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Enhanced Recruitment of CX3CR1+ T Cells by Mucosal Endothelial Cell–Derived Fractalkine in Inflammatory Bowel Disease

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Background & Aims: **Fractalkine (FKN/CX3CL1) is a unique chemokine combining adhesive and chemotactic properties. We investigated FKN production by the mucosal microvasculature in inflammatory bowel disease (IBD), its capacity for leukocyte recruitment into the gut, and the number of CX3CR1+ cells in the circulation and mucosa of IBD patients.** ***Methods:*** **The expression of FKN by human intestinal microvascular endothelial cells (HIMECs) and CX3CR1 by circulating cells was evaluated by flow cytometry, and mucosal CX3CR1+ cells were enumerated by immunohistochemistry. The capacity of FKN to mediate leukocyte binding to HIMECs was assessed by immunoblockade, and to induce HIMEC transmigration by a Transwell system.** ***Results:*** **The spontaneously low HIMEC FKN expression was enhanced markedly by tumor necrosis factor-α plus interferon-γ stimulation, or direct leukocyte contact. This effect was significantly stronger in IBD than control HIMECs. Up-regulation of HIMEC FKN expression was dependent on p38 and extracellular signal-regulated kinase phosphorylation, as was abrogated by selective mitogen-activated protein kinase inhibitors. Circulating T cells contained significantly higher numbers of CX3CR1+ cells in active IBD than inactive IBD or healthy subjects, and IBD mucosa contained significantly more CX3CR1+ cells than control mucosa. Antibody-blocking experiments showed that FKN was a major contributor to T- and monocytic-cell adhesion to HIMECs. Finally, FKN enhanced the expression of active β1 integrin on leukocytes and mediated leukocyte HIMEC transmigration.** ***Conclusions:*** **In view of the capacity of FKN to mediate leukocyte adhesion, chemoattraction, and transmigration, its increased production by mucosal microvascular cells and increased numbers of circulating and mucosal CX3CR1+ cells in IBD point to a significant role of FKN in disease pathogenesis.**

The migration of leukocytes from the vascular compartment into sites of inflammation is a dynamic process mediated by a complex series of interactions between leukocytes and the endothelium. Margination and rolling of leukocytes along the endothelial surface are followed by leukocyte activation, firm adhesion, and, finally, transmigration into the interstitium.[1] These cell–cell interactions are finely tuned by several cell adhesion molecules expressed on the surface of both endothelial cells and leukocytes.[2,3] Leukocyte migration also depends on the existence of a chemoattractant gradient across the blood vessel wall created by a large family of molecules known as *chemokines*.[4] Under physiologic conditions these substances selectively target various types of leukocytes, a process that ensures an appropriate distribution of discrete leukocyte subsets in all tissues and organs.[5] Under inflammatory conditions, including inflammatory bowel disease (IBD), altered levels and types of chemokines lead to improper leukocyte distribution and accumulation, accounting for the presence of inflammatory infiltrates in the target tissues.[6]

Unlike most chemokines that are small and exclusively secreted, fractalkine (FKN/CX3CL1) is a large transmembrane molecule consisting of a chemokine domain bound to the cell surface by a long mucin-like stalk.[7] Transfection studies have revealed that the key function of the mucin portion of FKN is to mediate cell adhesion.[8,9] This portion extends the chemokine domain away from the endothelial cell surface and enables its presentation to leukocytes, thus directly supporting their adhesion to the endothelium.[8,9] In addition to this membrane-bound form, a soluble form of FKN is secreted through cleavage at a membrane-proximal site,[10,11] and this soluble form shows efficient chemotactic activity for multiple cell types.[12] Therefore, because of its unique properties, FKN combines a dual function as an adhesion and chemotactic molecule.[13] FKN is produced by a large number of cell

Abbreviations used in this paper: Ab, antibody; ERK, extracellular signal-regulated kinase; FKN, fractalkine; HIMEC, human intestinal microvascular endothelial cell; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; IL, interleukin; MAP, mitogen-activated protein; MCP-1, monocyte chemoattractant protein 1; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule 1.

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types, including neurons,[14] glomeruli,[15] and epithelial cells,[16] but is particularly prominent in endothelial cells.[17,18]

In addition to its distinctive structure and function, FKN also differs from other chemokines because it has a single receptor, CX3CR1, which is expressed primarily on the surface of monocytes, natural killer cells, and CD8+ T cells, and mediates both adhesive and chemoattractive functions.[19,20] Similar to other chemokine receptors, CX3CR1 signals through pertussis toxin–sensitive G proteins to induce migration but not adhesion.[21]

Several reports have indicated that FKN is involved in the pathogenesis of numerous inflammatory conditions, including atherosclerosis,[22] glomerulonephritis,[23] cardiac allograft rejection,[24] psoriasis,[18,25] rheumatoid arthritis,[26] systemic sclerosis,[27] and primary biliary cirrhosis[28]; FKN also may play a role in IBD. Immunohistochemistry reveals increased FKN expression in Crohn's disease (CD) mucosa,[16] and studies with transformed epithelial cell lines have shown that CX3CR1 mediates epithelial cell activation and neutrophil migration.[29] In addition, a recent study showed that lamina propria dendritic cells require CX3CR1 to form transepithelial dendrites necessary to sample luminal antigens,[30] an event potentially relevant to intestinal inflammation. These studies have investigated the pathophysiologic role of FKN primarily in regard to epithelial cell function. The aim of this study was to investigate whether FKN contributes to IBD pathogenesis by mediating leukocyte adhesion to, or transmigration through, mucosal microvascular cells, or both.

Materials and Methods

Reagents, Antibodies, and Cell Lines

The following antibodies (Abs) were purchased from R&D Systems (Minneapolis, MN): blocking anti-FKN, biotinylated anti-FKN, anti-intercellular adhesion molecule 1 (ICAM-1) (CD54a), and anti–vascular cell adhesion molecule 1 (VCAM-1) (CD106). Antibodies against CX3CR1 were purchased from Torrey Pines Biolabs (Houston, TX) and from Abcam (Cambridge, MA). Phycoerythrin (PE)-conjugated anti-goat Ab was purchased from Caltag (Burlingame, CA) and fluorescein isothiocyanate–conjugated anti-rabbit Ab was purchased from BD Biosciences (San Jose, CA). Goat, sheep, and rabbit immunoglobulin (Ig) isotype controls were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human FKN was purchased from R&D Systems. PE-conjugated anti-CD3, PerCp-conjugated anti-CD4, and PE-conjugated anti-CD8 were purchased from Dako (Carpinteria, CA), and PE-conjugated anti-CD14 was purchased from BD Biosciences.

CD40L-positive D1.1 T cells[31] and CD40L-negative Jurkat T cells were obtained from the American Type Culture Collection (Rockville, MD), cultured in RPMI 1640 with 10% fetal bovine serum, and fed twice weekly. The monocytic cell line THP1 also was obtained from the American Type Culture Collection and cultured in medium containing antibiotics, 1% L-glutamine, 1% nonessential amino acids, 1% sodium piruvate, and 5×10^{-5} mol/L mercaptoethanol.

Isolation and Culture of Human Intestinal Microvascular Endothelial Cells and Peripheral Blood Mononuclear Cells

The isolation of human intestinal microvascular endothelial cells (HIMECs) was performed as previously reported.[32] Briefly, HIMECs were obtained from surgical specimens of patients with CD, ulcerative colitis (UC), and, as control, from normal areas of the intestine of patients admitted for bowel resection because of colon cancer, polyps, or diverticulosis. HIMECs were isolated by enzymatic digestion of intestinal mucosal strips followed by gentle compression to extrude endothelial cell clumps that adhered to fibronectin-coated plates, and subsequently were cultured in MCDB131 medium (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum, antibiotics, heparin, and endothelial cell growth factor. Cultures of HIMECs were maintained at 37°C in 5% CO_2, fed twice a week, and split at confluence. HIMECs were used between passages 3 and 12.

Blood samples were collected from patients with active and inactive UC and CD, and healthy subjects. All diagnoses were confirmed by clinical, radiologic, endoscopic, and histologic criteria, and clinical disease activity was assessed by the Harvey–Bradshaw Activity Index[33] and the Colitis Activity Index.[34] Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood, using a Ficoll–Hypaque density gradient, as previously reported.[35] This project was approved by the Institutional Review Board of University Hospitals of Cleveland.

Stimulation of HIMECs With Cytokines, Leukocytes, and CD40L

To study FKN expression on HIMEC surfaces, cells were plated on fibronectin-coated 24-well cluster plates at a density of 10^5 HIMECs/well. The effect of cytokines on FKN expression was assessed by stimulation of confluent HIMEC monolayers with 100 U/mL of interleukin (IL)-1β, 100 U/mL of tumor necrosis factor (TNF)-α, 500 U/mL of interferon (IFN)-γ, 20 U/mL of IL-4, 20 U/mL of IL-10, or a combination of the previous cytokines, for periods of time ranging from 4 to 72 hours. To evaluate whether the mitogen-activated protein (MAP) kinases p38 and ERK-1 and ERK-2 were involved in FKN up-regulation, additional experiments were performed in which HIMEC monolayers were pretreated for 1 hour with the p38-specific inhibitor SB203580 (Calbiochem, San Diego, CA) or the ERK-1– and ERK-2–specific inhibitor PD98059 (Calbiochem), both at 10 μg/mL,

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