Chemokine Fractalkine Mediates Leukocyte Recruitment to Inflammatory Endothelial Cells in Flowing Whole Blood
A Critical Role for P-Selectin Expressed on Activated Platelets

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Background—The membrane-bound chemokine fractalkine (CX3CL1) is expressed on various cell types such as activated endothelial cells and has been implicated in the inflammatory process of atherosclerosis. The aim of the present study was to dissect the role of fractalkine in leukocyte recruitment to inflamed endothelium under arterial shear forces.

Methods and Results—With the use of immunofluorescence and laminar flow assays, the present study shows that human umbilical vein endothelial cells stimulated with tumor necrosis factor-α and interferon-γ abundantly express CX3CL1 and promote substantial leukocyte accumulation under arterial flow conditions. In the presence of high shear, firm adhesion of leukocytes to inflamed endothelial cells is reduced by ~40% by a function-blocking anti-fractalkine antibody or by an antibody directed against the fractalkine receptor (CX3CR1). With the use of intravitral video-fluorescence microscopy we demonstrate that inhibition of fractalkine signaling attenuates leukocyte adhesion to the atherosclerotic carotid artery of apolipoprotein E–deficient mice, which suggests that the CX3CL1-CX3CR1 axis is critically involved in leukocyte adhesion to inflamed endothelial cells under high shear forces both in vitro and in vivo. Surprisingly, platelets were strictly required for fractalkine-induced leukocyte adhesion at high shear rates. Correspondingly, specific inhibition of platelet adhesion to inflamed endothelial cells also significantly reduced leukocyte accumulation. We show that both soluble and membrane-bound fractalkine induces platelet degranulation and subsequent surface expression of P-selectin, which thereby promotes direct platelet-leukocyte interaction.

Conclusion—Fractalkine expressed by inflamed endothelial cells triggers P-selectin exposure on adherent platelets, which thereby initiates the local accumulation of leukocytes under arterial shear, an essential step in the development of atherosclerotic lesions. (Circulation. 2007;116:764-773.)

Key Words: atherosclerosis • endothelium • inflammation • leukocytes • platelets

Atherosclerosis is a chronic inflammatory disease that involves the recruitment of leukocytes to the vascular endothelium.1 Accumulation of leukocytes in inflamed tissues engages a multistep cascade initiated by leukocyte capturing, with subsequent rolling and firm adhesion followed by extravasation into the perivascular tissue.2 Whereas the molecular determinants that regulate leukocyte adhesion under low-shear conditions in inflamed venules have been well characterized over recent decades,3,4 less is known about the signals that promote leukocyte adhesion in inflamed arteries.

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Fractalkine (CX3CL1) is a recently discovered chemokine of the CX3C family.5 It consists of a polypeptide chain that carries the chemokine domain on top of an extended mucin-like stalk, which allows the molecule to exist either as a...
membrane-anchored or soluble glycoprotein. Increasing evidence indicates that fractalkine is critically involved in the pathophysiology of different inflammatory diseases such as atherosclerosis.\textsuperscript{5,7} In fact, endothelial cells express substantial levels of fractalkine after in vitro stimulation with proinflammatory cytokines. In addition, fractalkine expression has recently been described on inflamed endothelial cells within the atherosclerotic plaque in vivo.\textsuperscript{8–10} The fractalkine receptor (CX\(_{3}\)CR1) is abundantly expressed on various cell types such as circulating leukocytes.\textsuperscript{10–12} Interaction between membrane-bound fractalkine and its receptor not only mediates chemotaxis but also promotes leukocyte adhesion.\textsuperscript{11,13,14} Correspondingly, leukocytes are recruited to fractalkine-coated surfaces under conditions of low shear stress.\textsuperscript{13} However, it remains unclear whether fractalkine also contributes to leukocyte adhesion to the inflamed endothelium at higher shear forces as they prevail in atherosclerosis-prone arteries.

In the present study, we show that fractalkine expressed on inflamed endothelial cells triggers the adhesion of leukocytes under arterial shear conditions both in vitro and in atherosclerotic murine arteries in vivo. Unexpectedly, platelets, which express CX\(_{3}\)CR1,\textsuperscript{12} were strictly required for fractalkine-induced leukocyte adhesion. We provide the first evidence that endothelial fractalkine induces activation of surface-adherent platelets with consecutive platelet degranulation and exposure of P-selectin. Furthermore, we show that fractalkine-dependent platelet expression of P-selectin is critical for leukocyte adhesion to inflamed endothelium under arterial shear conditions, a central step in the process of atherogenesis.

Evaluation of Leukocyte and Platelet Adhesion Under Flow Conditions
Flow experiments were performed as previously described.\textsuperscript{12} Glass coverslips for usage in a flow chamber were cultivated until confluence with either HUVEC or Flp-In CHO cells transfected with full-length human fractalkine or mock, respectively. Leukocytes and platelets were labeled in whole blood with rhodamine-6G and perfused for 10 minutes at 1000/s and visualized by fluorescence microscopy. Where indicated, HUVEC were incubated prior to perfusion with function-blocking goat anti-human fractalkine antibody (R&D Systems, Minneapolis, Minn) or a nonbinding goat anti-human control IgG for 30 minutes. Alternatively, whole blood was preincubated with antibodies against either the fractalkine receptor (TP502; Torrey Pines Biolabs, Houston, Tex) or anti-human control antibody. To define the role of platelet adhesion for leukocyte recruitment, monoclonal antibody (mAb) c7E3 (4 \(\mu\)g/mL), an anti-human glycoprotein (GP)IIb/IIIa mAb (10 \(\mu\)g/mL), or both mAbs in combination were added.

To define the contribution of platelets to leukocyte adhesion under high and low flow conditions, leukocyte accumulation on HUVEC and fractalkine-transfected CHO cells was determined at arterial (1000/s) and venous (100/s) shear rates with perfusion of isolated leukocytes in the presence or absence of platelets, or normal and platelet-depleted whole blood. Leukocytes were isolated from lysed whole blood by flow cytometric sorting of CD45 (leukocyte)-positive, CD41 (platelet)-negative cells. Platelet-depleted whole blood was generated with magnetic beads and anti-CD41-antibodies.

To address the role of fractalkine-induced platelet P-selectin exposure, whole blood was perfused for 10 minutes at 1000/s over fractalkine- or mock-transfected CHO cells in the presence of a neutralizing anti-P-selectin mAb (10 \(\mu\)g/mL) or mouse control IgG. Adherent leukocytes were identified with FITC-conjugated anti-CD45 mAb (Dako Cytomation, Hamburg, Germany).

Flow Cytometry
CX\(_{3}\)CL1 expression by cytokine-activated HUVEC (primarily isolated or cultivated) and by fractalkine-transfected Flp-In CHO cells was analyzed with an anti-human CX\(_{3}\)CL1 antibody (R&D Systems) and FITC-labeled secondary antibody. To analyze the expression of adhesion molecules on stimulated endothelium, we used antibodies directed either against CD106 (Serotec, Düsseldorf, Germany), CD62E, CD54, CD62P, CD11b, or a mouse IgG, isotype control (all from Beckman Coulter).

To assess platelet activation by soluble and membrane-bound CX\(_{3}\)CL1, washed human platelets were prepared as described.\textsuperscript{12} Platelet P-selectin surface expression was measured after incubation with soluble full-length recombinant human fractalkine (rhCX\(_{3}\)C; R&D Diagnostics), static adhesion to immobilized full-length fractalkine, or adhesion under flow to membrane-bound fractalkine expressed on transfected CHO cells as indicated.

To determine the effect of fractalkine on platelet-leukocyte interactions, whole blood was incubated with soluble rhCX\(_{3}\)C (final concentration, 1 \(\mu\)g/mL) in the absence or presence of function-blocking anti-human fractalkine (anti-CX\(_{3}\)CL1) or anti-P-selectin antibody as indicated, and leukocyte-platelet co-aggregation was assessed by flow cytometric analysis of platelet (CD41)-positive cells within the CD11b-positive leukocyte population.

Statistical Analysis
Data represent mean±SEM. Data were analyzed with 1-way ANOVA with a Tukey post hoc test. Probability values are reported as Tukey test adjusted probability values. \(P<0.05\) was considered to be significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Fractalkine Mediates Leukocyte Adhesion to Inflamed Endothelial Cells

To define the potential contribution of fractalkine to leukocyte-endothelium interactions at high shear, we first analyzed fractalkine expression on resting (nonstimulated) and inflamed endothelial cells. Primarily isolated or cultivated HUVEC were stimulated with IFN-γ (20 ng/mL) and TNF-α (50 ng/mL) for 20 hours. Fractalkine expression was assessed by flow cytometry and immunoblotting (Data Supplement Figure IA through ID). Whereas fractalkine was virtually absent in resting HUVEC, inflamed HUVEC abundantly expressed fractalkine (Data Supplement Figure IA). Notably, inflammation-induced fractalkine expression was similar on cultivated HUVEC as compared with primarily isolated HUVEC (Data Supplement Figure IA). Furthermore, combined stimulation with IFN-γ and TNF-α increased endothelial fractalkine expression significantly more efficiently than activation with each cytokine alone ($P<0.0001$) (Data Supplement Figure IB through ID).

Next we investigated the role of fractalkine, expressed on IFN-γ/TNF-α–activated endothelial cells, for leukocyte recruitment under arterial shear flow. Rhodamine-6G–labeled whole blood was perfused over nonstimulated or IFN-γ/TNF-α–activated HUVEC using a flow chamber. After 10 minutes of perfusion at 1000/s, we observed a marked increase in the number of firmly adherent leukocytes on activated but not on resting endothelial cells (Figure 1A and 1B). Importantly, endothelial integrity and confluence of the endothelial monolayer were not affected as indicated by confocal microscopy (not shown). May-Grunwald/Giemsa-staining of the leukocytes attached to inflamed HUVEC showed that monocytes and neutrophils represented the majority (70%) of recruited cells. Notably, leukocyte accumulation on inflamed endothelial cells exposed to arterial shear was substantially attenuated when we incubated the activated HUVEC monolayer with a function-blocking anti-fractalkine (anti-CX,C1L1) antibody. Likewise, the number of adherent leukocytes was reduced by $\approx 40\%$, when the whole blood was preincubated with a function-blocking anti-CX,C1R1 antibody prior to perfusion over IFN-γ/TNF-α–stimulated HUVEC ($P<0.05$) (Figure 1A). In contrast, the combined inhibition of the chemokine domain on HUVEC-expressed CX,C1L1 and its receptor CX,C1R1 had no additive effect, consistent with the notion that CX,C1L1 exerts its effects predominantly via CX,C1R1.

Enhanced fractalkine expression has been reported to occur in atherosclerotic arteries of humans and mice. Therefore, we next asked whether fractalkine might also contribute to leukocyte adhesion to the atherosclerotic vessel wall in vivo. To address this, murine monocytes (WEHI-274.1) that constitutively express the fractalkine receptor CX,C1R1 (not shown) were injected intravenously into 18-week-old ApoE$^{-/-}$ mice. Subsequently, interaction of WEHI-274.1 cells with the vascular endothelium was visualized in vivo by real-time fluorescence microscopy of the carotid artery. In line with previous reports, numerous monocytes were recruited to the atherosclerotic carotids in ApoE$^{-/-}$ mice. Notably, injection of a function-blocking anti-CX,C1L1 antibody but not a control antibody resulted in a significant reduction of leukocyte adhesion (Figure 1C). Together, the above findings indicate that fractalkine contributes substantially to leukocyte recruitment under arterial shear conditions both to inflamed endothelial cells in vitro and to atherosclerotic arteries in vivo.

P-Selectin Promotes Leukocyte Adhesion on Activated HUVEC Under Arterial Flow

We next dissected the cascade of events required for fractalkine-induced leukocyte adhesion to the vascular endothelium under arterial flow. With use of flow chamber assays we found that, apart from the CX,C1L1–CX,C1R1 axis, P-selectin is essential for leukocyte adhesion to inflamed endothelial cells exposed to high shear. Correspondingly, a function-blocking anti-CD62P mAb attenuated leukocyte adhesion to a similar degree as observed after inhibition of CX,C1L1 or CX,C1R1, respectively (Figure 1A and 1B). In contrast, anti-CD62E (E-selectin) mAb or irrelevant control antibodies did not reduce leukocyte adhesion to activated endothelium (Figure 1A). Together, this clearly indicated that endothelial inflammation triggers the recruitment of leukocytes in a process that involves both fractalkine and P-selectin. However, to our surprise P-selectin surface expression was low and remained unchanged on cultivated (Figure 1D) or primarily isolated HUVEC (Data Supplement Figure II) after 20 hours of incubation with IFN-γ and TNF-α, whereas we observed an increase in surface exposure of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (both of which mediate firm adhesion but not rolling of leukocytes under arterial shear) and of E-selectin when compared with resting endothelial cells ($P<0.05$). This implies that endothelial P-selectin could not account for P-selectin–dependent leukocyte accumulation on the inflamed endothelial monolayer.

Platelets Are Essential for Fractalkine-Induced Leukocyte Accumulation

Platelets are another rich source of P-selectin in the vascular system. We and others have reported earlier that, apart from leukocytes, platelets adhere to inflamed endothelial surfaces under arterial shear conditions in vitro and in vivo. Correspondingly, we found here that, in addition to leukocytes, platelets readily accumulate on the inflamed endothelium in the presence of arterial flow (Figure 2A and 2B). To determine the adhesion molecules involved in platelet recruitment to inflamed endothelial cells under arterial shear forces, washed human platelets were perfused over IFN-γ/TNF-α–stimulated HUVEC in the absence and presence of the mAb c7E3 and anti-GPIIb. In the presence of c7E3, which blocks the $\beta3$ integrin receptors $\alphaIIb$/$\beta3$ (GPIIbIIIa) and $\alphaV$/$\beta3$, firm platelet adhesion to the endothelium was strongly reduced. Likewise, inhibition of GPIb but not of CX,C1L1 substantially reduced platelet adhesion (Figure 2B). Thus, under inflammatory conditions, platelet adhesion to the endothelium involves both $\beta3$ integrins as well as GPIb.$\alpha$. 
Once they adhere to the inflamed endothelium, platelets degranulate and express P-selectin on the surface. Because IFN-γ/TNF-α-stimulated HUVEC did not show enhanced P-selectin surface expression, we hypothesized that platelets might provide the major source of P-selectin to promote leukocyte adhesion to the inflamed endothelial cell surface under arterial shear. Indeed, we observed that leukocyte rolling (not shown) and adhesion to activated endothelium primarily occurred at sites of platelet accumulation (Figure 2A). To determine whether platelet adhesion might in fact be...
blocking antibodies c7E3 and anti-GPIb were perfused with washed human platelets for 10 minutes at 1000/s in the presence and absence of c7E3, anti-GPIbα, or both mAbs. Where indicated, HUVEC were incubated with anti-CX3CL1 antibodies or control IgG prior to perfusion. The results are given as number of adherent platelets per mm² of HUVEC surface (n=4; *P<0.05, **P<0.01, ***P<0.001 versus IFN-γ/TNF-α-stimulation in the absence of antibody). C, IFN-γ/TNF-α-stimulated HUVEC were perfused with rhodamine-6G–labeled whole blood for 10 minutes at 1000/s in the absence or presence of c7E3, anti-GPIbα, or both mAbs. Firm leukocyte adhesion was quantified and is given as percentage of the adhesion achieved by IFN-γ-TNF-α-stimulation (without addition of antibodies) (n=3; *P<0.05 and **P<0.01 versus IFN-γ-TNF-α-stimulation in the absence of mAb). D and E, Isolated, buffer-resuspended leukocytes in the absence and presence of platelets (D) or normal and platelet-depleted whole blood (E) were perfused over IFN-γ-TNF-α-stimulated HUVEC at 1000/s (arterial shear) or 100/s (venous shear). Results are given as number of firmly adherent leukocytes per mm² of HUVEC surface (n=4; *P<0.05 versus leukocytes + platelets in D and nondepleted whole blood in E. Plt indicates platelet; Lc, leukocyte; and wb, whole blood.

Figure 2. Platelets adhere to inflamed endothelial cells and trigger leukocyte accumulation. A, Accumulation of platelet–leukocyte aggregates on IFN-γ/TNF-α-stimulated HUVEC. B, Untreated or IFN-γ/TNF-α-stimulated HUVEC were perfused with washed human platelets for 10 minutes at 1000/s in the presence and absence of c7E3, anti-GPIbα, or both mAbs. Where indicated, HUVEC were incubated with anti-CX3CL1 antibodies or control IgG prior to perfusion. The results are given as number of adherent platelets per mm² of HUVEC surface (n=4; *P<0.05, **P<0.01, ***P<0.001 versus IFN-γ-TNF-α-stimulation in the absence of antibody). C, IFN-γ/TNF-α-stimulated HUVEC were perfused with rhodamine-6G–labeled whole blood for 10 minutes at 1000/s in the absence or presence of c7E3, anti-GPIbα, or both mAbs. Firm leukocyte adhesion was quantified and is given as percentage of the adhesion achieved by IFN-γ-TNF-α-stimulation (without addition of antibodies) (n=3; *P<0.05 and **P<0.01 versus IFN-γ-TNF-α-stimulation in the absence of mAb). D and E, Isolated, buffer-resuspended leukocytes in the absence and presence of platelets (D) or normal and platelet-depleted whole blood (E) were perfused over IFN-γ-TNF-α-stimulated HUVEC at 1000/s (arterial shear) or 100/s (venous shear). Results are given as number of firmly adherent leukocytes per mm² of HUVEC surface (n=4; *P<0.05 versus leukocytes + platelets in D and nondepleted whole blood in E. Plt indicates platelet; Lc, leukocyte; and wb, whole blood.

To further substantiate the role of platelets for leukocyte accumulation under arterial shear, we next perfused isolated leukocytes (Figure 2D) or platelet-depleted whole blood (Figure 2E) over inflamed HUVEC. In the absence of platelets, few leukocytes attached to inflamed HUVEC when arterial flow was applied, whereas numerous leukocytes firmly adhered when platelets were present (Figure 2D and 2E). In contrast, under venous flow conditions (100/s), the number of adherent leukocytes was higher compared with arterial flow and did not require the presence of platelets (Figure 2D and 2E). Together, these findings indicate that, under arterial but not under venous shear rates, leukocyte recruitment to inflamed endothelial cells requires the action of both platelets and fractalkine.

To ascertain whether platelets and fractalkine act independently of each other to promote leukocyte adhesion under arterial flow, we generated CHO transfectants that stably express membrane-bound fractalkine. Fractalkine surface expression by the fractalkine transfectants but not by mock-transfected CHO cells closely resembled fractalkine expression on inflamed endothelial cells (Figure 3A). When we perfused isolated leukocytes over fractalkine-transfected CHO cells with or without addition of platelets, we found that under low venous shear conditions leukocytes readily adhered to fractalkine-transfected CHO cells even in the absence of platelets. This confirms previously published findings that fractalkine alone is sufficient to promote leukocyte capture at low shear rates13,14 (Figure 3B). However, when
arterial shear conditions were applied, substantial leukocyte adhesion to fractalkine-transfected CHO cells only occurred in the presence but not in the absence of platelets. In contrast, platelets did not affect leukocyte adhesion to mock-transfected CHO cells. This clearly indicates that platelets are required to allow fractalkine-induced leukocyte adhesion under arterial shear conditions.

**Fractalkine Induces Platelet Degranulation and P-Selectin Surface Expression**

Apart from leukocytes, platelets express the fractalkine receptor CX3CR1, and stimulation of platelet CX3CR1 in rats resulted in platelet activation.12 Given the role of platelets in fractalkine-induced leukocyte adhesion, we next investigated whether fractalkine present on the surface of inflamed endothelial cells might modulate the activation/degranulation of adherent platelets rather than act directly on leukocytes. In fact, we found that incubation of human platelets with soluble fractalkine (1 μg/mL) significantly and specifically enhanced surface expression of P-selectin to a similar extent as 1 μmol/L ADP (Figure 4A). Combined stimulation of platelets with fractalkine and ADP further increased platelet P-selectin expression (P<0.001) (Figure 4A). Because inflamed endothelial cells express membrane-bound fractalkine, we next investigated whether surface-adherent fractalkine might also induce platelet degranulation. After adhesion to immobilized full-length fractalkine for 10 minutes, platelets displayed concentration-dependent degranulation that resulted in the full-length fractalkine for 10 minutes, platelets displayed concentration-dependent degranulation that resulted in the

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**Figure 3.** Platelets mediate fractalkine-induced leukocyte adhesion at arterial shear rates. A, Representative FACS histograms show CX3CL1 expression on resting or IFN-γ/TNF-α-stimulated HUVEC and fractalkine- or mock-transfected CHO cells (overlay). B, Isolated, buffer-resuspended leukocytes (1×10⁶ cells/mL) were perfused in the absence or presence of platelets (1×10⁶ cells/mL) or control vector (mock) for 10 minutes at 1000/s (arterial shear) or 100/s (venous shear). Results are given as number of firmly adherent leukocytes per mm² of CHO surface (n=4; P<0.05 versus mock).

**Figure 4.** Fractalkine induces platelet degranulation and P-selectin surface expression. A, P-selectin (CD62P) surface expression was assessed on washed human platelets after stimulation with recombinant fractalkine (1 μg/mL) for 15 minutes in the absence or presence of an anti-CX3CL1 and 1 μmol/L ADP. Results are given as percentage of nonstimulated platelets (n=6; *P<0.001 versus baseline, **P<0.001 versus ADP and ADP+rhCX3C/anti-CX3CL1). B, CD62P expression was determined on washed platelets after adhesion to immobilized laminin (5 μg/mL), full-length fractalkine (rhCX3C, 1 or 5 μg/mL), fractalkine (5 μg/mL) preincubated with anti-CX3CL1, and anti-CX3CL1 alone for 60 minutes (n=3 to 6 experiments; *P<0.05, **P<0.01 versus laminin). Results are given as mean fluorescence intensity. C, CD62P expression was assessed on adherent platelets after perfusion over fractalkine-transfected or mock Flip-in CHO cells for 10 minutes at a shear rate of 1000/s. Adherent platelets were directly costained with anti-human CD62P-FITC and CD41-PE antibodies. CD62P expression on nonperfused platelets is indicated as baseline. Results are given as mean fluorescence intensity (n=3; *P<0.05 versus mock transfected cells). AU indicates arbitrary units.
surface expression of P-selectin (Figure 4B). This effect was specific because it was abolished by preincubation with a neutralizing anti-CX3CL1 antibody. To substantiate whether membrane-bound fractalkine is able to promote platelet degranulation at arterial shear rates, mock- or fractalkine-transfected CHO cells were cultivated on cover slides, and washed human platelets were superfused at arterial flow (1000/s) for 10 minutes. Subsequently, P-selectin expression on adherent platelets was determined by flow cytometry. Notably, platelet adhesion to fractalkine-transfected CHO cells but not to mock cells resulted in a significant increase in platelet P-selectin surface exposure (Figure 4C). These data show that membrane-bound fractalkine leads to the activation and degranulation of adherent platelets, which results in platelet P-selectin surface exposure. Although fractalkine expressed by inflamed endothelial cells therefore critically contributes to the activation of adherent platelets, it is not essential for platelet adhesion per se, because fractalkine blocking had no relevant effect on platelet adhesion (Figure 2B). Although fractalkine has been reported previously to enhance platelet adhesion to extracellular matrix proteins,\textsuperscript{12} it does not appear to play a role for platelet adhesion to the inflamed vascular endothelium.

Fractalkine-Induced Platelet Degranulation Triggers Platelet-Leukocyte Interactions

We then investigated whether fractalkine-induced platelet degranulation might trigger leukocyte adhesion. In fact, human recombinant fractalkine significantly and specifically enhanced platelet-leukocyte aggregate formation in human whole blood (\(P<0.05\)) (Data Supplement Figure IIIA and IIIB). No increase in the number of platelet-leukocyte coaggregates was detected in the presence of a function-blocking anti–P-selectin mAb, which implies that platelet P-selectin is essential for fractalkine-induced platelet-leukocyte interaction (Data Supplement Figure IIIC).

Next we determined whether fractalkine-induced platelet P-selectin exposure might also be sufficient to promote leukocyte adhesion under arterial shear forces. Monolayers of CHO cells stably transfected with full-length CX3CL1 or control vector (mock) were perfused in a flow chamber with whole blood at 1000/s in the presence or absence of anti–P-selectin mAb. Leukocyte adhesion on fractalkine-transfected cells was 6-fold increased compared with mock-transfected CHO cells (Figure 5A and 5B). Notably, preincubation of whole blood with function-blocking anti–P-selectin mAb substantially reduced the number of recruited leukocytes (Figure 5A and 5B). Because CHO cells do not express P-selectin (not shown), the present findings clearly demonstrate that fractalkine-induced degranulation of platelets, the major source of P-selectin in human whole blood, triggers the adhesion of leukocytes in a P-selectin–dependent manner under arterial shear conditions.

**Discussion**

In the present study, we show that CX3CL1 is expressed on the surface of IFN-\(\gamma\)/TNF-\(\alpha\)–activated HUVEC and promotes leukocyte adhesion under arterial flow in vitro and to atherosclerotic mouse arteries in vivo. We report that fractalkine does not act directly on leukocytes to promote adhesion in the presence of high shear. In contrast, fractalkine expressed by inflamed endothelial cells is recognized by CX3CR1 on activated platelets, which are recruited in large numbers on the surface of inflamed endothelial cells even under high arterial flow. Ligation of platelet CX3CR1 results in platelet activation and subsequent exposure of P-selectin on the surface of adherent platelets. In the presence of arterial shear...
conditions, this fractalkine-induced platelet P-selectin expression is critical for the initial tethering of leukocytes, which allows subsequent firm leukocyte adhesion and eventual leukocyte transmigration. In contrast, in the absence of platelets and platelet P-selectin, endothelial fractalkine is not sufficient to allow substantial leukocyte accumulation on endothelial monolayers exposed to arterial shear conditions. Hence we report a novel mechanism of fractalkine-induced platelet-dependent leukocyte accumulation to inflated endothelial cells that might play a key role in the chronic inflammatory process of atherosclerosis (Data Supplement Figure IV).

Chemotaxis, adhesion, and transmigration of leukocytes across the vascular endothelium represent critical steps during atherosclerotic lesion formation.1 Whereas the molecular determinants of leukocyte accumulation and emigration into inflamed tissues via postcapillary venules have been well characterized over recent decades,2 the mechanisms that underlie leukocyte recruitment to atherosclerotic endothelium within arteries under high shear rates are poorly understood. Only recently have inflamed or atherosclerotic arterial endothelial cells been reported to express large amounts of fractalkine.8–10 Fractalkine represents a novel type of chemo- kine that is released as a soluble form but is also expressed in a membrane-bound form.5 In addition to its functions in chemotaxis, fractalkine directly induces capture and firm adhesion of flowing leukocytes under conditions of low shear forces.13,14 In the present study we demonstrate that fractalkine is also able to promote leukocyte adhesion to inflamed endothelial cells exposed to high arterial shear rates, which indicates that fractalkine, which is abundantly expressed in atherosclerotic lesions, might contribute significantly to leukocyte accumulation in the arterial tree during atherosclerosis.

Although fractalkine directly promotes leukocyte arrest under low shear rates independent of additional adhesion receptors,13,14 it requires the action of additional adhesion receptors to initiate leukocyte adhesion under arterial shear. This is in line with a recent report by Kerfoot et al, which demonstrates that fractalkine promotes monocyte adhesion at increased shear rates only in the presence of additional trafficking molecules that promote initial leukocyte tethering.21 In their study, Kerfoot et al show that recombinant vascular cell adhesion molecule 1 coated at high density on cover slips was sufficient to promote initial leukocyte tethering, which allows subsequent fractalkine-induced leukocyte arrest. However, in our present model of endothelial inflammation, the increase in endothelial vascular cell adhesion molecule 1 and E-selectin expression was only moderate and not sufficient to promote substantial leukocyte capture under high flow conditions. In contrast, we show that another adhesion receptor, P-selectin, was absolutely mandatory for leukocyte accumulation to activated endothelial cells exposed to arterial shear.

P-selectin, released from endothelial Weibel-Palade bodies, has been well described as a major adhesion receptor that mediates the initial tethering and rolling of leukocytes under acute inflammatory conditions.22 However, to our surprise, we did not observe any substantial P-selection expression on the endothelial surface after the prolonged exposure to IFN-γ/TNF-α used in the present study. Hence, endothelium-expressed P-selectin was highly unlikely to contribute to P-selectin–dependent leukocyte accumulation on the inflamed endothelial cell surface.

Apart from endothelial cells, platelets provide another rich source of P-selectin in the vasculature. In fact, P-selectin expressed by surface-adherent activated platelets has been demonstrated to trigger significant leukocyte recruitment under flow even at a low density of surface coverage.23 We observed here that, together with leukocytes, substantial numbers of platelets attached to inflamed HUVEC after exposure to arterial shear stress. Importantly, leukocyte adhesion strictly colocalized with platelet adhesion, whereas few leukocytes were recruited to areas devoid of adherent platelets. In line with previous studies by our group and others that address platelet–endothelium interactions both in vitro15,20 and in vivo,17,18 platelet adhesion to activated endothelium involved β3 integrins and GPIbα. Remarkably, inhibition of αIIbβ3 integrin and/or GPIbα not only strongly reduced platelet adhesion to HUVEC but also resulted in a significant reduction of leukocyte rolling and adhesion. Because leukocytes do not express αIIbβ3 integrin or GPIbα, these findings clearly indicate that platelets are of paramount importance for leukocyte–endothelium interaction under arterial shear conditions. The reduction of leukocyte accumulation observed after inhibition of platelet adhesion was comparable to that achieved by interference with P-selectin–ligand interactions. In addition, endothelial inflammation resulted in substantial upregulation of P-selectin on adherent platelets but not on the endothelial cell surface. Hence, the present findings imply that P-selectin delivered by adherent platelets substantially contributes to leukocyte adhesion under conditions of arterial shear, most likely by binding to P-selectin glycoprotein ligand 1, the major ligand of P-selectin constitutively expressed by most leukocyte subsets.24 Although our data clearly show that platelet-derived but not endothelium-derived P-selectin promotes leukocyte adhesion under arterial flow in vitro, we cannot exclude that in vivo endothelial P-selectin might also serve as a P-selectin glycoprotein ligand 1 ligand to initiate monocyte adhesion under high shear rates in atherosclerotic arteries.

Notably, we show here that both soluble and membrane-bound fractalkine trigger platelet activation and induce platelet P-selectin exposure by action via platelet CX3CRI. Fractalkine-induced P-selectin expression is sufficient to initiate platelet-leukocyte interaction to facilitate leukocyte adhesion. Correspondingly, CHO cells that stably express fractalkine but not mock CHO triggered platelet P-selectin exposure and promoted subsequent leukocyte adhesion under arterial flow in the absence of additional inflammatory signals. Thus, in addition to its chemoattractive and leukocyte-binding properties at venous flow, fractalkine acts as a platelet-activating chemokine that induces subsequent leukocyte recruitment under arterial shear conditions. We therefore propose that fractalkine expressed on inflamed endothelial cells might act in a dual and sequential manner,
where the chemokine first binds to platelet CX3CR1 to increase platelet P-selectin expression and thereby promote initial leukocyte tethering and rolling. After capture and slowing of leukocytes, endothelial fractalkine may also act directly on CX3CR1 expressed by rolling leukocytes that trigger subsequent leukocyte arrest (Data Supplemental Figure IV).

We have reported earlier that platelets and leukocytes colocalize in areas of vascular injury and that platelet adhesion to the intact arterial wall clearly precedes leukocyte recruitment during initiation of atherosclerosis in vivo. Inhibition of platelet adhesion in a mouse model of atherosclerosis significantly reduces subsequent leukocyte recruitment and development of atherosclerotic lesions.

More recently, it was shown that leukocyte arrest in vivo is strongly dependent on the interaction between leukocytes and P-selectin derived from platelets as well as endothelium. Consistently, absence of P-selectin was shown to reduce and delay atherosclerotic lesion formation, and mouse bone marrow transplant experiments revealed that platelet P-selectin plays a critical role in this process. In fact, leukocyte adhesion to platelets is mediated through P-selectin, an interaction that facilitates leukocyte adhesion to endothelial cells and may finally support macrophage accumulation in the vessel wall. Apart from platelets, fractalkine has recently been implicated in the pathogenesis of atherosclerosis. Importantly, expression of fractalkine is also enhanced in human and murine atherosclerotic plaques. Correspondingly, 2 groups reported reduced lesion formation by deletion of CX3CR1 in a murine model of atherosclerosis. In the present study we provide further insight into the mechanisms by which the CX3C chemokine CX3CL1–CX3CR1 axis initiates leukocyte trafficking to inflamed endothelial cells and might lead to the development of novel strategies to treat and prevent atherosclerosis.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Atherosclerosis is a chronic inflammatory disease and the predominant cause in the Western world of morbidity and mortality that leads to atherothrombotic vascular events. Although leukocyte recruitment to the arterial vessel wall is critical for atheroprosess, the exact mechanisms that promote leukocyte adhesion to endothelial cells under arterial shear rates remain unclear. Increasing evidence implies a critical role for chemokines in the recruitment of leukocytes to inflamed endothelial cells. Fractalkine (CX₃CL1), a recently discovered chemokine of the CX₃C-family, is expressed in a membrane-bound form on inflammatory endothelium and within atherosclerotic plaques. Lesion formation is reduced by deletion of its receptor (CX₃CR1) in murine atherosclerosis. In humans, CX₃CR1-polymorphism (V249I) is associated with lower risk of coronary artery disease. In the present study, we show that fractalkine promotes substantial leukocyte adhesion under arterial flow to activated endothelium in vitro and to atherosclerotic mouse arteries in vivo. Platelets, which are critically involved in the initiation and progression of atherosclerotic vascular lesions, were mandatory for CX₃CL1-dependent leukocyte recruitment at arterial shear rates. We found that membrane-bound fractalkine acts directly on platelets to induce the activation and surface-expression of P-selectin. This subsequently triggers platelet-leukocyte aggregate formation and leukocyte recruitment under arterial flow conditions. Hence, fractalkine-mediated platelet activation and P-selectin surface-expression play a key role in the chronic inflammatory process of atherosclerosis. CX₃CL1/CX₃CR1 may serve as a novel and promising target for drug development in vascular disease to attenuate lesion formation and platelet activation and to prevent adverse clinical events such as myocardial infarction, stroke, or peripheral occlusive disease.
Chemokine Fractalkine Mediates Leukocyte Recruitment to Inflammatory Endothelial Cells in Flowing Whole Blood: A Critical Role for P-Selectin Expressed on Activated Platelets

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