Sequential adhesion of platelets and leukocytes from flowing whole blood onto a collagen-coated surface: Requirement for a GpVI-binding site in collagen

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Summary
The adhesion of leukocytes to immobilised platelets may contribute to inflammatory and thrombotic responses in damaged tissue. To investigate the conditions under which platelets and leukocytes might be deposited together in vessels, we perfused fluorescently-labelled whole blood through glass capillaries coated with various collagen preparations. Video-microscopic observations of the surfaces showed that platelets formed numerous, individual, rolling and stationary attachments to surfaces coated with acid-soluble, monomeric collagen. However, leukocyte interactions with the deposited platelets were rare. If the blood was washed out, the adherent platelets became more activated, and many rolling adherent leukocytes were observed if a second bolus of blood was perfused over them. This suggested that platelet activation had initially been inadequate to support leukocyte capture. Next, fibrillar collagen was adsorbed to the capillaries to present an ordered array of peptide motifs to platelet receptor glycoprotein (Gp)VI and transduce an activating signal. In this case, platelets were deposited in discrete, stable aggregates and the bound platelets captured many flowing leukocytes. Alternatively, acid-soluble collagen was seeded with collagen-related peptide (CRP) known to contain a GpVI-binding motif. Again, platelet adhesion became stable, and numerous flowing leukocytes were captured. Addition of antibody against GpVI or against P-selectin greatly reduced leukocyte adhesion to the platelets. Thus, in whole blood, platelets binding to exposed collagen need to be activated through GpVI in order to expose sufficient P-selectin to allow efficient capture of flowing leukocytes to take place.

Keywords
Platelet, neutrophils, adhesion, rheology, collagen

Introduction
Adhesion between leukocytes and platelets may occur within flowing blood or on the surface of vessels, and promote deposition and activation of either type of cell in damaged tissue. Platelets bound to the wall of vessels have been implicated in pro-inflammatory recruitment of leukocytes in atherosclerosis, following ischaemia and reperfusion, and in autoimmune glomerulonephritis (1–3). While platelets may bind to intact endothelium in some circumstances, they more typically bind to exposed matrix in damaged vessels. In this case, they first use their glycoprotein (Gp)Ib-IX-V complex to bind to von Willebrand factor (vWF) adsorbed to collagen, to form an unstable rolling form of adhesion (4). Signals transduced by the binding of platelet GpVI to collagen and by the GpIB-IX-V complex then activate platelet integrins, with \( \alpha_{2}\beta_{1} \)-integrin binding directly to collagen and \( \alpha_{m}\beta_{1} \)-integrin binding to vWF (5, 6). This results in stable attachment and spreading on the surface. Immobilised, activated platelets are able to transport P-selectin to their surface, which in turn is capable of capturing flowing leukocytes and supporting their rolling adhesion (7, 8). If sufficiently activated, the platelets can also present chemokines and lipid mediators that activate the leukocytes so that they too undergo integrin-mediated trans-stationary adhesion (9). Thus, in damaged vessels one might expect sequential adhesion of platelets and leukocytes to occur.

Studies of adhesion of flowing leukocytes to immobilised platelets have typically used models where the platelets are activated and bind first, and isolated leukocytes are perfused afterwards. Using such an approach, we and others have shown that
all the major classes of leukocytes can be captured by surface-bound, activated platelets through P-selectin (7, 8, 10, 11). Although various surfaces have been used, including collagen, these studies do not closely mirror the situation in vivo, where whole blood flows over exposed collagen, and platelets and leukocytes are delivered together. While it is well known that platelets adhere efficiently when whole blood is perfused over collagen, little is known of the conditions under which leukocytes can adhere to these platelets, and for instance, what is required to activate the platelets sufficiently to enable them to support capture of the flowing leukocytes. Hence we set out to directly record the essentially concurrent adhesion of platelets and leukocytes on collagen-coated surfaces for the first time. Using different collagens and related peptides, we examined the basic requirements for platelet adhesion to lead to leukocyte capture, and tested the hypothesis that GpVI-mediated activation was a necessary requirement.

Materials and methods

Collection and treatment of whole blood

Venous blood was collected from healthy adult volunteers into CPDA-1 (citrate phosphate dextrose adenine-1 blood bank storage anti-coagulant diluted 1:7 in blood, Baxter Healthcare Ltd, Thetford, UK) and was used within 3 hours (h). Platelets and leukocytes were fluorescently labelled by the addition of rhodamine 6G (R6G, 1 µg/ml; Sigma, Poole, UK) to whole blood 5 minutes (min) prior to assay. The leukocytes were counted using a Coulter Counter (Coulter Multisizer II, Coulter Electronics, Luton, UK). It may be noted that citrate chelates Ca$^{2+}$, and Mg$^{2+}$ incompletely and in equal proportions; therefore, at the concentrations used here, approximately 30–70 µM Ca$^{2+}$ and 25 µM Mg$^{2+}$ should remain (12). Previous studies have demonstrated that optimal levels of Ca$^{2+}$ are 100 µM for adhesion of isolated leukocytes to P-selectin. However, leukocytes do bind efficiently to P-selectin from flowing CPDA-1 blood (13). Occasionally, where stated, blood was instead anti-coagulated with D-Phe-Pro-Arg-chloromethylketone (PPACK; 2 µM; Merek Biosciences Ltd., Nottingham, UK), which does not modify concentrations of cations. In some experiments, blood was treated with rabbit polyclonal antibody against human P-selectin (10 µg/ml; a gift from Dr. Michael Berndt, University of Melbourne, Australia), or with control, non-specific rabbit immunoglobulin (10 µg/ml; Dako Cytomation, Ely, UK) for 20 min at room temperature before assay. These polyclonal reagents do not have a specific isotype or clone. In other experiments, blood was treated with a phage antibody against GpVI (single-chain variable fragment, scFv; clone 10B12; 50 µg/ml) previously shown to inhibit GpVI function (14). This antibody lacks any Fc portion. Irrelevant control scFv exist in principle but were not available here. Since the use of isotype-matched controls is substantial to control for effects of Fc-receptor-mediated processes, the need for such controls is largely eliminated by using a scFv. Comparison was thus made to paired, untreated samples.

Coating of microslides

Microslides (glass capillary tubes, 5 cm long with a rectangular cross-section of 3.0 x 0.3 mm; CamLab, Cambridge, UK), were acid-washed and coated with 3-aminopropyl-triethoxysilane (APES; Sigma, USA) to aid the binding of proteins as described (15). The microslides were filled with collagen preparations (500 µg/ml except where stated [see below]) and incubated at 37°C for 60 min. The collagen was replaced with 1% bovine serum albumin (BSA fraction V; Sigma, Poole, UK) in phosphate-buffered saline (PBS, Gibco, Paisley, UK), and microslides were incubated for a further 60 min to block any free binding sites. The following collagens were used at 0.5 mg/ml: acid-soluble, monomeric bovine collagen type I (in 0.5 M acetic acid, Chemicon International, Temecula, CA, USA); monomeric human collagen type I (in 0.5 M acetic acid; Sigma Chemical Co., Poole, UK); Horm collagen, a preparation of native collagen fibrils derived from equine tendon (Nycomed, Munich, Germany); fibrillar bovine collagen type I. Fibrillar collagen was obtained by dialysing monomeric bovine collagen type I overnight against buffer containing NaCl (136 mM), KCl (2.7 mM), NaH$_2$PO$_4$ (0.42 mM), NaHCO$_3$ (12 mM), glucose 0.5 mM) and HEPES (5 mM). The dialysed collagen was incubated at 37°C for 2.5 h to allow gelation (evidenced by increased turbidity), and centrifuged at 8,000 g for 1 h to collect fibrils. The pellet was re-suspended, homogenised in a rotating blade tissue homogeniser and its concentration measured (Sircol kit; Biocolor, Belfast, UK) and adjusted to 100 µg/ml. In some experiments, monomeric collagen (bovine or human) was combined with 10 or 100 µg/ml of one of the following peptides: collagen related peptide (CRP), GCP*(GCP)$^{10}$ GCP*G (single letter amino acid code: P* = hydroxyproline); $\alpha$2$\beta$1-integrin-binding peptide (GFO), GCP(GPP) (single letter amino acid code code: G = glutamic acid, P = proline); $\alpha$2$\beta$1 integrin fragment (GPP), GPC(GPP)$_{10}$GPC; control peptide (GPP), GPC(GPP)$_{10}$GPC; synthesised as described (16).

Flow-based adhesion assay

The adhesion assay was adapted from that previously described (13, 17). Collagen-coated microslides were glued to a glass microscope slide mounted on the stage of an inverted fluorescence video-microscope. The inlet end of the microslide was connected via silicone rubber tubing to an electronic valve, which allowed smooth switching between reservoirs containing either blood or PBS. The outlet end was connected to a Harvard Syringe pump that drew either blood or PBS through the microslide at a volumetric flow rate of 0.380 ml/min. This is equivalent to a wall shear rate of 140s$^{-1}$, assuming fully-developed Poiseuille (parabolic) flow of a Newtonian fluid. This is not an accurate representation of the flow profile when blood is perfused, where there is a tendency towards a blunted or ‘plug’ flow, and the wall shear rate is greater than calculated (18). In earlier studies we found that higher perfusion rates did not allow efficient leukocyte adhesion to whole blood onto surfaces coated with P-selectin (13).

Blood was perfused over the collagen-coated microslide for 4-10 min, and events at the surface of the collagen were recorded on video. Typically, analysis was based on a series of six video sequences, each with a duration of 20 seconds (sec), recorded along the centre line of the microslide between 2-4 min of inflow. Following the bolus, PBS (which was culture-tested, endotoxin-free grade) was perfused until red cells were washed out (~ 5 min), and in some experiments, a second bolus of blood was perfused. The PBS was Ca$^{2+}$- and Mg$^{2+}$-free to avoid clotting.
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during the washout and sudden increase in cations to which platelets were exposed.

In previous studies, we found that fluorescent dyes did not modify the adhesion of flowing leukocytes to selectins (19) but could modify their active, migration behaviour (20). Here, in early experiments, we noticed that during observation of adhesion of fluorescent platelets, their behaviour changed, e.g. unstable ‘rolling’ tended to convert to stable adhesion on acid-soluble collagen. After prolonged observation, it was clear that the level of adhesion was higher in the region of the circular, illuminated spot than in the area around it. This was not only true for R6G, but was also observed when we loaded platelets with calcine-AM or quinacrine. Thus, in subsequent experiments, we recorded first at the downstream end of the microslide, and then moved up the microslide so that fields were only illuminated once, and platelets were exposed for the shortest possible time.

**Determination of platelet coverage, leukocyte capture rate and duration of attachment**

Video recordings were digitised off-line using the computer programme Image Pro Plus (Media Cybernetics, Inc., Silver Spring, MD, USA). Images of fluorescent platelets were subject to thresholding at an operator-chosen level to obtain a binary (white on black) image. The percentage of the area that was white (i.e. covered with platelets) was determined and averaged for three consecutive fields.

Leukocyte capture rate was quantified by manually counting the number of leukocytes that were observed to appear and adhere to the platelets deposited on the collagen during each of the six 20-sec recordings of different fields. These counts were expressed as the mean number of leukocytes adhering per minute. From the known dimensions of the fields (calibrated with a stage micrometer), the number was converted to cells/min/mm². This value was divided by the flow rate (in ml/min) x the donor leukocyte count (in 10⁶/ml) to yield a “capture rate” (expressed as number mm²/10⁶ leukocytes perfused), which characterises the efficiency of capture allowing for inter-donor differences in leukocyte count. The captured leukocytes were clearly distinguishable from those which were free flowing, as even during stop motion video playback, the latter appeared as white streaks, while adhered cells had a clear, circular outline. The captured/adherent cells typically exhibited rolling adhesion or hopped across the platelets, demonstrating physical evidence of attachment and detachment, albeit briefly. A cell that attached and detached more

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**Figure 1:** Platelet adhesion to different collagen substrates. Digitised fluorescence video-micrographs are shown after blood was perfused for 4 minutes over: A) acid-soluble, monomeric bovine collagen type I; B) fibrillar collagen derived from the same source; C) Horm collagen. Percentage coverages of the surfaces with platelets are compared in D. Data are mean ± SEM from four or more experiments. Digitised phase contrast video-micrographs are shown after blood had been perfused for 4 minutes and then washed out for: E) acid-soluble, monomeric bovine collagen type I; F) fibrillar collagen derived from the same source. Magnification in E and F have been increased two-fold, compared to A-C. for clarity. The insert in E shows a further-magnified image of platelets, demonstrating various degrees of spreading.
than once while crossing the field was only counted as one capture event. The duration of individual attachments was quantified by counting the number of video fields (1/50th each) during which they remained attached to the platelets during field-by-field playback. The value for this parameter was taken from the first attachment of the cell, even if it went on to bind again further downstream.

Results

Platelet and leukocyte adhesion on surfaces coated with monomeric collagen

When blood was perfused over bovine acid-soluble, monomeric collagen type I, there was a steady build-up of platelet adhesion in the form of a uniform coverage of largely single platelets, some of which “rolled” slowly over the surface, and some of which became stationary (Fig. 1A). The platelets were easily distinguishable from leukocytes based on size and brightness, and indeed, although platelet surface coverage reached 5–10% (Fig. 1D) leukocytes were rarely observed attached to the surface (Fig. 2A). The few that did so made short-lived attachments, sometimes “skipping” across the surface, with few attachments lasting more than 100 ms (median attachment time = 65 ± 15 ms; mean ± SEM of median values from four experiments). In a limited number of experiments perfusion of blood over this monomeric collagen was continued for up to 20 min in case leukocyte adhesion could be induced by a slowly-occurring platelet activation. However, the frequency of leukocyte attachments remained low.

During washout of the blood with buffer, an increased number of rolling leukocytes was observed, which remained spherical and did not show any signs of activation. Examination of the washed surfaces by phase-contrast microscopy indicated that the platelets had become spread (Fig. 1E), suggesting that changes in platelet activation had occurred during the washout with buffer allowing adhesion, as opposed to any change in leukocyte state. Indeed, in previous work we have demonstrated that surfaces with comparable coverage of platelets deposited from flowing blood could subsequently bind large numbers of unstimulated, flowing isolated neutrophils (17). Thus, in order to investigate whether a lack of platelet activation was the limiting factor for leukocytes binding to platelets from flowing blood, a first bolus of blood was completely washed out and then a second bolus was perfused. In this case, leukocyte capture was observed much more frequently (>10-fold increase) compared to attachments during the first bolus (Fig. 2B). These results indicated that the non-fibrillar collagen supported adhesion of platelets that were not active enough themselves to capture flowing leukocytes. It showed that leukocyte adhesion to platelets could efficiently occur in whole blood if the platelets were sufficiently activated. We checked whether this lack of activation could be due to the relatively low cation concentration in CPDA-blood by conducting experiments with blood anti-coagulated with the specific thrombin inhibitor PPACK. However, although platelet coverage was slightly higher with PPACK compared to CPDA (12.3% vs. 9.3% after 4 min blood perfusion), leukocyte capture remained infrequent (28/mm²/10⁶ perfused; mean from two experiments) and comparable to that shown in Figure 2A.

Figure 2: Leukocyte capture by platelets binding to different collagen substrates, at different times after initiation of flow for: A) monomeric bovine collagen type I; B) Monomeric bovine collagen type I where a first bolus lasting 4 minutes was perfused and then washed out, and a second bolus of blood delivered. C) Fibrillar collagen derived from the same source. Data are mean ± SEM from four or more experiments. Analysis of variance was first carried out to test for the concurrent effects of time and treatment. First, we compared adhesion on monomeric collagen during the first bolus (A) and the second bolus (B), and found a significant effect of bolus number (p<0.01) but not of time. Post-hoc comparisons of the second bolus to the first bolus for specific times were carried out using the Dunnett test and showed significance as indicated (**=p<0.05; ***=p<0.01). Next, we compared adhesion to monomeric collagen (A) and to fibrillar collagen (C), and found a significant effect of collagen type (p<0.01) but not of time. Post-hoc comparisons of the fibrillar collagen to the monomeric collagen for specific times were carried out using the Dunnett test and showed significance as indicated (**=p<0.01).
Platelet and leukocyte adhesion on surfaces coated with fibrillar collagen

We hypothesised that the ability to sufficiently activate adherent platelets to capture leukocytes required a “correct” quaternary structure of collagen. Hence we tested “fibrillar” collagen made from the monomeric bovine collagen types I by dialysis, incubation and high-speed centrifugation. Platelets adhered readily from flowing blood but did not form a uniform dispersed layer. Many patchy aggregates could be seen gradually building up, with smaller stable adherences in between (Fig. 1B). Again, platelets were easily distinguishable from leukocytes, but in contrast to the monomeric collagen, many leukocytes were observed attaching to the surface soon after platelet deposition had started. The attachments were still of a transient nature, with some leukocytes rolling over short distances, and others skipping from point to point. The attachments tended to be of a longer duration than those detected on monomeric collagen (median attachment time = 300 ± 145 ms; mean ± SEM of median values from three experiments). However, we did not see any leukocytes coming to a halt or joining the platelet aggregates, and they did not add significantly to the assessed platelet coverage or appear on the surface after washout (Fig. 1F). Indeed, surface plateau coverage was similar to monomeric collagen after 5 min (Fig. 1D), even though there was a considerable increase in the number of leukocyte attachments (Fig. 2C). Nevertheless, we also checked for leukocytes by staining surfaces with the fluorescent nuclear stain bisbenzamide after washout of blood, and scanning the surface using fluorescence microscopy. We could not detect leukocyte nuclei. As a positive control we added isolated neutrophils to surfaces after washout of blood and fixed them in place using glutaraldehyde. The nuclei of these cells were clearly visible. Thus, we could reliably distinguish between adhering leukocytes and platelets during perfusion, and after washout.

We also coated surfaces with equine fibrillar (Horm) collagen, so large fibrils could be seen on the glass surface before perfusion of blood. Again, during perfusion of a bolus of blood, platelets adhered and formed many discrete aggregates (Fig. 1C), and leukocytes were seen to bind frequently but transiently to the platelets. In a series of experiments (including studies of blocking antibodies; see below), the rate of leukocyte capture during the period 2-4 min into the bolus was 285 ± 59 /mm²/10⁶ perfused (mean ± SEM from 21 experiments), which was comparable to the maximal rate for fibrillar bovine collagen (Fig. 2C). In a subset of paired experiments, the duration of attachment was analysed in order to obtain frequency distributions for the Horm collagen and for monomeric collagen (Fig. 3). The duration of attachment was approximately twice as long on the Horm collagen. We wondered whether the limited duration of attachment might arise from lack of Mg++ required for stable adhesion of flowing neutrophils to platelets (21). However, when 5 mM MgCl₂ was added to the CPDA-anticoagulated blood, adhesion was short-lived and the rate of leukocyte capture (134 ± 57 /mm²/10⁶ perfused; mean ± SEM from six experiments) was at the lower end of the range seen without added Mg++ (see above). We also checked whether flowing isolated neutrophils could bind directly to collagen (monomeric or Horm), but could not detect capture and only found an occasional cell (~2/mm²/10⁶ perfused) when the surface was extensively scanned after bolus perfusion.

Effect of seeding monomeric collagen with collagen receptor peptide (CRP)

The finding that fibrillar, but not acid-soluble, collagen type I supported sufficient platelet activation to induce leukocyte adhesion suggested that the latter lacked adequate presentation of ligand for GpVI. Hence we coated surfaces by incubation with a mixture of monomeric collagen and 10 or 100 µg/ml CRP to add a ligand for GpVI. Platelet adhesion was transformed from uniform adhesion to adhesion with aggregates, and leukocyte adhesion to the platelets was greatly increased by the presence of CRP (Fig. 4). The increase in leukocyte adhesion occurred even though platelet coverage was actually reduced by the addition of CRP to the monomeric collagen (e.g. from 7.0 ± 0.9% to 3.3 ± 0.7% when 100 µg/ml CRP was added; mean ± SEM from five experiments, p<0.05 by paired t-test). This reduction appeared to occur because the platelets were mainly in aggregates rather than being uniformly spread across the field. The combination of monomeric collagen with control peptides representing the β1-integrin binding site in collagen or a scrambled peptide did not cause any evident change in platelet behaviour or increase in leukocyte adhesion (Fig. 4). In this series of experiments, leukocyte adhesion was greater for monomeric collagen than previously, possibly because the stored collagen had undergone structural changes. However, further studies were not possible with this collagen, because supplies were no longer available.

Mixtures of monomeric human collagen type I and 100 µg/ml CRP were thus tested. The monomeric collagen supported lower coverage with platelets than with the bovine collagen (average coverage 1.5% in six experiments) and very little leukocyte adhesion (leukocyte capture 12 ± 7 /mm²/10⁶ perfused; mean ± SEM from six experiments). However, when co-coated with CRP platelet adhesion with aggregation occurred and coverage doubled to an average of 3%, and there were many adherent leukocytes (leukocyte capture was 219 ± 37 /mm²/10⁶ perfused; mean ± SEM from six experiments). Limited studies with
the control peptides did not show any obvious changes in either platelet or leukocyte adhesion (data not shown).

The aforementioned results show that there is little connection between changes in platelet coverage and increases in leukocyte adhesion. Combining CRP with bovine monomeric collagen caused leukocyte adhesion to go up nearly five-fold (Fig. 4), while platelet coverage actually decreased. Combining CRP with human monomeric collagen caused leukocyte adhesion to go up nearly 20-fold, while platelet coverage only doubled (for this initially very ineffective collagen). In earlier studies that compared acid soluble and fibrillar collagens, platelet coverage was similar, but leukocyte adhesion was much greater for the fibrillar collagen. These findings are consistent with our earlier publication on adhesion of isolated flowing neutrophils to surfaces sparsely covered with platelets (17), where the flux of adherent leukocytes was not increased with increasing coverage, although stability of adhesion was increased. Thus, it is the activation state of the adherent platelets (which itself depends on the nature of the collagen substrate) that is critical for leukocyte capture, and not the platelet area coverage.

**Effects of antibodies against GpVI or P-selectin on leukocyte adhesion**

The foregoing suggested that presentation of ligand for GpVI by fibrillar collagen or by sequences mimicking the “native” binding sites induced sufficient activation of platelets so that they presented P-selectin for leukocyte adhesion. We further tested the requirement for GpVI by treating blood with an antibody against this receptor (14) and by perfusing it over Horm collagen. We found a marked decrease in leukocyte adhesion (leukocyte capture was reduced by 90 ± 5% compared to untreated, paired control samples perfused over Horm collagen; mean ± SEM from three experiments; p<0.01 by paired t-test). However, after the 4-min bolus, the platelet coverage was nearly identical (5.6 ± 0.8% vs. 5.9 ± 0.6% coverage, for untreated and antibody-treated samples perfused over Horm collagen, respectively) and the degree of platelet aggregation was unchanged (judged qualitatively by a blinded, expert observer).

We tested the role of P-selectin by perfusing blood over the human monomeric collagen + CRP combination in the presence or absence of polyclonal rabbit anti-human P-selectin or non-specific rabbit immunoglobulin. In the presence of anti-P-selectin, adhesion of leukocytes to the surface was reduced by 74 ± 6.4% compared to untreated control blood (mean ± SEM from four experiments; p<0.01 compared to control by paired t-test). In the presence of the non-specific immunoglobulin there was a reduction of 21 ± 21% (mean ± SEM from five experiments) which was not statistically significant. In two separate experiments using Horm collagen as adhesive substrate, the anti-P-selectin reduced leukocyte adhesion by an average of 75%. We have previously demonstrated that ligation of GpVI in other models can lead to up-regulation of P-selectin expression (22). It must be noted that in the current experiments, there would be ongoing new exposure of P-selectin during blood perfusion as platelets continually arrive, bind to the fibrillar collagen and become activated. Antibodies which flowed in with the blood could not block the P-selectin in advance, and since antibody binding cannot be instantaneous, some leukocytes must be expected to bind before the receptor is blocked. Thus, it is not suprising that antibody blockade was not as effective as that which was observed (80–90% reduction) when we treated pre-deposited, activated platelets, and leukocytes were perfused afterwards (7, 21). Nevertheless, while P-selectin is clearly the major receptor for leukocyte capture, one cannot rule out contribution(s) from other receptors. However, this does not alter the conclusion that GpVI ligation is required for exposure of the capture receptor(s).

**Discussion**

We have described for the first time the conditions under which leukocytes can bind to collagen-adherent platelets in flowing whole blood, and have demonstrated that platelet activation through GpVI is required for efficient capture. When acid-soluble, monomeric collagen was used as the initial adhesive substrate, platelets bound predominantly in an unstable manner, and there was negligible adhesion of leukocytes. However, if the blood was washed out, the platelets became activated and spread, and numerous leukocytes adhered if a second bolus of blood was perfused. While this activation process was “non-physiological” and not linked to GpVI ligation, it did show that if platelets were non-specifically activated, then leukocyte capture could occur in flowing whole blood. In order to induce more physiologically-relevant activation of the platelets in blood perfused over collagen, we used fibrillar collagen which bears peptide motifs that are able to bind platelet GpVI (5). Not only was platelet adhesion transformed to a stable nature with formation of small aggregates, but leukocyte adhesion was also induced. The same was true when monomeric collagen was combined with CRP, a known ligand for GpVI (23, 24). The role of GpVI was further defined in studies where an antibody against GpVI was added to the blood. Platelet adhesion to fibrillar collagen was not affected (judged by percentage coverage or degree of aggregation), but...
leukocyte adhesion to the platelets was reduced by about 90%. Thus, at the low shear rates studied here (which allowed leukocyte adhesion), GpVI-ligation was not essential for efficient platelet adhesion to fibrillar collagen, but was necessary for the subsequent support of leukocyte capture. Even with fibrillar collagen or CRP, leukocyte capture was unstable, and the cells rolled or skipped across the platelets. Judging from experiments with blocking antibodies, these adhesive events were mediated by P-selectin, as might be expected from previous studies of adhesion of isolated leukocytes to immobilised platelets (7, 8, 10). A large proportion of the individual attachments lasted only ~100 ms, suggesting that they arose from only one or two bonds forming on discrete platelets (25). Unstable adhesion of this type was previously observed when we pre-coated surfaces very sparsely with platelets and perfused isolated neutrophils, whilst steady rolling required continual cycles of formation and shear-induced disruption of bonds over a more uniformly-coated surface (17). A few leukocytes rolled for longer periods of time in the current study, apparently over groups of adjacent platelets. Stationary adhesion would require activation of leukocyte β2-integrins, an event that can be induced for neutrophils rolling on pre-deposited platelets, e.g. by exogenous chemotactic agents (21). On the other hand, if platelets are sufficiently activated, e.g. with thrombin, then they themselves may generate chemokines or lipid mediators which are capable of activating neutrophils (9, 11).

While the model used here addresses the specific questions of the collagen structure required to induce platelets to support leukocyte capture, and the role of GpVI, it does not sufficiently represent the situation in vivo. The concentrations of Ca++ and Mg++ are lower than in non-anticoagulated blood. Although this did not appear to be a critical factor with regard to allowing platelet deposition and leukocyte capture, it is possible that additional adhesion processes could have contributed at higher cation levels. Moreover, on substrates that incorporate other proteins in addition to collagen, it is possible that ligation of other platelet receptors could signal to further promote leukocyte capture. Binding to vWF clearly does not activate platelets efficiently to induce leukocyte capture as this would have occurred in the experiments using monomeric collagen. Rolling of platelets characteristic to this ligand was observed, but there was very little leukocyte adhesion to these platelets. In different models, adherent, thrombin-stimulated platelets were not only able to capture isolated neutrophils, but also to activate the captured cells through presentation of CXC-chemokines (9). Presentation of platelet-derived CC-chemokine RANTES on endothelial cells has also been implicated in monocyte recruitment to arteries in vivo (26). Here, only selectin-mediated unstable adhesion of leukocytes was observed, indicating that chemokine-induced activation was absent. This suggests that binding to fibrillar collagen and signalling through GpVI was not adequate to induce chemokine exposure by the platelets (e.g. compared with thrombin activation). Another possibility was that there was not any stabilization because of inadequate Mg+2 to support integrin binding (21). However, the addition of extra MgCl2 to the CPDA blood did not have any discernible effect on adhesive behaviour. The aforementioned points do not influence our conclusion that GpVI ligation provides a signal adequate for induction of leukocyte capture by platelets, but they do suggest that GpVI alone might not drive the full potential interaction between platelets and leukocytes.

All major types of leukocytes were probably adhering to the platelets in the current study, although we could not identify them in the flowing whole blood. Most of them were probably neutrophils. Lymphocytes can adhere to immobilised platelets, but less efficiently than neutrophils (8), whereas monocytes adhere quite efficiently (27), but there are considerably fewer of them in the blood.

Considering the patho-physiological significance of these results, there is an indication that platelets which adhere to native collagen in vivo should be able to capture flowing leukocytes. This capture would be unstable unless platelets were additionally activated through some other agents, such as thrombin, which is generated locally during concurrent coagulation of blood. In addition, capture may be restricted to low shear regions, since we and others have shown that P-selectin-mediated adhesion is only operative over a restricted range of shear rates (7, 28), up to about 300s−1 in whole blood (13). In an earlier study which used a higher shear model, platelets could capture flowing leukocytes from blood in a region of disturbed flow where shear rate was locally reduced (29). This might allow adhesive interactions between leukocytes and platelets at bifurcations or bends in the arterial tree, and possibly promote them in a wider range of vessels if there is damage to the wall or a build up of a thrombus which distorts flow.

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