

Fractalkine Produced by Airway Smooth Muscle Cells Contributes to Mast Cell Recruitment in Asthma¹

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Human airway smooth muscle cells (HASM) secrete fractalkine (FKN), a chemokine the concentration of which is increased in asthmatic patients. HASM also induce mast cell chemotaxis, as a component of asthma inflammation. We therefore evaluated the role of smooth muscle-derived FKN in mast cell migration. We assessed the capacity of recombinant FKN to induce human mast cell chemotaxis. This effect implicates a calcium-independent pathway involving actin reorganization and protein kinase C- δ . We found that HASM constitutively produce FKN, the synthesis of which is reinforced upon proinflammatory stimulation. Under basal experimental conditions, FKN production by HASM is not sufficient to induce mast cell chemotaxis. However, pretreatment of mast cells with the neuropeptide vasoactive intestinal peptide (VIP) increases FKN potency to attract mast cells. Since we observed, in asthmatic patients, an increase in both FKN and VIP expression by airway smooth muscle and a positive correlation between VIP staining and mast cell infiltration of the smooth muscle layer, we conclude that HASM-derived FKN may contribute to mast cell recruitment in asthma. *The Journal of Immunology*, 2006, 176: 1860–1868.

Fractalkine (FKN),⁴ or CX3CL1, is a CX3C chemokine expressed as a membrane-bound form (1) by several cell types, including vascular smooth muscle cells (2, 3). Recently, it has been shown that human airway smooth muscle cells (HASM), also can produce FKN upon stimulation by proinflammatory cytokines (4). FKN interacts with its unique receptor CX3CR1 (5) on monocytes, NK cells, and T cells and mediates both adhesion and migration of leukocytes. In contrast, FKN is able to induce the migration of murine bone marrow-derived mast cells (6).

A recent study performed in asthmatic patients has reported that the plasma FKN level is higher than in normal subjects and that segmental allergen challenge up-regulates CX3CR1 (7). FKN was also elevated in bronchoalveolar lavage fluid and strongly expressed by epithelial and endothelial cells. Asthma is characterized by bronchial hyperresponsiveness and infiltration of airway mucosa by several cell types, including eosinophils and activated mast cells (8). It has been clearly demonstrated that inflammatory infiltration also concerns the smooth muscle layer and that the number

of mast cells infiltrating the bronchial smooth muscle is higher in asthmatic patients than in normal subjects and closely related with hyperresponsiveness (9). Mast cells can produce a variety of lipid mediators, proteases, and cytokines that interact with smooth muscle cells and induce both contraction and proliferation (10) phenomena that are deleterious in limiting airway narrowing reversibility (9). HASM have the capacity to produce a variety of cytokines and chemokines that may attract and retain mast cells within the smooth muscle layer (11). Among these chemotactic agents, stem cell factor (SCF) is the more specific for mast cell, but previous data obtained in vitro have failed to demonstrate a strong effect (10). TGF- β 1 is more potent as a chemotactic factor in vitro (10) but the role of this immunosuppressive cytokine in the pathophysiology of asthma remains controversial. Since, on the one hand, FKN can be produced by smooth muscle cells and, in contrast, FKN is overexpressed in asthma, it was our hypothesis that HASM could synthesize FKN and specifically attract mast cells through this production.

The aim of the current study was thus to examine the role of FKN in airway inflammation in asthma. Specifically, we have investigated 1) the effect of FKN on mast cell attraction within the smooth muscle layer and 2) the signal transduction involved upon CX3CR1 receptor activation.

Materials and Methods

Cell cultures

The human mast cell line HMC-1 (12) was cultured in 10% FCS/DMEM and was passaged every 3–5 days with trypsin-EDTA (Invitrogen Life Technologies). HMC-1 was challenged with recombinant human FKN at the concentration of 1/500 ng/ml, TGF- β 1 at the concentration of 1 ng/ml, or SCF at the concentration of 30 ng/ml (all from R&D Systems) for 5–60 min. Priming of HMC-1 was done using pretreatment of cells with 10^{-7} M vasoactive intestinal peptide (VIP; Sigma-Aldrich) for 30 min at 37°C and subsequent rinsing.

HASM were derived by primary culture from bronchial tissue as described previously (13). Smooth muscle squares were cultured and maintained in DMEM (Invitrogen Life Technologies) containing 10% (v/v) FCS (Invitrogen Life Technologies). Only cells passaged two to four times with trypsin-EDTA (Invitrogen Life Technologies) were used for this study. Cells were seeded in 24-well plates at a density of 2×10^5 cells/ml and grown in 10% FCS/DMEM. Confluent cells were then rinsed twice with

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⁴ Abbreviations used in this paper: FKN, fractalkine; HASM, human airway smooth muscle cell; SCF, stem cell factor; VIP, vasoactive intestinal peptide; hp, high-power field; IP-10, IFN-inducible protein 10.

HBSS and growth was arrested using serum-free DMEM for 72 h as previously described (10). Cells were then challenged with 100 ng/ml IL-1 β or TNF- α (both from R&D Systems) for 3–72 h.

RNA extraction, reverse transcription, and real-time quantitative PCR

The RNA was extracted as described previously using TRIzol (Invitrogen Life Technologies) and chloroform (Sigma-Aldrich) (10). Total pure RNA (1 μ g) was reverse transcribed into cDNA using AMV reverse transcriptase (Promega), RNase inhibitor, and oligo(dT) as a primer at 42°C for 60 min, followed by heating at 94°C for 3 min. Real-time quantitative PCR was performed on a Rotor-Gene 2000 (Corbett Research) as described previously (10). Briefly, appropriate primers were designed using the primer analysis software (Oligo 6.6; Molecular Biology Insights) and ordered from Sigma-Genosys. Primers sense and antisense were: GAPDH (NM_002046) forward, 5'-CTGACTTCAACAGCGACACC-3' and reverse, 5'-AGCCAAATTCGTTGTCATACC-3'; FKN (NM_002996) forward, 5'-CGCAATCATCTTGGAGACGAG-3' and reverse, 5'-CGC CATTTCGAGTTAGG-3'; and CX3CR1 (NM_001337) forward, 5'-CCCTTGGCAGTCCACGC-3' and reverse, 5'-GTGAGGGCAAACAC TACCAAC-3'. The RT-PCR expression of the target gene was presented as a ratio, normalized to an endogenous reference (GAPDH) and relative to a calibrator (control condition) (14).

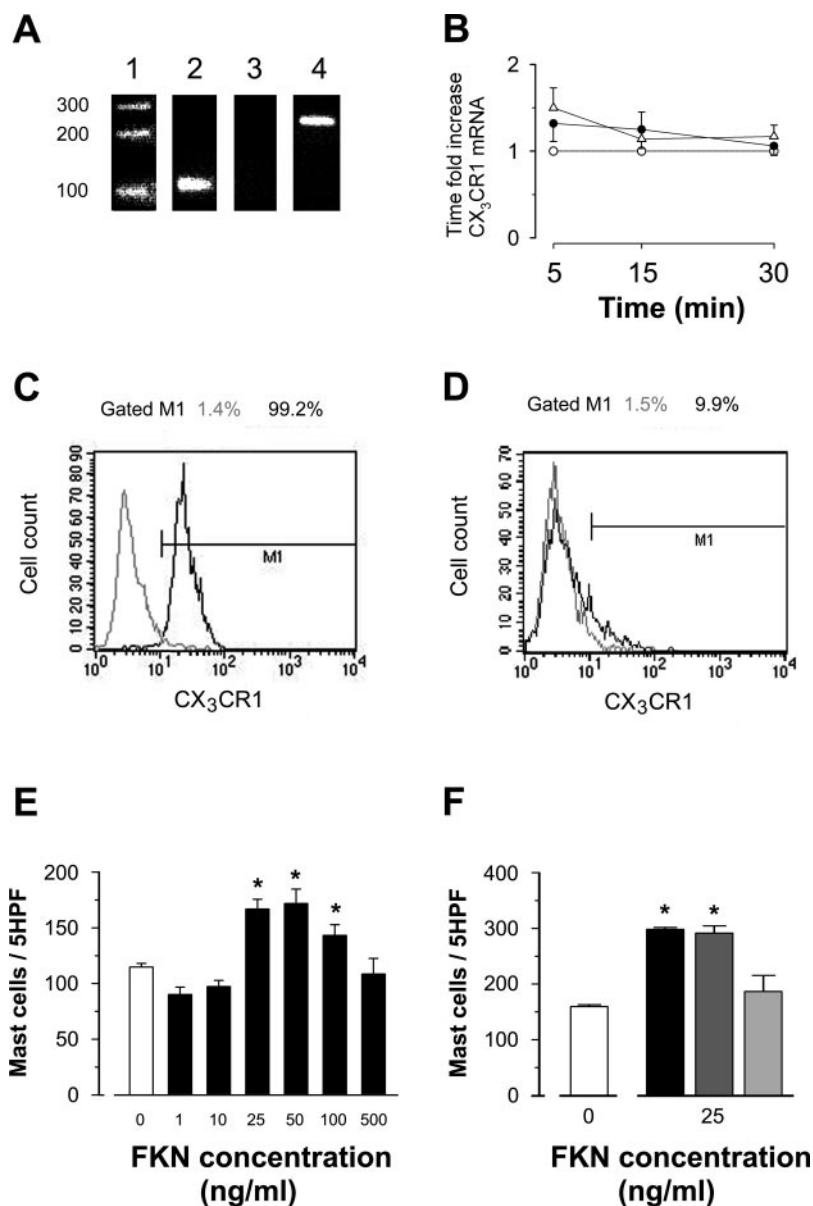
FACS analysis

CX3CR1 expression was analyzed by FACS on HMC-1 or HASMC. Stimulated or unstimulated cells were fixed with 4% paraformaldehyde (Fisher Chemicals) in the presence (total expression) or in the absence (surface expression) of 0.1% saponin (Sigma-Aldrich) for 15 min in ice. The cells were then washed twice and incubated with mouse anti-human CX3CR1-FITC (MBL; Clinisciences) Ab for 30 min in ice. After two additional washes, cells were analyzed for their fluorescence intensity using a FACSCalibur (BD Biosciences). Additional experiments were done to analyze Fc ϵ RI and CD117 expression on HMC-1 after FKN and/or VIP incubation. For this purpose, we used either mouse anti-human CD117-PE (DakoCytomation) or mouse anti-human anti-Fc ϵ RI (Upstate Biotechnology) with a secondary goat anti-mouse IgG-PE (Beckman Coulter).

Chemotaxis assay

The migration of HMC-1 cells against recombinant human FKN (R&D Systems), HASMC supernatants, or VIP was assayed using a 48-well microchemotaxis chamber (NeuroProbe) described previously (10). The number of migrating cells per well was counted at five selected high-power fields (hpf) areas (five hpf; magnification, $\times 400$). Each experimental condition was processed in triplicate.

FIGURE 1. CX3CR1 expression and function on mast cells. RT-PCR (A and B) performed from HMC-1 mRNA. A, Gel electrophoresis conducted for GAPDH (lane 2), FKN (lane 3), and CX3CR1 (lane 4) from one representative patient. B, Relative mRNA expression obtained from RT-PCR of CX3CR1 on GAPDH in HMC-1 stimulated for 5–30 min with recombinant 1 ng/ml TGF- β 1 (Δ), 25 ng/ml FKN ($\bullet$), or nonstimulated ($\circ$). Data are mean $\pm$ SEM from five experiments. Results compared using paired Student's *t* test (*, *p* < 0.05). FACS representative histograms (C and D) obtained from CX3CR1 expression on HMC-1. C, Total expression. D, Surface expression. Cell migration toward FKN (E and F) presented as the number of mast cells per five hpf (original magnification, $\times 400$). E, Mast cell migration toward a range of concentrations of recombinant FKN ($\blacksquare$). D, Mast cell migration toward recombinant FKN assessed in the absence ($\blacksquare$) or in the presence of 10 μ g/ml irrelevant (dark gray bars) or blocking anti-FKN (light gray bars) Abs. Data are mean $\pm$ SEM from six experiments. Results compared with controls ($\square$) using paired Student's *t* test (*, *p* < 0.05).



In another set of experiments, HASMC supernatants or FKN in optimal conditions were incubated for 15 min at room temperature with 0.1–10 $\mu\text{g/ml}$ blocking anti-FKN, anti-IFN-inducible protein 10 (IP-10), anti-TGF- β 1, or anti-SCF Abs (all from R&D Systems) before inducing mast cell chemotaxis. As for signal transduction experiments, mast cells were pretreated by incubation with various concentrations of different kinase inhibitors for 1–2 h before the induction of chemotaxis by 25 ng/ml FKN. The following inhibitors were used at the concentration of 10^{-7} to 10^{-5} M: tyrosine kinase inhibitor (genistein; Sigma-Aldrich), protein kinase (PK) A inhibitors (H-89, HA-100, both from Sigma-Aldrich), PKC inhibitors (HA-100, staurosporin, calphostin C, all from Sigma-Aldrich), MEK 1 and 2 inhibitor of the ERK MAPK pathway (PD98059; Calbiochem), MAPK p38 inhibitor (SB203580 and SB202190, both from Calbiochem).

Finally, mast cells were primed with 10^{-7} M VIP for 30 min at 37°C and subsequent rinsing in insulin-transferrin-sodium selenite (ITS) medium. Chemotaxis of primed mast cells was then assessed as described above. Before VIP priming experiments, mast cells were initially incubated with either VIP receptor antagonist ([D-p-Cl-Phe⁶,Leu¹⁷]-VIP; Sigma-Aldrich) (15) at the concentration of 10^{-7} to 10^{-5} M for 15 min at 37°C or the different kinase inhibitors used above for 1–2 h at 37°C .

Actin reorganization assessment with phalloidin-FITC

After each challenge, HMC-1 were fixed in cold methanol for 20 min and permeabilized with 0.1% saponin (Sigma-Aldrich) for another 30 min. The cells were then stained with phalloidin-FITC labeled (Sigma-Aldrich) for 1 h in the dark, in ice, and analyzed by FACS or confocal microscopy.

Microspectrofluorimetry

Changes in HMC-1 intracellular calcium concentration were assessed using the Ca^{2+} -sensitive probe indo-1 as described previously (13, 16). Briefly, cells were loaded with indo-1 (Calbiochem) and mounted in a perfusion chamber continuously perfused. Mast cells were stimulated with either 1–50 ng/ml FKN, 10^{-9} to 10^{-7} M VIP, or 10^{-5} M ATP (Sigma-

Aldrich), and calcium levels were monitored continuously. In VIP priming experiments, mast cells were first incubated with 10^{-7} M VIP for 30 min at 37°C and then challenged with FKN. Experiments were done at room temperature (22 – 25°C).

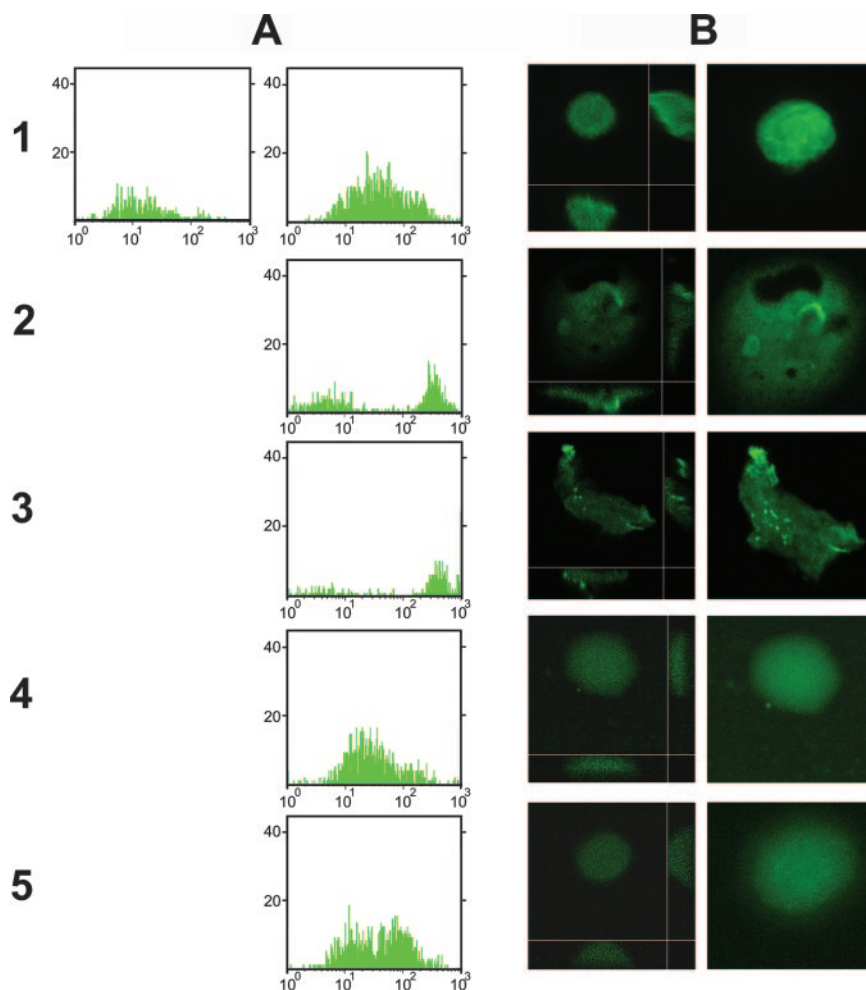
Immunocytochemistry and confocal microscopy

Growth-arrested HASMC or HMC-1 after various challenges were rinsed in PBS and then fixed with cold methanol (VWR International) for 20 min on ice. After drying, the cells were treated with 3% BSA (Sigma-Aldrich) and incubated with primary Abs including goat anti-human FKN (R&D Systems), rabbit anti-human PKC- δ , rabbit anti-human PKC- ϵ (Santa Cruz Biotechnology), or isotype controls. After rinsing, cells were further incubated with either anti-goat Ig-FITC (DakoCytomation) or anti-rabbit IgG-rhodamine conjugate (Santa Cruz Biotechnology) secondary Ab. After washing, the slides were mounted with 10% glycerol. Confocal differential interference-contrast images were obtained using a Fluoview laser scanning microscope (Nikon) and $\times 60$ oil immersion objective. Z-series sections were recorded in successive z-axis serial sections at $0.5\text{-}\mu\text{m}$ intervals and were composed of optical sections in the x-y optical plane. Sections were reconstituted in three-dimensional (3D) images using Imaris software (Bitplane).

Immunoblotting

Whole lysates from HMC-1 incubated with buffer only or with 25 ng/ml FKN were collected using 1% Triton X-100 lyses buffer for 15 min in the presence of pervanadate, 1 mM sodium orthovanadate, 4 mM EDTA, 100 mM NaF, 50 $\mu\text{g/ml}$ aprotinin, 200 $\mu\text{g/ml}$ leupeptin, 50 $\mu\text{g/ml}$ pepstatin A, and 1 mM PMSF (all from Sigma-Aldrich). The supernatant was reduced with 2-ME, subjected to electrophoresis on a 10% acrylamide reducing gel, and transferred to Immobilon TM-P polyvinylidene difluoride membranes (Millipore). The immunoblots were then developed using rabbit polyclonal anti-CX3CR1 (MoBiTec) and goat polyclonal anti-human PKC- ϵ or PKC- δ (Santa Cruz Biotechnology). A biotinylated swine secondary Ab

FIGURE 2. Actin reorganization and cell shape changes induced by FKN on mast cells. HMC-1 stained with phalloidin-FITC. F-Actin content was analyzed by FACS (A) and cell shape changes by confocal microscopy (B). Mast cells were stimulated by recombinant FKN for 0 min (1), 5 min (2), or 15 min (3–5) in the absence (1–3) or presence of the PKC inhibitors staurosporin (4) and calphostin C (5). Representative confocal images (B) presented as three axis slices (left panel) and after 3D reconstruction (right panel).



(DakoCytomation) and a streptavidin- biotinylated HRP complex (DakoCytomation) were used for amplification. Immunoblots were revealed by ECL (Uptima; Interchim). For quantification we used BioCaptMW software (Fischer Bioblock Scientific). The experiments were repeated three times with the same protein isolation.

Immunometric measurement of FKN content in HASMC supernatants

Levels of immunoreactive FKN were assayed in the supernatants of HASMC by ELISA according to the manufacturer's instructions (R&D Systems). Absorbance was measured at 450 nm in a microplate reader in duplicate.

Immunohistochemistry

Bronchial biopsies obtained from control subjects ($n = 5$), or persistent asthmatic patients ($n = 9$) were recovered using fiberoptic bronchoscopy. All patients gave their written informed consent to participate in the study after the nature of the procedure had been fully explained. The study received the approval from the local ethics committee (Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale). Specimens were then embedded in glycolmethacrylate and processed for immunohistochemistry as previously described (10). Primary Abs included mouse anti-human tryptase (AA1), goat anti-human FKN (R&D System), goat anti-human VIP (Santa Cruz Biotechnology), or the appropriate unrelated Ab. The number of mast cells (positive for AA1 Ab) and the staining intensity (of anti-FKN or anti-VIP Ab) was automatically assessed by Quancoul software at a magnification of $\times 200$ (17). Cell counts were expressed as number of cells per square millimeter of each bronchial layer and staining intensity as percentage of a tissue area positive for an Ab.

Statistical analysis

Results are expressed as the mean $\pm$ SEM. Statistical significance was analyzed by ANOVA, Kruskal-Wallis ANOVA, and paired Student's *t* test. Spearman's coefficient was used to evaluate the correlation between mast cell counts and FKN or VIP staining intensity. A $p < 0.05$ was considered to be statistically significant.

Results

FKN induces human mast cell chemotaxis through a calcium-independent PKC pathway

Human mast cells from the HMC-1 cell line expressed significant levels of CX3CR1 mRNA but did not express FKN mRNA (Fig. 1, *A* and *B*). Mast cells also expressed CX3CR1 at the protein level as assessed by Western blot (data not shown) or FACS (Fig. 1, *C* and *D*). The total and cell surface expression of the protein were 93.1 ± 4.4 and $9.4 \pm 0.6\%$, respectively. None of these expressions was altered by the stimulation of mast cells with optimal concentrations of TGF- $\beta 1$ (1 ng/ml), SCF (30 ng/ml), or FKN (25 ng/ml) (data not shown). CX3CR1 was functional since recombinant human FKN induced a mast cell chemotaxis in a concentration-dependent manner (Fig. 1*E*). This effect was maximal using 25–50 ng/ml FKN and was similar to that obtained with an optimal concentration of SCF (30 ng/ml). FKN-induced mast cell migration

was significantly inhibited by 10 μ g/ml a blocking anti-FKN Ab (Fig. 1*F*). To differentiate chemotaxis from chemokinesis, FKN was placed either in the lower wells or in both the upper and the lower wells. FKN at the concentration of 50 ng/ml induced a significant mast cell migration when it was placed in the lower wells (239 ± 1.2 vs 136 ± 1.6 mast cells per 5 hpf; $p < 0.00001$), and no migration was detected when FKN was placed in both the upper and the lower wells (122 ± 3.7 mast cells per 5 hpf). The effect of FKN on mast cell shape changes and cytoskeleton reorganization was next evaluated using confocal microscopy and FACS. Mast cell stimulation with 25 ng/ml FKN induced the development of pseudopods associated with an increase in F-actin content reorganized mainly to the cell periphery (Fig. 2). The shape changes started as early as 1 min and increased in intensity for at least 15 min.

Inhibition of PKC by pretreatment of mast cells with staurosporin or calphostin C resulted in a dose-dependent inhibition of FKN-induced mast cell chemotaxis (Table I) and actin reorganization (Fig. 2). Thus, mast cell migration in response to FKN involved PKC but did not involve MAPK, PKA, or tyrosine kinases (Table I). Complete chelation of the extracellular calcium from the culture medium did not affect mast cell chemotaxis in response to FKN (Fig. 3*A*). Intracellular basal calcium concentration (75 ± 1.3 nM; $n = 125$) was not significantly changed after stimulation with FKN at the concentration of 25 ng/ml (89 ± 3.3 nM; $n = 27$) or 50 ng/ml (87 ± 3.5 nM; $n = 28$) (Fig. 3*B*). In contrast, mast cell stimulation with 10 μ M ATP, used as positive control, significantly increased calcium concentration (408 ± 43.5 nM; $n = 28$, $p < 0.05$; Fig. 3*C*). Among the different PKC subtypes, δ and ϵ isoenzymes are calcium independent (18). Mast cell stimulation by FKN resulted in a PKC- δ intracellular increase as assessed by Western blot (Fig. 4*A*) and in PKC- δ reorganization within pseudopods as assessed by confocal microscopy (Fig. 4, *B* and *C*). By contrast, both the content and the subcellular distribution of PKC- ϵ were not altered.

Mast cell attraction by HASMC-derived FKN

HASMC expressed significant levels of FKN mRNA as assessed by RT-PCR (Fig. 5, *A* and *B*). HASMC also expressed intracellular FKN protein (Fig. 5*C*) and secreted soluble FKN in the supernatant (Fig. 5*D*). The stimulation of HASMC by the proinflammatory cytokines TNF- α and, to a lesser extent, IL-1 β induced both mRNA FKN increase with a maximal effect at 6 h and a FKN protein secretion (Fig. 5, *B* and *D*). HASMC did not express the FKN receptor (CX3CR1) at the transcriptional (Fig. 5*A*) or at the protein level, in the presence or in the absence of proinflammatory cytokines (data not shown).

Table I. Mast cell chemotaxis transduction pathway^a

FKN	–	+		–	+	
Inhibitors	–	–	<i>p</i> ^b	+	+	<i>p</i> ^b
Genistein (10^{-5} M)	113 $\pm$ 4	170 $\pm$ 11	0.002	142 $\pm$ 11	206 $\pm$ 3	0.001
HA-89 (10^{-6} M)	127 $\pm$ 2	207 $\pm$ 12	0.002	169 $\pm$ 6	249 $\pm$ 6	<0.0001
HA-100 (10^{-6} M)	105 $\pm$ 18	203 $\pm$ 19	0.004	120 $\pm$ 12	151 $\pm$ 2	NS
Staurosporin (10^{-6} M)	86 $\pm$ 5	156 $\pm$ 2	<0.0001	99 $\pm$ 2	85 $\pm$ 3	0.01
Calphostin C (10^{-6} M)	91 $\pm$ 2	138 $\pm$ 2	<0.0001	87 $\pm$ 3	90 $\pm$ 5	NS
PD98059 (10^{-5} M)	97 $\pm$ 1	150 $\pm$ 1	<0.0001	96 $\pm$ 1	149 $\pm$ 2	<0.0001
SB203580 (10^{-5} M)	117 $\pm$ 2	155 $\pm$ 1	<0.0001	90 $\pm$ 1	164 $\pm$ 1	<0.0001
SB202190 (10^{-5} M)	122 $\pm$ 5	158 $\pm$ 1	0.002	98 $\pm$ 1	155 $\pm$ 1	<0.0001

^a Values are mean $\pm$ SEM of mast cells' number per five hpf ($n = 6$, each in triplicates) in the absence (–) or in the presence (+) of various inhibitors of transduction pathways. Lower wells contained ITS medium alone (–) or 25 ng/ml FKN (+).

^b Indicates the probability of paired *t* tests.

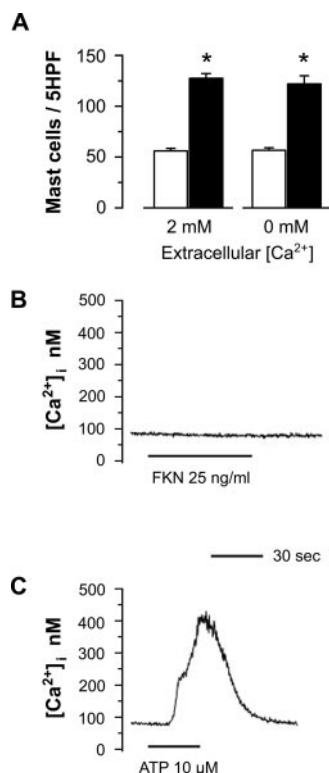


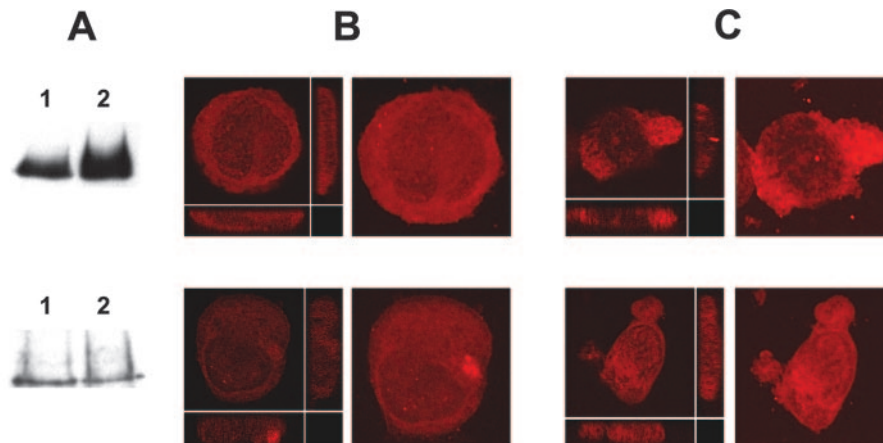
FIGURE 3. Role of calcium in FKN-induced mast cell chemotaxis. Mast cell migration (A) was assessed toward 25 ng/ml FKN (■) in the presence (2 mM) or absence of extracellular calcium (0 mM). Data are mean $\pm$ SEM of mast cell number per five hpf (original magnification, $\times 400$) from six experiments. Results compared with controls (□) using the paired Student's *t* test (*, $p < 0.05$). Typical intracellular calcium ($[Ca^{2+}]_i$) responses evoked by a short stimulation of mast cells with 25 ng/ml FKN (B) or 10 μ M ATP (C). Each trace is representative of experiments performed in 27–125 cells.

HASMC supernatant induced a significant mast cell chemotaxis when compared with negative control (buffer only), but this effect was not affected by the presence of a blocking anti-FKN Ab (Fig. 5E). However, the presence of blocking anti-TGF- β 1 decreased unstimulated HASMC-induced mast cell chemotaxis (Table II). Challenging HASMC with 100 ng/ml TNF- α or IL-1 β for 72 h significantly increased the supernatant-induced mast cell chemotaxis, when compared with supernatants collected from unchallenged cells. This increased mast cell chemotaxis was not due to a

direct effect of TNF- α or IL-1 β since no difference was obtained when these cytokines were added in the culture medium in the absence of HASMC (data not shown). Again, the presence of blocking anti-FKN Ab did not alter mast cell migration (Fig. 5E). However, the presence of blocking anti-IP-10, anti-TGF- β 1, or anti-SCF decreased TNF- α -stimulated HASMC-induced mast cell chemotaxis (Table II).

It was then our hypothesis that another local product, the neuropeptide VIP, could amplify the FKN effect. VIP is highly expressed in the airways and detected within the smooth muscle layer of asthmatic patients (19). Several reports have also demonstrated its role in immunomodulation (20). In the present study, VIP potentiated the chemoattractive effect of FKN on mast cells. VIP, by itself, did not induce any chemotaxis and even slightly decreased mast cell migration (Fig. 6A). Priming of mast cells with VIP resulted in a significant increase in FKN-induced chemotaxis (Fig. 6B) that was abolished by the specific inhibition of VIP receptor expressed by mast cells (Fig. 6C). Chemotaxis was differentiated from chemokinesis using VIP-primed mast cells in a checkerboard analysis. FKN at the concentration of 10 ng/ml induced a significant VIP-primed mast cell migration when it was placed in the lower wells (255 ± 2.9 vs 151 ± 2.0 mast cells per 5 hpf; $p < 0.00001$) and a significantly lower migration was detected (197 ± 4.5 mast cells per 5 hpf) when FKN was placed in both the upper and the lower wells ($p = 0.0002$). However, FKN only placed in the upper wells still induced a mast cell migration (182 ± 1.9 vs 151 ± 2.0 mast cells per 5 hpf; $p < 0.00001$). Therefore, a low concentration of FKN induces a chemotaxis of VIP-primed mast cells and, to a lesser extent, a chemokinesis. We then evaluated the role of priming mast cells with VIP in smooth muscle-induced mast cell migration. For this purpose, we used a blocking anti-FKN Ab in HASMC supernatants before chemotaxis assay. Blocking FKN significantly altered the VIP-primed mast cell chemotaxis when HASMC were stimulated with TNF- α (Fig. 6D), whereas it was ineffective using naive mast cells (Fig. 5E) or unstimulated HASMC (Fig. 6D). The role of FKN in smooth muscle-induced mast cell chemotaxis was thus dependent on both the preincubation of mast cells with VIP and the stimulation of HASMC by proinflammatory cytokines. Such a model, including both neurovegetative and inflammatory stimulations, can be considered more relevant regarding the asthmatic pathophysiology. We also checked VIP effects directly on HASMC. Various concentrations of VIP (from 10^{-9} to 10^{-7} M), for various incubation times (from 24 to 72 h), did not alter FKN synthesis by HASMC in terms of mRNA using quantitative RT-PCR or secreted protein using

FIGURE 4. Effect of FKN on calcium-independent PKC- δ and ϵ isoforms on mast cells. Expression of PKC- δ (top panels) or PKC- ϵ (bottom panels) assessed in HMC-1 by Western blot (A) and confocal microscopy (B and C). Mast cells were either nonstimulated (A, lane 1, and B) or stimulated by 25 ng/ml FKN (A, lane 2, and C). Representative confocal images (B and C) are presented as three axis slices (left panels) and after 3D reconstruction (right panels).



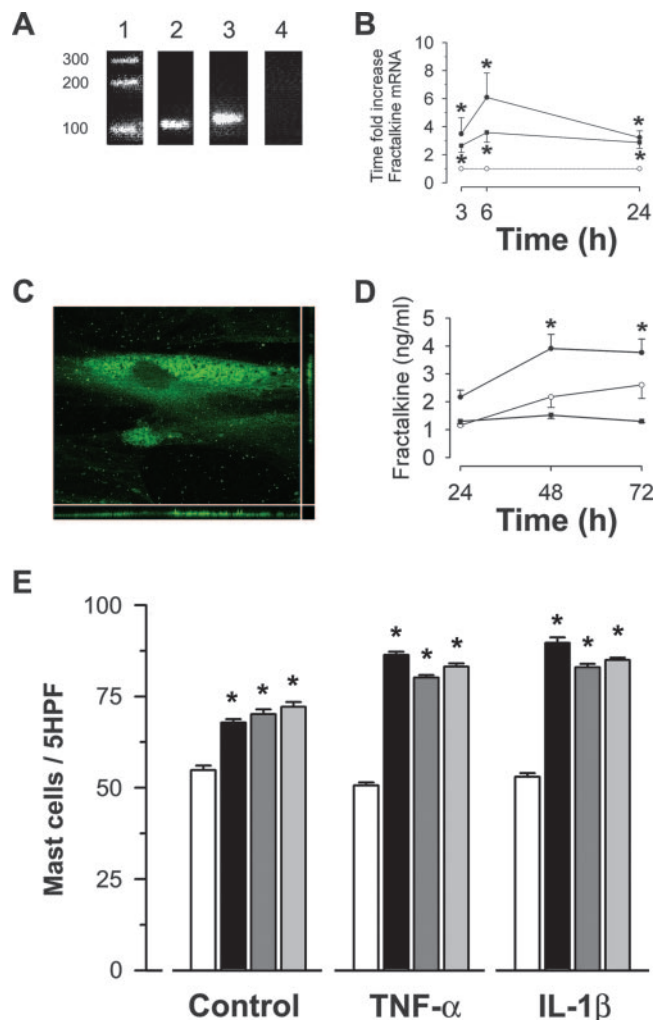


FIGURE 5. Expression and function of FKN secreted by HASMC. RT-PCR (A and B) performed from HASMC mRNA. A, Gel electrophoresis was conducted for GAPDH (lane 2), FKN (lane 3), and CX3CR1 (lane 4) from one representative patient. B, Relative mRNA expression obtained from RT-PCR of FKN on GAPDH in HASMC stimulated for 3–24 h with 100 ng/ml rTNF- α (●), 100 ng/ml IL-1 β (■), or ITS medium (○). Data are mean $\pm$ SEM from five experiments. Results compared using the paired Student's *t* test (*, $p < 0.05$). FKN protein expression assessed by confocal microscopy (C) or ELISA (D). C, Representative three axis slices image with nonstimulated HASMC. D, Supernatants were collected from HASMC nonstimulated (○) or stimulated for 24–72 h with either 100 ng/ml rTNF- α (●) or 100 ng/ml IL-1 β (■). Data are mean $\pm$ SEM from four experiments. Results compared using the paired Student's *t* test (*, $p < 0.05$). Cell migration toward supernatants from HASMC (E) presented as the number of mast cells per five hpf (original magnification, $\times 400$). Mast cell migration toward supernatants was assessed in the absence (black bars) or the presence of 10 μ g/ml irrelevant (dark gray bars) or blocking anti-FKN (light gray bars) Abs. Data are mean $\pm$ SEM from six experiments. Results compared with controls (open bars) using the paired Student's *t* test (*, $p < 0.05$).

ELISA in the supernatants (data not shown). However, using confocal microscopy, VIP appeared to change the subcellular distribution of FKN within the HASMC. Compared with nonstimulated HASMC (Fig. 6E), FKN was more frequently localized to the plasmatic membrane in VIP-stimulated cells (Fig. 6F). This effect was maximal after a cell stimulation of 48 h with VIP at the concentration of 10^{-9} M.

The interaction of VIP with its receptor on mast cells did not alter CX3CR1 mRNA and protein surface expression on mast cells

(data not shown). Neither VIP nor FKN altered Fc ϵ RI and CD117 expression on mast cells (data not shown). Regarding the signaling transduction in VIP-primed mast cells migration toward FKN, significant blockade was obtained with PKC inhibitors (HA-100, staurosporin, calphostin C; Table III). These inhibitors also blocked the migration induced by FKN in the absence of VIP (Table I). However, after VIP priming, a significant blockade of chemotaxis required higher concentrations of calphostin C (10^{-5} M). VIP neither mobilized mast cell calcium nor changed the intracellular calcium response to FKN. After VIP priming, the basal calcium concentration (87 ± 2.0 nM; $n = 87$) or calcium response to 10 ng/ml FKN (90 ± 2.7 nM; $n = 27$) was not significantly changed. Thus, a calcium-independent PKC was still involved in VIP-primed mast cells. The PKA inhibitor (HA-89) and the ERK inhibitor (PD98059) were still ineffective (Table III); however, the inhibitors genistein, SB203580, and SB202190 significantly blocked FKN-induced migration of VIP-primed mast cells, suggesting the involvement of tyrosine kinases and p38 MAPK in VIP receptor signal transduction.

Tissue expression of VIP and FKN in airways from asthmatic and healthy subjects

Mast cell infiltration and expression of FKN and VIP were examined in the bronchial mucosa from asthmatic patients and control subjects using immunohistochemistry. The number of mast cells within the airway smooth muscle layer was higher in tissue from asthmatic patients than in control subjects (Table IV and Fig. 7, A and B). Immunostaining intensity for anti-FKN Ab was significantly higher within the whole bronchial wall from asthmatic patients than that measured in controls (Table IV and Fig. 7, C and D). Immunostaining intensity for anti-VIP Ab was significantly and selectively increased within the smooth muscle layer from asthmatic patients but not within the others layers of the bronchial wall (Table IV and Fig. 7, E and F). Smooth muscle mast cell number significantly correlated with VIP smooth muscle staining intensity ($r = 0.74$; $p = 0.009$) but not with FKN smooth muscle staining intensity ($r = 0.52$; $p = 0.08$). However, FKN staining intensity significantly correlated with VIP staining intensity ($r = 0.59$; $p = 0.03$).

Discussion

Our results demonstrate that FKN can contribute to airway infiltration by mast cell since 1) recombinant FKN induces a human mast cell chemotaxis involving CX3CR1, calcium-independent PKC- δ activation, and actin reorganization and 2) HASMC produce FKN, but VIP priming is necessary for mast cell attraction by smooth muscle-derived FKN.

Regarding the mechanism of the FKN effect on mast cells, our results demonstrate a calcium-independent activation of CX3CR1. Using the mast cell line HMC-1, FKN failed to induce any change in cytosolic calcium concentration, whereas CX3CR1 expressed in rat microglia (21) or stably transfected in a Chinese hamster ovary cell line (22) induced, upon FKN stimulation, a rapid rise in the concentration of intracellular calcium. Although several chemokine receptors are usually linked to calcium signaling, it has been shown that MCP-1 induces monocyte chemotaxis through a calcium-independent pathway (23). In our hands, FKN binding to CX3CR1 did not mobilize calcium but was sensitive to PKC inhibitors. We therefore hypothesized that one of the calcium-independent isoforms (δ or ϵ) of the PKC family was involved upon CX3CR1 activation. We showed that PKC- δ is involved since the total amount of PKC- δ increased and PKC- δ was localized in the mast cell pseudopods. Recent data suggest that PKC- δ is a negative regulator of Ag-induced mast cell degranulation. The

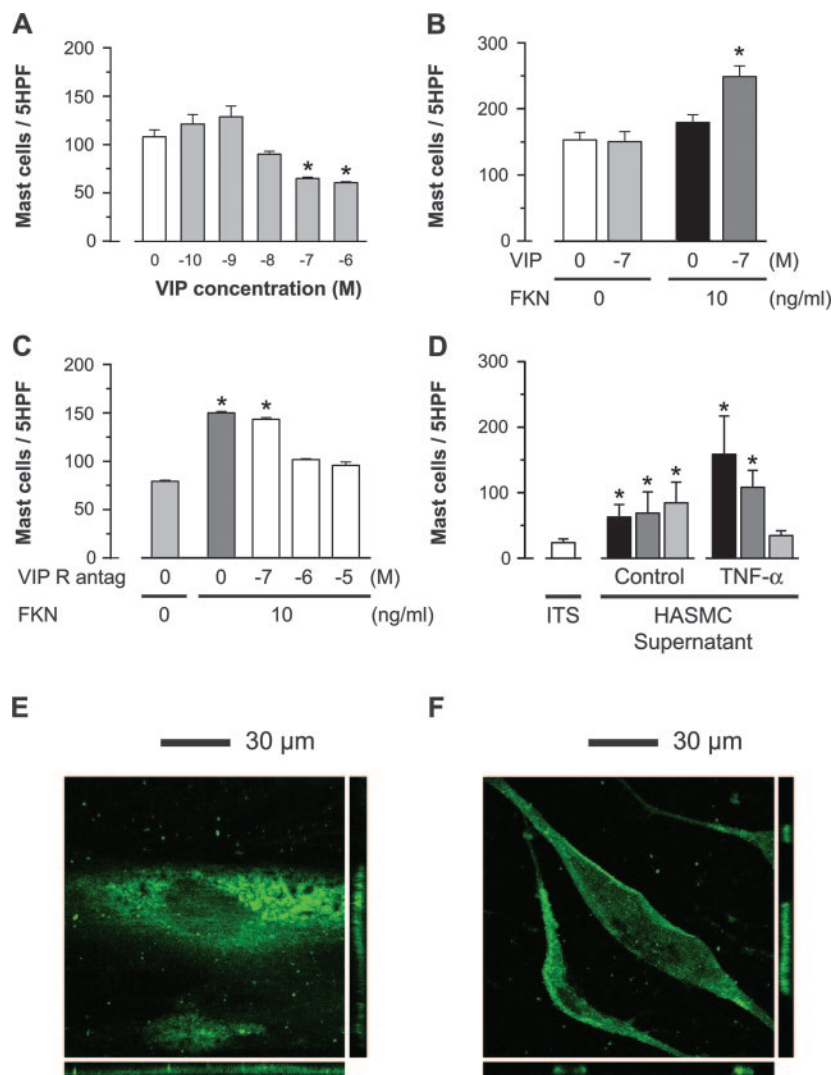
Table II. Mast cell chemotaxis in the absence of VIP^a

	No Ab	Irrelevant Ab	Anti-IP-10-Blocking Ab	Anti-TGF- β 1-Blocking Ab	Anti-SCF-Blocking Ab
ITS	12 $\pm$ 3.4	11 $\pm$ 2.2	13 $\pm$ 4.9	ND	ND
IP-10	75 $\pm$ 14.9	70 $\pm$ 15.4	34 $\pm$ 10.1*	ND	ND
Uns SMC	60 $\pm$ 11.6	69 $\pm$ 13.2	56 $\pm$ 21.4	23 $\pm$ 7.2*	66 $\pm$ 12.6
TNF- α SMC	110 $\pm$ 19.4	123 $\pm$ 28.2	44 $\pm$ 12.9*	54 $\pm$ 10.5*	62 $\pm$ 8.0*

^a Values are mean $\pm$ SEM of mast cells number per five hpf ($n = 6$, each in triplicates) in the presence of various blocking Abs. Lower wells contained ITS medium, recombinant IP-10, unstimulated smooth muscle cell supernatant (Uns SMC), or TNF- α -stimulated smooth muscle cell supernatant (TNF- α SMC).

*, $p < 0.05$ of paired t tests vs no Ab or irrelevant Ab.

FIGURE 6. Role of VIP as a primer for FKN-induced mast cells chemotaxis and HASMC-derived FKN subcellular expression. Mast cell migration assessed toward a range of concentrations of VIP (A), 10 ng/ml FKN (B and C), or HASMC supernatants (D). B, Effect of priming mast cells with 10^{-7} M VIP assessed on HMC-1 chemotaxis induced by a suboptimal concentration of FKN. C, Before chemotaxis assay, all HMC-1 were primed with 10^{-7} M VIP, and migration was assessed in the absence (gray bars) or in the presence (open bars) of increasing concentrations of VIP receptor antagonist [D-p-Cl-Phe⁶,Leu¹⁷]-VIP. D, Similarly, all HMC-1 were primed with 10^{-7} M VIP, and migration toward supernatants was assessed in the absence (black bars) or in the presence of 10 μ g/ml irrelevant (dark gray bars) or blocking anti-FKN (light gray bars) Abs. Data are mean $\pm$ SEM of mast cell number per five hpf (original magnification, $\times 400$) from six separate experiments. Results compared with adapted controls using the paired Student's t test (*, $p < 0.05$). FKN subcellular expression assessed in HASMC by confocal microscopy (E and F). After serum deprivation, HASMC were nonstimulated (E) or stimulated with 10^{-9} M VIP for 48 h. Representative images are presented as three axis slices.

Table III. Mast cell chemotaxis transduction pathway in VIP primed mast cells^a

	FKN	+	+	+	
	VIP	−	+	+	
	Inhibitors	−	−	+	p^b
Genistein (10^{-5} M)	158 ± 1.2	333 ± 1.8	241 ± 3.8	0.000001	
HA-89 (10^{-6} M)	218 ± 2.1	302 ± 2.2	312 ± 5.5	0.11	
HA-100 (10^{-6} M)	87 ± 2.0	131 ± 1.8	82 ± 1.7	0.00002	
Staurosporin (10^{-6} M)	90 ± 1.5	145 ± 2.3	118 ± 2.6	0.002	
Calphostin C (10^{-5} M)	87 ± 1.8	126 ± 1.4	79 ± 2.3	0.00002	
PD98059 (10^{-5} M)	176 ± 2.9	239 ± 16.1	216 ± 1.4	0.21	
SB203580 (10^{-5} M)	170 ± 2.7	220 ± 2.8	202 ± 1.4	0.0006	
SB202190 (10^{-5} M)	173 ± 3.0	218 ± 4.7	189 ± 2.2	0.002	

^a Values are mean $\pm$ SEM of mast cells' number per five hpf ($n = 6$, each in triplicates) in the absence (-) or in the presence (+) of various inhibitors of transduction pathways. Mast cells were primed or not primed (-) with 10^{-7} M VIP. Lower wells contained ITS medium alone (-) or 10 ng/ml FKN (+).

^b The probability of paired t tests vs FKN plus VIP in the absence of inhibitors.

Table IV. Immunohistochemistry in airways from control subjects and asthmatic patients^a

	Control	Asthma	<i>p</i> ^b
Number (male/female)	5 (3/2)	9 (5/4)	
Mast cell number (cells/mm ²)			
Total	53.2 ± 28.4	91.7 ± 28.1	0.15
Epithelium	53.9 ± 45.1	125.1 ± 45.6	0.15
Submucosa	65.4 ± 31.3	98.9 ± 30.6	0.46
Smooth muscle	15.5 ± 4.0	65.0 ± 15.2	0.004
FKN staining intensity (%)			
Total	2.4 ± 1.6	16.3 ± 3.2	0.004
Epithelium	4.3 ± 3.1	31.3 ± 4.7	0.002
Submucosa	2.5 ± 2.0	9.5 ± 3.6	0.04
Smooth muscle	0.9 ± 0.4	13.2 ± 4.1	0.002
VIP staining intensity (%)			
Total	1.1 ± 0.5	15.7 ± 6.2	0.11
Epithelium	1.9 ± 0.9	31.0 ± 10.6	0.07
Submucosa	0.9 ± 0.5	10.5 ± 4.0	0.08
Smooth muscle	0.1 ± 0.1	12.7 ± 6.4	0.03

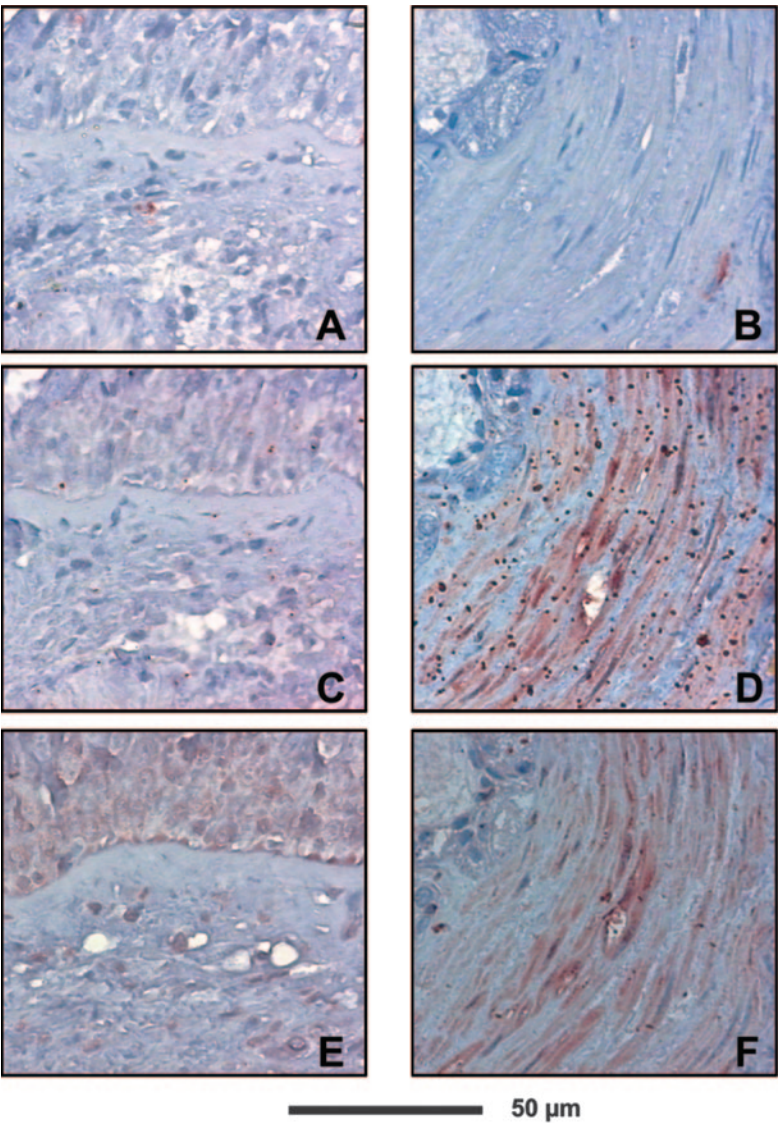
^a Number of cells and staining intensity were assessed by the Quancoul software. Values are mean ± SEM.

^b The probability of unpaired Wilcoxon rank test.

stimulation of bone marrow-derived mast cells from PKC- δ ($^{-/-}$) knockout mice with Ag has been shown to sustain calcium mobilization and increase degranulation compared with wild-type cells

(24). Therefore, it is likely that FKN possesses a specific chemotactic activity on mast cells without inducing degranulation. FKN-CX3CR1 may thus be viewed as a mechanism contributing

FIGURE 7. Relationship between mast cell numbers and FKN or VIP expression within the smooth muscle layer from asthmatic patients. Representative serial sections stained with anti-human tryptase (A and B), anti-human FKN (C and D), or anti-human VIP (E and F). Human bronchial tissue was obtained from a control subject (A, C, and E) or a persistent asthmatic patient (B, D, and F) and observed at $\times 400$ magnification.



to smooth muscle-induced mast cell chemotaxis in addition to the cytokines TGF- β 1 and SCF (10) and to the chemokine CXCL10 or IP-10 (25). This latter chemokine has recently been shown to induce mast cell chemotaxis and to be preferentially expressed by asthmatic smooth muscle. In the present study, we show that, besides the chemotactic activity of TGF- β 1 and SCF, IP-10 also accounts for the mast cell chemotactic activity of stimulated HASMC in the absence of VIP priming. Therefore, it appears that multiple cytokines/chemokines and specific receptors contribute to an autoactivation loop involving mast cell and airway smooth muscle.

Although substance P is a well-known activator for mast cell functional responses such as chemotaxis or degranulation, the effect of VIP on human mast cell is not fully characterized. Nevertheless, it has been shown that VIP is also produced and secreted by Th2 cells following Ag stimulation (26) and can be now considered as a real Th2 cytokine (27). In this respect, VIP up-regulates the Th2 chemokine CCL22 and down-regulates Th1 chemokine CXCL10 (28). With regard to leukocyte migration, VIP inhibits the chemotaxis of monocytes or T lymphocytes in response to CCL3, CCL4, and CCL5 (29), and its closely related peptide PACAP is able to inhibit neutrophil chemotaxis (30). In our study, VIP was necessary to the chemoattractive effect of FKN on mast cells, reinforcing the idea that VIP strengthens the consequences of Th2 response. Moreover, whereas VIP did not alter FKN production, there was a clear membrane redistribution of FKN within the HASMC. Membrane-bound FKN is thus able to bind to CX3CR1 on mast cells as on NK cells (5). FKN is quite unique among chemokines to display such adhesion properties combined with a chemotactic effect after being released as a soluble form (31). This cleavage is PKC dependent and requires proteases such as TNF- α -converting enzyme (31), the concentration of which has been suggested to increase in asthma (32). In our study, we found an increase in FKN expression within the airway smooth muscle of asthmatics, and a recent report demonstrated an increased secretion of FKN in the lavage fluid upon allergen stimulation (7), suggesting the activation of FKN cleavage in asthma. Finally, the importance of VIP expression by airway smooth muscle and its relationship with mast cell infiltration and FKN expression suggest a close interaction among nerves, smooth muscle, and mast cells in airway inflammation in asthma.

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Disclosures

The authors have no financial conflict of interest.

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