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Fractalkine Is an Epithelial and Endothelial Cell-Derived Chemoattractant for Intraepithelial Lymphocytes in the Small Intestinal Mucosa


Fractalkine is a unique chemokine that combines properties of both chemoattractants and adhesion molecules. Fractalkine mRNA expression has been observed in the intestine. However, the role of fractalkine in the healthy intestine and during inflammatory mucosal responses is not known. Studies were undertaken to determine the expression and function of fractalkine and the fractalkine receptor CX3CR1 in the human small intestinal mucosa. We identified intestinal epithelial cells as a novel source of fractalkine. The basal expression of fractalkine mRNA and protein in the intestinal epithelial cell line T-84 was under the control of the inflammatory mediator IL-1β. Fractalkine was shed from intestinal epithelial cell surface upon stimulation with IL-1β. Fractalkine localized with caveolin-1 in detergent-insoluble glycolipid-enriched membrane microdomains in T-84 cells. Cellular distribution of fractalkine was regulated during polarization of T-84 cells. A subpopulation of isolated human intestinal intraepithelial lymphocytes expressed the fractalkine receptor CX3CR1 and migrated specifically along fractalkine gradients after activation with IL-2. Immunohistochemistry demonstrated fractalkine expression in intestinal epithelial cells and endothelial cells in normal small intestine and in active Crohn’s disease mucosa. Furthermore, fractalkine mRNA expression was significantly up-regulated in the intestine during active Crohn’s disease. This study demonstrates that fractalkine–CX3CR1-mediated mechanism may direct lymphocyte chemoattraction and adhesion within the healthy and diseased human small intestinal mucosa. The Journal of Immunology, 2000, 164: 3368–3376.

The epithelium layer forms the interface between the external and the internal environments of the gastrointestinal tract. The intestine-associated lymphoid tissue serves as an immunological barrier against a wide range of infectious agents. It has become clear that intestinal mucosal barrier function is regulated by interaction of intestinal epithelial cells with intestinal lymphocytes.

Fractalkine (Neurotactin/NKAF) is a novel chemokine that is characterized by a CX3C spacing of the cysteine motif and has a unique membrane-bound structure (1–3). The domain organization includes a 37-residue intracellular tail, a short membrane-spanning region, and an extended, mucin-like stalk, which presents the N-terminal chemokine domain at the cell surface. This unique architecture represents a novel mechanism of cell adhesion that differs from soluble CXC, CC, and C chemokines, which have heparin-binding domains that may promote immobilization by cell surface proteoglycans or extracellular matrix components (4). Also, the juxtamembrane part of fractalkine contains a dibasic motif (Thr-Arg-Arg-Gln) that can be cleaved, to yield chemoattractant soluble fractalkine. Thus, fractalkine represents a new class of chemokines that exhibits properties of both traditional chemokines and adhesion molecules (3). Fractalkine binds specifically to the chemokine receptor V28, now termed CX3CR1 (5, 6). CX3CR1 appears to be a highly specific receptor for fractalkine, since no additional chemokines that bind or compete for the receptor have been identified, with the exception of the HHV-8-derived virus chemokine vMIP-II (6–8). Surface expression of the CX3CR1 has been demonstrated in NK cells, monocytes, CD8+ T cells, and to a lesser extent in CD4+ T cells (5).

Intestinal intraepithelial lymphocytes (IEL) are thought to represent a first-line defense against pathogens and may be critical for intestinal integrity. Most IEL express the CD8+ phenotype with either CD8 heterodimeric αβ-chains or homodimeric αα-chains. IEL exhibit a number of important immunological functions, including cytotoxic activity (9, 10); secretion of cytokines, including IL-2, IL-3, IL-5, TNF-α, TGF-β, and IFN-γ (11); and may regulate epithelial cell proliferation and regeneration (12). In addition, intestinal epithelial cells are capable of expressing MHC class II molecules and can act as effective APCs that can induce and activate T cells in vitro (13–15). Furthermore, intestinal epithelial cells may selectively activate T cells with suppressor function via CD1d molecule, which subsequently down-regulate immune responses (16–20). Intestinal epithelial cells also express IL-15 (21),

4 Abbreviations used in this paper: IEL, intestinal intraepithelial lymphocyte; LPL, lamina propria lymphocyte.
which is a strong inducer of iIEL activation and proliferation (22). Together, these observations suggest that intestinal epithelial cells can provide regulatory signals for T cells to maintain appropriate homeostasis in the gastrointestinal tract. However, it is not clear how iIEL are continually attracted toward intestinal epithelial cells and then retained within the highly dynamic intestinal epithelium.

To determine the functional role of fractalkine expression in the intestinal mucosa, we have examined the source and function of the fractalkine-CXCR1 ligand-receptor system in the healthy and diseased small intestinal mucosa.

Materials and Methods

Abs and cytokines

The following Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti-fractalkine C-20, directed against the intracellular domain of fractalkine; anti-caveolin-1; and anti-E-cadherin. Anti-goat HRP was obtained from Amersham (Piscataway, NJ); anti-goat biotin, anti-mouse/rabbit/biotin, and streptavidin-FITC were obtained from Dako (Carpenteria, CA); Streptavidin-PE, streptavidin-rhodamine, anti-rabbit biotin, anti-CD4 FITC, anti-CD8 biotin, and anti-CD8 PE used for FACS analysis were obtained from PharMingen (San Diego, CA). CXCR1 expression constructs and antisera were a gift from P. M. Murphy (National Institute of Allergy and Infectious Disease, Bethesda, MD). Fractalkine, IL-1β, and IL-2 were obtained from R&D Systems (Minneapolis, MN).

Cell culture

The human intestinal epithelial cell line T-84 was obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM/Ham F12 (50:50 v/v) medium (Cellgro; Mediatech, Herndon, VA) supplemented with 10% heat-activated FCS (Sigma), 1% penicillin, and streptomycin (Life Technologies, Gaithersburg, MD). Cells were grown at 37°C in a 5% CO2 atmosphere within a humidified incubator. Cells were always used for experiments at 70% confluence.

RT-PCR for the detection of CXCR1 mRNA

The nucleotide sequence of CXCR1 (GenBank accession number U28934) was analyzed and two primer generated: 5′-TTG AGT ACG ATG ATT TGG CTG A-3′ and 5′-GGC TTT GGC TTT CTT GTG G-3′. PCR conditions were 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C for 40 cycles.

Northern blot analysis

Total RNA was extracted from T-84 cells and intestinal biopsy specimens or resected intestinal tissues using Trizol reagent (Life Technologies), according to the manufacturer’s instructions. Crohn’s disease, ulcerative colitis, or normal intestinal control tissues were obtained from the tissue bank of the Center for the Study of Inflammatory Bowel Disease at Massachusetts General Hospital, according to the guidelines for the use of discarded human material at Massachusetts General Hospital. Resected tissue from three patients with Crohn’s disease and three specimens from control patients were analyzed.

Fractalkine cDNA was generated by RT-PCR using the following primers: 5′-ACT CCT GCC CAC CCT CAG C-3′, and 5′-TGG AGG GAG GCA CTC-3′. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. cDNA probes were labeled with [α-32P]dCTP by a random hexamer priming method using the Rediprime random primer labeling kit (Amersham Life Science, Arlington Heights, IL). Membranes were hybridized in Quickhyb solution (Stratagene, La Jolla, CA) at 68°C for 1 h. The membranes were washed and the blots analyzed by autoradiography. Expression of fractalkine mRNA was corrected for GAPDH expression and quantitated densitometrically with the NIH Image 1.6.1 analysis software.

Western blot analysis

Cells were placed in lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM NaF, 10 mM aprotinin, 10 mg/ml leupeptin, 1 mM PMSF, and 1 mM sodium-orthovanadate (pro tease/phosphatase-inhibitor mixture)). After 20 min on ice, cell lysates were cleared by centrifugation at 20,000 × g for 15 min at 4°C. Protein concentration in each sample was quantitated by the Bradford method, and 20 μg protein was used for Western blot analysis. Proteins were separated by SDS-PAGE, using 10% Tris-glycine gels, and subsequently transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated in blocking solution (1× TBS, 0.025% Tween 20 (TBS-T), 1% BSA) at room temperature for 1 h. After an overnight incubation with the first Ab (anti-fractalkine C-18 1:1000 or C-20 1:200 in blocking solution) at 4°C, membranes were washed four times in TBS-T. The HRP-labeled second Ab was dissolved (1:6000) in TBS-T, supplemented with 5% dry milk. After incubation with the second Ab for 1 h at room temperature, the membranes were washed four times in TBS-T and proteins were detected by the ECL method (Amersham, Piscataway, NJ), according to the instructions of the manufacturer. Western blots were quantitated densitometrically with NIH Image 1.6.1 analysis software.

Immunoprecipitation from T-84 cytosol protein and membrane protein fraction

T-84 cells were washed three times with ice-cold PBS, and 6 ml hypotonic lysis buffer (10 mM Tris, pH 7.4, 1 mM MgCl2, supplemented with the protease/phosphatase inhibitor mixture) was added to two 10-cm dishes. After incubation on ice for 20 min, the cells were disrupted by douncing for 15 times. By adding 5 mM NaCl, iso-osmolar conditions were obtained. The cell lysate was cleared by centrifugation twice for 10 min at 1000 × g. The supernatant was then centrifuged at 100,000 × g for 35 min at 4°C. The supernatant (cytosolic fraction), was adjusted to a final concentration of 0.1% sodium deoxycholate, 0.1% SDS, and 0.1% Triton X-100. The pellet was solubilized in 1% sodium deoxycholate, 1% SDS, and 1% Triton X-100, and after an incubation for 20 min on ice, diluted to obtain identical conditions as in the cytosolic fraction. The lysate was dounced 10 times and cleared by a centrifugation at 10,000 × g for 15 min at 4°C, and resuspended in the membrane fraction. Equal (600 μg) amounts of protein, as determined by the Bradford method, were subjected to immunoprecipitation. After preclearing for 2 h with protein A/G beads (Calbiochem, La Jolla, CA), the lysates were incubated overnight with 2 μg/ml C anti-fractalkine Ab. After washing twice with washing buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.5% Triton X-100), the immunoprecipitate was denatured by boiling in Laemmli buffer and subjected to Western blot analysis.

Isolation of detergent-insoluble glycolipid-enriched membrane microdomains

Glycolipid-enriched membrane microdomains or detergent-insoluble glycolipid rafts were isolated as described before (23) (24), with minor modifications. Four dishes of T-84 cells (10 cm diameter) were washed three times with ice-cold PBS, and the cells were solubilized in 1 ml of lysis buffer (25 mM MES, pH 6.8, 150 mM NaCl, 1% Triton X-100 supplemented with the protease/phosphatase inhibitor mixture). After incubation for 30 min on ice, the membrane fraction was dounced gently 10 times and centrifuged at 100,000 × g for 35 min at 4°C. The supernatant was transferred to the bottom of a centrifugation tube and adjusted to 40% sucrose with an equal volume of 80% sucrose in MBS (25 mM MES, pH 6.8, 150 mM NaCl supplemented with the protease/phosphatase inhibitor mixture). A sucrose step gradient with 30%, 25%, 20%, 15%, and 5% in MBS (2 ml each) was layered on top. This combination of sucrose concentrations gave the best resolution between 15% and 25% (data not shown). After a centrifugation in a SW 41 rotor (Beckman Coulter, Fullerton, CA) at 39,000 rpm for 14–16 h at 4°C, fractions of 1 ml each were taken, starting from the top (fraction 1), going to the bottom (fraction 12). For the preparation of cytosol and membranes, two dishes of T-84 cells (10 cm diameter) were three times washed with ice-cold PBS and taken up in 2 ml buffer A (20 mM Tris, pH 7.8, 1 mM EDTA, 0.25 M sucrose supplemented with the protease/phosphatase inhibitor mixture). The lysate was sheared 10 times and cleared by a centrifugation for 5 min at 1000 × g. One-fourth of the supernatant was taken for preparation of the cytosol. Four milliliters of buffer A were added, and a centrifugation for 5 min at 100,000 × g was performed. The middle, clear fraction referred to the cytosolic fraction. For membrane isolation, the remaining three-fourths of the cleared supernatant were layered on top of a 30% Percoll solution in buffer A, supplemented with 8.5% sucrose. After centrifugation for 30 min at 100,000 × g, the resulting interface, the membrane fraction, was isolated. Protein measurement of the cellular fractions was performed with BCA Protein Assay Reagent, according to the manufacturer’s instructions (Pierce, Rockford, IL). Equal amounts of protein were subjected to Western blot analysis, as described above.

Immunofluorescence staining for the detection of fractalkine

Intestinal biopsy specimen was obtained during diagnostic endoscopy after prior patients’ consent. The tissue was fixed with OCT-mounting medium (Miles, Elkhart, IN), immediately frozen in liquid nitrogen, and stored at −80°C until use. The tissue was cut at −22°C into 5-μm slices and put on
FIGURE 1. Basal fractalkine mRNA expression in T-84 cells is up-regulated by IL-1β. Northern blot analysis of fractalkine mRNA expression in RNA from T-84 cells (20 μg of total RNA/lane). Cells were incubated for the time indicated with 10 ng/ml IL-1β. Fractalkine mRNA expression was quantified with a densitometer (Molecular Dynamics, Sunnyvale, CA) and NIH Image 1.61 (National Institutes of Health, Bethesda, MD). A, Represents the densitometrical analysis of fractalkine mRNA expression in A, normalized to GAPDH mRNA expression in B, expressed in median density/area. One representative experiment of four conducted is shown.

FIGURE 2. Induced expression of membrane-bound fractalkine by IL-1β is accompanied by secretion of soluble fractalkine. Western blot analysis of fractalkine protein expression in T-84 cell lysates after stimulation with 10 ng/ml IL-1β. A total of 20 μg of protein per lane was resolved on 4–12% SDS gel and immunoblotted with Abs recognizing the intracellular region of fractalkine (A) or the fractalkine chemokine domain (C). B and D, Represent the densitometrically analysis of membrane-bound fractalkine in A or soluble fractalkine in C, respectively. Fractalkine was immunoprecipitated from cytosolic or membrane fractions of T-84 cells with an Ab directed against the cytoplasmic region of fractalkine and resolved by SDS-PAGE, followed by immunoblotting for the presence of fractalkine (E). A total of 100 ng/ml of glycosylated recombinant fractalkine was used as a control (E, lane 5). F, Represents densitometric analysis of fractalkine protein concentration in E expressed as mean density/area. One representative experiment of three conducted is shown.

positively charged glass slides and stored at −80°C until use. The slides were thawed for 5–10 min, dipped into acetone (−20°C) for 30 s, and air dried. T-84 cells were seeded at a density of 10,000 cells/ml and grown on coverslips (Lab-Tek, Naperville, IL) for 3 days. Cells were fixed as the intestinal biopsy specimens. After air drying, the cells or biopsy specimens, respectively, were blocked for 20 min in 5% (v/v) donkey serum (Santa Cruz Biotechnology) in PBS (blocking solution). All steps were conducted at room temperature in a humidified, light-protected chamber. After an incubation with anti-fractalkine C-20 (2.5 μg/ml), anti-cadherin, or anti-caveolin-1 (all from Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution for 2 h, slides were washed with PBS (2 × 2 min) and incubated with anti-goat biotin Ab in blocking solution (1:50) for 30 min. After an additional washing step, the slides were incubated with streptavidin-FITC (1:50) for 1 h. After washing, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Staining was analyzed with an IX70 Olympus-fluorescent microscope.

Isolation of iIEL and FACS analysis
Intestinal jejunal tissue was obtained during gastric bypass surgery for obesity. iIEL were isolated as described earlier (25). iIEL were either used freshly for FACS analysis or kept in culture, as described before (26). FACS analysis was performed, as described before (25), using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The anti-CXCR1 Ab containing antisera was used at a dilution of 1/50; negative control experiments were performed with the preimmune serum also at a 1/50 dilution.

iIEL migration assay
iIEL were stimulated with IL-2 (10 ng/ml) for 3 days. Using a chemotaxis chamber (Neuroprobe, Cabin John, MD), the lower well was filled with medium with or without fractalkine, and, in some instances, anti-fractalkine Ab at concentrations indicated. A 5-μm polycarbonate membrane separated the lower from the upper well, which was filled with 50 μl medium, containing 100,000 iIEL. Each assay was performed in triplicate. The cells were allowed to migrate for 8 h at 37°C. Cells in the lower well were counted, and the migration index (migrated cells divided by the number of cells that migrated without fractalkine) was calculated.

Results
IL-1β up-regulates basal expression of fractalkine mRNA in T-84 cells
Fractalkine mRNA expression has been found in different tissues, including small intestinal and colonic tissue (3). The latter

prompted us to investigate whether fractalkine mRNA is expressed in intestinal epithelial cells. Northern blot analysis demonstrated that fractalkine mRNA is expressed in the intestinal epithelial cell line T-84 (Fig. 1). Incubation with the NF-κB-inducing cytokine IL-1β was able to induce a transient 4-fold increase in basal steady state expression of fractalkine transcripts within 2 h (Fig. 1).

IL-1β-induced expression of membrane-bound fractalkine in T-84 cells is accompanied by secretion of soluble fractalkine
To determine whether the induction of fractalkine mRNA expression in T-84 cells resulted in increased production of fractalkine protein, Western blot and immunoprecipitation analysis were conducted with Abs specifically directed against the chemokine domain and the intracellular region of fractalkine. As demonstrated in
5-fold over 24 h after induction by IL-1β expression of fractalkine in T-84 cell media supernatants increased expression in T-84 cell lysates was followed by an elevation of demonstrated in Fig. 2, C.

Fraction, which corresponded to the size of full-length glycosylated cipitates from the cytosol as well as the transmembrane protein fractalkine Abs hybridized to a protein band in the immunoprecipitate domain. As demonstrated in Fig. 2, Western blotting with Abs directed against the fractalkine chemokine region, we therefore determined whether fractalkine was released from T-84 cells upon stimulation by IL-1β. As demonstrated in Fig. 2, C and D, the increase of fractalkine protein expression in T-84 cell lysates was followed by an elevation of soluble fractalkine in the T-84 cell media supernatants. The basal expression of fractalkine in T-84 cell media supernatants increased 5-fold over 24 h after induction by IL-1β (Fig. 2, C and D).

To confirm that the fractalkine protein detected in T-84 cell lysates was membrane bound, T-84 membranes were separated from cytosol protein fractions before and after stimulation with IL-1β, followed by immunoprecipitations with anti-fractalkine Abs directed against the intracellular region of fractalkine and Western blotting with Abs directed against the fractalkine chemokine domain. As demonstrated in Fig. 2, E and F, the anti-fractalkine Abs hybridized to a protein band in the immunoprecipitates from the cytosol as well as the transmembrane protein fraction, which corresponded to the size of full-length glycosylated recombinant fractalkine (Fig. 2F, lane 5). More than 82% of the fractalkine expressed in nonstimulated T-84 cells localized in the T-84 cell membrane fraction. Stimulation with IL-1β for 12 h increased the fractalkine content in the cytosol fraction 2.5-fold and by 1.3-fold in the membrane fraction (Fig. 2, E and F).

Fractalkine is localized in caveolin-1 containing detergent-insoluble glycolipid-enriched membrane microdomains in T-84 cells

To further characterize the membrane compartment containing fractalkine, we separated postnuclear membrane protein fractions by sucrose density-gradient centrifugation. This method separates detergent-insoluble functional distinct membrane fractions characterized by their specific lipid composition (24, 27, 28). As demonstrated in Fig. 3A, fractalkine was present in low density membrane fractions from T-84 cells. Fractalkine was present in membrane fractions corresponding to sucrose concentrations of 15% to 24%, with the highest concentration in the fraction corresponding to 22% (Fig. 3A, lane 5). These low density membrane fractions have been associated with detergent-insoluble glycolipid-enriched membrane fractions or rafts (29). In epithelial cells, these membrane fractions are characterized by the presence of a distinct scaffolding protein caveolin (30). As demonstrated in Fig. 3B,
Western blot analysis of these cellular compartments revealed that the expression of caveolin-1 was restricted to same sucrose gradients as the fractalkine expression T-84 cells. Caveolin-1 concentration was maximal at apparently 22% sucrose (Fig. 3), consistent with a previous report (31). This seems to be cell specific for T-84 cells, as maximal amounts of caveolin are typically detected at sucrose concentrations of 15–20% in other cell lines (24). Collectively, these data indicate that fractalkine is largely sequestered in a microdomain of T-84 cell membranes that display the biophysical characteristics of caveolae (29).

Fractalkine expression is regulated during polarization of the intestinal epithelial cells

The human intestinal epithelial cell line T-84 provides a well-established model for the assembly of intercellular junctions and the development of apical-basolateral polarity (32). Immunofluorescence staining revealed specific staining for fractalkine in T-84 cells (Fig. 4A). Fractalkine was mainly expressed in undifferentiated proliferating T-84 cells that are localized at the edges of monolayers with developed apical-basolateral polarity (Fig. 4). Polarized T-84 cells still retained some fractalkine expression, which appeared to be localized to cell-cell contact regions. In contrast, fractalkine expression in unpolarized T-84 cells demonstrated a strong granular cytoplasmic and cell surface expression pattern (Fig. 4, A and B). Because fractalkine demonstrated a strong enrichment in caveolin-1-containing membrane fractions, we determined the expression of caveolin-1 in polarized and unpolarized T-84 cells. As shown in Fig. 4, C and D, caveolin-1 was strongly expressed in unpolarized T-84 cells. The specific expression pattern of fractalkine and caveolin-1 in unpolarized T-84 was not due to restricted access to the basolateral surface of the polarized epithelial cells because anti-E-cadherin Abs were able to detect E-cadherin on the surface of unpolarized as well as at the basolateral surface of polarized T-84 cells (Fig. 4, E and F).

**Human iIEL express functional fractalkine receptor CX3CR1**

To determine the potential target cells of fractalkine expression by intestinal epithelial cell in the intestinal mucosa, we assessed expression of CX3CR1 transcripts in RNA isolated from lymphocyte population obtained from normal human small intestine. As shown in Fig. 5, iIEL from three independent cell isolations and two of three lamina propria lymphocyte isolations demonstrated CX3CR1 mRNA expression. In addition, CX3CR1 mRNA expression was detected in the iIEL cell line EIE10.

Because iIEL are most likely to interact with intestinal epithelial cells and both freshly and cultivated iIEL demonstrated a strong expression of CX3CR1 mRNA, we determined whether iIEL expressed CX3CR1 protein on the cell surface using flow cytometry. As demonstrated in Fig. 6A, the freshly isolated human iIEL consisted of 78% CD8-positive (Fig. 6A) and 7.6% CD8-positive phenotype (D) in IL-2-activated iIEL were confirmed by flow cytometry. In C, the dotted line represents the fluorescence intensity after incubation of iIEL with preimmune sera; the solid line after incubation with anti-CX3CR1 antisera. D, Demonstrates the expression of CD8 on iIEL without (dotted line) or after stimulation for 3 days with IL-2 (solid line).

**Fractalkine mediates chemoattraction of human iIEL**

To determine whether the CX3CR1 receptor expressed on iIEL is able to mediate chemotraction, the migratory response of isolated human iIEL was determined in the chemotaxis chamber multwell
system, as described (33), and expressed in fold increase of the basal cell migration (Migration Index) (Fig. 7A). Since fresh isolated iIEL do not migrate efficiently toward chemokine gradients, iIEL were stimulated with IL-2 (10 ng/ml) for 3 days, as previously described (34). As demonstrated in Fig. 7A, soluble fractalkine induced the migration of IL-2-activated iIEL in a dose-dependent fashion. Highest induction was found at a fractalkine concentration of 1 ng/ml, and activity decreased at higher concentrations. Similar dose-response curves have been reported for RANTES in the induction of migration of iIEL (33). The fractalkine-induced migration of iIEL could be significantly inhibited in a dose-dependent fashion by Abs against the chemokine domain (C-18), but not the carboxy terminus of fractalkine (C-20) (Fig. 7B, p < 0.001). Flow cytometry confirmed the presence of CX3CR1 on iIEL (Fig. 7C) after incubation with IL-2. Furthermore, iIEL retained their CD8-expressing phenotype during IL-2 activation (Fig. 7D). Together, these experiments demonstrate that iIEL express functional CX3CR1 receptors, and can be chemoattracted by soluble fractalkine when activated by IL-2.

Fractalkine is expressed by intestinal epithelial cells and endothelial cells within the small intestinal mucosa and up-regulated in active Crohn’s disease

Immunohistochemistry was utilized to determine whether fractalkine is expressed in human normal or diseased small intestinal mucosa. Frozen sections of normal and inflamed small intestinal mucosa (Fig. 8) were stained for the presence of fractalkine with a combination of Abs against the intracellular (C-18) and extracellular (C-20) regions of fractalkine, followed by incubation with biotinylated anti-rabbit Abs and FITC-coupled streptavidin. As demonstrated in Fig. 8A, intestinal epithelial cells in normal small intestinal tissues stained positive for fractalkine. In addition, a punctuated positive staining for fractalkine was present in endothelial cells outlining small mucosal blood vessels (Fig. 8A, indicated by white arrows). Fractalkine expression was highly up-regulated in endothelial cells outlining all small blood vessels observed within the inflamed intestinal mucosa during Crohn’s disease even in areas of the mucosa that did not show infiltration...
Crohn’s disease tissues after correction for GAPDH expression density/area of 32 lanes 4–6 associated in Crohn’s disease (Fig. 9, migration of intestinal lymphocytes in healthy and diseased intestine was observed fractalkine expression by intestinal epithelial cells and lymphocyte regulation by intestinal epithelial cells. Thus, we have ligand-receptor system provides a novel mechanism of intestinal

Fractalkine mRNA expression is up-regulated in the intestine

To determine whether fractalkine expression is involved in intestinal inflammation, we assessed fractalkine mRNA expression in normal small intestine, as well as in intestinal tissues resected from patients with active Crohn’s disease. As demonstrated in Fig. 9, fractalkine mRNA was expressed in the normal small intestine (Fig. 9, lanes 1–3). Fractalkine mRNA was significantly up-regulated in Crohn’s disease (Fig. 9, lanes 4 and 5). The expression of fractalkine mRNA in small intestine increased from a mean density/area of 32 ± 4.5 in normal intestine up to 85 ± 16.2 in Crohn’s disease tissues after correction for GAPDH expression (p < 0.01).

Discussion

In this study, we have determined that the fractalkine-CX$_3$CR1 ligand-receptor system provides a novel mechanism of intestinal lymphocyte regulation by intestinal epithelial cells. Thus, we have observed fractalkine expression by intestinal epithelial cells and endothelial, which can contribute to regulation of the attraction and migration of intestinal lymphocytes in healthy and diseased intestinal mucosa. In the intestine, lymphocytes constantly migrate through the endothelial cell layer of high endothelial venules and organize within the lamina propria (35, 36). Some of the lymphocytes continue through the epithelial basal membrane and settle within the intestinal epithelial cell monolayer forming the iIEL compartment. During intestinal inflammation, neutrophils are also able to cross the epithelial barrier. The composition of migrating intestinal leukocyte populations is dynamically regulated as a reflection of the immunological status of the intestinal mucosa. Chemokine expression by intestinal epithelial cells has been suggested to contribute to chemotactic gradients necessary to direct leukocyte migration into the intestinal mucosa (37–40). 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 the adhesive capacity of integrins (41, 42). However, fractalkine is the first chemokine, which is able to mediate leukocyte attraction, capture, firm adhesion, and activation even in the absence of integrins (43, 44).

The detection of fractalkine transcripts in mRNA isolated from small and large intestine (3) prompted us to determine whether intestinal epithelial cells are a source of fractalkine mRNA and protein expression. Our experiments demonstrate that the human intestinal epithelial cell line T-84 is able to express membrane-bound and soluble fractalkine under the control of the inflammatory mediator IL-1β, suggesting that fractalkine may be involved in directing intestinal epithelial cell-lymphocyte interactions as well as the attraction of lymphocytes into the intestinal lamina propria. Induction of fractalkine expression in intestinal epithelial cells may mediate long lasting effects. Whereas basal expression of fractalkine mRNA was maximally induced within 4 h after a single induction by IL-1β, membrane-bound fractalkine protein levels increased over 12 h, and the amount of soluble fractalkine increased steadily over 24 h. Furthermore, the expression of fractalkine in intestinal epithelial cells was regulated during polarization and differentiation of T-84 cells. Immunohistochemistry demonstrated that fractalkine was highly expressed in nonpolarized T-84 cells, which are still able to migrate and to proliferate, whereas fractalkine expression decreased in T-84 cells after polarization and differentiation and localized to the basolateral cell surface. Fractalkine expression in undifferentiated and migrating intestinal epithelial cells may indicate a role of fractalkine in intestinal wound healing.

Membrane-bound fractalkine colocalized with caveolin-1 in a specialized membrane compartment characterized by its specific lipid composition, which allowed separation on a sucrose gradient from other membrane protein fractions. This indicates that fractalkine may be specifically expressed in caveolae on the surface of intestinal epithelial cells. Caveolae are vesicular invaginations of the plasma membrane characterized by the expression of the caveolin family of scaffolding proteins (30). Caveolins can assemble signaling molecules into preassembled signaling complexes, and an increasing number of signaling pathways have been shown to be associated with caveolae, such as those mediated by G protein-coupled receptors, the ras-raf pathway, and pathways involving the activation of src family tyrosine kinases (30). Signal transduction events important in cell-cell adhesion, such as integrin signaling, have been shown to be dependent on the presence of caveolins (45). Consistent with the expression of fractalkine in caveolae-like structures, the same nonpolarized T-84 cell population stained positive for the expression of caveolin-1. Targeting of fractalkine to glycolipid-enriched microdomains may be mediated by the myristylation of cysteines in the membrane or juxtamembrane region of fractalkine (C353, C367, and C386), analogous to the membrane targeting of LAT (linker for activation of T cells).
(46). Furthermore, the punctuated expression pattern of fractalkine was observed in primary intestinal epithelial cells as well as on the surface of endothelial cells in the intestinal mucosa. The significance of the expression of fractalkine in specialized membrane fractions is not clear, but may aid the close attraction and interaction of leukocytes with signaling molecules on the surface of intestinal epithelial cells.

Cell populations regulated by fractalkine within the intestinal mucosa may include iIEL as well as lamina propria lymphocytes. CX₃CR1 mRNA expression could be detected in RNA prepared from isolated human small intestinal IEL as well as lamina propria mononuclear cells. Flow cytometry revealed that a subpopulation of fresh isolated human iIEL expressed CX₃CR1 on its surface. Consistent with these results, fractalkine was able to specifically chemotaxt-activated isolated human iIEL. In these experiments, isolated human iIEL required stimulation with IL-2 to migrate within fractalkine gradients. Although the IL-2-stimulated iIEL retained their CD8⁺ phenotype, it is not clear whether iIEL require additional signals by other chemokines or adhesion molecules to migrate in vivo. Fractalkine has been shown to mediate leukocyte capture and firm adhesion of nonstimulated and activated peripheral CD8⁺ T cells, monocytes, and CD16/56 NK cells (43). Although CX₃CR1 mRNA was also expressed by IL-2-activated CD4⁺ T cells, these cells did not firmly adhere to immobilized fractalkine (5). Cultured human iIEL have been demonstrated to retain their capability to migrate into polarized epithelial cell monolayers in vitro, where they assume a subapical function, identical to that observed in vivo (47). This migration was partially inhibited by pertussis toxin, suggesting a potential mechanism for iIEL migration by chemokine receptor-mediated signaling (47).

Furthermore, several chemokines, including IL-8 and RANTES, have been shown to be able to direct the migration of cultured intraepithelial lymphocytes (34). In these experiments, as in ours, cultured iIEL had to be activated by IL-2R signaling to migrate in response to chemokines. This may be due to the abrogation of chemokine receptor expression by PHA, used in the protocols to propagate iIEL, because PHA has been shown to down-regulate CX₃CR1 expression (5) as well as CCR1 and CCR2 expression (48).

The mobilization of CD8⁺ T cells into intestinal mucosa has been proposed to be cooperatively regulated by integrins and chemokines (34, 49, 50). The α₄β₁ integrin is strongly expressed by iIEL (51–53). α₇β₇ is involved in iIEL binding to epithelial cells via interaction with E-cadherin (54). The iIEL deficiency associated with a lack of α₇ or β₇ expression would suggest that α₄β₁ is required for entry and/or retention of T cells into the intestinal epithelium (55, 56). Because fractalkine has been shown to mediate strong integrin-independent adhesion in vitro (5, 44), fractalkine expressed by intestinal epithelial cells may not only attract iIEL toward the epithelium, but may also contribute to the retention of iIEL within the intestinal epithelial cell layer.

In addition to the attraction of iIEL into the small intestinal mucosa, fractalkine may play an important role during intestinal inflammation. Fractalkine mRNA expression was strongly up-regulated in Crohn’s disease mucosa, suggesting an involvement of the fractalkine-CX₃CR1 ligand receptor system in recruitment of leukocytes during intestinal inflammation. Our data demonstrate that both iIEL and LPL can express CX₃CR1 mRNA. The leukocyte cell populations attracted by intestinal epithelial as well as endothelial cell-derived fractalkine in intestinal inflammation need to be determined. Fractalkine expression has been demonstrated in HUVEC after induction with IL-1 or TNF-α, and therefore a role of fractalkine has been proposed in the regulation of inflammation (3). However, to date, no direct demonstration of the involvement of fractalkine in inflammatory disease has been reported. The best evidence that fractalkine is involved in the attraction of leukocytes during disease was demonstrated in experiments in which fractalkine-mediated migration of granulocytes isolated after Ab-induced glomerulonephritis could be inhibited by vMIP-2 (7).

The mechanism of lymphocyte migration into healthy and diseased intestinal mucosa is not well understood. Together, our studies support a model in which fractalkine may be involved in the attraction and retention of iIEL into the intestinal epithelium. Fractalkine expression within the intestinal mucosa may have an important function in intestinal inflammation. Endothelial-expressed fractalkine may initiate capture of lymphocytes by firm adhesion to the activated endothelium, and epithelial cell-derived soluble fractalkine may direct lymphocytes to the site of inflammation and tissue repair. Membrane-bound fractalkine expressed by intestinal epithelial cells may then help to retain iIEL within the regenerating intestinal epithelium.

References


