Endothelial cell-borne platelet bridges selectively recruit monocytes in human and mouse models of vascular inflammation

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Aims
Cells of the monocyte lineage are the most abundant inflammatory cells found in atherosclerotic lesions. Dominance of the inflammatory infiltrate by monocytes indicates that there is a disease-driven mechanism supporting their selective recruitment. Previous studies have demonstrated that interactions between endothelial cells (ECs) and platelets may promote monocyte recruitment. In this study, we sought to expand on this knowledge using a complex coculture model of the diseased vessel wall.

Methods and results
Using primary human cells in an in vitro flow-based adhesion assay, we found that secretory arterial smooth muscle cells (SMCs), cocultured with ECs, promote preferential recruitment of monocytes from blood in a TGF-β1-dependent manner. Approximately 85% of leucocytes recruited to the endothelium were CD14+. Formation of adhesive platelet bridges on ECs was essential for monocyte recruitment as platelet removal or inhibition of adhesion to the ECs abolished monocyte recruitment. Monocytes were recruited from flow by platelet P-selectin and activated by EC-derived CC chemokine ligand 2 (CCL2), although the presentation of CCL2 to adherent monocytes was dependent upon platelet activation and release of CXC chemokine ligand 4 (CXCL4). In an intravital model of TGF-β1-driven vascular inflammation in mice, platelets were also necessary for efficient leucocyte recruitment to vessels of the microcirculation in the cremaster muscle.

Conclusions
In this study, we have demonstrated that stromal cells found within the diseased artery wall may promote the preferential recruitment of monocytes and this is achieved by establishing a cascade of interactions between SMCs, ECs, platelets, and monocytes.

Keywords
Monocytes • Adhesion molecules • Platelets • Endothelial cells • Atherosclerosis

1. Introduction

The recruitment of monocytes into the arterial intima occurs at all stages in the development of the atherosclerotic plaque. Indeed, in early lesions (fatty streaks), monocytes constitute the bulk of cellular infiltrate, and differentiation of monocytes into foam cells is thought to establish a chronic inflammatory environment within the artery.1 The mechanisms supporting the recruitment of monocytes are not fully understood. The expression of adhesion receptors such as VCAM-1 and E- and P-selectin has been reported on plaque endothelial cells (ECs), and these molecules can support leucocyte adhesion.2 However, these molecules are utilized by numerous leucocyte subsets and therefore this does not explain the specific recruitment of monocytes to atherosclerotic lesions. Presumably other signalling molecules must be present to promote preferential recruitment of monocytes, although this has not been demonstrated in a model representing the diseased artery wall.

Leucocyte–EC interactions are not the only route by which monocytes might be recruited in atherosclerosis and the ability of platelets to recruit leucocytes has recently received considerable attention. Activated platelets express adhesion receptors and chemotactic agents that can support the recruitment, activation and migration of

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leucocytes including monocytes.3–7 Interestingly, platelets are strongly implicated in animal models of atherosclerosis. In the ApoE−/− mouse, prolonged inhibition of platelet adhesion to the artery wall by infusion of antibodies against GPIb or GPIIb/IIIa dramatically reduced atheroma formation, while the infusion of activated platelets significantly increased plaque burden.8,9 Platelet expressed P-selectin has been reported to play a major role in atherosclerotic lesion development.10 Likewise the genetic ablation of the platelet-borne chemokine CXCL4 was shown to be partially protective against atherosclerosis in mice, although no molecular or cellular mechanisms underlying this observation were provided.11 Taken together, these data strongly imply that platelets contribute to atherogenesis by supporting leucocyte recruitment. However, a role for platelets in the selective recruitment of monocytes has yet to be demonstrated.

We have recently shown that in cocultures of ECs and secretory smooth muscle cells (SMCs; a phenotype localized in the diseased intima), a matrix of von Willebrand Factor (vWF) is presented to flowing blood so that platelet adhesion and activation occurs on the EC surface.12 In the current study, we show that these surface-bound platelets form an adhesive bridge between flowing blood and the EC surface. By presenting P-selectin to blood-borne leucocytes, platelets preferentially recruit monocytes from whole blood to the EC surface. The release of CXCL4 from platelets promotes the activity of EC-derived CCL2, which activates monocytes and supports their trans-endothelial migration. This leads to a dramatic enrichment of monocytes on and under the EC monolayer when compared with their numbers in the circulating blood.

2. Methods

2.1 Preparation of blood and isolated blood cells

This investigation conforms with the principles outlined in the Declaration of Helsinki and approval was granted from the University of Birmingham Ethical Review Committee. Blood was collected from healthy donors and anticoagulated with 5 U/mL heparin. Platelets were isolated from whole blood and fluorescently stained with calcein-AM (5 
M, Sigma). Platelet poor plasma (PPP) and washed RBC (WRBC) were pre-
pared from blood anticoagulated with 5 U/mL heparin. Platelets were isolated and characterized as described previously.3–7 Each experiment used first passage ECs from a different donor and with cells cultured either on gelatin-coated glass capillaries (microslides)13 or cocultured with SMCs on opposite sides of a porous transwell inserts for 24 h as previously published.12 EC cultures in microslides were either untreated or stimulated with 10 ng/mL TGF-β1 for 24 h or 100 U/mL TNF-α for 4 h prior to adhesion assay.

2.2 Preparation of platelet-depleted whole blood

Whole blood was centrifuged at 300 g for 10 min and platelet-rich plasma was removed. The original blood volume was reconstituted using calcium and magnesium-free PBS (Sigma) containing 1% BSA, fraction IV (Sigma) and gently mixed. The centrifugation and removal of the platelet-rich top layer was then repeated twice. At the end of the protocol leucocytes and platelets counts were made and the blood was restored to its original volume with autologous plasma either with or without autologous washed platelets (see Supplementary material online, Section 1.2).

2.3 Culture and coculture of SMCs and ECs

Human umbilical vein ECs and SMCs were isolated and characterized as described previously.13–15 Each experiment used first passage ECs from a different donor and with cells cultured either on gelatin-coated glass capillaries (microslides) or cocultured with SMCs on opposite sides of a porous transwell inserts for 24 h as previously published.12 EC cultures in microslides were either untreated or stimulated with 10 ng/mL TGF-β1 for 24 h or 100 U/mL TNF-α for 4 h prior to adhesion assay.

2.4 Adhesion assay using whole blood or isolated leucocytes

Adhesion assays on EC cultured on glass microslides or to EC/SMC cocul-
tures were conducted at a wall shear rate of 140 s−1 for whole blood or a wall shear stress of 0.1 Pa for isolated populations of leucocytes. Blood or leucocytes were perfused at 37°C for 4–12 min, followed by a 9 min washout. Following washout, the level of leucocyte adhesion was assessed by phase contrast microscopy or the levels of platelet adhesion were assessed by fluorescent microscopy as previously reported (see Supplementary material online, Section 1.3),12

2.5 Identifying adherent leucocytes by flow cytometry

Following whole-blood perfusion and washout with cell-free buffer, EC monolayers were treated for 5 min with Accutase® (Innovative Cell Technologies, San Diego, CA, USA) and disassociated cells were col-
lected. Cells were stained with fluorescent antibodies for CD14 and CD45 and analysed by flow cytometry. The number of CD45 positive cells co-expressing CD14 was used to determine the percentage of monocytes recruited to the ECs (see Supplementary material online, Section 1.4).

2.6 Intravital microscopy on the murine cremaster muscle

Male mice were injected intrascrotally with 80 μg/kg TGF-β1. Donor platelets collected from a littermate control were labelled with CFSE (10 μM) before being introduced into recipient mice via the carotid artery. To visualize leucocyte adhesion, mice were injected with rhodamine 6G (1 μg in 100 μL). Post-capillary venules with a diameter of 20–30 μm were selected for study and at least three vessels in each mouse were monitored for platelet and leucocyte adhesion at 4 h follow-
ing TGF-β1 stimulation. Data are expressed as adhesive interactions of platelets or leucocytes/mm of vessel segment (see Supplementary material online, Section 1.5).

3. Results

3.1 Coculture of SMCs and ECs induces the recruitment of leucocytes from flowing whole blood

We have previously demonstrated that TGF-β1, produced in cocul-
tures of ECs and secretory SMCs, promotes platelet adhesion to the EC surface.12 Here, we demonstrate that the TGF-β1 produced in this coculture environment is also sufficient to promote the concoc-
mitant recruitment of leucocytes from the same flowing whole blood. Thus, ECs cultured alone did not support leucocyte adhesion (Figure 1A), but leucocytes were efficiently recruited by ECs cocul-
tured with SMCs for 24 h (Figure 1A). A TGF-β1 function neutralizing antibody added to cocultures greatly reduced leucocyte adhesion while control antibody had no effect (Figure 1A). In addition, when ECs cultured alone were stimulated with 10 ng/mL recombinant
human TGF-β1, they could also support the adhesion, activation and trans-EC migration of leucocytes from whole blood (Figure 1B and C).

Interestingly, TGF-β1-stimulated ECs did not support the adhesion of purified suspensions of neutrophils, monocytes, or lymphocytes although ECs stimulated with TNF-α as a positive control could recruit all of these leucocytes (Figure 1D). Therefore, TGF-β1 was a
sufficient stimulus to promote the adhesion of leucocytes to ECs from whole blood, but importantly, other elements within flowing blood were required for efficient recruitment.

### 3.2 The recruitment of leucocytes from whole blood requires the adhesion and activation of platelets

To investigate the requirement for platelet adhesion in the secondary recruitment of leucocytes, function neutralizing antibodies against the platelet adhesion receptors GPIb or GPIIb/IIIa were added to whole blood prior to perfusion across TGF-β1 stimulated ECs. We found that both platelet coverage (Figure 2A) and leucocyte recruitment (Figure 2B) were dramatically reduced. This strategy was also effective in reducing leucocyte adhesion to ECs cocultured with SMCs (Figure 2C). To demonstrate that platelet activation was also necessary, whole blood was pre-treated with the phosphodiesterase inhibitor theophylline, which has previously been shown to inhibit platelet activation.\(^{12,16}\) Again, both leucocyte and platelet adhesion from whole blood were greatly reduced (Figure 3A and B). ECs treated with theophylline alone prior to perfusion of whole blood did not recruit flowing leucocytes or platelets in the absence of TGF-β (Figure 3A and B). As an additional control ECs treated with theophylline were unable to recruit isolated populations of leucocytes (i.e. neutrophils or mononuclear cells) in the absence of platelets (data not shown). To substantiate the observation that platelet activation was required, we selectively removed platelets from whole blood while leaving the leucocyte populations unchanged (see Supplementary material online for details). When the platelet count

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**Figure 2** The effects on leucocyte and platelet recruitment of inhibiting platelet adhesion to ECs cocultured with SMCs, or to monocultured ECs stimulated with TGF-β1. Effects of antibodies against platelet GPIb or GPIIb/IIIa on the adhesion of (A) platelets to ECs stimulated with TGF-β1; (B) leucocytes to ECs stimulated with TGF-β1; (C) leucocytes to ECs cocultured with SMCs. Data are mean ± SD of four experiments; *P < 0.05; **P < 0.01 for comparison of adhesion on ECs treated with blocking or control antibody.

**Figure 3** The effects of inhibiting platelet activation or depleting platelet numbers, on leucocyte and platelet recruitment to ECs stimulated with TGF-β1. The adhesion of (A) leucocytes to ECs stimulated with TGF-β1 in the presence or absence of theophylline; Data are mean ± SD of four experiments; **P < 0.01 for comparison of adhesion to TGF-β1 stimulated platelets in the presence or absence theophylline. (B) Platelets to ECs stimulated with TGF-β1 in the presence of absence of theophylline; Data are mean ± SD of four experiments; **P < 0.01 for comparison of adhesion to TGF-β1 stimulated platelets in the presence or absence theophylline. (C) Leucocytes to ECs stimulated with TGF-β1 after whole blood had been depleted of platelets or subsequently reconstituted with washed platelets. Data are mean ± SD of four experiments; **P < 0.01 for comparison of adhesion before and after platelet depletion. ++ P < 0.01 for comparison of adhesion after platelet depletion and after reconstitution.
was reduced to ~3% of the constitutive population, ECs stimulated with TGF-β1 were unable to recruit leucocytes (Figure 3C). However, if the blood was reconstituted with washed isolated platelets, the ability to recruit leucocytes was restored. Taken together these lines of evidence imply that platelets adherent to ECs form adhesive bridges between the EC surface and the flowing blood which are essential for the recruitment of flowing leucocytes.

### 3.3 Adhesive platelet bridges selectively recruit monocytes from flowing whole blood

Dual staining of recruited leucocytes for the pan-leucocyte marker CD45 and the monocyte-specific marker CD14 showed that monocytes constituted 84 ± 4.3% of the CD45 positive population (mean ± SD of four experiments). This represents approximately a nine-fold enrichment for monocytes (data not shown).

### 3.4 The recruitment of monocytes from whole blood requires P-selectin, CXCL4, and CCL2

When function-neutralizing antibodies against P-selectin and CXCL4 were added to whole blood prior to perfusion across TGF-β1-stimulated ECs, monocyte adhesion was significantly reduced (Figure 4). Antibodies against P-selectin or CXCL4 did not significantly alter the levels of platelet adhesion (data not shown), indicating that monocytes capture from flow to ECs required platelet P-selectin and CXCL4. The receptor for CXCL4 (CXCR3) has not been demonstrated on monocytes, while ECs are reported to possess a spliced variant of CXCR3 (CXCR3b), which has high affinity and therefore specificity for, CXCL4. We therefore considered that CXCL4 released from platelets may be activating ECs. When ECs were pre-treated with anti-CXCR3 blocking antibodies for 15 min prior to perfusion of blood, we observed significant inhibition of leucocyte adhesion, which strongly implies that the EC pool of CXCR3 is the target of platelet-derived CXCL4 which promotes EC activation and monocyte recruitment. On the basis of this assumption, we incorporated a function neutralizing antibody against CCL2 (MCP-1) into blood prior to perfusion. Blocking CCL2 function significantly inhibited leucocyte adhesion (>65%), implying that CCL2 was presented to adherent monocytes in response to CXCL4 release from adherent platelets.

To confirm that the same molecular explanation for leucocyte recruitment was pertinent to EC–SMC cocultures, whole blood containing antibodies against CXCL4 or P-selectin was perfused across cocultured ECs. Both antibodies dramatically reduced the levels of leucocyte recruitment in this model (data not shown).

### 3.5 TGF-β1 induces platelet and leucocyte adhesion in vivo

To confirm that TGF-β1 could promote platelet-dependent recruitment of leucocytes in vivo, we used intravital microscopy on the microcirculation of the cremaster muscle in treated mice. Following TGF-β1 stimulation, we observed a dramatic increase in the number of platelet interactions with the endothelium compared with untreated mice (Figure 5A). This was paralleled by an increase in leucocyte adhesion (Figure 5A). Indeed, analysis by linear regression showed that leucocyte adhesion correlated significantly with platelet adhesion, showing that TGF-β1 could promote both platelet and leucocyte recruitment in vivo (Figure 5B). To determine whether leucocyte adhesion was dependant on the recruitment of platelets, mice were treated with blocking antibody against GPIb that reduced leucocyte adhesion by 40.1 ± 18.9% (P < 0.01 for comparison of levels of adhesion between anti-GPIb and control antibody by t-test) compared with an IgG control in TGF-β1-treated mice (data are a comparison between six mice in each treatment group; Figure 5C). Therefore, platelet adhesion was required for efficient leucocyte recruitment in this model.

### 4. Discussion

The mechanism by which inflammatory leucocytes are recruited to the artery wall and enriched within the atherosclerotic environment is poorly described. Current observations using coculture and animal models of the diseased artery wall, coupled with data from our previous studies, demonstrate that interactions between cells of the diseased artery wall can generate signals that coordinate the preferential recruitment of monocytes from flowing blood (Figure 6). This process depends upon crosstalk between secretory SMCs and ECs, which leads to the plasmin-dependent generation of active TGF-β1. This agent induces vWF expression on ECs so that platelets are recruited and activated and act as an adhesive bridge between the EC surface and the flowing blood. Monocytes are preferentially recruited from blood by tethering to platelet P-selectin and preferentially activated by CCL2 (MCP-1), which is released from ECs by the action of the platelet chemokine CXCL4 stimulating the EC receptor CXCR3b. This process greatly enriches for monocytes at the EC surface and supports monocyte transmigration. Thus, for the first time, we describe a series of cellular and molecular interactions initiated by cells resident within the diseased stroma that result in the specific recruitment of the predominant population of inflammatory leucocytes recruited during atherogenesis (Figure 6).
models of atherosclerosis inhibition of platelet adhesion utilizing anti-
during plaque development has received great interest. In murine
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The effects of TGF-β1 administration on platelet and leucocyte adhesion in the microcirculation of the murine cremaster
muscle. Four hours after interscrotal administration of TGF-β1 (A) the levels of both platelet and leucocyte adhesion were higher compared with vehicle-treated animals. Data are from six mice in each experimental group; *P < 0.05, **P < 0.01 for comparison of adhesion in TGF-β1 and vehicle-control-treated animals. (B) Analysis of platelet and leucocyte adhesion by linear regression showed that the levels of adhesion of both cell types were significantly correlated. (C) Intravenous instillation of an anti-GPIb antibody significantly (***P < 0.01) reduced the levels of leucocyte recruitment in TGF-β1 stimulated animals. Data are from six mice in each experimental group.

Figure 5

Recently, the concept that platelets might bind to EC monolayers during plaque development has received great interest. In murine models of atherosclerosis inhibition of platelet adhesion utilizing antibodies against GPIb or in mice lacking GPIIb, the burden of atheroma is dramatically reduced, while systemic administration of activated platelets during early disease increases the rate of plaque formation. In our study, we found that platelet adhesion and activation were required for the recruitment of leucocytes and that both GPIb and GPIIb/Illa were needed to sustain platelet interactions with ECs. These data imply a requirement for stable interactions between platelets and ECs in order to facilitate leucocyte recruitment. Interestingly, intravital studies of carotid arteries in ApoE−/− mice indicate that stable interactions between platelets and the vessel wall are rare, although transient interactions occur regularly.

In our intravital analysis, we also see many transient interactions, although the small population of labelled tracer platelets utilized for this analysis was not representative of the endogenous platelet population. It is possible that short-lived interactions contribute to disease, for example, by allowing the deposition of chemokines in a manner that promotes recruitment of monocytes. However, it is likely that less frequent longer-lived platelet–EC interactions, which support the direct recruitment and activation of leucocytes, may be sufficient to support a chronic disease process.

In the current study, we found that P-selectin was essential for leucocyte adhesion. There are two potential pools of P-selectin that could mediate this process: platelet or endothelial expressed. Our studies utilizing purified suspensions of monocytes, lymphocytes, and neutrophils, all of which failed to interact with TGF-β1 stimulated ECs, discounting the EC pool of P-selectin. Endothelial P-selectin has also been implicated in the recruitment of platelets to ECs and it was a possibility that this cellular pool was required for the adhesion of platelets in our experiments. However, blockade of leucocyte adhesion with anti-P-selectin did not have a significant effect on platelet adhesion. This is consistent with observations in the current and previous studies that GPIb and GPIIb/Illa support platelet adhesion to vWF on the surface of ECs. Taken together, these data demonstrate that in SMC–EC cocultures, or on ECs stimulated with TGF-β1, platelet P-selectin supported leucocyte tethering. Interestingly, studies using ApoE−/− mice in which P-selectin had also been genetically deleted, showed significant reduction in the burden of atheroma. In that study, the construction of bone marrow chimeras showed that both platelet and endothelial pools of P-selectin contributed to plaque formation. Thus, there is growing evidence that P-selectin is a molecule important for the development of atheroma, and the pool localized within platelets appears to play an important role in the progression of disease.

Activated immobilized platelets have been reported to be a substrate for the recruitment of flowing leucocytes. Platelet P-selectin supports the tethering of a number of leucocyte subsets as well as being required for the formation of platelet satellites on monocytes in the peripheral circulation. In addition, platelet granules also contain large amounts of the chemokine CXCL4. Here, we have demonstrated that CXCL4 plays a role in the recruitment of monocytes by platelet bridges formed on ECs. Others have proposed that CXCL4 may stabilize monocyte adhesion to ECs after an initial activating stimulus delivered by CCL5. However, CXCL4 alone has also been reported to directly activate monocytes. Importantly, CXCL4 is found in human atherosclerotic lesions, and expression has been shown to correlate with the severity of atherosclerosis. Moreover, the ablation of CXCL4 in the LDLR−/− mouse leads to a significant reduction in atherosclerosis, although the molecular and cellular mechanism supporting this observation have not been reported. Interestingly, the repeated administration of activated platelets to the ApoE−/− mouse accelerated the formation of atherosclerosis in a process that was associated with the deposition of CXCL4 on to the ECs overlying the plaque. Here, we have shown that platelet CXCL4 interacts with EC CXCR3b and does not directly...
stimulate monocytes. In fact CXCR3b is reported to show specificity for CXCL4, while CXCR3 on leucocytes is promiscuous, also binding the interferon-inducible CXC chemokines CXCL9, 10, and 11. Thus, there is now substantial evidence that CXCL4 is an important chemokine involved in the cellular pathology of atherosclerosis, and here, we provide evidence that CXCL4 may operate as part of the molecular machinery utilized by platelets and ECs to selectively recruit monocytes from flowing blood.

When we characterized the identity of the leucocytes recruited in our models, we were surprised to observe a dramatic enrichment for monocytes, all the more so, as platelet P-selectin can be utilized by lymphocytes, neutrophils, and monocytes. It is possible that monocytes can utilize P-selectin more efficiently than lymphocytes, indeed we have observed that monocytes are more highly enriched from a mixed mononuclear cell preparation (≏75% lymphocytes: 25% monocytes) at low densities of immobilized P-selectin (unpublished observations). Thus, the ability of monocytes to utilize platelet P-selectin efficiently may confer a degree of specificity to the recruitment process. However, it is likely that the chemokine(s) operative in this system also contribute to the preferential recruitment of monocytes. Here, we show that CCL2 derived from ECs was required for efficient monocyte recruitment. CCL2 is relatively specific for monocyte activation and has been widely reported to be released from ECs. It is interesting that CCL2 in our assay appears to be released rapidly and possibly quite locally in response to platelet adhesion and degranulation. We are not aware that this CXCR3b-mediated pathway of CCL2 presentation has been previously reported and the intracellular mechanisms underlying this phenomenon remain to be described.

In conclusion, we propose that the recruitment of leucocytes to atherosclerotic plaques can occur through a cascade of inflammatory interactions that originate within the arterial stroma and are functionally evident on the surface of the ECs which form the interface between the diseased vessel wall and the flowing blood. Moreover, the platelet-dependent mechanism of leucocyte recruitment described here contributes to the increasing body of evidence that blood platelets are important in early atherosclerotic disease. It also demonstrates that complex inflammatory pathways can be initiated from within the atherosclerotic plaque that provide a highly specific gateway for the entry of blood monocytes into the disease environment.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**

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