at 4 h, and cells were stimulated 48 h, after transfection.

2.8. Statistical analysis

Data are mean ± standard error. One-way ANOVA was used for statistical analysis with significance accepted at $P<0.05$.

3. Results

3.1. Effect of cytokine stimulation on VCAM-1 shedding from MCEC-1 cells

Elevated levels of sVCAM-1, demonstrated by Western blot and ELISA, were observed in the conditioned media of MCEC-1 cells treated with TNF-$\alpha$ and IL-1-$\beta$ either individually or in combination (Fig. 1). 1–8 h following the manufacturer’s instructions. Conditioned media were diluted between 1:30 and 1:70 and lysates (standardized according to protein concentration) 1:100 before assay.

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treatment, levels of sVCAM appear to be elevated (TNFα and IL-1β at 4 h, \( P < 0.01 \)). At 24 hrs, levels of sVCAM-1 produced by all treated cells were significantly greater than controls (\( P<0.05; \) Fig. 1B). Hence, we elected to study VCAM-1 shedding at 24 h, as stimulation resulted in robust increases in sVCAM-1 at this time point. Treatment for 24 h with both TNFα and IL-1β cause increased expression of VCAM-1 in cell lysates (Fig. 2). TNFα increased VCAM-1 protein expression by approximately 2.5-fold whilst IL-1β induced a 1.6-fold increase, whilst in combination the cytokines caused similar increase in VCAM-1 expression as to TNFα alone (Fig. 2B). TNFα and IL-1β, alone or in combination, produced a 2.5-fold increase in sVCAM-1 in the conditioned media corresponding to these lysates, but the percentage of total VCAM-1 released (assuming no loss of VCAM-1 other than ectodomain release) remained similar whether cytokine stimulated or unstimulated (11%, 14% 10% and 10% for TNFα, IL-1β, TNFα and IL-1β or control respectively).

To further characterize the mechanisms by which the cytokines increase VCAM-1 shedding in the MCEC-1 cells, we adopted a pharmacological approach using inhibitors of several key kinases. The increased VCAM-1 shedding induced by TNFα and IL-1β was significantly reduced (3-fold) by the p38 MAPK inhibitor SB202190 (Fig. 3A). Expression of VCAM-1 in the cell lysates was not significantly decreased by SB202190 (Fig. 3B). There is little change in percentage of total VCAM-1 released, however (8%). The MEK/ERK inhibitor UO126 also significantly decreased VCAM-1 shedding (\( P<0.05; \) Fig. 3C), without a concomitant significant decrease in VCAM-1 expression in the cell lysates (Fig. 3D). As a percentage of total VCAM-1 this equated to a reduction of 2%. Similar results were obtained with these inhibitors when cells were stimulated with individual cytokines (not shown). The PI3K inhibitor LY294002 (25–50 \( \mu \)M) and the PKC inhibitor GF109203X (1–5 \( \mu \)M) had no effect on VCAM-1 shedding or expression in the presence or absence of TNFα and IL-1β (data not shown).

### 3.2. Inhibition of VCAM-1 shedding by the hydroxamate inhibitor BB94

BB94 is a well characterized synthetic hydroxamate inhibitor of all metzincin family proteases including all MMPs and ADAMs [23,24]. Treatment of cells with 5 \( \mu \)M BB94 prior to stimulation with TNFα and IL-1β

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**Fig. 3.** Effect of treatment of MCEC-1 with the p38 MAPK inhibitor SB202190 (5 \( \mu \)M) for 24 h in the presence and absence of a combination of TNFα and IL-1β (both at 10 ng/ml), on sVCAM-1 release into the media (A) and VCAM-1 expression in cell lysates (B). Similarly the effect of the MEK/ERK inhibitor UO126 on sVCAM-1 release into the media (C) and VCAM-1 expression in cell lysates (D) was determined following 24 h in the presence and absence of a combination of TNFα and IL-1β (both at 10 ng/ml). VCAM-1 and sVCAM concentrations were measured by sandwich ELISA (\( n \geq 4 \)).
resulted in complete suppression of any increase in sVCAM-1 (Fig. 4A). BB94 had no effect on VCAM-1 levels measured in cell lysates (data not shown). PMA was also shown to stimulate shedding of VCAM-1 in this system and again BB94 reduced this induced shedding to basal levels (Fig. 4B). Interestingly, in contrast to TNFα or IL-1β stimulated VCAM-1 shedding, there was no significant increase in VCAM-1 in cell lysates 24 h after PMA stimulation (data not shown).

3.3. TIMP-3 regulates cytokine stimulated VCAM-1 ectodomain release

Preincubation with TIMP-3 before addition of TNFα and IL-1β significantly decreased the amount of sVCAM-1 released into the media after 24 h stimulation (Fig. 5), whilst TIMP-1 or TIMP-2 had no effect on VCAM-1 ectodomain shedding. Interestingly, the constitutive control levels of sVCAM-1 shedding were not inhibited by TIMP-3; in fact addition of TIMP-3 significantly increased the amount of sVCAM-1 release. Similarly, addition of TIMP-1 and 2 also had a tendency to increase basal shedding. To investigate this further, and to address the fact that the TIMP-3 added to the culture was of human rather than mouse origin, we cultured cells from TIMP-3 deficient mice and compared them to wild-type littermates, to examine the effect of loss of endogenous TIMP-3.

Cells were cultured from aortic explants taken from timp-3 null and wild-type mice, and then stimulated with TNFα and IL-1β in the same manner as the MCEC-1 cells in the previous experiments. After 4 h stimulation with TNFα and IL-1β or PMA, a significant increase in sVCAM-1 release into the media, of approximately 4-fold, was observed in the timp-3 null cells, compared to a less than 2-fold increase in shedding in wild-type cells (Fig. 6A). This trend could also be observed at 24 h after stimulation (Fig. 6B). It can be seen that VCAM-1 expression in the cell lysates in response to each treatment was comparable, indicating similar cell numbers in each culture (Fig. 6C). Moreover, the elevation in VCAM-1 expression following TNFα and IL-1β stimu-
population was comparable to that observed for the MCEC-1 cell line.

3.4. **ADAM17 and TIMP-1, 2 and 3 expression following cytokine stimulation**

Since this inhibitory effect of TIMP-3 implicated it as a potential regulator of VCAM-1 ectodomain release, its expression was profiled in MCEC-1 cells using real-time PCR. Expression of TIMP-3 was decreased at 8 and 24 h post-stimulation with TNFα and IL-1β but was unaffected by PMA (Fig. 7A). Concurrent with cytokine stimulated down-regulation of TIMP-3, ADAM17 was up-regulated by a small but significant extent (Fig. 7B). Interestingly TIMP-1 was up-regulated in response to cytokine stimulation (Fig. 7C), whilst TIMP-2 was down-regulated in a similar fashion to TIMP-3 (Fig. 7D). Treatment with PMA had no effect on TIMP-2, TIMP-3 or ADAM17 expression but did cause significant up regulation of TIMP-1 at 8 h.

3.5. **ADAM17 mediates cytokine induced VCAM-1 ectodomain release**

ADAM10, ADAM12 and ADAM17 have all previously been implicated in the ectodomain release of cell surface molecules and are all inhibited by TIMP-3, in a manner consistent to the TIMP-3 sensitivity of VCAM-1 shedding following cytokine stimulation demonstrated in this paper. Therefore we chose to study the effect of siRNA knock-down of the ADAM10, ADAM12 and ADAM17 genes. MCEC-1 cells were transfected with ADAM17 targeting siRNAs, which caused significant reduction in ADAM17 RNA levels at 24 (Fig. 8A) and 48 h (Fig. 8B), as determined by real-time PCR. As an additional negative control, it was demonstrated that the ADAM17 siRNAs had no effect on ADAM10 or ADAM12 expression (data not shown). It can be seen by dot blot (Fig. 8C) that the reduction in ADAM17 mRNA was accompanied by a decrease in ADAM17 protein in cell lysates harvested at 48 h. Although the extent of the gene silencing varied, it appeared to be consistently greater at 48 h and therefore we elected to study the cells at this time point.

Significant reduction in ADAM10 and ADAM12 RNA levels (Fig. 9), compared to the non-targeting scrambled siRNA control, was achieved prior to stimulation of cells for 4 h with TNFα and IL-1β or PMA, which resulted in a significant increase in VCAM-1 shedding over basal levels in transfected and untransfected cells (Fig. 9D). This cytokine induced VCAM-1 release was significantly lower in ADAM17 siRNA transfected cells but not in ADAM10 or ADAM12 siRNA transfected cells. Similarly, after 24 h of TNFα and IL-1β stimulation, ADAM17 siRNA transfected cells exhibited a significantly reduced level of VCAM-1 shedding, whilst that of ADAM10 and ADAM12 siRNA transfected cells did not differ from the scrambled control (Fig. 9E). Transfection with the ADAM10, ADAM12 or ADAM17 siRNAs had no effect on levels of VCAM-1 in the cell lysates at 24 h under any of the condition tested (Fig. 9F).

4. **Discussion**

During atherosclerosis and other inflammatory conditions, the endothelium becomes activated in response to proinflammatory factors including TNFα and IL-1β, which results in the induction of cell surface adhesion molecules such as VCAM-1. It appears that the shedding of VCAM-1 may represent an important mechanism by which its functions on the cell surface can be down-regulated. We have shown that the MCEC-1 endothelial cell line exhibits ectodomain release of VCAM-1 and that 24 h stimulation...
with TNFα and IL-1β up-regulates both sVCAM-1 generation and expression of VCAM-1. Up-regulation of cell surface VCAM-1 expression in response to TNFα and IL-1β is seen in endothelial cells derived from a variety of vascular beds [3, 25–27]. The responses to TNFα may vary between different endothelial cell types, with microvascular endothelial cells reported to have a relatively transient increase in VCAM-1 expression compared to human umbilical vein endothelial cells, whilst iliac arterial endothelial cells exhibit no increased VCAM-1 expression [25, 27]. However, there is little data regarding the effects of cytokines on the shedding of VCAM-1, despite a strong association between elevated serum levels of sVCAM-1 and inflammatory disorders [28, 29], implicating ectodomain release as a mechanism of regulation of VCAM-1 action.

In our study, elevated levels of VCAM-1 shedding were observed within 8 h of cytokine stimulation which may indicate an up-regulation of a proteolytic mechanism. Stimulation with TNFα and IL-1β increases cell surface expression of VCAM-1, which may lead to more VCAM-1 being accessible to proteases at the cell surface rather than up-regulating activation of proteolysis. This in turn, is consistent with the observation of increased cell surface levels of VCAM-1 in the MCEC-1 line (as well as an up-regulation of ICAM-1, P and E-selectin) following a 6 h incubation with TNFα and IL-1β [17]. We have shown that at later time-points, following cytokine stimulation, the amount of VCAM-1 ectodomain release as a proportion of total VCAM-1 present is similar to unstimulated levels. In human brain endothelial cells TNFα has been shown to induce an approximately 2-fold increase in membrane VCAM-1, whilst sVCAM-1 levels increased by up to 4-fold [3].

The increased VCAM-1 shedding induced by TNFα and IL-1β was shown to be dependent on p38 MAPK and the MEK/ERK pathway but independent of PI3K and PKC, whilst there was no significant difference in VCAM-1 expression in the presence of these inhibitors. This seems likely to conform to our hypothesis that cytokines are elevating VCAM-1 shedding by increasing the availability

Fig. 7. Expression of TIMP-3 (A), ADAM17 (B), TIMP-1 (C) and TIMP-2 (D) measured by real-time quantitative PCR and normalised for 18-S ribosomal RNA, in MCEC-1 cells 8 and 24 h following stimulation with TNFα and IL-1β (both at 10 ng/ml), PMA (20 ng/ml) or unstimulated. TIMP-3 and TIMP-2 expression is reduced following TNFα and IL-1β stimulation whilst ADAM17 and TIMP-1 expression is increased. *Significantly different, \( P<0.05 \), from unstimulated control at same timepoint \( (n=3) \).
of VCAM-1 to protease(s) resident at the cell surface, since stimulation with TNFα increases endothelial cell surface VCAM-1 in a p38 MAPK dependent and post-transcriptional manner [30]. The regulation of MP and TIMP expression by MAP kinase pathways is known to be complex in other cell types [31] and so future studies will reveal the precise role(s) of the p38 MAP kinase pathway in the regulation of VCAM-1, ADAM17 and TIMP-3.

Ablation of VCAM-1 ectodomain release by BB94 provides strong evidence that a metalloproteinase (MP) is the sheddase within our system following cell stimulation. Measurement of VCAM-1 in cell lysates indicated that BB94 had not perturbed the increased VCAM-1 expression induced by the cytokines, and so any decrease in sVCAM-1 is due to inhibition of VCAM-1 proteolysis. BB94 reduced stimulated ectodomain release to levels observed without stimuli, as previously shown in human cerebral endothelial cells following TNFα stimulation [32]. In common with shedding of other cell surface proteins it appears that basal ectodomain release of VCAM-1 is not an MP mediated mechanism.

The inhibitory profile of sVCAM-1 generation was suggestive of involvement of ADAM family proteinases (TIMP-3 inhibitable) rather than MMPs (TIMP-1 and/or TIMP-2 inhibitable). Several ADAMs are inhibited by TIMP-3, including ADAM12, ADAM17, ADAM19, ADAMTS-4 and ADAMTS-5 [33–35] implicating one, or several, of these enzymes in VCAM-1 shedding. Human ADAM10 has been shown to be inhibited by TIMP-3, but also by TIMP-1, and so it is not a prime candidate [36]. The tendency for basal shedding to be elevated following TIMP treatment may perhaps be due to protection of the shed VCAM-1 from further MP mediated proteolysis after release. Stimulation of timp-3 null cells resulted in greater levels of VCAM-1 shedding than observed in wild-type cells, which supports a role for TIMP-3 in regulation of this system.

Our data show that TIMP-3 is expressed in MCEC-1 cells and that this expression is reduced following stimulation with TNFα and IL-1β in a manner concurrent with increased levels of VCAM-1 shedding. It has previously been shown that concentrations of TNFα similar to those used in the experiments we have performed decrease both TIMP-3 transcription and protein synthesis in ventricular myocytes and almost completely block TIMP-3 production in brain endothelial cells [37,38]. Therefore it is possible that the increase in VCAM-1 proteolysis observed following treatment with cytokines is, in part, due to down-regulation of TIMP-3 causing an increase in availability of active sheddases. This is consistent with data from one of our laboratories demonstrating increased ADAM17 activity in timp-3 null mice [39]. Recently TNFα has been shown to increase ADAM17 expression in murine brain endothelial cells [40] and in the MCEC-1 cells we demonstrate TNFα and IL-1β to increase expression of ADAM17. Taking these data together, it is likely that there are elevated levels of ADAM17 available for sVCAM-1 generation following TNFα and IL-1β stimulation. It is of interest to note the down-regulation of TIMP-2 and up-regulation of TIMP-1, following treatment with TNFα and IL-1β, which indicates differential regulatory roles for these inhibitors in endothelial activation, whereas expression of TIMP-2 is largely constitutive in several other systems (reviewed in [41]).

ADAM17 has been implicated in the proteolysis of many cell surface molecules, including VCAM-1, in response to PMA simulation [42–44]. We also investigated the effects of other ADAMs, since in some systems more than one has been implicated, such as for fractalkine where ADAM17 has been shown to be responsible for PMA-induced shedding and ADAM10 for basal shedding [16,45]. Interestingly PMA had no effect on expression of ADAM17 or TIMP-3, which further distinguishes the action of this non-physiological molecule from that of the cytokines. Garton et al. [42] have reported that PMA-induced shedding of VCAM-1 from endothelial cells is mediated by ADAM17, where low levels of VCAM-1 shedding were observed 45 min following PMA stimulation. A physiological counterpart for such rapid VCAM-1 ectodomain release remains to be determined. Gene knockdown of ADAM17 (but not ADAM10 or ADAM12) was shown in the current work to reduce cytokine stimulated VCAM-1 ectodomain release. This clearly indicates a role for ADAM17 in the ectodomain release of VCAM-1 in response to cytokine stimulation,
although it does not necessarily rule out the involvement of other enzymes.

In conclusion, we have shown that cytokine-induced sVCAM-1 is generated by a p38 MAPK and MEK/ERK dependent mechanism that involves ADAM17. Since consideration is being given to ADAM17 as an upstream target for intervention in TNF signaling it is important to consider all its functions, such as regulation of cell surface

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**Fig. 9.** MCEC-1 cells were transfected siRNAs targeting ADAM10 (A), ADAM12 (B) and ADAM17 (C), which caused significant reduction in mRNA levels for each of the respective genes after 48 h relative to the non-targeting scrambled control RNA (SCR), as measured by quantitative PCR. Cells were transfected with siRNA 48 h prior to stimulation. Release of sVCAM-1 into the media was measured following 4 (D) or 24 (E) h incubation in the presence and absence of TNFα and IL-1β (both at 10 ng/ml) or PMA (20 ng/ml), and as well as levels of VCAM-1 in the cell lysates after 24 h (F). ADAM17 siRNA significantly reduced TNFα and IL-1β stimulated VCAM-1 release at 4 and 24 h, in addition to reducing PMA stimulated VCAM-1 release at 4 h after stimulation. Soluble and cell associated VCAM-1 was measured by ELISA. *P<0.01, **P<0.01. (A), (B) and (C) n=3; (D), (E) and (F) n≥8.
VCAM-1 expression, which may be anti-inflammatory in a pathophysiological setting. The inhibitory action of TIMP-3 upon VCAM-1 ectodomain shedding, and the down-regulation of TIMP-3 following cytokine stimulation, indicate that it may have a regulatory role in endothelial activation. Future studies of circulating TIMP-3 levels in patients with atherosclerotic disease might substantiate its involvement in such conditions. Importantly we demonstrate sVCAM-1 generation to occur continuously for 24 h after cytokine stimulation, mimicking the chronic nature of this process in inflammatory disorders and highlighting the need to use physiological tools to address the mechanisms involved.

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