Population of the Vessel Wall by Leukocytes Binding to P-Selectin in a Model of Disturbed Arterial Flow

Christopher Skilbeck, Susan M. Westwood, Peter G. Walker, Tim David, Gerard B. Nash

Abstract—We examined the hypothesis that disturbance of laminar flow promotes the attachment of leukocytes to the vessel wall in regions where the wall shear stress is otherwise too high. Isolated neutrophils, lymphocytes, or monocytes were perfused through chambers with backward-facing steps so that vortices occurred with well-defined reattachment of flow. Wall shear stresses downstream in reestablished flow equaled 0.07 Pa (low shear) or 0.3 Pa (high shear). In chambers coated with P-selectin, adherent leukocytes rolled. By use of a P-selectin–Fc fragment chimera, adhesion was predominantly stationary, enabling definition of initial attachment sites. Neutrophils adhered in all regions of the low-shear chamber, with a local maximum around the reattachment point. However, in the high-shear chamber, adhesion was restricted to the recirculation zone and immediately downstream from the reattachment point. Rolling at high shear stress allowed a population of regions where initial attachment could not occur. At high shear, lymphocytes and monocytes also formed attachments restricted to the region of the reattachment point. The results imply that all types of leukocytes might bind to a capture receptor in high-shear vessels with discontinuities in the wall and might then spread to other regions. 

Key Words: adhesion ■ leukocytes ■ rheology ■ disturbed flow ■ P-selectin

During the protective inflammatory response, leukocytes typically bind to the endothelium of postcapillary venules and migrate into tissue.1 Leukocytes do not normally adhere to the walls of arteries, possibly because local wall shear stresses are too high or because arterial levels of inflammatory cytokines are too low to upregulate the adhesion molecules required for attachment. However, blood-derived monocyte/macrophages and T lymphocytes are found within atheromatous plaques and are important promoters of the development of these lesions.2,3 Moreover, endothelial cells covering plaque do express selectins and vascular cell adhesion molecule (VCAM)-1, which are receptors associated with the capture and rolling adhesion of flowing leukocytes, which are absent from healthy arteries.4,5 Plaques form preferentially at sites in arteries where the laminar pattern of blood flow is disturbed as a result of a change in blood vessel geometry, e.g., at bifurcations and regions of vessel curvature.6 In these regions, the laminar flow detaches from the vessel wall at a point where vortices develop and reattaches where the vortices terminate. Characteristic of these vortices are regions of backflow (recirculation), stagnation, and low wall shear stress (eg, see Reneman et al7 and Goldsmith and Turitto8). Disturbed flow may also occur at stenoses in advanced plaques, at anastomoses of surgical arterial reconstructions, and at places where the vessel wall has been disrupted by angioplasty. Such disturbances may facilitate adhesion by bringing leukocytes into contact with the vessel wall at sites of relatively low shear stress.

We wished to investigate whether recirculating flow could promote the adhesion of human granulocytes, lymphocytes, or monocytes to the wall in regions where shear imposed by simple laminar flow in straight sections of the vessel would be too high. Previous studies using flow through annular expansions9,10 or over a backward-facing step11 demonstrated that the adhesion of platelets or of monocyte cell lines was promoted in the downstream region of flow disturbance. In the present study, rectangular flow channels with backward-facing steps12 were used; coating was with P-selectin. The present study differed from previous reports in several important respects: (1) freshly isolated human neutrophils, monocytes, and lymphocytes were compared at shear stresses above and below those at which adhesion could occur without recirculation; (2) P-selectin was used because it is a receptor found over plaque, it is able to capture flowing leukocytes of all types, and it is also implicated in leukocyte recruitment in thrombotic vessels4,13,14; (3) a chimeric P-selectin–Fc fragment fusion protein was used to cause rapid immobilization of cells bearing the Fc receptor, so that they remained where they had been initially captured; and (4) patterns of adhesion were characterized over a wide area upstream and downstream from the reattachment point, and a distinction was made between regions in which capture initially occurred and...
regions that became populated by rolling cells that had initially adhered elsewhere.

Methods
For a full description of the methods used, see online supplement, which can be accessed at http://www.atvb.ahajournals.org.

Preparation of Leukocytes
Venous blood was collected from healthy volunteers into EDTA. Neutrophils, mononuclear cells (mixed lymphocytes and monocytes), and monocytes were isolated as described and suspended in PBS containing 1 mmol/L Ca\(^{2+}\), 0.5 mmol/L Mg\(^{2+}\), and 0.1% BSA. On the basis of cell volume distribution, neutrophils contained <5% lymphocytes, and mononuclear suspensions contained 82% lymphocytes and 18% monocytes, whereas monocytes contained 20% lymphocytes and 80% monocytes (means from 3 experiments each). The ratio of platelets to leukocytes averaged <1 for neutrophil preparations, 7 for mononuclear cells, and 66 for monocytes. In all preparations, >95% of leukocytes were viable.

Flow System and Calculation of Wall Shear Stress
The vertical-step flow chamber is illustrated in Figure 1 and was based on the design of Chiu et al. Coverslips forming the base were coated with 1 µg/mL P-selectin (R&D Systems) or 10 µg/mL P-selectin–Fc chimera (extracellular region of P-selectin fused with the Fc portion of human IgG; gift of Dr Susan Watson, Genentech, San Francisco, Calif) in PBS. Remaining protein-binding sites were blocked with 1% BSA. Controls were coated with albumin alone.

Perfusion was via a flow system that was maintained at 37°C and mounted on a video microscope, as described. Two different sets of gaskets, which were cut from 200- to 230-µm-thickness or 250- to 300-µm-thickness silicone rubber (ESCO, Bibby Sterilin Ltd), were used in the flow chamber. The shallower channel had depths of 260 µm and 450 µm upstream and downstream, respectively, from the step (190-µm step height). The deeper channel had respective depths of 300 µm and 600 µm (300-µm step height). Channel width was 10 mm. The shallower channel provided a higher wall shear stress.

Flow expands as it passes over the backward facing step, and a vortex or recirculating flow is set up (Figure 1B). A reattachment point can be defined as the place on the wall at which forward and backward flow separate. We used Computational Fluid Dynamics (CFD) (Fidap 8, Fluent Inc) to predict wall shear stresses and distances to the reattachment points (Figure 1C). Downstream from a reattachment point, parabolic flow becomes reestablished, and wall shear stress increases to a constant level within ~1 mm. We chose rates of flow so that the predicted downstream wall shear stresses were 0.07 Pa in the deep channel and 0.3 Pa in the shallow channel. These “low” and “high” shear stresses were chosen because adhesion of flowing isolated leukocytes to immobilized selectins is efficient at ~0.1 Pa but rare at 0.3 Pa. Comparative studies used chambers of uniform depth but the same wall shear stresses.

Adhesion Assay
Typically, 20 mL of neutrophils at 2.5×10⁶/mL, 20 mL of mononuclear cells at 10⁷/mL, or 10 mL of monocytes at 2×10⁶/mL was perfused. Video fields (400 µm along axis, 300 µm across axis) were recorded, starting from the step and moving 400 µm at a time to 12 mm downstream. In studies with P-selectin, 10-second recordings allowed discrimination between stationary or rolling adherent cells. With the P-selectin chimera, 5 separate series (1 second for each field) were recorded at different positions across the chamber, and data were averaged. Video recordings were analyzed offline to obtain the following, described below.

Total Adherent Leukocytes
Total adherent leukocytes, both stationary or rolling, were easily distinguished from nonadherent leukocytes flowing at several hundred micrometers per second. Numbers were normalized per square millimeter per 10⁻⁶ perfused. In the first 2 fields downstream from each step, numbers were counted within consecutive strips that were 50 µm in length.

Figure 1. Diagram showing flow chamber (A), pattern of flow downstream from the step (B), and variation in predicted wall shear stress with distance downstream from the step (C). In panel A, the protein-coated coverslip (c) was fit into a recess in the lower plate, and gaskets (a and b) were placed on top and held by the upper plate, bolted in place with 8 screws. Inlet and outlet ports were drilled through the upper plate. In panel B, R represents the reattachment point, at which flow separates either to flow backward in the vortex or downstream. In panel C, CFD was used to calculate stress on the basis of chamber geometry and flow rate for the deeper, low-shear chamber (broken line) and the shallower, high-shear chamber (solid line). A negative value indicates that flow near the wall was back toward the step. The locations at which the shear stress is zero represent the reattachment points.

Percentage of Adherent Cells Rolling and Rolling Velocity
A sequence of video frames was digitized, and the distance moved and the velocity of adherent cells were measured. Cells moving steadily downstream at a velocity >1 µm/s were considered rolling; otherwise, they were classified as stationary.

Distance to Reattachment Point
From recordings of cells revolving in the vortex, the distance was measured from the step to furthest point that cells in the outer orbits

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From recordings of cells revolving in the vortex, the distance was measured from the step to furthest point that cells in the outer orbits
reached. Experimental values were 244 ± 4 and 303 ± 3 μm for low- and high-shear channels, respectively (mean ± SEM, n = 5). The CFD-predicted values were 250 and 265 μm, respectively. The discrepancy in the higher value is not due to grid spacing in CFD, because tests for grid independence were carried out for all simulations. Grids of increasing density were used until the difference in the solution was of the order of the truncation error of the scheme. Discrepancy may reflect the difficulty in measuring the channel depth accurately. If the step was higher than expected, then the recirculation area would be bigger. The fact that the measured step in the high-shear channel was slightly less than half the chamber height supports this idea. This would not alter the downstream uniform shear stress for a given total depth.

Results

Patterns of Rolling Adhesion of Neutrophils on P-Selectin

The patterns of adhesion of neutrophils to P-selectin downstream from the step are shown in Figure 2. Neutrophils were observed to adhere to P-selectin either side of the reattachment point in the deep and shallow channels (Figure 2A and 2C). Further downstream, adhesion gradually increased for the deeper, low-shear channel (Figure 2B). In contrast, adhesion decreased steadily downstream for the shallower, high-shear channel, and few cells adhered in the region of

| Numbers of Adherent Leukocytes in Different Regions Downstream From a Step |
|---|---|---|---|
| **Surface** | **Step (τw)** | **Distance From Step** |
| | | 0–0.8 mm | 0.8–8 mm | 8–12 mm |
| Neutrophils | | | |
| P-selectin (n = 3) | Deep (0.07 Pa) | 335±99 | 455±192 | 962±254 |
| P-selectin (n = 3) | Shallow (0.3 Pa) | 30±7.0 | 12±5.0 | 0.2±0.2 |
| P-selectin–Fc (n = 4) | Deep (0.07 Pa) | 303±66 | 409±59 | 715±28 |
| P-selectin–Fc (n = 4) | Shallow (0.3 Pa) | 183±71 | 1.5±0.7 | 0.4±0.4 |
| Albumin (n = 3) | Deep (0.07 Pa) | 212±59* | 1.5±0.4 | 1.5±0.4 |
| Albumin (n = 3) | Shallow (0.3 Pa) | 0.3±0.3 | <0.1 | <0.1 |
| Mononuclear cells | P-selectin–Fc (n = 3) | Shallow (0.3 Pa) | 74±38 | 1.9±0.6 | <0.1 |
| Monocytes | P-selectin–Fc (n = 3) | Shallow (0.3 Pa) | 98±12 | 5.4±3.6 | <0.1 |

*τw indicates wall shear stress far downstream from the step. Data are mean ± SEM numbers of adherent cells/mm² per 10⁷ perfused in n experiments. Deep and shallow step heights were 300 and 190 μm, respectively. ANOVA showed significant effect of (1) wall shear stress on adhesion of neutrophils on all surfaces (P<0.01), (2) distance from step on adhesion to P-selectin–Fc for neutrophils and monocytes (P<0.01), and (3) distance from step on adhesion to P-selectin at 0.3 Pa (P<0.05).

*94±4% of adherent cells counted in this region were within 40 μm of the step and may not have been attached to the surface (see text).
fully established flow (Figure 2D). The Table summarizes absolute levels of adhesion averaged over areas close to the step (0 to 0.8 mm), in the mid range (0.8 to 6 mm, where flow becomes reestablished), and far downstream (8 to 12 mm, where flow is fully established). Absolute levels of adhesion were higher in all regions for the low-shear channel (Table).

The great majority of adherent cells were rolling, eg, 87±2% (n=4) and 87±4% (n=3) of adherent cells downstream from the reattachment point for the low-shear and high-shear channels, respectively. In the fully established downstream flows, neutrophils had a rolling velocity of 3.3±0.5 μm/s (n=4) at a wall shear stress of 0.07 Pa (deep chamber) or 16.8±3.4 μm/s (n=3) at 0.30 Pa (shallow chamber). Inside the recirculation zone, the velocities were 3.8±1.2 μm/s (deep channel) or 9.7±1.9 μm/s (shallow channel). The predominance of rolling adhesion meant that the patterns of adhesion had spread out with time. Cells rolled away from the step if they adhered downstream from the reattachment point, thus “smearing out” the adhesion pattern, especially at high shear. Cells rolled toward the step if they adhered within the recirculation zone. Thus, rolling might populate areas with leukocytes, where they might not otherwise attach. It also makes it difficult to separate patterns of attachment from patterns of mass transport. To clarify where attachment was initiated, we used channels coated with a P-selectin–Fc chimera, which immobilized neutrophils in preliminary experiments.

Localized Attachment of Neutrophils to P-selectin–Fc

Neutrophils adhered to the chimeric protein in numbers similar to those for P-selectin (Table), but they remained localized. Cells were observed to roll a short distance (~1-cell diameter) before all became stationary, and >90% spread out on the surface. In the deep channel and the shallow channel, there were local maxima for adhesion around the flow reattachment point, and adhesion tailed off upstream and downstream (Figure 3A and 3C). In the deep (low-shear) channel, there was an additional peak in cell adhesion within 50 μm of the step. This probably represented the accumulation of largely nonadherent cells in the stagnant region in the shadow of the step (see below).

Figure 3B and 3D shows the larger scale patterns of adhesion. In the shallow channel, almost all adhesion occurred within 800 μm of the step. Only a few percent of the adherent neutrophils were further downstream. This is consistent with results from uniform channels (depth 450 μm) at a wall shear stress of 0.30 Pa, a level at which neutrophil adhesion was rare (3.4±0.03 cells/mm² per 10⁷ perfused, n=3). In contrast, in the deep channel, adhesion decreased to a minimum beyond the reattachment point and then increased ~5-fold to plateau at ~8 mm from the step. Again, the level of adhesion seen in this fully established flow (Table) was of the same order as that observed in uniform channels (depth 600 μm) at 0.07 Pa (532±28 cells/mm² per 10⁷ perfused, n=3). The depression in adhesion between ~1 and 8 mm downstream, as illustrated in Figure 3D and quantified in the Table, can be explained by the initial low density of cells near the wall, with the gradual increase in adhesion caused by steady sedimentation of flowing cells into that region. By using a theory described by Munn et al., calculations of the sedimentation rate of neutrophils and fluid flow velocity near the wall predict that cell density within 10 mm of the wall would increase 5-fold over a distance of ~5 mm. Given that some of the cells adhere continually, this is in fair agreement with the observation that adhesion increased 5-fold over ~7 mm. To obtain steady levels of adhesion, arrival and attachment must then be balanced.

Specificity of Neutrophil Adhesion

When neutrophils were perfused over surfaces coated with albumin alone, adhesion in the shallow chamber was negligible (Table). In the deeper, low-shear channel, adhesion outside the recirculation zone was <1% that seen when
P-selectin was present (Table). In the recirculation zone, relatively large numbers of neutrophils could be counted (Table). However, $94\pm6\%$ ($n=3$) of these cells were within 40 $\mu$m of the step, and many appeared not to be truly adherent. Cells flowing toward the step were seen either to slowly rise out of focus and enter the vortex or to slowly rise and then to sediment down onto the surface. Thus, there was a region at the foot of the step that was essentially “stagnant.” Cells collected there only in the low-shear channel. In this channel, we also treated surfaces coated with P-selectin with polyclonal rabbit anti-human P-selectin (20 $\mu$g/mL for 15 minutes; gift of Dr Mike Berndt, Baker Institute, Melbourne, Australia). Adhesion downstream from the recirculation zone was reduced by $90.4\pm5.6\%$ (mean±SEM, 3 experiments).

The region upstream from the step (viewed through 1 gasket) was poorly visible. Variable and sparse adhesion was observable in this region, but quantitative estimates were not made. No adherent cells were detected in this region in the high-shear channel. Thus, overall, a specific capture receptor, P-selectin, was required for adhesion in most zones and for all zones at higher shear stress.

**Adhesion of Mononuclear Cells to P-Selectin–Fc**

We tested whether lymphocytes and monocytes could bind only in the region of disturbed flow in the high-shear channel. The patterns of adhesion of mononuclear cells on P-selectin–Fc close to the step (Figure 4A) and over a wider area (Figure 4B) are similar to patterns for neutrophils. They are somewhat more spread out because an average of 30% ($n=3$) of mononuclear cells rolled. Nevertheless, adhesion was negligible in the fully established flow with wall shear stress 0.3 Pa. The rolling cells were small round cells, probably representing lymphocytes lacking the Fc receptor. Approximately 40% of the adherent cells were small, round, and stationary. The remaining 30% of the adherent cells were large and spread out on the surface, probably representing monocytes. Because discrimination between adherent lymphocytes and monocytes was not reliable on the basis of morphology, we studied monocytes separately. Purified monocytes did not roll on the chimera but rapidly immobilized and flattened on the surface. They gave adhesion patterns (Figure 5) very close to those for neutrophils, with a sharp fall off outside the first 1 mm downstream from the step. Platelets, which were numerous in mononuclear and monocyte preparations, rarely adhered to the surface, although we cannot exclude the possibility that platelets adherent to mononuclear cells or monocytes promoted their adhesion.

Comparing absolute levels of adhesion for the different types of leukocytes, for the same surface (P-selectin–Fc) and flow chamber (reestablished wall shear stress 0.3 Pa), they adhered in the following order: neutrophils > monocytes > lymphocytes (Table). This order is in agreement with previous studies using uniform channels, in which flowing leukocytes adhered to P-selectin presented by immobilized platelets.$^{13,14}$

**Discussion**

These studies indicate that when laminar flow is disturbed, all the major types of human leukocytes may bind to the wall of a vessel in which shear stress would otherwise be too high for adhesion. Using an experimental model of flow over backward-facing steps of different height, we could generate stable vortices. When the wall shear stress was too high for adhesion in the region at which laminar flow was reestablished far downstream, binding occurred upstream and downstream from the reattachment point, where relatively low wall shear stresses existed. Cells attaching to P-selectin in these areas rolled upstream or downstream and thus gradually
populated the regions at which direct adhesion from flow could not occur. When the wall shear stress was lower, then adhesion could occur over the whole downstream region, but there was a local maximum around the reattachment point.

We could distinguish the regions at which primary capture had occurred from those into which adhesion had spread by comparing adhesion to P-selectin with adhesion to a chimeric P-selectin–Fc fusion protein. Neutrophils, monocytes, and a proportion of lymphocytes became immobilized on the chimeric receptor. Ligation of Fc receptors (eg, FcyRIIA) by IgG is known to activate neutrophils and monocytes.18,19 B lymphocytes and NK cells express FcγRIIA but not the more numerous T cells.20 In a recent study, we found that antibody against FcyRIIA inhibited integrin-mediated immobilization of rolling neutrophils caused by anti-neutrophil cytoplasm antibodies.18 In the present study, we treated neutrophils with the same antibody at equal and greater concentrations than those used previously (1 to 4 μg/mL antibody IV.3, Medarex Inc) and found that they no longer spread out on the chimera-coated surface but that they still became stationary. Although Fc receptor ligation appears to have been responsible for the activation of neutrophils, the basis of leukocyte immobilization remains uncertain in these experiments. In atherosclerotic or thrombotic arteries, one would not expect activation via the Fc receptor. However, integrin-mediated immobilization of rolling cells might be induced by locally presented chemokines, such as monocyte chemotactic protein-1, which is found in plagues, or by activating agents from codeposited platelets.21 Neutrophils are not found in plaque, possibly because neutrophil-attracting chemokine(s) is lacking, because neutrophils cannot use the adhesion receptor VCAM-1 (which is effective for T cells and monocytes), or because neutrophils are recruited but rapidly cleared by apoptosis and phagocytosis.

These data represent the first detailed analyses of attachment patterns of freshly isolated leukocytes in recirculating flow. At higher Reynold’s numbers, downstream from a sudden axisymmetric tubular expansion, Karino and Goldsmith9 observed that platelet adhesion was promoted at either side of the reattachment point. Pritchard et al10 found that a monocyte-like cell line (U937) and monocytes adhered preferentially downstream from the reattachment point. The flow model was of larger dimension and Reynold’s number than that used in the present study, but it had much lower wall shear stress and was not coated with a specific adhesion receptor. Recently, Barber et al11 used a backward-facing step to study adhesion of U937 cells to human umbilical vein endothelial cells (HUVECs). Interactions were concentrated around the reattachment point. Only transient attachments of unknown molecular specificity were observed, even if HUVECs had been pretreated with tumor necrosis factor-α. Because HUVECs treated with tumor necrosis factor can support rolling and stable adhesion of flowing monocytes,22,23 it seems that the behavior of the unstimulated U937 does not resemble the behavior of blood monocytes closely. Moreover, in the study of Barber et al, the step height was much greater than the upstream channel depth (≈10:1 compared with 1:1 in the present study), and the wall shear stress was actually higher around the reattachment point than downstream, where it fell to <0.1 Pa. That adhesion was not seen in the low-shear downstream regions again indicates a functional difference between the U937 and the “fresh” leukocytes in the present study.

The wall shear stress in arteries varies with time during the cardiac cycle and between different sites, even those quite close together, eg, in the carotid bifurcation.24 In small arteries and arterioles, wall shear stress averages ≈2.5 to 5.0 Pa, but in large arteries, it is estimated to be 0.5 to 1.0 Pa, which is equivalent to a wall shear rate of 150 to 300 s⁻¹.25 The wall shear rate in our higher shear channel was ≈400 s⁻¹. The shear stress of 0.3 Pa was at the low end of values expected in arteries because the suspending phase viscosity was lower than that of blood. Thus, our findings are most directly relevant to large-vessel disease. Recent studies of ex vivo–perfused carotid arteries in animals with early atherosclerotic lesions support this contention. P-selectin–mediated capture and VCAM-1–mediated immobilization of mononuclear cells were localized to the region of the bifurcation at a wall shear stress of 0.3 Pa.26,27 It has also been shown that P-selectin supports the adhesion of monocytes to sections of diseased human artery in vitro.28 Thus, promotion of leukocyte adhesion by disturbed flow may be a factor in the localization of atheroma to low-shear regions of vortices and the recirculation associated with arterial junctions and curvature.6,8 It is also possible that adhesion of leukocytes, promoted by discontinuity in the vessel wall, could influence pathology, such as restenosis in vessels subjected to angioplasty, or thrombosis downstream from anastomoses after surgical reconstruction of arteries. In other circumstances, hemodynamic promotion of the delivery and adhesion of leukocytes and platelets to points at which the vessel wall is damaged might actually assist hemostasis and wound healing.

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References


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