Fractalkine and CX_3CR1 Mediate a Novel Mechanism of Leukocyte Capture, Firm Adhesion, and Activation under Physiologic Flow

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Summary

Leukocyte migration into sites of inflammation involves multiple molecular interactions between leukocytes and vascular endothelial cells, mediating sequential leukocyte capture, rolling, and firm adhesion. In this study, we tested the role of molecular interactions between fractalkine (FKN), a transmembrane mucin-chemokine hybrid molecule expressed on activated endothelium, and its receptor (CX_3CR1) in leukocyte capture, firm adhesion, and activation under physiologic flow conditions. Immobilized FKN fusion proteins captured resting peripheral blood mononuclear cells at physiologic wall shear stresses and induced firm adhesion of resting monocytes, resting and interleukin (IL)-2–activated $CD8^+$ T lymphocytes and IL-2–activated NK cells. FKN also induced cell shape change in firmly adherent monocytes and IL-2–activated lymphocytes. CX_3CR1-transfected K562 cells, but not control K562 cells, firmly adhered to FKN-expressing ECV-304 cells (ECV-FKN) and tumor necrosis factor α–activated human umbilical vein endothelial cells. This firm adhesion was not inhibited by pertussis toxin, EDTA/EGTA, or antiintegrin antibodies, indicating that the firm adhesion was integrin independent. In summary, FKN mediated the rapid capture, integrin-independent firm adhesion, and activation of circulating leukocytes under flow. Thus, FKN and CX_3CR1 mediate a novel pathway for leukocyte trafficking.

Key words: leukocyte migration • chemokines • cell adhesion • fractalkine • chemokine receptors

The recruitment of leukocytes from the circulation into sites of inflammation is a dynamic process involving multiple regulated steps (1, 2). The initial step involves leukocyte contact and rolling on endothelium, and is predominantly mediated by the selectins (3, 4). Subsequently, activation through pertussis toxin (PTX)[1]-sensitive G protein–coupled receptors leads to upregulation of integrin adhesiveness and activation-dependent stable arrest (5–7). Chemokines are soluble, cell-selective molecules that regulate the activation step of leukocyte migration (5–7).

Fractalkine (FKN) is a unique, transmembrane, mucin/chemokine hybrid molecule expressed on the cell surface of IL-1– and TNF-activated endothelium (8). FKN shares high homology with the CC family of chemokines, but has an insert of three amino acids between the two NH_2-terminal cysteine residues, conferring a CX_3C structural motif (8). In vitro, FKN has been shown to have multiple activities including signal transduction through the PTX-sensitive G protein–coupled receptor CX_3CR1 (also called V28) (9) and adhesion of monocytes, NK cells, and T cells in static binding assays (8, 9). In addition, the soluble form of FKN is chemotactic for monocytes, NK cells, and T lymphocytes (8–10). FKN's unique structure and multiplicity of molecular activities led us to hypothesize that it may regulate several pathways involved in leukocyte migration.

Here, we report that FKN on endothelium interacting with CX_3CR1 on leukocytes can mediate the initial capture, firm adhesion, and activation of circulating leuko-

[1]*Abbreviations used in this paper:* ELC, EBI1 ligand chemokine; FKN, fractalkine; HUVECs, human umbilical vein endothelial cells; IF, immunofluorescence; MCP, monocyte chemotactic protein; PTX, pertussis toxin; SEAP, secreted placental alkaline phosphatase; TARC, thymus and activation regulated chemokine.

A.M. Fong and L.A. Robinson contributed equally to this work.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/10/1413/07 $2.00
Volume 188, Number 8, October 19, 1998 1413–1419
http://www.jem.org

cytes. Thus, we describe a new pathway for leukocyte migration.

Materials and Methods

Cells, Transfections, and Culture Conditions. Resting PBMCs were isolated from whole blood by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, NC) as described previously (11). IL-2–activated PBLs were generated by culturing PBMCs in RPMI containing 10% FBS and 400 U/ml IL-2 (R & D Systems, Inc., Minneapolis, MN) for 5 d as described previously (9) and harvesting the nonadherent cells.

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics Corp. (San Diego, CA), and grown in EGM™ medium (Clonetics Corp.). ECV-304 cells were obtained from the American Type Culture Collection (Rockville, MD) and the generation of FKN-expressing ECV-304 cells (ECV-FKN) has been described previously (9). ECV-304 and ECV-FKN cells were maintained in M199 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS. K562 cells were obtained from the American Type Culture Collection and maintained in RPMI supplemented with 10% FBS. Cells were grown to confluence on 25-mm glass coverslips before use in flow assays. HUVECs used in flow assays were activated with TNF-α (100 ng/ml; R & D Systems, Inc.) for 12 h.

For stable transfection of CX_3CR1 in K562 cells, the expression plasmid pCAGGS-Neo-V28 was transfected into K562 cells as described previously (9). Control neomycin-expressing cells were generated by transfecting pcDNA3.1$^-$ (Invitrogen, Carlsbad, CA) into K562 cells. After selection with 500 μg/ml G418 for 3 wk, drug resistant cells were used unselected. Expression of CX_3CR1 was assessed by RNAse protection using the hCKR-5 and hCKR-6 probe sets (PharMingen, San Diego, CA) as described by the manufacturer. CX_3CR1 is identical to V28 in the probe set. K562 cells expressed no mRNA for chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR5, CCR7, CCR8, CXCR1, CXCR2, CXCR3, CXCR4, and CX_3CR1. Cell surface expression of CX_3CR1 was also tested by indirect immunofluorescence (IF) as described below. To inhibit signal transduction through CX_3CR1, cells were treated with PTX at 500 ng/ml for 30 min at 37°C. To inhibit integrin function, cells were preincubated with 5 mM each of EDTA and EGTA in flow buffer (see below) for 15 min before use in flow assays, and both EDTA and EGTA were added to the flow buffer. Integrins were also blocked by preincubation of K562-neo or K562-CX_3CR1 cells for 10 min with the anti-β1 antibody 4B4 (anti-CD29, IgG1) (12) or the anti-β2 antibody H52 (anti-CD18, IgG1) (13) before use in flow assays. Antiintegrin antibodies were used as ascites fluid diluted 1:100.

Recombinant Proteins. FKN, thymus and activation regulated chemokine (TARC), and EBI1 ligand chemokine (ELC) fused with secreted placental alkaline phosphatase (FKN-SEAP, TARC-SEAP, and ELC-SEAP), and a control SEAP protein were produced as described previously (9, 14, 15). Monocyte chemotactic protein (MCP)-1–SEAP was also produced essentially as described previously (14). In brief, the DNA fragment containing the coding region of MCP-1 was amplified from MCP-1 cDNA by PCR. After digestion with SalI and XbaI, the MCP-1 fragment was subcloned into SalI–XbaI sites of pDREF-SEAP$(His)_6$-Hyg vector in order to express MCP-1 as a soluble fusion protein linked through five amino acid residues (Ser-Arg-Ser-Ser-Gly) with SEAP tagged with six histidine residues [$(His)_6$]. 293/EBNA-1 cells (Invitrogen) were transfected with the expression vector pDREF–MCP-1–SEAP$(His)_6$ using lipofectamine (GIBCO BRL). The transfected cells were incubated for 3–4 d in DMEM supplemented with 10% FBS. The supernatant containing each SEAP$(His)_6$ chimera was collected by centrifugation, filtered (0.45 μm), and stored at 4°C after adding 20 mM Hepes (pH 7.4) and 0.02% sodium azide. 25-mm round glass coverslips were coated with fusion proteins as described previously (9). In brief, 10 μg/ml anti-SEAP antibody 8B6 (Sigma Chemical Co., St. Louis, MO) in buffer (50 mM Tris, pH 9.5) was placed on the coverslip and incubated overnight at 4°C. The coverslips were blocked in PBS with 1% BSA at room temperature for 2 h. After washing, 10 nM FKN-SEAP, SEAP, MCP-1–SEAP, TARC-SEAP, or ELC-SEAP was added and incubated at room temperature for 1 h. Coverslips were washed three times before use in the flow chamber.

Hydrodynamic In Vitro Flow Chamber Experiments. Parallel plate flow chamber assays were performed as described previously (16). In brief, protein-coated glass coverslips were assembled in a parallel plate laminar flow chamber and placed on the stage of an inverted phase-contrast microscope. K562-neo or K562-CX_3CR1 cells were suspended at a density of 10^6 cells/ml in flow buffer (PBS containing 0.75 mM $CaCl_2$, 0.75 mM $MgCl_2$, and 0.5% bovine serum albumin). Cell suspensions were perfused through the chamber for a 5- or 10-min period at a wall shear stress of 0.25 dynes/cm^2 via a syringe pump (Harvard Apparatus, South Natick, MA). At the end of the 5- or 10-min perfusion period, flow buffer was perfused through the chamber at 1.85 dynes/cm^2 for 3 min and at 10 dynes/cm^2 for 3 min, and multiple $\times$100 fields were visualized. The entire experiment was recorded using a CCD video camera (Hitachi Denshi, Ltd., Woodbury, NY) and a Sony SuperVHS video recorder (Sony Electronics, Inc., San Jose, CA). For some experiments, cell suspensions were initially passed through the chamber at a low shear stress (0.25 or 0.5 dynes/cm^2), followed by flow buffer at progressively increasing wall shear stresses of 0.5, 1.0, 1.85, 5.0, 10, and 20 dynes/cm^2 at 2-min intervals to determine the stability of adhesive interactions. The number of adherent cells was determined by analyzing the videotapes and counting 16 fields each with a 0.16-mm^2 area.

Immunophenotypic Analysis. PBMCs and IL-2–activated PBLs were characterized by three-color flow cytometry as described previously (17) on a FACStarPlus® (Becton Dickinson, San Jose, CA) using CD3-FITC, CD4-PE, CD8-Cy5, CD14-PE, CD16/56-PE, and CD19-FITC directly conjugated antibodies generously provided by Coulter Corp. (Hialeah, FL). Resting PBMCs and IL-2–activated PBLs that bound to FKN-SEAP–coated coverslips were characterized by two-color IF using CD3-FITC, CD4-PE, CD8-FITC, CD14-PE, CD16/56-PE, and CD19-FITC antibodies. In brief, coverslips were incubated with 50 μl of mAbs diluted in PBS with 1% BSA for 15 min at room temperature and washed. Coverslips were mounted on glass slides and analyzed by IF on an epifluorescent microscope.

Cell surface expression of FKN was assessed by indirect IF using mAb 1D6 to the mucin domain of FKN as described previously (9). Expression of FKN on HUVECs was determined by two-color flow cytometry using mAb 1D6 and directly conjugated CD31-PE mAb kindly provided by Coulter Corp. (Hialeah, FL) as described previously (17). Cell surface expression of CX_3CR1 was also determined by indirect IF. In brief, 10^6 cells were incubated with 5 nM chemokine-SEAP fusion proteins in PBS containing 0.05% NaN_3 for 30 min at room temperature and washed. After incubation with anti–alkaline phosphatase mAb 8B6 and washing, cells were incubated with fluorescein-conjugated goat anti–mouse Ig (Cappell, Gaithersburg, MD), washed, and analyzed by flow cytometry.

Results

FKN Mediates Leukocyte Capture. To determine whether FKN can mediate capture of free flowing leukocytes under conditions of shear stress encountered in vivo, freshly isolated PBMCs were perfused over immobilized FKN-SEAP fusion proteins at physiologic shear stresses. FKN-SEAP (but not SEAP alone) captured leukocytes at wall shear stresses up to 1.85 dynes/cm^2 (Fig. 1). The capture of leukocytes by FKN was rapid ($<$33 ms), and no rolling was observed. Despite diminished efficiency of capture with increasing wall shear stress, there was no significant difference in the rate of capture of monocytes compared with lymphocytes. Thus, FKN-mediated leukocyte capture at physiologic wall shear stresses.

PBMCs and IL-2–Activated PBLs Firmly Adhere to and Are Activated by FKN. To test if FKN could support the firm adhesion of normal human leukocytes, both resting PBMCs and IL-2–stimulated PBLs were perfused over immobilized SEAP fusion proteins at 0.25 dynes/cm^2 and allowed to capture for 5 min. Firm adhesion was defined as the ability of cells to resist detachment at a wall shear stress of 10 dynes/cm^2. TARC-SEAP and ELC-SEAP were used as controls. CCR4, the receptor for TARC, is selectively expressed on CD4$^+$ T cells (14) and CCR7, the receptor for ELC/MIP-3β, is selectively expressed on activated T and B cells (15, 18, 19). Although resting PBMCs and IL-2–stimulated PBLs adhered firmly to purified FKN-SEAP (Fig. 2 *B*), they were neither captured by (data not shown) nor adherent to (Fig. 2 *B*) control proteins. Virtually all captured cells remained firmly adherent throughout the experiments ($>$10 min in duration). As determined by IF, the majority of firmly bound PBMCs were either CD14$^+$ monocytes (62.5%) or CD8$^+$ T cells (30%; Fig. 2 *C*). Resting NK cells bound to immobilized FKN, but the percentage of bound NK cells varied widely between experiments (2.5–15%). Of the IL-2–activated PBLs bound to FKN, 37.5% were NK cells (CD16$^+$ and/or CD56$^+$), 56.3% were CD8$^+$ T cells, and only 6.3% were CD4$^+$ T cells. Thus, FKN mediated the firm adhesion of resting monocytes, rest-

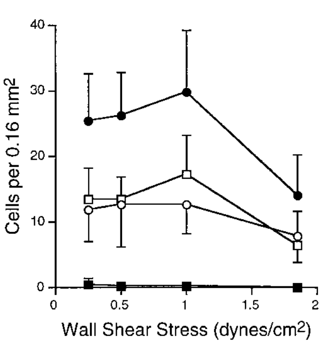

Figure 1. PBMCs are captured by immobilized FKN-SEAP under physiologic wall shear stresses. Freshly isolated PBMCs were perfused over immobilized FKN-SEAP and SEAP fusion proteins at wall shear stresses of 0.25, 0.5, 1, or 1.85 dynes/cm^2 for 5 min. After perfusing the chamber with flow buffer at 10 dynes/cm^2 for 3 min, the number of adherent cells was determined. Also shown are the numbers of PBMCs captured by FKN-SEAP (●), monocytes captured by FKN-SEAP (○), lymphocytes captured by FKN-SEAP (□) and PBMCs captured by control SEAP fusion protein (■). The error bars represent the mean ± SD of the number of cells bound. Data are representative of three independent experiments.

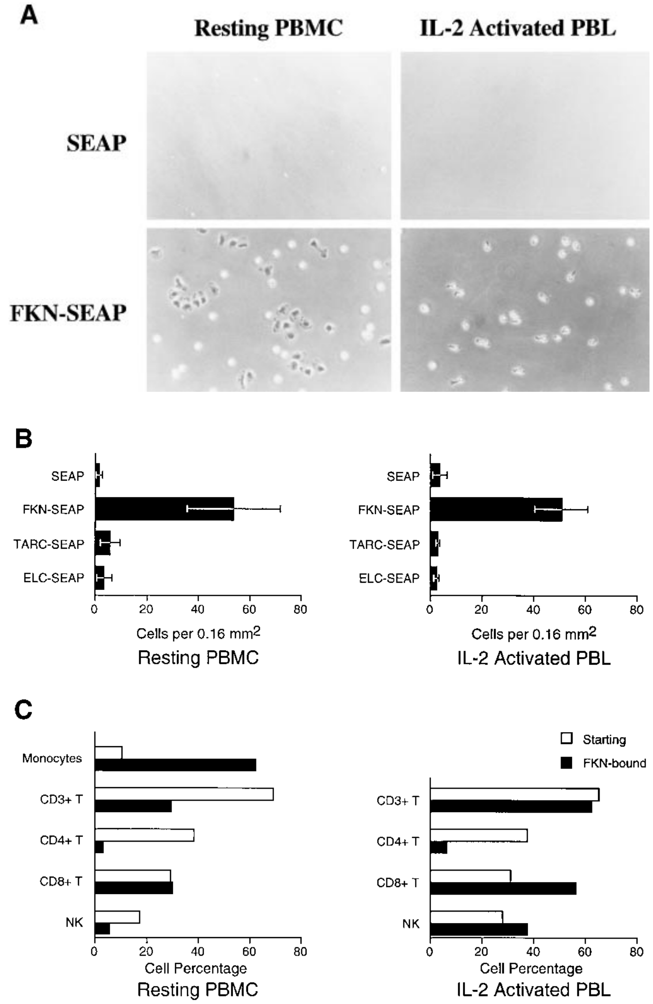

Figure 2. Resting monocytes, resting and IL-2–activated CD8$^+$ T cells, and IL-2–activated NK cells firmly adhere to FKN under flow. Immobilized FKN was tested for its ability to capture and induce the firm adhesion of freshly isolated PBMCs in the parallel plate flow chamber. Cells were perfused over immobilized proteins at 0.25 dynes/cm^2 for 5 min and washed at 10 dynes/cm^2. Resting PBMCs and IL-2–activated PBLs were characterized by three-color flow cytometry and firmly adherent cells were characterized by two-color IF microscopy. (*A*) Photomicrographs of PBMCs and IL-2–activated PBLs bound to immobilized SEAP and FKN-SEAP. (*B*) Numbers of PBMCs and IL-2–activated PBLs remaining bound to immobilized SEAP, FKN-SEAP, TARC-SEAP, and ELC-SEAP at 10 dynes/cm^2. (*C*) Percentages of leukocyte cell types binding to FKN-SEAP under flow. The cell types measured and quantified were: CD14$^+$ monocytes, CD3$^+$CD16/56$^-$ T cells, CD3$^+$CD4$^+$ T cells, CD3$^+$CD8$^+$ T cells, and CD16/56$^+$ NK cells. The percentage of leukocyte subsets in the starting material as measured by multicolor flow cytometry is depicted by white bars, and the percentage of cell subsets in the FKN-bound fraction as measured by two-color IF microscopy is depicted by black bars. Leukocyte subsets from both resting PBMCs and IL-2 activated PBLs bound firmly and specifically to FKN-SEAP under flow. FKN preferentially bound resting monocytes, resting and IL-2–activated CD8$^+$ T cells, and IL-2–activated NK cells. The majority of FKN-bound, IL-2–activated PBLs formed pseudopods. Data are representative of three experiments performed. The error bars represent the mean ± SD of the number of cells bound.

ing and activated $CD8^+$ T cells, and resting and activated $CD16/56^+$ NK cells under physiologic flow conditions.

Soluble FKN-SEAP fusion proteins bind to and transduce signals through CX_3CR1 to mediate a Ca^{2+} flux and chemotaxis (9). Immobilized FKN induced the spreading of bound monocytes (Fig. 2 *A*), but had no effect on the shape of resting PBLs. Furthermore, FKN induced pseudopod formation in the majority of IL-2–activated PBLs. Thus, FKN can activate resting monocytes and IL-2–activated PBLs.

CX_3CR1 Is the Leukocyte Receptor for FKN-mediated Capture and Firm Adhesion. Normally, CX_3CR1 mRNA is expressed primarily by $CD16^+$ NK cells and at low levels by $CD8^+$ T cells and $CD14^+$ monocytes (9). As for other chemokine receptors, IL-2 enhances CX_3CR1 expression on both $CD4^+$ and $CD8^+$ T cells (9). Since these were the very cell types that were captured by and firmly adherent to purified FKN before becoming activated, FKN-CX_3CR1 interactions probably mediated these events. Thus, the ability of FKN-transfected ECV-304 transformed endothelial cells (ECV-FKN; Fig. 3) to capture and induce stable arrest of CX_3CR1-transfected K562 erythroleukemia cells (K562-CX_3CR1; Fig. 3) was examined. CX_3CR1-transfected and control cells were allowed to bind to ECV-FKN cells at 0.25 dynes/cm^2 for 10 min and exposed to increasing shear stresses up to 20 dynes/cm^2 (Fig. 4 *A*). K562-CX_3CR1 cells remained bound to ECV-FKN monolayers at wall shear stresses up to 10 dynes/cm^2, and began to release at 20 dynes/cm^2. Thus, FKN on endothelium interacts with CX_3CR1 on leukocytes to mediate their capture and firm adhesion.

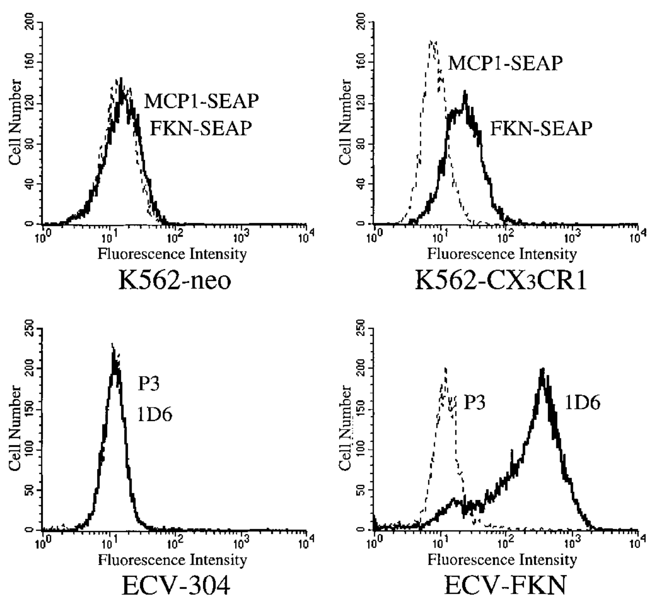

Figure 3. Expression of FKN in ECV-304 cells and CX_3CR1 in K562 cells. The top shows histograms depicting the binding of anti-FKN mAb 1D6 (*solid lines*) and control mAb P3 (*dashed lines*) to untransfected ECV-304 cells and to transfected ECV-FKN cells. The bottom shows histograms depicting the binding of FKN-SEAP (*solid lines*) and MCP-1–SEAP (*dashed lines*) to control K562-neo cells and transfected K562-CX_3CR1 cells.

FKN-mediated Firm Adhesion Is Integrin Independent. To test the role of integrins and signal transduction through CX_3CR1, the effect of PTX, EDTA/EGTA, and antiintegrin antibodies on FKN-mediated firm adhesion was tested. Neither PTX (known to inhibit Ca^{2+} mobilization and chemotaxis in CX_3CR1 expressing cells; 9) nor EDTA/EGTA (to disrupt integrin function; 20, 21) had an effect on FKN-mediated capture or firm adhesion (Fig. 4 *A*). Function-blocking antiintegrin mAbs to $\beta1$ (4B4; 12) and $\beta2$ (H52; 13) subunits also had no effect on the firm adhesion of K562-CX_3CR1 cells to ECV-FKN cells under flow (Fig. 4 *B*). Therefore, FKN-mediated capture and firm adhesion occurred independently of integrin activation and PTX-sensitive signal transduction through CX_3CR1.

To further confirm the specificity of FKN-CX_3CR1–mediated adhesive interactions, the ability of immobilized FKN-SEAP to induce the firm adhesion of CX_3CR1-expressing versus nonexpressing cells was compared. FKN-SEAP, but not SEAP or MCP-1–SEAP, could support firm adhesion of K562-CX_3CR1 under physiologic wall shear stresses (Fig. 4 *C*). Thus, FKN–CX_3CR1 interactions alone are sufficient to mediate firm adhesion under flow.

TNF-α–activated HUVECs Support FKN-mediated Leukocyte Arrest. Although the above studies indicate that FKN can support firm adhesion of leukocytes under conditions of flow, they were performed with either purified fusion proteins or with transfected cells. To determine whether the FKN interaction is physiologically relevant, the level of FKN expression on TNF-activated primary HUVECs was tested. TNF-α induced the expression of FKN on the surface of a subset of $CD31^+$ HUVECs (Fig. 5, *A* and *B*). FKN expression was induced by 1 h, and was maximal by 6–12 h, in a dose-dependent manner with peak expression at a dose of 100 ng/ml TNF-α (data not shown). Subsets of TNF-activated HUVECs and ECV-FKN cells expressed similar levels of FKN (Fig. 5 *C*), indicating that ECV-FKN cells express physiologically relevant levels of FKN. To further confirm this point, the ability of TNF-activated HUVECs to induce stable arrest of K562-CX_3CR1 cells was tested. CX_3CR1-transfected and control cells were allowed to bind to resting and TNF-activated HUVECs at 0.5 dynes/cm^2 for 5 min and were exposed to increasing shear stresses up to 20 dynes/cm^2 (Fig. 5, *D* and *E*). K562-CX_3CR1 cells did not bind to unactivated HUVECs (Fig. 5 *D*), but did bind to a subset of TNF-activated HUVECs (data not shown). Although some K562-CX_3CR1 cells began to release from TNF-activated HUVECs at a shear stress of 5 dynes/cm^2, a subset of cells remained bound at wall shear stresses up to 20 dynes/cm^2 (Fig. 5 *E*). Thus, HUVECs can be induced to express sufficient levels of FKN to mediate leukocyte stable arrest.

Discussion

In the current models of leukocyte migration, chemokines and their receptors transduce signals to the rolling leukocyte to induce cell arrest and firm adhesion by activating

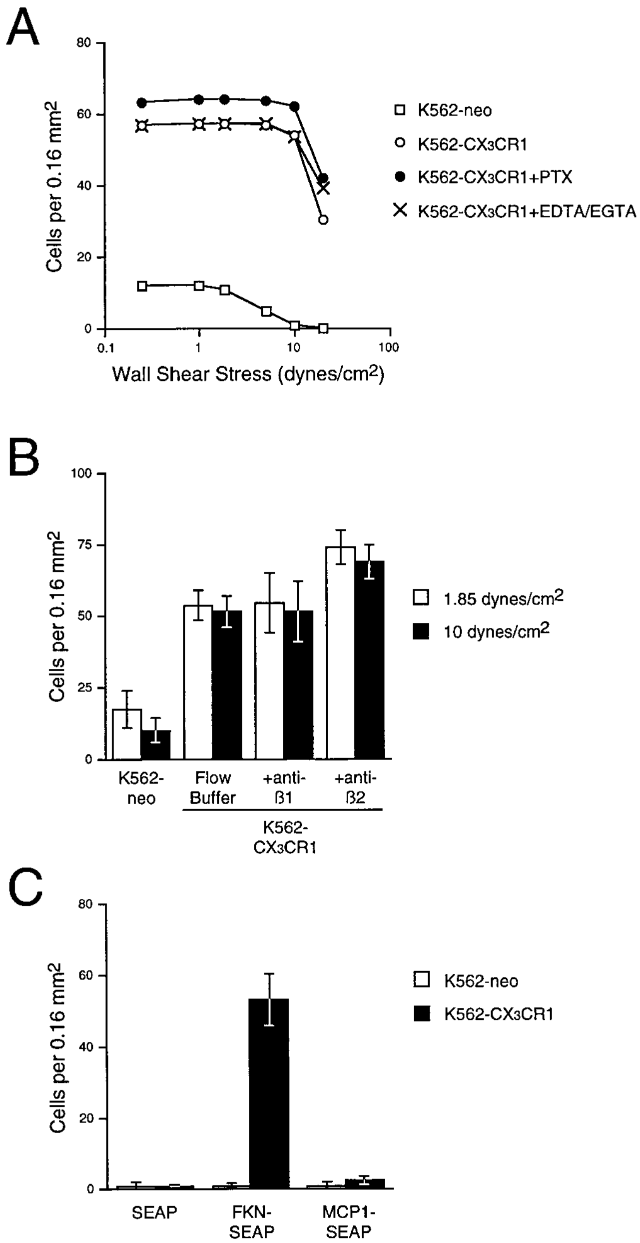

Figure 4. Integrin-independent firm adhesion of K562-CX_3CR1 cells to ECV-FKN cells and immobilized FKN-SEAP under physiologic flow conditions. (*A*) K562-CX_3CR1 cells remain firmly adherent to ECV-FKN cells under physiologic wall shear stresses, and this FKN-mediated firm adhesion is PTX insensitive and integrin independent. K562-neo cells and K562-CX_3CR1 cells (± EDTA/EGTA and ± PTX treatment) were perfused over ECV-FKN monolayers for 10 min at 0.25 dynes/cm^2 and subjected to increasing wall shear stresses up to 20 dynes/cm^2. Shown are the numbers of firmly adherent cells at various shear stresses. (*B*) Anti-β1 and β2 integrin mAbs have no effect on the firm adhesion of K562-CX_3CR1 cells to ECV-FKN cells. The numbers of adherent K562-neo and K562-CX_3CR1 cells at 1.85 dynes/cm^2 and 10 dynes/cm^2 in the absence and presence of anti-β1 and β2 integrin mAbs are shown. (*C*) K562-CX_3CR1 cells bind to immobilized FKN under flow. FKN-SEAP, MCP-1–SEAP, and SEAP fusion proteins were immobilized by binding to glass coverslips coated with anti–alkaline phosphatase mAbs and were tested for their ability to support firm adhesion of K562-CX_3CR1 cells under flow. Cells were perfused over immobilized SEAP fusion proteins for 10 min at 0.25 dynes/cm^2, and exposed to a wall shear stress of 10 dynes/cm^2. Shown are the numbers of K562 and K562-CX_3CR1 cells remaining bound to immobilized chemokine-SEAP fusion proteins at 10 dynes/cm^2. Error bars represent the mean ± SD. Data are representative of three experiments performed.

the adhesive capacity of integrins (1, 2, 5–7). This study demonstrates new roles for chemokines and their receptors in leukocyte migration, and describes a novel mechanism of leukocyte capture, firm adhesion, and activation mediated by the interactions of FKN with CX_3CR1.

We have shown that FKN alone on the endothelium can mediate leukocyte capture and firm adhesion of monocytes, $CD8^+$ T cells, and $CD16/56^+$ NK cells. Although CX_3CR1 mRNA is also expressed by IL-2 activated $CD4^+$ T cells (9), they did not firmly adhere to immobilized FKN in these studies. The reason for this is not clear. Although CX_3CR1 on the leukocyte can serve as the receptor for FKN to mediate all of these functions, FKN may also interact with other receptors. In the current models of leukocyte recruitment, selectins capture circulating leukocytes in the primary adhesion step (1–4). The selectins recognize distinct, but closely related, sialylated carbohydrates on their receptors (22–25). For example, L-selectin recognizes mucin-like molecules with extensive *O*-glycosylation (22–26). Since FKN has a mucin-like domain with many potential *O*-glycosylation sites, it may interact with selectins. However, in this study, selectins were not necessary for FKN-CX_3CR1 adhesion since K562 cells do not express detectable levels of L-, E-, or P-selectin (27 and data not shown). Thus, in this system, leukocyte capture by FKN was selectin independent.

Leukocyte firm adhesion, in the multistep model of leukocyte migration, requires activation by chemokines and is mediated primarily by the integrin family of adhesion molecules and their receptors (1, 2). In this study, FKN interactions with CX_3CR1 were sufficiently strong to mediate firm adhesion under conditions of high wall shear stresses (up to 20 dynes/cm^2), without the involvement of integrins or other adhesion molecules. Combined with the ability of FKN to capture free-flowing leukocytes at physiologic wall shear stresses (1.85 dynes/cm^2), this indicates that FKN and CX_3CR1 mediate leukocyte capture and firm adhesion under flow. However, FKN and CX_3CR1 may also act in concert with other effectors of the classical multistep pathways of leukocyte migration.

FKN appears to be unique amongst the chemokines in its ability to induce leukocyte firm adhesion. Neither TARC nor ELC fusion proteins were able to induce the firm adhesion of flowing PBMC. FKN naturally has a mucin stalk, whereas the other chemokines do not. The negative results with TARC and ELC may have been due to the fact that they lack mucin stalks. The extension provided by the mucin stalk may be a key factor in allowing FKN to mediate firm adhesion.

FKN expression is induced on primary cultures of human umbilical vein and pulmonary artery endothelium by the proinflammatory cytokines IL-1 and TNF-α (8, 10).

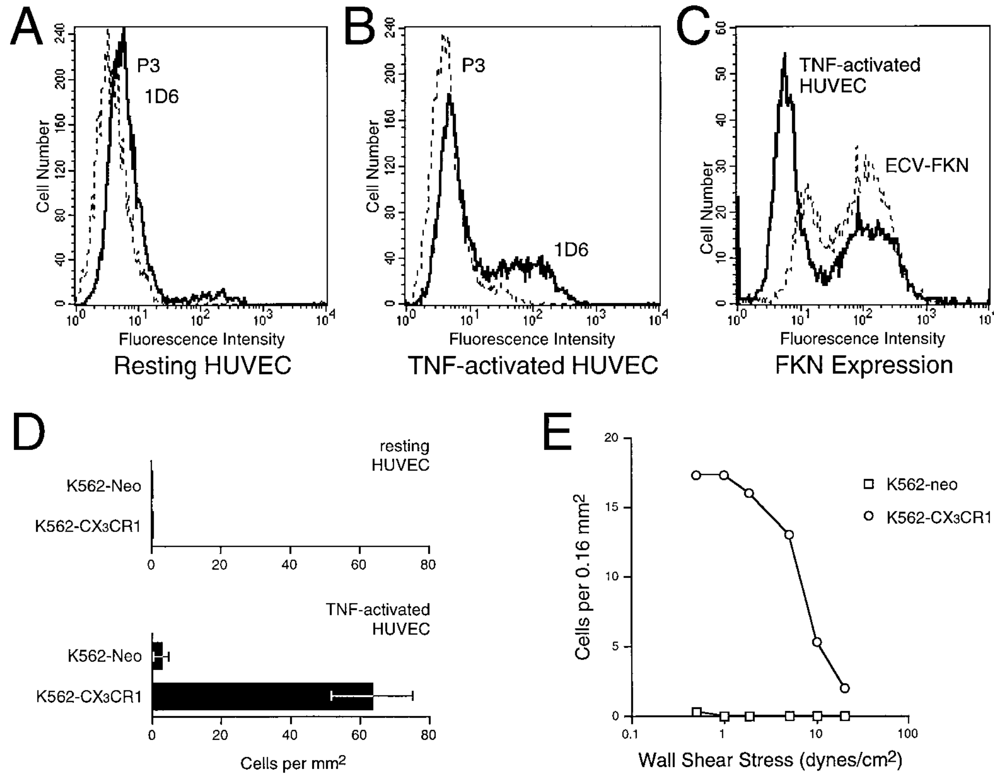

Figure 5. Expression of FKN by TNF-activated HUVECs and their ability to support arrest of K562-CX_3CR1 cells under flow conditions. FKN expression on HUVECs, either resting (*A*) or stimulated with 100 ng/ml TNF-α for 12 h (*B*), was measured by IF staining with mAb 1D6. Shown are histograms of the reactivity of anti-FKN (1D6) and control (P3) mAbs. Cells were also counterstained with anti-CD31-PE to ensure they were endothelial cells. FKN was expressed on a subset of $CD31^+$ TNF-activated HUVECs. (*C*) Comparison of the level of FKN expression by TNF-activated HUVECs and ECV-FKN cells. Shown are histograms of the reactivity of mAb 1D6 with 12-h TNF-activated HUVECs and ECV-FKN cells. (*D*) K562-CX_3CR1 cells bind to TNF-activated HUVECs but not to resting HUVECs. K562-neo cells and K562-CX_3CR1 cells were perfused over HUVECs and TNF-activated HUVEC monolayers for 5 min at 0.5 dynes/cm^2, and subjected to a wall shear stress of 1.85 dynes/cm^2. Shown are the numbers of firmly adherent cells to HUVECs and TNF-activated HUVECs at 1.85 dynes/cm^2. Error bars represent the mean ± SD. (*E*) K562-CX_3CR1 cells bind firmly to TNF-activated HUVECs at high wall shear stresses. K562-neo cells and K562-CX_3CR1 cells were perfused over TNF-activated HUVECs monolayers for 5 min at 0.5 dynes/cm^2 and were subjected to increasing wall shear stresses up to 20 dynes/cm^2. Shown are the numbers of firmly adherent cells to TNF-activated HUVECs at various shear stresses from a representative experiment. All data are representative of at least three independent experiments.

Further, the mouse homologue of FKN, neurotactin, is expressed at low levels on brain endothelium in normal mice and upregulated on endothelium in inflamed brain in allergic encephalomyelitis (10), suggesting that the FKN pathway may be functional in leukocyte trafficking to inflamed brain. Although trafficking of lymphocytes to brain during peak inflammation in Sindbis virus–infected mice was blocked by antibodies to the β2 integrin LFA-1, lymphocyte entry into the brain during the early inflammatory response was not affected by antibodies to β1 integrins, VLA-4, or CD44 (28), indicating that the lymphocytes migrating to brain during early Sindbis virus infection used a different pathway of leukocyte migration. It is intriguing to hypothesize that the FKN pathway of circulating leukocyte adhesion to endothelium may play an important role in the accumulation of leukocytes on the endothelium of inflamed brain, facilitating subsequent diapedesis and migration.

In summary, we have shown that FKN can capture circulating leukocytes and induce their firm adhesion and activation. Furthermore, FKN-mediated firm adhesion is dependent on neither chemokine receptor signaling nor on integrins or other cell adhesion molecules, suggesting an alternate and novel regulatory mechanism for leukocyte trafficking. This novel FKN-mediated pathway may be particularly relevant for the recruitment of monocytes, $CD8^+$ T cells, and NK cells to sites of inflammation.

We thank Leona P. Whichard, Jonathan Baron, and Dawn M. Jones for their technical assistance. We also thank Barton F. Haynes and Michael Krangel for helpful discussion and a critical review of this manuscript.

This work was supported by National Institutes of Health grants AR-39162, AI-26872, CA-54464, and HL-50985 and by the Shionogi Institute for Medical Science. L.A. Robinson is supported by the Pediatric Scientist Development Program through a grant from St. Jude Children's Research Hospital.

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Received for publication 17 March 1998 and in revised form 31 July 1998.

References

1. Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration, the multistep paradigm. *Cell.* 76:301–314.
2. Butcher, E.C., and L.J. Picker. 1996. Lymphocyte homing and homeostasis. *Science.* 272:60–66.
3. Ley, K., and T.F. Tedder. 1995. Leukocyte interactions with vascular endothelium: new insights into selectin mediated attachment and rolling. *J. Immunol.* 155:525–528.
4. Tedder, T.F., D.A. Steeber, A. Chen, and P. Engel. 1995. The selectins: vascular adhesion molecules. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 9:866–873.
5. Bargatze, R.F., and E.C. Butcher. 1993. Rapid G protein–regulated activation event involved in lymphocyte binding to high endothelial venules. *J. Exp. Med.* 178:367–372.
6. Honda, S., J.J. Campbell, D.P. Andrew, B. Engelhardt, B.A. Butcher, R.A. Warnock, R.D. Ye, and E.C. Butcher. 1994. Ligand-induced adhesion to activated endothelium and to vascular cell adhesion molecule-1 in lymphocytes transfected with the N-formyl peptide receptor. *J. Immunol.* 152:4026–4035.
7. Campbell, J.J., J. Hedrick, A. Zlotnik, M.A. Siani, D.A. Thompson, and E.C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science.* 279:381–384.
8. Bazan, J.F., K.B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D.R. Greaves, A. Zlotnik, and T.J. Schall. 1997. A new class of chemokine with a CX_3C motif. *Nature.* 385: 640–644.
9. Imai, T., K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T.J. Schall, and O. Yoshie. 1997. Identification and molecular characterization of fractalkine receptor CX_3CR1, which mediates both leukocyte migration and adhesion. *Cell.* 91:1–20.
10. Pan, Y., C. Lloyd, H. Zhou, S. Dolich, J. Deeds, J.A. Gonzalo, J. Vath, M. Gosselin, J. Ma, B. Dussault, et al. 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature.* 387:611–617.
11. Denning, S.M., D.T. Tuck, K.H. Singer, and B.F. Haynes. 1987. Human thymus epithelial cells function as accessory cells for autologous mature thymocyte activation. *J. Immunol.* 138:680–686.
12. Nojima, Y., M.J. Humphries, A.P. Mould, A. Komoriya, K.M. Yamada, S.F. Schlossman, and C. Morimoto. 1990. VLA-4 mediates CD3-dependent $CD4^+$ T cell activation via the CS1 alternatively spliced domain of fibronectin. *J. Exp. Med.* 172:1186–1192.
13. Hildreth, J.E.K., and J.T. August. 1985. The human lymphocyte function-associated (HLFA) antigen and a related macrophage differentiation antigen (Hmac-1): functional effects of subset-specific monoclonal antibodies. *J. Immunol.* 134: 3272–3280.
14. Imai, T., T. Yoshida, M. Baba, M. Nishimura, M. Kakizaki, and O. Yoshie. 1996. Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein-Barr virus vector. *J. Biol. Chem.* 271:21514–21521.
15. Yoshida, R., T. Imai, K. Hieshima, J. Kasuda, M. Baba, M. Kitaura, M. Nishimura, M. Kakizaki, H. Nomiyama, and O. Yoshie. 1997. Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *J. Biochem.* 272:13803–13809.
16. Luscinskas, F.W., G.S. Kansas, H. Ding, P. Pizcueta, B. Schleiffenbaum, T.F. Tedder, and M.A. Gimbrone, Jr. 1994. Monocyte rolling, arrest and spreading on IL-4–activated vascular endothelium under flow is mediated via sequential action of L-selectin, β1-integrins, and β2-integrins. *J. Cell Biol.* 125:1417–1427.
17. Bruggers, C.S., D.D. Patel, R.M. Scearce, L.P. Whichard, B.F. Haynes, and K.H. Singer. 1995. AD2, a human molecule involved in the interaction of T cells with epidermal keratinocytes and thymic epithelial cells. *J. Immunol.* 154:2012–2022.
18. Birkenbach, M., K. Josefsen, R. Yalamanchili, G. Lenoir, and E. Kieff. 1993. Epstein-Barr virus-induced genes: first lymphocyte-specific G-protein–coupled peptide receptors. *J. Virol.* 67:2209–2220.
19. Rossi, D.L., A.P. Vicari, K. Franz-Baron, T.K. McClanaham, and A. Zlotnik. 1997. Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3α and MIP-3β. *J. Immunol.* 158:1033–1036.
20. Humphries, M.J. 1996. Integrin activation: the link between ligand binding and signal transduction. *Curr. Opin. Cell Biol.* 8:632–640.
21. Tozer, E.C., P.E. Hughes, and J.C. Loftus. 1996. Ligand binding and affinity modulation of integrins. *Biochem. Cell Biol.* 74:785–798.
22. Lasky, L.A. 1992. Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science.* 258:964–969.
23. Rosen, S.D. 1993. Cell surface lectins in the immune system. *Semin. Immunol.* 5:237–247.
24. McEver, R.P. 1994. Selectins. *Curr. Opin. Immunol.* 6:75–84.
25. Varki, A. 1994. Selectin ligands. *Proc. Natl. Acad. Sci. USA.* 91:7390–7397.
26. Fuhlbrigge, R.C., R. Alon, K.D. Puri, J.B. Lowe, and T.A. Springer. 1996. Sialylated, fucosylated ligands for L-selectin expressed on leukocytes mediate tethering and rolling adhesions in physiologic flow conditions. *J. Cell Biol.* 135:837–848.
27. Shaw, S., G.G. Luce, W.R. Gilks, K. Anderson, K. Ault, B.S. Bochner, L. Boumsell, S.M. Denning, E.G. Engleman, T. Fleisher, et al. 1995. Leukocyte differentiation antigen database. *In* Leukocyte Typing V. S.F. Schlossman, L. Boumsell, W. Gilks, J.M. Harlan, T. Kishimoto, C. Morimoto, J. Ritz, S. Shaw, R. Silverstein, T. Springer, et al., editors. Oxford University Press Inc., New York. 16–198.
28. Irani, D.N., and D.E. Griffin. 1996. Regulation of lymphocyte homing into the brain during viral encephalitis at various stages of infection. *J. Immunol.* 156:3850–3857.