Cellular Pathology of Atherosclerosis
Smooth Muscle Cells Prime Cocultured Endothelial Cells for Enhanced Leukocyte Adhesion

G. Ed Rainger, Gerard B. Nash

Abstract—During the development of an atherosclerotic plaque, mononuclear leukocytes infiltrate the artery wall through vascular endothelial cells (ECs). At the same time, arterial smooth muscle cells (SMCs) change from the physiological contractile phenotype to the secretory phenotype and migrate into the plaque. We investigated whether secretory SMCs released cytokines that stimulated ECs in a manner leading to increased leukocyte recruitment and thus might accelerate atheroma formation. SMCs and ECs were established in coculture on the opposite sides of a porous membrane, and the cocultured cells were incorporated into a flow-based assay for studying leukocyte adhesion. We found that coculture primed ECs so that their response to the inflammatory cytokine tumor necrosis factor-α was amplified. ECs cocultured with SMCs supported greatly increased adhesion of flowing leukocytes and were sensitized to respond to tumor necrosis factor-α at concentrations 10 000 times lower than ECs cultured alone. In addition, coculture altered the endothelial selectin adhesion molecules used for leukocyte capture. EC priming was attributable to the cytokine transforming growth factor-β1, which was proteolytically activated to a biologically active form by the serine protease plasmin. These results suggest a new role for secretory SMCs in the development of atheromatous plaque. We propose that paracrine interaction between ECs and SMCs has the potential to amplify leukocyte recruitment to sites of atheroma and exacerbate the inflammatory processes believed to be at the heart of disease progression. (Circ Res. 2001;88:615-622.)

Key Words: endothelial cells ■ smooth muscle cells ■ leukocyte adhesion ■ transforming growth factor-β1 ■ tumor necrosis factor-α

Atherosclerotic plaque typically contains abnormal deposits of mononuclear leukocytes and secretory smooth muscle cells (SMCs). Ordinarily, SMCs are confined to the medial layer of the artery wall, where, as contractile cells, their physiological role is the maintenance of mechanical structure and vascular tone. Early in the genesis of atherosclerotic plaque, medial SMCs undergo mitogenic stimulation and change phenotype, losing contractile elements and acquiring the ability to replicate and migrate within the artery wall. In consequence, SMCs invade the intimal layer of the diseased artery, where they contribute to progressive arterial hyperplasia by inappropriate replication and deposition of a fibrotic connective tissue matrix. Intimal SMCs also acquire a well-developed rough endoplasmic reticulum and alter their secretory profile, generating several proinflammatory agents. We wished to examine the possibility that secretory SMCs could release agents that promote the recruitment of mononuclear leukocytes to the developing plaque.

Adhesion between leukocytes in the arterial blood and activated endothelial cells (ECs) overlying atherosclerotic plaque seems to represent the major route of entry into the artery wall for T lymphocytes and monocytes. The recruitment of blood-borne leukocytes to inflamed tissue normally occurs across the ECs of postcapillary venules. When activated by cytokines, such as tumor necrosis factor-α (TNF-α), ECs express specialized adhesion receptors (E- and P-selectin and vascular cell adhesion molecule-1 [VCAM-1]), which have rapid forward kinetics for bond formation essential for binding flowing cell. These molecules also support subsequent rolling adhesion, allowing the slow-moving leukocytes to assimilate signals from the EC-borne chemoktractants that promote immobilization and transendothelial migration via integrin adhesion receptors. ECs of the healthy human artery do not express selectins or VCAM-1, but their expression has been demonstrated on the ECs overlying atherosclerotic plaque.

Secretory SMCs generate several agents that could promote leukocyte infiltration of the artery wall. They release potent leukocyte chemoattractants, such as interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1). They also release the proinflammatory cytokines TNF-α and IL-1, which induce the expression of adhesion
molecules and leukocyte chemoattractants on ECs. The cytokine transforming growth factor-β1 (TGF-β1) is also generated in biologically active form when SMCs and ECs are cultured together. Although TGF-β1 does not induce leukocyte adhesion to ECs in a manner comparable to TNF or IL-1, it can modulate the effects of other proinflammatory agents. The ability of TGF-β1 to either inhibit or promote leukocyte adhesion to ECs has been reported depending on the type and conditions of growth of the ECs.

In the present study, we have developed an in vitro model of the artery wall to investigate interactions between secretory SMCs, endothelial cells, and flowing purified leukocytes in a physical milieu resembling the circulation. Our studies show that secretory SMCs can greatly potentiate adhesion of leukocytes to ECs. The results suggest a new role for SMCs in the cellular pathology of atherosclerosis.

Materials and Methods

Leukocyte Isolation

Mononuclear leukocytes (29% monocytes and 71% lymphocytes), purified monocytes (91% monocytes and 9% lymphocytes), peripheral blood lymphocytes (PBLs) (95% lymphocytes and 5% monocytes), and purified neutrophils (>95% granulocytes) were prepared from EDTA anticoagulated blood by density gradient centrifugation, cell passing, or magnetic bead separation (for more information, see the online data supplement available at http://www.circresaha.org). To aid visualization, leukocytes were loaded with the fluorochrome calcein-AM (5 μg/mL), which did not affect adhesion.

Primary Culture of ECs, SMCs, and Fibroblasts

Human ECs were isolated from the veins of umbilical cords, as described elsewhere. Each experiment used first-passage ECs from a different donor. Human SMCs were explanted from the arteries of umbilical cords. SMCs from 8 different donors were used. Skin fibroblasts were explanted from normal human foreskin. For details, see the online data supplement.

ECs and SMCs Coculture on Porous Inserts

SMCs were cultured for 24 hours on the inside of culture inserts (Becton Dickinson). ECs were then added to the outside face of the monocentrone and cultured alone or with SMCs for 24 hours. Coculture inserts or inserts bearing ECs alone were treated with TNF-α at 0 to 100 U/mL for an additional 24 hours before adhesion assay. See the online data supplement for details. In some experiments, inserts bearing ECs were treated for 24 hours with 1 or 10 ng/mL recombinant TGF-β1 before addition of TNF.

Flow Adhesion Assay

Adhesion assays were conducted in a novel parallel-plate flow chamber engineered to incorporate the insert and allow perfusion of leukocytes over the ECs on the outer face of the culture membrane at 37°C. The chamber was placed on the stage of a fluorescence microscope and connected to a withdrawal syringe pump. Fluorescently labeled leukocytes were perfused over the ECs at a wall shear stress of 0.05 or 0.1 Pa, and adhesion was assessed by fluorescence microscopy. See the online data supplement for details.

Antibodies and Inhibitors

ECs or mononuclear leukocytes were treated with monoclonal adhesion-blocking antibody (mAb) against E-selectin, P-selectin, VCAM-1, or α5 integrin for 15 minutes before adhesion assay. See the online data supplement for details.

Results

Secretory Smooth Muscle Cells Prime but Do Not Activate Cocultured Endothelial Cells for Leukocyte Adhesion

When ECs and secretory SMCs were cultured on the opposite sides of porous culture membranes for 48 hours, the ECs barely supported the adhesion of isolated mononuclear leukocytes from flow (Figure 1). The level of binding to cocultured ECs was no greater than for ECs cultured alone. Thus, secretory SMCs did not activate endothelial cells.

In contrast, cocultured ECs markedly altered their response to the cytokine TNF-α. When ECs were treated alone with TNF-α for 24 hours at concentrations between 0 and 100 U/mL, a dose-dependent increase in their ability to capture mononuclear leukocytes from flow between 1 and 100 U/mL was observed (Figure 1). A qualitatively similar response to TNF-α was observed on endothelial cells cocultured with SMCs (Figure 1). However, the presence of SMCs greatly sensitized the ECs for response to TNF-α. Significant adhesion of leukocytes was inducible with a concentration of TNF-α as low as 0.0001 U/mL on cocultured ECs, whereas the response of ECs alone was extinct below 1 U/mL TNF (Figure 1). With TNF-α at or above 1 U/mL, ECs cocultured with SMCs supported levels of mononuclear cell adhesion between 100% and 200% higher than ECs cultured alone (Figure 1).
Secretory SMCs Prime Cocultured ECs for Enhanced Adhesion of Purified Neutrophils, Monocytes, and Peripheral Blood Lymphocytes
When ECs were cocultured with SMCs and then treated with either 0.1 or 100 U/mL TNF, the adhesion of purified neutrophils, monocytes, or PBL was increased compared with adhesion on ECs cultured alone (Figure 2). As was the case for the mixed mononuclear cell preparation, adhesion to ECs cultured alone was negligible with 0.1 U/mL TNF, whereas ECs cocultured with SMCs demonstrated a significant increase in leukocyte adhesion at both concentrations of TNF-α (*P<0.05; **P<0.01 compared with untreated EC control by Student’s paired t test). At both doses of TNF, adhesion to cocultured ECs was significantly greater than to ECs alone (+P<0.05 by Student’s paired t test).

Contractile SMCs or Skin Fibroblasts Do Not Prime Cocultured ECs
We cocultured ECs with contractile SMCs to determine whether SMCs in their physiological phenotype had any priming effect. To culture contractile SMCs, they must be maintained in medium devoid of growth factors and containing only 0.5% serum. ECs cannot ordinarily be maintained under this regimen and have been reported to undergo apoptosis on exposure to TNF after serum-free culture.28 In only 1 of 4 experiments did ECs maintain a monolayer suitable for adhesion studies. When cultured with contractile SMCs and 0.1 or 100 U/mL TNF, ECs showed no increase in the ability to bind flowing purified PBL compared with ECs cultured alone (Figure 3). Thus, although secretory SMCs could prime ECs for sensitized responses to TNF, this was not a characteristic of all stromal cells.

Coculture of ECs With SMCs Alters the Patterns of EC Adhesion Molecules Used to Recruit Flowing Mononuclear Cells
We used a panel of function-blocking antibodies to investigate the roles of the endothelial receptors E-selectin, P-selectin, and VCAM-1 and the leukocyte integrin α4β1 integrin (ligand for VCAM-1) in adhesion observed in our model. In studies on ECs cultured alone and stimulated with 100 U/mL TNF-α, antibodies against P-selectin, VCAM-1, or α4 integrin inhibited leukocyte attachment by ≈50% whereas antibody against E-selectin had no significant effect (Figure 4a). Antibodies against α4 integrin and P-selectin had an additive effect and blocked >80% of adhesion (Figure 4a).
Thus, mononuclear leukocytes used a combination of VCAM-1 and P-selectin to bind to ECs cultured alone.

A different pattern was seen when ECs were cocultured with SMCs. When cocultured ECs were stimulated with 100 U/mL TNF-α, antibodies against E-selectin, VCAM-1, or α5 integrin inhibited ∼50% of adhesion. Combination of antibodies against α5 integrin and E-selectin blocked >90% of adhesion (Figure 4b). Antibody against P-selectin had no effect. Thus, VCAM-1 provided a common route for leukocyte adhesion under both culture regimes, but EC/SMC coculture altered the pattern of selectin adhesion molecules used for leukocyte adhesion. We determined whether the same adhesive mechanisms were functional in cocultures at lower concentrations of TNF-α. Using 0.1 U/mL TNF-α (a concentration that was stimulatory for ECs only when cultured with SMCs), leukocytes used the same combination of receptors, ie, VCAM-1 and E-selectin (Figure 4c) as with 100 U/mL TNF-α. Antibody studies also showed that purified monocytes and PBL both used VCAM-1 and P-selectin on ECs cultured alone but VCAM-1 and E-selectin on cocultured ECs (data not shown) (see the online data supplement for details). Thus, in coculture, both E-selectin and VCAM-1 supported adhesion at TNF-α concentrations, where they were not functional on ECs cultured alone.

Immunofluorescence labeling of ECs with antibodies against E-selectin or VCAM-1 and flow cytometry demonstrated that E-selectin and VCAM-1 expression were significantly increased on cocultured ECs compared with ECs cultured alone when both were stimulated with 100 U/mL TNF-α (data not shown) (see the online data supplement for details). This strongly indicated that increased expression of EC adhesion receptors contributed to the adhesion of leukocytes to primed ECs.

**Endothelial Cell Priming Is Dependent on Proteolytically Activated TGF-β1**

Modulation of EC responses to TNF has been observed by some laboratories using recombinant, biologically active TGF-β1. To investigate whether TGF-β1 primed ECs in our model, we used a TGF-β1 function neutralizing antibody in cocultures of ECs and SMCs. The antibody greatly inhibited increases in adhesion in cocultures exposed to TNF-α (Figure 5). Adhesion to ECs cocultured in the presence of 0.1 U/mL TNF was nearly abolished (Figure 5a), whereas the adhesion to ECs cocultured in the presence of 100 U/mL TNF was reduced to the level seen on ECs cultured alone (Figure 5b).

TGF-β1 is secreted in a biologically inert form bound to latency-associated peptide. Biologically active TGF-β1 is released from this complex by an acid environment or by proteolytic cleavage. The serine protease plasmin, derived from plasminogen, processes TGF-β1 from the inert to the biologically active form in cocultures of ECs with pericytes or with SMCs. When we added the serine protease inhibitor aprotinin or the plasmin inhibitor α2-antiplasmin to cocultures, EC priming was completely inhibited (Figure 5). Thus, at 0.1 U/mL TNF, the adhesion of mononuclear cells to cocultured ECs was virtually abolished by both agents (Figure 5a). In the presence of 100 U/mL TNF, mononuclear cell adhesion to cocultured ECs was reduced nearly to the levels seen on ECs alone (Figure 5b).

To confirm that ECs could be primed in coculture by TGF-β1, we inhibited endogenous plasmin activity (and thus the activation of endogenous TGF-β1) with α2-antiplasmin but also added biologically active recombinant TGF-β1. Recombinant TGF-β1 reconstituted priming of cocultured ECs in a dose-dependent manner (Figure 6). In the presence
of 0.1 U/mL TNF (Figure 6a) or 100 U/mL TNF (Figure 6b), α2-antiplasmin reduced the level of mononuclear cell adhesion to cocultured ECs to the levels seen on ECs cultured alone. When α2-antiplasmin and 1 ng/mL of recombinant TGF-β1 were simultaneously added to the cocultures, EC priming was partially restored and adhesion was higher than on ECs cultured alone (Figures 6a and 6b). The addition of α2-antiplasmin and 10 ng/mL recombinant TGF-β1 fully restored EC priming, and mononuclear cell adhesion was equivalent to that on EC/SMC cocultures (Figures 6a and 6b).

Recombinant TGF-β1 Can Prime ECs in the Absence of Secretory SMCs

We conducted a series of experiments to determine if recombinant TGF-β1 could prime ECs cultured alone for increased levels of leukocyte adhesion. ECs were cultured for 24 hours on inserts in the absence of SMCs but in the presence of recombinant TGF-β1, at concentrations of 0, 1, or 10 ng/mL. TGF-β1 dose-dependently primed ECs for increased adhesion of purified flowing mononuclear cells in response to stimulation with TNF at 100 U/mL (Figure 7). ECs also responded to TNF at 0.1 U/mL after priming with 1 ng/mL recombinant TGF-β1. ECs bound 36±3 compared with 2±0.4 leukocytes on primed and unprimed ECs, respectively (mean±SEM of 3 matched experiments; P<0.05 by Student’s paired t test).

**Figure 5.** Effect of neutralizing TGF-β1 activity on the adhesion of mononuclear leukocytes to ECs cocultured with SMCs. ECs were cultured alone (EC) or with SMCs (EC/SMC) for 24 hours in the presence of either a function-neutralizing antibody against TGF-β1, control antibody, the serine protease inhibitor aprotinin, or the plasmin inhibitor α2-antiplasmin. Cultures were then stimulated for 24 hours in the continued presence of these agents with TNF-α at 0.1 U/mL (a) or 100 U/mL (b). Neutralizing the activity of TGF-β1 with antibody reduced leukocyte adhesion nearly to the levels for ECs cultured alone. Control antibody had no significant effect. Inhibition of the proteolytic capacity of plasmin in cocultures with aprotinin or α2-antiplasmin also reduced adhesion of leukocytes toward levels for ECs alone. +P<0.05; ++P<0.01 for treated EC/SMC cocultures compared with untreated EC/SMC by Student’s paired t test.

**Figure 6.** Recombinant, biologically active TGF-β1 reconstitutes EC priming in cocultures after plasmin inhibition by protease inhibitors. ECs were cultured alone or with SMCs for 24 hours in the presence of α2-antiplasmin (to inhibit the activation of endogenously generated TGF-β1) and recombinant, biologically active TGF-β1 at concentrations of 0, 1, or 10 ng/mL. Cocultures were then stimulated for 24 hours in the continued presence of the agents with TNF-α at either 0.1 U/mL (a) or 100 U/mL (b). ANCOVA showed a significant effect on leukocyte adhesion of the priming concentration of TGF-β1 (P<0.05) and of the stimulatory concentration of TNF-α (P<0.01). +P<0.05 for comparison of EC/SMC cultures treated with α2-antiplasmin and TGF-β1, compared with cocultures treated with α2-antiplasmin alone by Student’s paired t test.

**Figure 7.** Effect of recombinant TGF-β1 on adhesion of purified mononuclear leukocytes to ECs treated with TNF-α. ECs were grown alone on the porous membranes of culture inserts for 24 hours. Inserts were then treated for 24 hours with 0, 1, or 10 ng/mL recombinant TGF-α followed by 24 hours of stimulation with 0 or 100 U/mL TNF. Culture inserts were incorporated into a flow chamber, and the adhesion of flowing mononuclear cells was assessed. Untreated ECs did not support the adhesion of leukocytes. ECs treated with 100 U/mL TNF supported significantly increased leukocyte adhesion (**P<0.01 compared with untreated EC control by Student’s paired t test). Leukocyte adhesion was significantly increased in a dose-dependent manner when the ECs had been pretreated with 1 or 10 ng/mL TGF-α1 (+P<0.05 by Student’s paired t test).
**Discussion**

Using a novel, multicellular, flow-based model of the artery wall, we demonstrated a paracrine interaction between ECs and secretory SMCs, which may influence the progression of atherosclerosis. ECs cultured with secretory SMCs were primed for increased responsiveness to the inflammatory cytokine TNF-α. At equal concentrations of TNF-α, ECs cocultured with SMCs supported greatly increased adhesion of flowing leukocytes compared with ECs cultured alone. Cocultures were also sensitized to respond to much lower concentrations of TNF-α compared with untreated EC control by Student’s paired t test, whereas E-selectin was not detected at any concentration of TNF. In the presence of 10 ng/mL recombinant TGF-β1, both VCAM-1 and E-selectin were detectable at 0.1 and 100 U/mL TNF, and expression was significantly increased compared with ECs cultured in the absence of TGF-β1 (*<0.05 compared with ECs with out TGF-β1; by Student’s paired t test).

ng/mL TGF-β1 demonstrated increased expression of both VCAM-1 and E-selectin after stimulation with TNF at 100 or 0.1 U/mL (Figure 8).

The present study improves our understanding of how interactions between cells of the artery wall may promote recruitment of leukocytes to developing atheroma. During inflammation, leukocytes adhere to selectins or VCAM-1 expressed by cytokine-stimulated ECs of postcapillary venules and migrate under the action of local chemoattractants. Leukocytes do not usually adhere to the wall of healthy arteries, but monocytes and T lymphocytes are found in atherosclerotic plaque. Studies using animal models or human tissue have revealed the cellular composition of plaque and the local patterns of expression of adhesion molecules and chemoattractants. However, in these systems, it is difficult to dissect the complex interactions between cellular components and cytokines leading to leukocyte recruitment. To overcome these limitations, we developed an in vitro model incorporating chosen cellular elements of the atherosclerotic lesion. Flow was included both to mimic the in vivo requirement for fast-acting capture receptors (selectins or VCAM-1) and enable washout of secreted chemotactic agents that may otherwise accumulate in static systems. Thus, we identified a novel pattern of adhesion molecule usage on human umbilical vein ECs (HUVECs) responding to TNF-α in the presence of SMCs.

Ordinarily, E-selectin expression on HUVECs peaks after 4 hours and is absent after 24 hours of exposure to TNF-α. In the present study, E-selectin was functional after 24 hours on ECs cocultured with SMCs, whereas P-selectin was functional on ECs cultured alone. VCAM-1 was functional for either type of culture stimulated with TNF-α. A switch from P-selectin to E-selectin could influence the leukocytes found in atherosclerotic plaque, for example, because the ability to use P- or E-selectin differs between subsets of T lymphocytes. We have not described the intracellular mechanisms that underlie prolonged E-selectin expression. Others, using dermal microvascular ECs that demonstrate prolonged expression of E-selectin in response to TNF, have described a role for sustained activity of nuclear factor-κB (NF-κB), the nuclear factor responsible for transcriptional activity of the E-selectin gene. NF-κB activity was sustained by reduction of cytoplasmic levels of IkB-β, an inhibitor of NF-κB function. Alterations in the membrane turnover and intracellular degradation of E-selectin protein may also contribute to prolonged E-selectin expression in dermal microvascular ECs.

ECs cocultured with SMCs were primed for increased adhesion of all leukocyte types tested, including neutrophils. However, neutrophils are not present in atherosclerotic plaque, even though receptors that support neutrophil capture and adhesion are known to be expressed on diseased arteries. The inability of arterial ECs to generate chemotactic stimuli, which activate neutrophils and drive migration into the artery wall, could account for the observed lack of plaque resident neutrophils. Alternatively, neutrophils may infiltrate the artery wall but undergo rapid clearance, for example, by apoptosis.

We demonstrated that EC priming was abolished by a neutralizing mAb against TGF-β1 or by inhibitors of plasmin-mediated activation of TGF-β1. EC priming in cocultures could be reconstituted with exogenous recombinant TGF-β1, when the activation of endogenous cytokine was inhibited by antiproteases. Recombinant TGF-β1 also effectively primed ECs cultured in the absence of SMCs. However, TGF-β1 has previously been reported to inhibit as well as prime ECs for the recruitment of leukocytes. A series of studies described TGF-β1-dependent inhibition of leukocyte adhesion to E-selectin expressed on subconfluent HUVECs.
using ECs specialized for lymphocyte trafficking during immune surveillance, eg, high endothelial venule ECs and dermal microvascular ECs, also showed inhibition of leukocyte adhesion in response to TGF-β1. Alternatively, TGF-β1–mediated priming of confluent HUVECs for increased VCAM-1 expression by lipopolysaccharide has been demonstrated. Also, injection of TGF-β1 into the skin of rabbits greatly increased the infiltration of neutrophils in response to a secondary challenge with TNF 24 hours later. Thus, we believe that TGF-β1 should be considered a regulator of leukocyte adhesion, with the nature of the response depending on the phenotype of the responding ECs. From our own studies and those of others, we believe that in the context of inflammation, preexposure of ECs to TGF-β1 can sensitize ECs to subsequent exposure to TNF.

ECs or SMCs cultured alone do not generate biologically active TGF-β1, but cocultures of these cells produce the bioactive cytokine, which has been demonstrated to influence cellular functions, such as EC proliferation and motility. TGF-β1 is secreted in complex with a latency-associated peptide by both ECs and SMCs in monoculture. Thus, additional production of latent TGF-β1 is unlikely to be a prerequisite for priming in coculture. Rather, it is the proteolytic processing of the latent cytokine to a biologically active form by plasmin that occurs in coculture but not in EC and SMC monocultures. It is probable that plasmin is activated in EC/SMC coculture by plasminogen activator (PA), because the inclusion of a function neutralizing antibody directed against urokinase-PA inhibits the functional activation of TGF-β1 in EC/SMC cocultures. Both ECs and SMCs in monoculture produce PA, and it is thus unlikely that TGF-β1 activation requires induction of this system in coculture. Why PA is not functional in EC and SMC monocultures is not clear, but alterations in the expression of receptors required to colocalize plasminogen and PA to the cell surface where plasmin activation occurs or alterations in the function or production of PA-inhibitor have been postulated.

Conclusions

Our results suggest a new role for secretory SMCs in the development of atheromatous plaque. SMCs contribute to arterial thickening by invading the intima and depositing a fibrotic connective tissue matrix. They have not previously been directly implicated in the recruitment of inflammatory mononuclear leukocytes. These leukocytes may not normally be recruited, because arterial endothelium may be exposed only to low levels of cytokines or have low responsiveness to them. We describe a mode of paracrine interaction between ECs and SMCs whereby ECs are rendered highly sensitive to TNF-α. The priming, attributable to TGF-β1, has the potential to amplify leukocyte recruitment to sites of established atheroma and, therefore, to exacerbate the inflammatory processes believed to be at the heart of disease progression. EC priming could potentiate the inflammatory effects of cytokines generated by the cells of the lesion. Moreover, primed ECs would have a heightened sensitivity to cytokines released elsewhere into the systemic circulation and typically found at relatively low concentration in a range of disorders with an inflammatory component. This heightened sensitiv-
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1. Material and Methods

1.1 Leukocyte isolation.

Blood for leukocyte isolation was taken from normal donors and anticoagulated with EDTA. Mixed mononuclear cells (monocytes and peripheral blood lymphocytes [PBL]) were isolated by centrifugation on Histopaque 1077 (Sigma, Poole, UK), washed twice using low speed centrifugation (200g) to remove contaminating platelets and resuspended at a concentration of $10^6$ /ml in phosphate buffered saline containing 0.5mM Mg$^{++}$/ 1mM Ca$^{++}$ and 0.1% bovine serum albumin (PBS/Alb)$^1$. Mononuclear cell preparations were 29% monocytes and 71% lymphocytes assessed by cell volume distribution on a Coulter Counter Multisizer II (Coulter Electronics, Luton, UK) (means from 10 experiments).

In some experiments purified monocytes or PBL were used. PBL were purified by panning of contaminating monocytes on culture plastic. Washed mononuclear cell mixtures were placed in to culture plastic petri dishes at 37°C for 30 min in which time monocytes adhered to the plastic. Non-adherent PBL were washed from the petri dishes, resuspended at a concentration of 1 x $10^6$/ml in PBS/Alb and utilised for adhesion assay. PBL preparations were 95% lymphocytes and 5% monocytes$^2$.

Monocytes were purified using the magnetic bead MACS negative selection monocyte isolation kit from Miltenyi Biotech (Bisley, Surrey, UK). Washed mononuclear cell mixtures were resuspended in PBS/Alb containing 2mM EDTA. Contaminating cells (lymphocytes and residual platelets) were labelled with a cocktail of antibodies conjugated with hapten. After washing to remove excess antibody,
tagged cells were labelled with anti-hapten magnetic microbeads and removed from the cell mixture by passage down a ferrous column suspended in a magnetic field. Preparations were typically 91% monocytes and 9% lymphocytes (mean 4 experiments).

Neutrophils were separated using two-step density gradients of Histopaque as previously described\textsuperscript{1-3}. Cells were washed, counted and adjusted to 1 x 10\textsuperscript{6} / ml in PBS/Alb. Preparations were greater than 95% neutrophilic granulocytes\textsuperscript{1-3}.

To aid visualisation in the adhesion assay, leukocytes were incubated with the fluorochrome calcein-AM at 5\textmu g/ml for 10 minutes. At this concentration calcein-AM does not affect adhesion of mononuclear cells or neutrophils to EC stimulated with TNF-\textalpha\textsuperscript{3}.

1.2 Culture of EC, SMC and skin fibroblasts.

Human EC were isolated from the veins of umbilical cords as described\textsuperscript{1} and cultured in Medium 199 containing 28\textmu g/ml gentamycin (Roussel Laboratories Ltd, Uxbridge, UK), 20\% fetal bovine serum (Labtech International, Ringmer, UK), 10ng/ml epidermal growth factor (Sigma) and 1\mu g/ml hydrocortisone (Sigma) until confluent (approximately 5-7 days). Each experiment utilised first passage EC cultures from a different donor.

Human SMC were explanted from the arteries of umbilical cords. 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 12.5\mu g/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. Alternatively, isolated human umbilical artery SMC were purchased from Promocell and treated identically to our own explanted cells. Experiments in the current study used explanted SMC from 8 different donors (3 Promocell isolates and 5 of our own isolates). We used SMC in the secretory phenotype which were positive for secreted interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) (tested by commercial ELISA, R&D systems, Abingdon, UK) but which were negative for SMC actin (tested by microscopy after immunofluorescence staining) (data not shown). SMC could readily be converted to a ‘contractile’ phenotype (i.e. negative for IL-8 and MCP-1 secreted protein but positive for SMC actin) by removal of growth factors and the use of 0.5% FBS in the Promocell culture medium (data not shown).

Skin fibroblasts were kind gift from Dr C.D Buckley (Dept Rheumatology, The University of Birmingham) and were cultured from human foreskin. Normal human tissue was finely chopped and subject to enzymatic digestion by collagenase. Fibroblasts were cultured out in RPMI medium containing 28μg/ml gentamycin and 20% fetal bovine serum. Cells were maintained in rapid growth phase and serially passaged until use (between passage 10-20).
1.3 Coculture on porous inserts.

EC/SMC or EC/skin fibroblast cocultures were established on the opposite sides of porous polyethylene terephthalate (PET) culture plastic inserts (Becton Dickinson, New Jersey, USA). The membranes on the inserts had an effective culture area of 0.3 cm², a pore size of 0.4 μm and a pore density of 1 x 10⁸ /cm². Cells on the inserts were maintained in culture medium in 24 well plates specifically manufactured for the purpose (Becton Dickinson). Briefly, 1 x 10⁴ secretory SMC, contractile SMC or skin fibroblasts were cultured in the well of the inserts for 24h. Inserts were then inverted in sterile moist boxes and 2.5 x 10⁴ EC (sufficient cells to produce a confluent monolayer, verified at time of experiment by fluorescence microscopy) pipetted on to the opposite face of the inserts. EC were allowed to settle and adhere for 1h and then the cocultures were returned to the plates for a further 23h. Coculture inserts or inserts bearing EC alone were treated with TNF-α at concentrations between 0-100 U/ml for a further 24h prior to adhesion assay.

1.4 Flow adhesion assay

Adhesion assays were conducted in a novel parallel plate flow chamber (Fig 1) engineered to allow perfusion of leukocytes over the EC on the ‘outside’ of culture inserts. Inverted inserts fitted into a slot so that the EC monolayer formed part of the bottom of a channel of defined dimensions (100 μm deep by 5mm wide by 5cm long). The chamber was placed on the stage of a fluorescence microscope and connected to a withdrawal syringe pump as previously described¹. Fluorescence microscopy was used due to the poor optical qualities of the coculture membrane. Fluorescently-
labelled leukocytes were perfused over the EC for 3 minutes at a constant wall shear stress of either 0.05 or 0.1 Pa. All experiments using adhesion blocking Mab were conducted at 0.1Pa. Where numerous experimental variations were used in matched experiments (i.e. in titrating EC responses to TNF-α, studies with plasmin inhibitors and reconstitution experiments with recombinant TGFβ1), 0.05 Pa was used in order to limit the volume of blood collected for leukocyte isolation. Non-adherent cells were removed by 2 minutes of wash with cell free buffer. The fluorescent adherent cells were counted in a number of complete microscope fields of known dimensions and binding expressed as number / mm² / 10⁶ perfused (Fig 2).

1.5 Adhesion blocking antibodies.

Monoclonal antibodies were all mouse IgG1. Antibody against VCAM-1 was clone 4B2 used at 50µg/ml and against α4-integrin was clone 2B4 used at 5µg/ml (both gifts of Dr John Clements, British Biotech). mAb against E-selectin was F(ab’)2 of clone ENA2 used at 1µg/ml (Bradshaw Biologicals, Chepshet, UK)³. Antibody against P-selectin was clone G1 used at 50µg/ml (gift of Dr Rodger McEver, University of Oklahoma College of Medicine, Oklahoma)⁴. Isotype matched anti-P-selectin and anti-E-selectin and anti VCAM-1 were used in the same experiments. Thus, the P-selectin antibody is a functional control antibody for the E-selectin and VCAM-1 antibody and E-selectin antibody was control for P-selectin and VCAM-1 antibodies.
2. Results

2.1 Coculture of EC with SMC alters the patterns of EC adhesion molecules used to recruit flowing mononocytes or PBL.

Experiments using mixed mononuclear cells indicated that E-selectin and VCAM-1 were responsible for the capture of nearly all flowing leukocytes on cocultured EC, but VCAM-1 and P-selectin mediated capture on EC cultured alone. We used the same adhesion blocking antibodies to investigate routes of adhesion of purified monocytes and PBL. On EC cultured alone, both cell types utilised VCAM-1 and P-selectin to adhere to the EC from flow (Fig 3). However, both monocytes and PBL utilised a combination of VCAM-1 and E-selectin to adhere to EC cocultured with SMC (Fig 3). This again indicated that adhesion observed in mixed populations of mononuclear cells was not due to the preferential recruitment of a particular leukocyte subset, and that monocytes and PBL could utilise the same routes for adhesion from flow.

2.2 Secretory SMC prime cocultured EC for increased expression of E-selectin and VCAM-1.

Experiments utilising monoclonal antibodies to inhibit mononuclear cell adhesion indicated that increased expression of E-selectin and/or VCAM-1 was likely to underlie the increased adhesion observed on EC cells cocultured with SMC.
compared to EC cultured alone. We measured the expression of both these molecules as well as another EC adhesion receptor, inter-cellular adhesion molecule-1 (ICAM-1), by immunofluorescence and flow cytometry. Table 1 shows that the VCAM-1 expression was significantly increased by 100U/ml TNF on EC irrespective of the culture regime and there was a significant increase in VCAM-1 on cocultured EC compared to EC cultured alone. In contrast no E-selectin could be detected on EC cultured alone with TNF for 24h, but again, coculture induced a small but significant increase in expression in response to 100U/ml TNF. ICAM-1 was expressed at significant levels under all culture regimes. Expression of ICAM-1 increased in the presence of 100U/ml TNF although there was no measurable difference in expression between cocultured EC and EC cultured alone. However, using immunofluorescent flow cytometry, differences in adhesion molecule expression could not be measured on EC stimulated with 0.1U/ml TNF.

Alternatively we found that ELISA was suitable for measuring adhesion receptor expression on EC cultured in microtitre plates and primed with recombinant TGFβ1 in the absence of SMC at TNF concentrations of 0.1 and 100 U/ml TNF. These experiments are detailed in the main text. We have also attempted to utilise ELISA to detect E-selectin and VCAM-1 on EC cultured on porous membranes. However the porous nature of the culture inserts trapped ELISA reagents and introduced a large background signal over which changes in EC receptor expression were undetectable. ELISA is unsuitable for the measurement of VCAM-1 anyway, as SMC express this receptor abundantly, thus confounding attempts to measure EC expression in their presence. Using trypsin for proteolytic release of EC from the
culture substrate before immunofluorescent flow cytometry possibly removed antibody specific epitope from the cell surface by trypsin resulting in reduced sensitivity of this methodology compared to ELISA in microtitre plates.

**ODS References**


ODS Figure legends

Figure 1

**Flow chamber incorporating culture inserts.** The chamber comprised rigid perspex plates, sealed with silicon rubber gaskets, and held together by locating screws ‘a’ and ‘k’. The upper plate, ‘b’, was made from polished perspex to facilitate microscopic observation. A machined groove in its lower surface ‘c’ (depth 100μm, width 5mm, length 5cm) formed the flow channel. Plate ‘e’ accommodated the coculture insert, ‘h’, in a receiving slot ‘f’ of complementary dimensions. The coculture insert was held in position from behind by polished perspex plate ‘j’ and gasket ‘i’. When the coculture insert was in place it protruded through gasket ‘d’, butted onto plate ‘b’, and formed part of the bottom of the flow channel. Fluid was perfused via an inflow conduit ‘g’ in plate ‘e’ and an identical outflow conduit at the opposite end of the flow channel. Bright field, transmission, light microscopy and incident illumination fluorescence microscopy were possible using objectives viewing from above. The light scattering characteristics of the porous filters on the inserts did not allow phase contrast microscopy.

Figure 2

**Fluorescent microscopy images of EC and adherent leukocytes.** Mononuclear leukocytes adherent to EC/SMC cocultures are shown using fluorescent microscopy in a) the absence of TNF; b) in the presence of 0.1U/ml TNF. The EC monolayer was visible after a bolus of leukocytes, including the fluorochrome calcein-AM in
soloution, had been perfused (a). Brightly fluorescent, adherent leukocytes were readily distinguished from the EC monolayer (b).

Figure 3

The effect of monoclonal antibodies against adhesion molecules on adhesion of monocytes or PBL to EC. Monocytes or PBL were treated with function-blocking monoclonal antibody against α4-integrin and/or EC were treated with antibodies against E-selectin or P-selectin and adhesion was measured for: a) monocytes adhering to EC alone treated with 100U/ml TNFα; b) monocytes adhering to EC/SMC cocultures treated with 100U/ml TNFα; c) PBL adhering to EC treated with 100U/ml TNFα; d) PBL adhering to EC/SMC cocultures treated with 100U/ml TNFα. Antibody blockade demonstrated that both monocytes and lymphocytes utilised a combination of VCAM-1 and P-selectin to bind to EC monocultures but utilised VCAM-1 and E-selectin to adhere to EC/SMC cocultures. * P < 0.05; ** P < 0.01 for leukocyte adhesion in the presence of adhesion blocking antibodies compared to adhesion without antibodies by paired t test.
Table 1. **Expression of E-selectin, VCAM-1 and ICAM-1 on EC cocultured with SMC or EC cultured alone.** EC were cocultured with SMC or cultured alone for 24h in the presence of 0, 0.1 or 100 U/ml TNF. EC were isolated by trypsin digestion and then labelled with a primary antibody specific for an adhesion receptor. A secondary fluorescently conjugated anti-immunoglobulin antibody was used to label the primary antibody. Levels of adhesion molecule expression were assayed by flow cytometry and expressed as fluorescence intensity relative to controls labelled with isotype matched non-specific antibody or in the case of cocultured EC, relative to fluorescence intensity on EC cultured alone. * $P < 0.05$; ** $P < 0.01$ for comparison of relative fluorescence intensity to 1.0 by student $t$-test. + $P < 0.05$; ++ $P < 0.01$ for comparison between 100U/ml and 0U/ml TNF by paired $t$-test.

<table>
<thead>
<tr>
<th>Mab specificity</th>
<th>Fluorescence of EC cultured alone relative to IgG1 control</th>
<th>Fluorescence of cocultured EC relative to IgG1 control</th>
<th>Fluorescence of cocultured EC relative to EC cultured alone</th>
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<tbody>
<tr>
<td></td>
<td>0U/ml TNF</td>
<td>0.1U/ml TNF</td>
<td>100U/ml TNF</td>
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<tr>
<td>E-selectin</td>
<td>0.97 ± 0.06</td>
<td>1.03 ± 0.03</td>
<td>1.07 ± 0.04</td>
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<tr>
<td>VCAM-1</td>
<td>0.97 ± 0.6</td>
<td>1.08 ± 0.01</td>
<td>1.95** ± 0.13</td>
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<tr>
<td>ICAM-1</td>
<td>3.59* ± 0.77</td>
<td>4.00** ± 0.44</td>
<td>6.86** ± 0.99</td>
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