Cellular Pathology of Atherosclerosis

Smooth Muscle Cells Promote Adhesion of Platelets to Cocultured Endothelial Cells


Abstract—Although platelets do not ordinarily bind to endothelial cells (EC), pathological interactions between platelets and arterial EC may contribute to the propagation of atheroma. Previously, in an in vitro model of atherogenesis, where leukocyte adhesion to EC cocultured with smooth muscle cells was greatly enhanced, we also observed attachment of platelets to the EC layer. Developing this system to specifically model platelet adhesion, we show that EC cocultured with smooth muscle cells can bind platelets in a process that is dependent on EC activation by tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β1. Recapitulating the model using EC alone, we found that a combination of TGF-β1 and TNF-α promoted high levels of platelet adhesion compared with either agent used in isolation. Platelet adhesion was inhibited by antibodies against GPIb-IX-V or αIIbβ3 integrin, indicating that both receptors are required for stable adhesion. Platelet activation during interaction with the EC was also essential, as treatment with prostacyclin or theophylline abolished stable adhesion. Confocal microscopy of the surface of EC activated with TNF-α and TGF-β1 revealed an extensive matrix of von Willebrand factor that was able to support the adhesion of flowing platelets at wall shear rates below 400 s⁻¹. Thus, we have demonstrated a novel route of EC activation which is relevant to the atherosclerotic microenvironment. EC activated in this manner would therefore be capable of recruiting platelets in the low-shear environments that commonly exist at points of atheroma formation. (Circ Res. 2006;98:98-104.)

Key Words: smooth muscle cells ▪ endothelial cells ▪ coculture ▪ transforming growth factor-β1 ▪ platelet adhesion

Adhesion of platelets to the artery wall and formation of mural thrombi occur in the late stages of atherosclerosis and underlie cardiovascular pathology.¹ This process requires exposure of thrombogenic subendothelial materials which contact arterial blood on rupture of mechanically compromised, “mature” plaques.¹–³ The idea that platelets might additionally adhere to endothelial cells (EC) during earlier stages of plaque development and contribute to disease progression has also been proposed.¹,²,⁴,⁵ Further, animal models show that endothelium supports adhesion of platelets at sites prevalent to formation of atherosclerosis through a mechanism that is not understood.⁶,⁷ The platelet receptors GPIb-IX-V and integrin αIIbβ3 are implicated in this process,⁶ but the events that initiate platelet adhesion are unclear.

Ordinarily, EC present an antithrombotic surface to flowing blood.⁴,⁵ This is achieved by constitutive production of NO and the lipid prostaglandin prostacyclin.⁸–¹⁰ However, a substantial number of studies report that platelets bind EC with compromised antithrombotic properties, although the pathophysiological significance of many of these reports is unclear, as powerful stimulatory agents were used to activate EC or platelets.⁵,¹¹–²² Recently, experiments in mice report platelet adhesion to mesenteric venules following treatment with calcium ionophore.²³ The adhesion of platelets to arteries at sites of atheroma formation has also been visualized in apolipoprotein E (apoE) knockout mice.⁶,²⁴ The major route of platelet adhesion in these models is via bridging of platelet αIIbβ3 integrin¹⁶–¹⁸,²⁰,²¹ to endothelial cell α,β₁ integrin¹⁸,¹⁹ by von Willebrand factor (VWF), with a possible contribution from P-selectin.²⁵ However, the molecular basis of the change in EC reactivity that supports platelet adhesion has not been identified and cannot be readily mapped using animal models.

We and others have previously shown that cells known to be present within the atherosclerotic environment interact with EC, so that their inflammatory phenotype is markedly altered.²⁶–²⁹ For example, crosstalk between monocytes and EC may establish a self-perpetuating and escalating cycle of EC activation and leukocyte recruitment.²⁶,²⁷ Additionally, crosstalk between secretory smooth muscle cells (SMC) and EC “primes” EC, so that they are hypersensitive to inflam-
matory stimulation by tumor necrosis factor (TNF) and can support significantly increased levels of leukocyte adhesion.\cite{28,29} Under the latter conditions, platelets, which were present in low numbers in the preparations of monocytes, also adhered to TNF-stimulated, cocultured EC. Thus, here we set out to examine the hypothesis that transcellular cross talk between EC and SMC altered the ability of EC to bind platelets, a result that has important implications for the events that initiate atheroma formation.

### Materials and Methods

#### Platelet Isolation and Preparation

Human platelet-poor plasma and washed red blood cells were prepared from blood anticoagulated with 5 U/mL heparin. Washed human platelets were prepared from platelet-rich plasma produced from blood anticoagulated with citrate phosphate dextrose adenine in the presence of theophylline. Platelets were fluorescently labeled with calcein-acetylmethylester (5 μg/mL), washed, and resuspended in medium 199 (Invitrogen, Paisley, UK) containing 20% autologous platelet-poor plasma and 5 U/mL heparin. In some experiments, autologous washed red blood cells were added to obtain a hematocrit of 20%. Where stated, platelets were activated with 5 μmol/L ADP immediately before addition to adhesion assay. For details, see Section 1.1 in the online data supplement available at http://circres.ahajournals.org.

#### Culture and Coculture of EC and SMC

HUVEC were isolated and cultured as described.\cite{30} Human SMC were explanted from the arteries of umbilical cords as previously described.\cite{28,29} Each experiment used first passage EC from a different donor. EC were cocultured with SMC on the opposite sides of porous polyethylene terephthalate culture plastic inserts.\cite{28,29} Alternatively, EC were cultured in gelatinized 24-well tissue culture plates or in gelatinized glass capillaries (microslides) until confluent.\cite{30} For details, see Sections 1.2 and 1.3 in the online data supplement.

#### Platelet Adhesion Assays

Adhesion of platelets to EC cultures on filters or in plastic dishes or to EC cocultured with SMC was quantified under static conditions. Calcein-acetylmethylester–labeled platelets were added to the EC surface and allowed to adhere for 1 hour at 37°C. Nonadherent cells were removed by washing with PBS/BSA and the EC monolayers fixed. Platelets were observed in situ by fluorescent microscopy and video recordings made for analysis of platelet adhesion.

The adhesion of flowing platelets was assayed in microslides at a wall shear rate of 100 or 400 s⁻¹. In some experiments, video recordings of platelets binding to EC were made in real time during platelet perfusion. In other experiments, the system was not illuminated until nonadherent cells had been removed with wash buffer. Platelet adhesion was quantified using Image Pro Plus software (Media Cybernetics).

In some experiments platelets were treated with antibodies against GPIb, αIIbβ₃, or control antibody against vascular cell adhesion molecule (VCAM)-1. Alternatively, platelet activation was inhibited with prostacyclin or theophylline. In coculture experiments, function-neutralizing antibody against transforming growth factor (TGF)-β₁ was included in the culture medium on the addition of EC to the insert. For details, see Section 1.4 in the online data supplement.

#### Visualization and Quantification of VWF on EC

To visualize VWF, confluent monolayers of EC were grown on glass coverslips in 24-well plates. Labeled VWF was detected on live cells using confocal microscopy and fluorescence quantified by integrated pixel intensity determination over an entire field. For details, see Section 1.5 in the online data supplement.

#### Results

**Endothelial Cells Cocultured With Secretory Smooth Muscle Cells Support the Adhesion of Platelets in the Presence of TNF**

Unstimulated EC cultured in isolation or cocultured with SMC on porous transwells for 48 hours did not support adhesion of isolated washed platelets (Figures 1a, 1b, and 2a). Moreover, the addition of TNF to EC cultured alone did not promote platelet adhesion (Figure 2a). However, when EC cocultured with SMC for 24 hours were stimulated for a further 24 hours with TNF, they supported significant levels of platelet adhesion (Figures 1c and 2a). We have previously shown that biologically active TGF-β₁, generated by the proteolytic action of plasmin in cocultures, regulates the inflammatory phenotype of EC.\cite{28} Taken with the current data, this raised the possibility that exogenous TNF combined with released TGF-β₁, promoted platelet adhesion. Consistent with this hypothesis, we found that neutralizing the activity of

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**Figure 1.** Fluorescently labeled platelets adherent to EC cultured in isolation or cocultured with SMC. Endothelial cells were cultured on porous transwell membranes in isolation (a), in coculture with SMC (b), in coculture with SMC in the presence of 100 U/mL TNF-α (c), or in coculture with SMC in the presence of TNF-α and an antibody against TGF-β₁ (d). Alternatively, EC were cultured in 24-well plates and were untreated (e), treated with 100 U/mL TNF-α (f), treated with 10 ng/mL TGF-β₁ (g), or treated with a combination of TNF-α and TGF-β₁ (h). Bar=50 μm.
TGF-β₁ using an antibody abolished platelet adhesion (Figures 1d and 2b), whereas a control antibody had no effect. Moreover, platelet adhesion was greatly reduced if aprotinin, a plasmin inhibitor, was added to coculture supernatants (Figure 2b). Thus, coculture promoted interactions between platelets and EC that depended on a novel route of EC activation, which required a combination of TGF-β₁ and TNF-α.

Antibodies against the platelet receptors GPIb or α₁bβ₃ inhibited adhesion of platelets to cocultured EC indicating that both adhesion molecules were required for binding (Figure 2c). Platelet adhesion to the cocultured EC also depended on activation of the platelets, as prostacyclin inhibited the response (Figure 2c).

**TGF-β₁ and TNF Promote Platelet Adhesion to EC Cultured Alone**

The role of TGF-β₁ and TNF was confirmed using purified recombinant reagents to reconstitute the coculture environment. Untreated EC grown in 24 well plates (Figures 1e and 3) or cells exposed to TNF (Figures 1f and 3), interleukin (IL)-1β, or a combination of these proinflammatory cytokines did not support adhesion of unactivated platelets or platelets that had been activated with ADP (Figure 3). However, after treatment with TGF-β₁, platelets bound to EC and the level of this was greatly increased if platelets were activated with ADP (Figures 1g and 3). When the combination of TGF-β₁ and TNF was used to stimulate EC, we found that adhesion of platelets increased dramatically to levels comparable with those seen on cocultured EC (Figures 1h and 3). Interestingly, there was no requirement for exogenous activation of the platelets when EC were activated with the combination of TGF-β₁ and TNF, as activating platelets with ADP did not cause increased adhesion (Figure 3). We verified that TNF, TGF-β₁, or a combination of these agents did not directly activate platelets by conducting aggregation assays in their presence (supplemental Figures I and II).

To confirm that the same receptors were used for platelet adhesion, we blocked GPIb and α₁bβ₃. Platelet adhesion to EC stimulated with TGF-β₁ and TNF was abolished in the presence of antibody against either receptor, but a control antibody had no consistent effect (Figure 4). Platelet activation by the EC was essential for binding in this system, because treatment with either prostacyclin or theophylline inhibited platelet adhesion (Figure 4). To determine the nature of the activating stimulus we conducted experiments using indomethacin and antagonists of ADP receptors. Both strategies significantly reduced platelet adhesion, strongly implying that thromboxane and ADP were necessary for stable platelet adhesion (supplemental Figure III).

These observations imply that exposure of EC to TGF-β₁ induces expression of receptor(s) that supports platelet adhesion in the presence of an exogenous platelet agonist such as ADP. However, in the presence of TGF-β₁ and TNF, EC also provide an endogenous activator of platelets, which leads to stabilization of adhesion.

**Combined Stimulation With TGF-β₁ and TNF Induces the Expression of a Matrix of VWF on the Surface of Endothelial Cells**

As VWF is a ligand for both GPIb and α₁bβ₃, and is abundant within EC, we determined whether it was expressed on the

**Figure 2.** The adhesion of platelets to EC cocultured with SMC in the presence or absence of inhibitors of platelet adhesion. The effect on platelet adhesion to EC of coculture with SMC in the presence or absence of TNF-α (a); the addition to the EC/SMC coculture medium of antibodies against TGF-β₁, VCAM-1 or the inactivation of plasmin by aprotinin (b); or of antibodies against GPIb, GPIIb/IIIa or prostacyclin added to platelet preparations (c). Data are mean ± SEM of at least 4 experiments. *P<0.05 for comparison of treated EC/SMC cocultures compared with untreated cocultures by paired t test.

**Figure 3.** The adhesion of platelets to EC stimulated with cytokines. The adhesion of untreated platelets (□) or platelets activated with 5 μmol/L ADP (●) to EC that were unactivated or stimulated with TNF-α, IL-1β, TGF-β₁, TNF-α, and IL-1β or TNF-α and TGF-β₁. Data are mean ± SEM of at least 4 experiments. *P<0.05, **P<0.01 for comparison of platelet adhesion to untreated and cytokine treated EC; +P<0.05 for comparison of adhesion of unactivated and ADP activated platelets to TGF-β₁ stimulated EC.
surface of EC exposed to TGF-β and TNF. Using immunofluorescent staining and confocal microscopy, we could not detect VWF on unstimulated (Figure 5a) or on TNF stimulated EC (Figure 5b). Exposure of EC to TGF-β induced a small amount of surface VWF (Figure 5c). However, coexposure of EC to TGF-β and TNF induced the expression of a matrix of VWF across the EC monolayer (Figure 5d), which was statistically significant (Figure 6).

High molecular weight VWF is an effective ligand for platelet GPIb at both venous and arterial rates of shear. Surprisingly, we found that stimulated EC were only able to support platelet adhesion at modest wall shear rates (100 s⁻¹, Figures 7 and 8a), and we verified that this was supported by both GPIb and α₄β₃ integrin (supplemental Figure IV). At 400 s⁻¹, platelet adhesion was not observed (Figure 8a). It is possible that GPIb-VWF interactions, which usually support platelet tethering and rolling, were occurring in our system but that stable adhesion via α₄β₃ was not being achieved under flow. To investigate this, we measured the number of platelet-EC interactions that lasted for greater than 40 ms in our experiments. This analysis demonstrated that even tethering interactions between platelets and VWF were evident at a much reduced incidence on EC exposed to flowing platelets at 400 s⁻¹ (Figure 8b). Thus, although we could induce EC coverage with VWF, this matrix of protein did not bind platelets at higher rates of shear. Interestingly, platelets did bind to EC at a shear rate of 400 s⁻¹ when experiments were conducted in the absence of the plasma borne protease, ADAMTS-13, which has been described to process ultra-large VWF into less adhesive units. Thus, in the absence of autologous plasma or in the presence of heat inactivated plasma (which neutralizes ADAMTS-13) or an antibody against ADAMTS-13, we saw the formation of platelet

Figure 4. The effects of inhibitors on the adhesion of platelets to EC stimulated with TNF-α and TGF-β. The effect of antibodies against VCAM-1, GPIb or α₄β₃ integrin, or addition of theophylline or prostacyclin on the adhesion of platelets to EC activated with TNF-α and TGF-β. Data are mean±SEM of at least 4 experiments. **P<0.01 for comparison of untreated and treated platelets.

Figure 5. Photomicrographs of surface VWF on EC. Confocal images of immunofluorescently labeled VWF on untreated EC (a), EC treated with TNF-α (b), EC treated with TGF-β (c), or EC treated with a combination of TNF-α and TGF-β (d). Bar=50 μm.

Figure 6. Semiquantitative fluorimetry of surface VWF on EC. The expression of immunofluorescently labeled surface VWF on untreated EC or EC treated with TGF-β, or a combination of TNF-α and TGF-β. Data are expressed relative to fluorescent intensity of EC immunofluorescently labeled with a control antibody and is the mean±SEM of 4 experiments. *P<0.05 for comparison of untreated and cytokine treated EC by paired t test.

Figure 7. Photomicrographs of flowing platelets adherent to cytokine treated EC. a, Phase contrast image of adherent platelets perfused at a wall shear rate of 100 s⁻¹ across EC treated with TNF-α and TGF-β. Nonadherent cells were removed by perfusion of cell free buffer. b, The same field viewed using fluorescent microscopy. Bar=50 μm.
leukocyte recruitment and activation themselves.39–47 IL-1 activated with TNF-support platelet adhesion. For example, platelets bind EC and SMC, TGF-1 and TNF-high shear. It has also been reported that platelets activated with thrombin can bind to confluent unstimulated monolayers of EC.18,50,51 In our hands, activation of platelets with a relatively weak agonist, ADP, did not result in adhesion to unstimulated or cytokine stimulated EC, although it did potentiate adhesion of platelets in response to EC activation with TGF-1. It should also be noted that the interpretation of results using a strong agonist such as thrombin may be hampered by the formation of thrombi in suspension.

Here, TNF and TGF-1 promoted the expression of VWF that covered the EC monolayer. It is likely that this VWF was the ligand for platelet adhesion to EC, as platelet GPIb and a(m)β3 integrin were both required in our system and are known to be receptors for VWF.52 Additionally, VWF supports platelet adhesion to EC in a number of in vitro studies that report VWF bridged platelet a(m)β3 integrin and endothelial cell α,β3 integrin or P-selectin.16–18,20,21 Several in vivo studies implicate VWF in adhesion of platelets to EC. For example, crossing mice lacking VWF with mice lacking low-density lipoprotein receptors significantly reduced the size of atherosclerotic lesions.7 Platelet adhesion may also play a key role in the process of very early atherogenesis in the apoE knockout mouse.6 Using intravital techniques, these authors showed that fluorescent platelets bound preferentially to the EC of atherosclerosis prone areas of the carotid artery. Furthermore, chronic treatment with antibodies against GPIb or a(m)β3 inhibited platelet adhesion and significantly reduced lesion formation. We also found that GPIb and a(m)β3 were essential for platelet binding to EC in static and flow based systems. However, GPIb–VWF interactions do not directly support platelet immobilization. Rather, platelets tether to VWF through GPIb, which results in the mobilization of intracellular calcium stores that activate a(m)β3 and thereby promote “stable” integrin mediated adhesion.54,55 Our observations that platelet adhesion can be blocked with antibodies against either receptor imply that integrin mediated adhesion is essential for stable adhesion and that this is disrupted by directly hindering a(m)β3–VWF interactions or by removing the integrin activating signal by blocking interactions between GPIb and VWF.

When we conducted flow experiments, we were surprised that the VWF on the surface of EC was not an efficient ligand for platelets at high shear rate. In fact, EC activated with TGF-1 and TNF-high shear. This strongly implies that VWF on EC was not the large molecular weight multimeric form reported to be an efficient ligand for rapidly flowing platelets (wall shear rates >1000 sec1).56 Similar observations have been reported in an intravital model (the microvasculature of the mouse cremaster muscle) where topical
application of calcium ionophore induced expression of VWF on venous EC. This supported GPIb-mediated platelet adhesion at shear rates up to 100 s⁻¹. As stated above, EC also express strings of high molecular weight VWF in response to histamine, TNF-α, IL-6, or IL-8, which support the adhesion of flowing platelets at high shear rates. However, these experiments were conducted in the absence of plasma. If plasma was added, VWF was rapidly processed by the proteolytic activity of ADAMTS-13 to lower molecular weight units, which did not support platelet adhesion at high shear. It is probable, therefore, that plasma is present, VWF is rapidly degraded so that it less efficiently supports platelet adhesion and can only do so at low wall shear rates. This would not preclude the adhesion of platelets to developing or established atheroma, as blood flow is often disturbed so that flow separation, eddies, flow reversal, and even stasis of flow can occur where shear rates are markedly reduced.

The observation that TGF-β1 regulates platelet adhesion to EC indicates a complex role for this agent in the evolution of atheroma. For example, TGF-β1 can inhibit the responses of cultured EC to cytokines although we and others have reported that in multicellular culture systems or whole tissues, TGF-β1 primes EC for increased sensitivity to TNF-α or lipopolysaccharide. Interestingly, in rodent models of atherosclerosis and in human atheroma, the overexpression of TGF-β1 appears to stabilize complex plaques. Additionally, the loss of TGF-β signaling via TGF-β-RII in murine T cells greatly exacerbates atheroma formation in apoE knockout mice. Thus, it is possible that TGF-β1 has antiinflammatory, proinflammatory, and prothrombotic roles in atherogenesis. The balance of these signals may vary at different stages of plaque formation and may be critical in orchestrating the evolution of the plaque.

In conclusion, we have demonstrated that SMC in a phenotype relevant to the atherosclerotic microenvironment can activate EC to support platelet adhesion. This novel route of transcellular crosstalk between secretory SMC and EC may promote the adhesion of platelets and leukocytes may be critical for the development of atheromatous disease.

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References


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1. Expanded Materials and Methods.

1.1 Platelet isolation and preparation.

Blood for platelet isolation was collected from healthy volunteer donors and anticoagulated with citrate-phosphate-dextrose-adenine (1:9 CPDA: blood; Sigma, Poole, UK) in the presence of theophylline (1.3 mg/ml; Sigma) to maintain the platelets in a quiescent state. Platelet rich plasma (PRP) was produced by centrifugation at 300g for 10 min. Washed platelets were produced by centrifuging PRP at 800g for 10 min and resuspending the platelets in calcium and magnesium-free phosphate buffered saline (PBS; Sigma) containing 0.15% bovine serum albumin (BSA fraction IV; Sigma) and 1.3 mg/ml theophylline (Sigma). The washed platelets were fluorescently labelled with calcein-AM (5µg/ml; Molecular Probes, Eugene, Oregon, USA) for 20 min in the dark and then washed twice using the above buffer to remove excess fluorochrome. Platelets were finally resuspended in medium 199 (Invitrogen, Paisley, UK) containing 20% autologous platelet poor plasma (PPP; see below for preparation) and 5 U/ml heparin. In some experiments autologous washed red blood cells (RBC) were added to obtain an haematocrit of 20% (see below for preparation). Where stated, platelets were activated with 5µM ADP (Sigma) immediately prior to addition to adhesion assay.

Platelet poor plasma and washed RBC were prepared from blood anticoagulated with 5U/ml heparin (Sigma). After centrifugation at 1300g for 20
min, PPP was collected from the top of the sedimented blood cells. RBC were prepared by centrifuging blood at 1300g for 10 mins and removing the leukocyte rich layer (approximately 10% by volume). The original volume was reconstituted with PBS/BSA and after thorough mixing the washing process was repeated twice. The haematocrit of the final RBC pellet was measured using a microhaematocrit centrifuge and RBC reconstituted to a final haematocrit of 40% in PBS/BSA.

1.2 Culture of EC and SMC.

Human EC were isolated from the veins of umbilical cords as described\(^1\) and cultured in Medium 199 containing 28µg/ml gentamycin (Roussel Laboratories Ltd, Uxbridge, UK), 20% fetal bovine serum (Labtech International, Ringmer, UK), 10ng/ml epidermal growth factor (Sigma) and 1µg/ml hydrocortisone (Sigma) until confluent (approximately 5-7 days). Each experiment utilised first passage EC cultures from a different donor. Primary cultures were subcultured either into porous polyethylene terephthalate (PET) culture plastic inserts (Becton Dickinson, New Jersey, USA) to establish cocultures with smooth muscle cells (see below), into gelatinized 24 well tissue culture plates or into gelatinized glass capillaries (microslides) and cultured until confluent as previously described\(^1\).

Human SMC were explanted from the arteries of umbilical cords\(^2,3\). Arteries were excised from the cord and cut into 1mm rings. 6-10 rings were put
into plastic culture dishes with Promocell SMC medium (basal medium and all
additions from Promocell, Heidleberg, Germany) containing 12.5µg/ml
gentamycin, 12.5ng/ml amphotericin B, 5% foetal bovine serum, 10ng/ml
epidermal growth factor, 2 ng/ml basic fibroblast growth factor and 0.4µg/ml
dexamethasone. Rings remained in culture for 3-5 weeks until substantial SMC
migration onto culture plastic occurred. Primary SMC were passaged 4 times and
used for experiment at fifth passage. SMC were in the secretory phenotype and
secreted interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1)\(^3\).

1.3 Coculture on porous inserts.

EC/SMC cocultures were established as previously described\(^2,3\) on the
opposite sides of porous polyethylene terephthalate (PET) culture plastic inserts
which had an effective culture area of 0.3cm\(^2\), a pore size of 0.4µm and a pore
density of 1 \(\times\) 10\(^8\) /cm\(^2\) (Becton Dickinson, New Jersey, USA). Briefly, inserts
were inverted in sterile moist boxes and 1 \(\times\) 10\(^4\) secretory SMC were pipetted on to
the external face of the membrane of the inserts. SMC were allowed to settle and
adhere for 1h and then inserts were returned to 24 well companion plates for a
further 23h. 2.5 \(\times\) 10\(^4\) EC (sufficient cells to produce a confluent monolayer) were
seeded on the opposite side of the porous membrane and cultured for a further 24
hours.
1.4 Platelet adhesion assays.

Adhesion of platelets to EC cultures on filters or in plastic dishes or to EC cocultured with SMC on filters, was quantified under static conditions. Prior to performing the adhesion assay, cocultures were either left unstimulated or stimulated for 24h with 100U/ml TNF. EC alone were either left unstimulated or stimulated for 24h with either 100U/ml TNF, 0.5 ng/ml IL-1; 10ng/ml TGFβ1; a combination of TNF and IL-1 or a combination of TNF and TGFβ1. Prior to the addition of platelets cytokines were removed from the culture systems with several washes in PBS/BSA. Calcein-AM labeled platelets were added to the EC surface and allowed to adhere for 1h at 37°C. Non-adherent cells were removed by 4 washes with PBS/BSA and the EC monolayers fixed in 2% formaldehyde for 10 min. Platelets adherent to EC in 24 well dishes were observed in situ using an Olympus Reflected Fluorescence System microscope (model IX70) using an LCPlanFL 20X objective lens with a numerical aperture of 0.4. The microscope was fitted with a video camera, video monitor and video recorder and 10 fields of view selected at random were taped for analysis of platelet adhesion. In the case of cocultured EC, porous membranes were cut out of the inserts and mounted under glass coverslips for observation by fluorescence microscopy.

The adhesion of flowing platelets was assayed in microslides containing confluent monolayers of EC which were unstimulated or activated with a combination of 100 U/ml TNF and 10 ng/ml TGFβ1. Platelets at a concentration
of 2 x 10^8/ml in the presence of 20% washed red blood cells were perfused through the microslide at a wall shear rate of 100s^{-1} or 400s^{-1} for 2 min. In some experiments video records of fluorescently labeled platelets binding to EC were made in real time during the platelet perfusion period by illuminating 6 different and widely spaced fields for 10 sec. In other experiments the system was not illuminated until non adherent cells had been removed by perfusing wash buffer for 8 min when adhesion of platelets was visualized and video records made at the end of the wash period (i.e. after 10 min perfusion). In experiments to further characterize the nature of platelet interactions with EC at high shear we conducted experiments at 400s^{-1} in conditions lacking ADAMTS-13. This was achieved by either omitting autologous plasma from the perfusion buffer or by heat inactivating ADAMTS-13 in the plasma. Alternatively a function neutralizing rabbit polyclonal antibody (kind gift of Dr. J. Crawley, Haematology Dept. Imperial College, London, UK) or polyclonal non-immune serum as control, were used to treat platelet preparations prior to adhesion assay.

Platelet adhesion was quantified using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). For the analysis of platelet adhesion to EC in static assays, or after washout in flow assays, single video images from the records were digitized and platelet adhesion calculated as percentage of the total area of the video field that was fluorescently labeled and bright against a dark background (% coverage). In flow, to measure the number of platelet-EC
interactions that lasted for greater than 40ms, real time video records of platelet perfusion were analysed frame by frame so that a total of 30 sec of the 1 min record (equivalent to 750 individual frames) was assessed. Platelets that were already adherent to the EC in the first frame of analysis were ignored and only platelets that made contact during the flow period were scored. Platelets were scored only once upon first contact and were not scored in subsequent frames if adhesive interactions lasted longer than 40ms. The number of interactions was normalized per unit area and for the number of platelets perfused (interactions / mm²/10⁸ platelets perfused).

In some experiments platelets were treated with antibodies against GPIb (clone SZ2/0409; 10 µg/ml; Immunotech, or clone 6D1; 10 µg/ml; Kind gift of Prof. B. Coller, Mount Sinai Medical Centre, New York, USA.), αIIbβ3 (clone 7E3; [Reopro; abciximab]; 10 µg/ml; Centocor/Eli Lilly) or control antibody against VCAM-1 (clone 1.4C3; 10 µg/ml; Dako, Glostrup, Denmark). In other experiments, platelet activation was inhibited by adding 0.5 µg/ml prostacyclin (Alexis Ltd, Bingham, UK) or 1.25 mg/ml theophylline (Sigma), indomethacin or the ADP receptor antagonists MRS2179 (conc; against P₂Y₁) or ARC67085 (conc; against P₂Y₁₂) to platelet preparations. In coculture experiments, function-neutralising antibody against TGFβ1 (9016.2; 100 µg/ml; R&D Systems Ltd, Abingdon, UK), was included in the culture medium upon the addition of EC to the insert.
1.5 Visualisation and quantification of VWF on EC.

To visualise VWF, confluent monolayers of EC were grown on glass coverslips in 24 well plates or in microslides. EC were activated with either 100 U/ml TNFα, 10 ng/ml TGFβ1 or a combination of these agents. To ensure that only surface presented VWF was fluorescently labeled, live cells were labeled with Rabbit anti-human VWF antibody (P0226; Dako) or a rabbit immunoglobulin control antibody (Dako) for 1 hr at 4°C. Secondary labeling with a sheep-anti-Rabbit-FITC antibody for 1 hr at 4°C (PF310; Dako) was used to visualise VWF. VWF was detected using a confocal microscope (Leica DMIRE2, TCS SP-2, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) (Objectives HCPL FLUOTAR: 20X0.5-Dry) and fluorescence was quantified by integrated pixel intensity determination over an entire field of view (Leica TCS SP-2 software, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

1.6 Platelet aggregation assay.

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation of heparinised blood from healthy volunteers. The aggregometer (460VS dual channel lumi-aggregometer; Chrono-log Corp, Havertown, P.A., U.S.A.) trace was calibrated using unstimulated stirred PRP (at 37°C) which
Online Data Supplement

represents minimum light transmission and PPP which represents maximum light transmission. Platelet aggregation was then followed over time in the presence of 10ng/ml TGF-β1, 100U/ml TNFα or a combination of these agents. ADP at concentrations between 1 and 100μM was used as positive control. Data are expressed as % aggregation (mean ± SEM of 2 experiments).

2. Results.

2.1 Recombinant TNF-α and TGFβ-1, singly or in combination do not promote platelet aggregation.

To ensure that the adhesion of platelets to cocultured endothelial cells or to endothelium stimulated with recombinant TNF-α and/or TGFβ-1 was not promoted by recombinant cytokines, we utilised a standard platelet aggregometry assay in the presence of these agents. Treatment of platelets with either agent singly or in combination did not promote aggregation, although ADP, used as a positive control, could dose dependently induce the aggregation of platelets (Figs 1 and 2).

2.2 ADP receptor antagonists and indomethacin inhibit the activation (and therefore the adhesion) of platelets to TNF-α and TGFβ-1stimulated EC.
To determine the nature of the activating stimulus that promoted stable platelet adhesion to TNF-α and TGFβ-1 stimulated EC, we conducted experiments using indomethacin and antagonists of ADP receptors. Both of these strategies significantly reduced the adhesion of platelets to EC, strongly implying that thromboxane and ADP were necessary for stable platelet adhesion in this system (ODS Fig 3).

2.3 The adhesion of flowing platelets requires both GPIb and αIIbβ3 integrin.

To verify that flowing platelets utilized the same adhesion receptors to bind to EC as were described under static conditions (Main Text Fig 4) we used the same antibodies against GPIb and αIIbβ3 integrin to block the adhesion of flowing platelets (wall shear rate = 100s⁻¹). Blockade of either receptor resulted in substantial inhibition of platelet adhesion (ODS Fig 4).

2.4 Platelets adhere at a shear rate of 400s⁻¹ to EC stimulated with TNF-α and TGFβ-1 in the absence of ADAMTS-13 activity.

When experiments were conducted in the presence of autologous plasma we found that the matrix of VWF elaborated by EC did not support the adhesion of flowing platelets at shear rates above 100s⁻¹ (Main Text Fig 6). We postulated that this was due to the proteolytic processing of ultra-large VWF molecules by
ADAMTS-13 into less adhesive and smaller units. Thus, when these experiments were conducted in the presence of autologous plasma in which ADAMTS-13 activity had been neutralized by heat inactivation or by treatment with a monoclonal antibody, we found that platelets did adhere to the EC at shear rates of 400s$^{-1}$. Interestingly, we observed the formation of long strings of platelets (ODS Figs 3 and 4), which have been previously described$^{4,5}$ and are supported by the expression of ultra-large VWF. Conducting these experiments in the absence of autologous plasma also supported platelet chain formation at 400s$^{-1}$ (data not shown from 2 independent experiments).

3. References


**Online Figure Legends**

Online Figure 1. *Aggregometer traces of platelets stirred in the presence of recombinant cytokines.* TNF-α, TGF-β1 or a combination of TNF-α and TGF-β1 did not promote the aggregation of platelets. ADP (1-100 µM) dose dependently promoted the aggregation of platelets.

Online Figure 2. *The effects of recombinant cytokines and ADP on the aggregation of platelets.* ADP dose dependently increased the aggregation of stirred platelets. TNF-α, TGF-β1 or a combination of TNF-α and TGF-β1 did not promote the aggregation of platelets. Data is the mean ± SEM of 2 experiments.
Online Figure 3. **The effects of inhibitors of platelet activation on the adhesion of platelets to endothelial cells.** The cyclooxygenase inhibitor, indomethacin, and the ADP receptor antagonists MRS2179 (P2Y1) or ARC67085 (P1Y12), inhibited the adhesion of platelets to EC activated with TNF-α and TGF-β1. Data are mean ± SEM of 3 experiments. * = P < 0.05; ** = P < 0.01 for comparison by Students t-test between untreated and inhibitor treated platelets.

Online Figure 4. **The effects of inhibitors on the adhesion of platelets to EC stimulated with TNF-α and TGFβ1.** The effect of antibodies against VCAM-1, GPIb (SZ2), or αIIbβ3 integrin on the adhesion of platelets to EC activated with TNF-α and TGFβ1. Data are the mean ± SEM of 2 experiments.

Online Figure 5. **Photomicrographs of the adhesion of flowing platelets to EC in the absence of the activity of ADAMTS-13.** a) A phase contrast photomicrograph and b) a fluorescent image of the same field, of platelets bound to EC at a wall shear rate of 400 s⁻¹ when the activity of ADAMTS-13 was neutralized. Platelets bound in characteristic ‘strings’ to the EC surface.

Online Figure 6. **The effects of inhibiting ADAMTS-13 activity on the adhesion of platelets to EC stimulated with TNF-α and TGFβ1.** a) the number of platelet strings and b) the number of platelets in strings on EC stimulated with TNF-α and
TGFβ1 in the presence or absence of autologous plasma (AP) heat inactivated AP, polyclonal antibody against ADAMTS-13 or control serum. Data is mean ± SEM of 3 experiments. * = P < 0.05; ** = P < 0.01 for comparison by students t-test between experiments with active and inactive ADAMTS-13.
ODS Figure 1

1 Min

TNF-\(\alpha\), TGF-\(\beta_1\)
or TNF-\(\alpha\) + TGF-\(\beta_1\)

1 \(\mu\)M ADP

10 \(\mu\)M ADP

100 \(\mu\)M ADP
ODS Fig 3

Platelet adhesion (% coverage)

Inhibitor Treatment

- None
- Indomethacin
- MRS2179 (P₂Y₁)
- ARC67085 (P₂Y₁₂)

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ODS Fig 4

Platelet adhesion (% coverage)

- Untreated
- Anti-VCAM-1
- Anti-GPlb
- Anti-αIIb/β3
ODS Figure 5