ROLE(S) OF *FORMYL*-PEPTIDE RECEPTOR EXPRESSED IN NASAL EPITHELIAL CELLS

N. PREVETE[1], F.A. SALZANO[2], F.W. ROSSI[1], F. RIVELLESE[1], M. DELLEPIANE[3], L. GUASTINI[3], R. MORA[3], G. MARONE[1], A. SALAMI[3] and A. DE PAULIS[1]

[1]Department of Clinical Immunology and Allergy, University of Naples Federico II, Naples; [2]Sperimental Biomedical and Neuroscience Department (BIONEC), University of Palermo, Palermo; [3]ENT Department, University of Genoa, Genoa, Italy

Received July 14, 2011 – Accepted October 28, 2011

Chronic rhinosinusitis is one of the most frequent chronic diseases in humans. Little is known about stimuli initiating tissue remodeling process that determines the morphological expression of the disease. *N-formyl* peptide receptors (FPRs) are innate immunity receptors important in tissue remodeling of gastric and intestinal epithelium. The expression and functions of FPRs in nasal epithelial cells were examined to evaluate whether they could be important in the remodeling of nasal mucosa. The aim of this study is to examine FPR expression in a nasal epithelial cell line (RPMI-2650) at mRNA and protein levels. To determine whether FPRs were functional, chemotaxis experiments were carried out. In addition the effects of FPRs agonists on the expression (PCR and ELISA) of VEGF-A and TGF-β, two key mediators of tissue remodelling, were examined. Here we demonstrate that RPMI-2650 express FPR and FPRL2, but not FPRL1. fMLP, a bacterial product active on FPR, and $uPAR_{84-95}$, an inflammatory mediator agonist for FPRL2, stimulated migration of nasal epithelial cells. fMLP and $uPAR_{84-95}$ induce expression and secretion of VEGF-A and TGF-β. Our results suggest a possible mechanisms initiating tissue remodeling observed during chronic rhinosinusitis. This study provides further evidence that FPRs play a more complex role in human pathophysiology than bacterial recognition.

Chronic rhinosinusitis (CRS) is presently classified into two subgroups: CRS with and without nasal polyps. CRS may occur in macroscopically different morphological variants such as: i) a simple hyperplasia of the sinus mucosa; ii) polypoid degeneration; iii) polyposis (1).

A variety of inflammatory mediators, including cytokines and chemokines, as well as adhesion molecules and matrix metalloproteinases, are upregulated in both subgroups of CRS; remodeling is also observed. These conditions are extremely clinically variable; differences are in part due to the existence of different clinical subgroups, and in part to factors not yet defined (2).

Recent studies have advanced knowledge about the different profiles of cytokines related to nasal chronic inflammation and polyps and assessed the degree of involvement of different elements (cell immunity, infectious agents, etc.) in the pathogenesis of these processes. In this regard, the hypothesis that the formation of biofilms of microbic agents (bacterial, fungal, etc) could promote the chronic

Key words: chronic rhinosinusitis, formyl-peptide receptors, fMLP, uPAR

Mailing address: Dr Nella Prevete,
Department of Clinical Immunology and Allergy,
University of Naples Federico II, Via S. Pansini 5
80131 Napoli, Italy
Tel: +39 081 7462219
Fax: +39 081 7462219
e-mail: nellaprevete@gmail.com

0393-974X (2011)
Copyright © by BIOLIFE, s.a.s.
This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.
Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

PROOF

inflammation and tissue remodeling is particularly interesting (3).

Some authors have also suggested the involvement of innate immunity receptors (Toll-like receptors) in the development of chronic inflammation and in the increased expression of cytokines and growth factors (TGF-β, IL-4, and VEGF-A). In particular, Lane et al. demonstrated increased levels of expression of TLR2 and a variety of inflammatory genes in sinonasal mucosa of CRS patients compared with controls (4). In order to clarify some of the mechanism underlying the process of tissue remodeling responsible for such clinical conditions, we investigated the expression and functions of a class of receptors of innate immunity (N-formyl peptide receptors) in nasal epithelium.

N-formyl peptide receptors (FPRs), which are seven transmembrane G-protein-coupled-receptors, regulate innate inflammatory responses. Three FPRs have been identified in humans, each encoded by a different gene, namely FPR, FPR-like 1 (FPRL1), and FPRL2 (5-6). The FPR family has evolved as chemoattractant receptors that assist the organism in counteracting bacterial infections (5). Moreover, FPRs also participate in essential pathophysiologic processes, including intestinal epithelial cell restitution (7).

FPR has a high affinity for the bacterial-derived peptide *formyl*-methionyl-leucyl-phenylalanine (fMLP) and is activated by nanomolar concentrations of fMLP. Cyclosporin H (CsH) blocks FPR-evoked responses. FPRL1 is a promiscuous receptor activated in response to high concentrations of fMLP, the prion peptide ($PrP_{106–126}$), lipoxin A_4, Hp(2-20), serum amyloid A, and various synthetic peptides. Three natural ligands for FPRL2 have been described: Hp(2-20), $uPAR_{84-95}$ peptide and F2L (5). Recently, we demonstrated that gastric epithelial cells express FPR, FPRL1, and FPRL2 at mRNA and protein level. We also provided evidence that a peptide derived from *H. pylori*, i.e. Hp(2-20), stimulates gastric epithelial cell migration, proliferation and up-regulates VEGF-A expression through the interaction with FPRL1 and FPRL2 (8).

Our study with gastric epithelial cells supports the findings that activation of FPRs could positively influence the remodeling phase of gastric mucosal healing. In fact, revascularization of damaged tissue through the process of neoangiogenesis is a necessary part of wound healing (9) and deranged angiogenesis leads to abnormal healing of ulcers (10).

The hypothesis of this study is that FPRs might play a role in regulating the process of nasal epithelium remodeling by facilitating epithelial cell migration, growth factor production and neoangiogenesis. For this purpose we investigated the effects on nasal epithelium of two ligands of FPRs: fMLP, a bacterial peptide mimicking the antigenic stimulation induced *in vivo* by external pathogens; and $uPAR_{84-95}$, a chemotactic epitope exposed after urokinase plasminogen activator receptor (uPAR) cleavage, found in tissues and biological fluids during inflammatory conditions (11).

Therefore, this study was designed: 1) to assess whether nasal epithelial cell lines express FPRs; 2) to investigate whether FPR ligands stimulate cell migration; 3) to evaluate whether FPR agonists could induce the expression in nasal epithelial cell lines of TGF-β and VEGF-A, two key mediators of tissue remodeling.

MATERIALS AND METHODS

Peptides and chemicals

fMLP was purchased from ICN Biomedicals Inc. (Aurora, OH). CsH was obtained from Novartis Pharmaceuticals (Basel, Switzerland). The hexapeptide WKYMVm was synthesized by Innovagen (Lund, Sweden); the peptide $uPAR_{84-95}$ (AVTYSRSRYLEC) and its scrambled version (TLVEYYSRASCR) were synthesized by PRIMM (Milan, Italy). CsH was obtained from Drs. D. Romer and E. Rissi (Novartis, Basel, Switzerlan).

Cell culture

PMI-2650 cell line derived from a squamous cell carcinoma of the nasal septum. RPMI-2650 cells were grown as monolayer in Minimum Essential Medium Eagle (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum (12). To induce VEGF-A protein, cells were incubated in medium supplemented with 5% FCS, in order to maintain stability of the protein (13).

Isolation of cellular mRNA and RT-PCR analysis

Total RNA was isolated from nasal epithelial cell

PROOF

and retrotranscribed according to the manufacturer's instructions (Promega, Madison, WI). Equivalent templates of cDNAs were amplified for FPR, FPRL1, FPRL2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using specific primers. The primers for FPR were 5' sense ATGGAGACAAATTCCTCTCTC and 3' antisense CACCTCTGCAGAAGGTAAAGT; for FPRL1 were 5' sense CTTGTGATCTGGGTGGCTGGA and 3' antisense CATTGCCTGTAACTCAGTCTC; for FPRL2 were 5' sense ATTAAATCCAGCCGTCCCTT and 3' antisense ACTAGTGGGCAAAGAGCGAA. The primers for GAPDH were 5' sense GCCAAAGGGTCATCATCTC and 3' antisense GTAGAGGCAGGGATGATGTTC. PCR products, together with a DNA ladder as a size standard, were separated on a 1% agarose gel, stained with ethidium bromide, and visualized with the image analysis system ChemidocXRS (Bio-Rad Laboratories, Hercules, CA) (14)

Real-time quantitative PCR was performed on the iCycler (Bio-Rad) by using the PE SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA). Target-specific primers for GAPDH, VEGF-A, and TGF-β suitable for real-time PCR were designed by using Beacon Designer 3.0 software (Bio-Rad; GAPDH: 5' sense GCCAAAGGGTCATCATCTC and 3' antisense GTAGAGGCAGGGATGATGTTC; VEGF-A: 5' sense GTGAATGCAGACCAAAGAAAG and 3' antisense AAACCCTGAGGGAGGCTC; TGF-β: 5' sense AGCCTGAGGCCGACTACTAC and 3' antisense TGTTAAGGACCGCTATGGAG). Each cDNA sample was analyzed in triplicate, and the corresponding no-RT mRNA sample was included as a negative control. The data were analyzed with iCycler iQ analysis software (Bio-Rad), and the changes in VEGF-A and TGF-β mRNAs were expressed as the ratio versus GAPDH (8).

Flow cytometric analysis

Anti-FPR, -FPRL1 and -FPRL2 antibodies recognize intracellular epitopes. Therefore, a cell membrane permeabilization assay was carried out using the Citofix/Cytoperm kit (BD Biosciences Pharmigen, San Diego, CA, USA) before staining, according to the manufacturer's instructions. Briefly, cells were incubated for 30 min at 4°C with specific or isotype control Abs. For indirect staining, this step was followed by a second incubation for 20 min at 4°C with appropriate anti-isotype FITC-conjugated Abs. Finally, cells were washed and analyzed with a FACSCalibur cytofluorimeter using CellQuest software (BD Bioscience, San Ferdinando, CA, USA). A total of 10^4 events for each sample were acquired in all cytofluorimetric analysis (8).

Chemotaxis assay

Nasal epithelial cell chemotaxis was performed using a modified Boyden chamber technique. Briefly, 25 µl of complete cell culture medium or various concentrations of the chemoattractants in the same buffer were placed in triplicate in the lower compartment of a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD). The lower compartments were covered with 8-µm-pore polycarbonate membranes (Nucleopore, Pleasanton, CA, USA), which were coated with fibronectin 10 µg/ml (Sigma, St. Louis, MO). Fifty microliters of the cell suspensions (5 x 10^4/well) resuspended in complete cell culture medium were pipetted into the upper compartments. The chemotactic chamber was then incubated for 6 h at 37°C in a humidified incubator with 5% CO_2 (Automatic CO_2 Incubator, model 160 IR; ICN Flow, Milan, Italy). At the end of incubation, the membrane was removed; the upper side was washed with PBS, and the filter was fixed, stained with May-Grunwald-Giemsa, and mounted on a microscope slide with Cytoseal (Stephens Scientific, Springfield, NJ, USA). Nasal epithelial cell chemotaxis was quantified microscopically by counting the number of cells attached to the lower surface of the 8-µm cellulose nitrate filter. In each experiment, 10 fields per triplicate filter were measured at a x40 magnification. The results were compared with buffer controls. Checkerboard analyses were performed to discriminate between chemotaxis and non-directed migration (chemokinesis) of nasal epithelial cell. In these experiments, cells were placed in the upper chemotactic chambers and various concentrations of stimuli or buffer were added to the upper or lower wells or to both. Spontaneous migration (chemokinesis) was determined in the absence of chemoattractant or when stimuli were added to either the lower or upper chambers. The cell migratory response to chemotactic stimuli was largely due to chemotaxis and not to chemokinesis. Indeed, a checkerboard analysis, in which chemoattractants above and below the filters varied, resulted in significant migration only when there was a gradient of the factor below the filters (15). In desensitization experiments RPMI-2650 cells were treated with buffer or with $uPAR_{84-95}$ (10^{-10} M), or the WKYMVm peptide (10^{-8} M) in culture medium containing EDTA (4 mM) for 1 h at 37°C. At the end of incubation, cells were washed and resuspended in MEM containing 10% FCS and allowed to migrate in response to $uPAR_{84-95}$ (10^{-10} M).

Scratch motility assay

Confluent monolayers of RPMI-2650 cells were treated with mitomycin (2 µg/ml for 2 h) (Sigma, St. Louis, MO) to inhibit cell growth. Monolayers were then wounded with a single-edge razor blade; three wounds, 10 to 12 mm across and separated by 1.5 cm, were made in each dish as previously described (8).

PROOF

After wounding, cells were washed with fresh serum-free medium, and the wounded monolayers were incubated for 24 h with medium alone, as control, fMLP (10^{-8} M), $uPAR_{84-95}$ (10^{-10} M). After fixation in absolute methanol and staining with hematoxylin and eosin, migration was assessed in a blinded fashion to avoid observer bias by counting the number of RPMI-2650 cells that crossed the wound border. The mean number of cells that migrated per centimeter in six 1-cm wound segments within each triplicate dishes was calculated; results are expressed as percentage of control treated with unstimulated cells.

VEGF-A and TGF-β ELISA

VEGF-A and TGF-β release in the culture supernatants of nasal epithelial cells was measured in duplicate determinations with a commercially available ELISA (R&D Systems, Minneapolis, MN, USA).

Statistics

The results are expressed as mean ± SEM. Values from groups were compared using the paired Student's *t* test (16). Differences were considered significant when *p* was less than 0.05.

RESULTS

Expression of FPR, FPRL1 and FPRL2 in human nasal epithelial cells

We examined FPR, FPRL1 and FPRL2 expression at mRNA and protein levels in human nasal epithelial cells derived from a squamous cell carcinoma of the nasal septum (i.e. RPMI-2650 cells). PBMC were used as a control. The analysis of the PCR product by electrophoresis in agarose gel showed FPR and FPRL2 mRNA expression in RPMI-2650 cell line and PBMC (Fig. 1A). By contrast, RPMI-2650 cells do not express FPRL1 mRNA whereas PBMC, used as a positive control, express FPRL1 mRNA at high levels. We then confirmed FPR and FPRL2 expression in nasal epithelial cell lines at protein level using flow cytometry. Fig. 1B shows FPR and FPRL2 expression on the vast majority of RPMI-2650 cells. These experiments demonstrate that this cell line synthesize mRNA for FPRs and translate it into proteins.

Effects of fMLP, WKYMVm and $uPAR_{84-95}$ on chemotaxis of human nasal epithelial cells

Because chemotaxis is believed to be a crucial step for accumulation of epithelial cells and subsequently for tissue remodeling (17), we tested the capacity of the bacterial chemotactic peptide fMLP to induce directional migration of RPMI-2650. Nasal epithelial cells (Fig. 2A) migrated significantly toward fMLP (10^{-10}-10^{-6} M) with a bell-shaped dose-response curve, similar to the typical response to fMLP observed in inflammatory cells (18). Checkerboard analysis showed that cell migration induced by fMLP is mainly due to a chemotactic effect with a minor contribution of chemokinesis (data not shown). In these experiments, the hexapeptide WKYMVm (10^{-10}-10^{-6} M), which activates FPR, FPRL1, and FPRL2 with different affinities (5), proved to be a very potent chemotactic stimulus plateauing at 10^{-8} M (Fig. 2B). These results support the concept that RPMI-2650 cells carry FPRs whose activation stimulates chemotaxis.

We next evaluated the *in vitro* effects of a wide range of concentrations (10^{-12}-10^{-8} M) of $uPAR_{84-95}$ on chemotaxis of nasal epithelial cells. uPAR (CD87) is the specific high affinity cell surface receptor for the urokinase plasminogen activator (uPA). The uPAR-derived peptide fragment $uPAR_{84-95}$ induces basophil migration by binding to FPRL2. Fig. 2C shows the results of five experiments in which subnanomolar concentrations of $uPAR_{84-95}$ caused RPMI-2650 chemotaxis, which plateaued at 10^{-10} M. The $uPAR_{84-95}$ scrambled peptide was not active (Fig. 2C). We carried out a checkerboard analysis, which showed that $uPAR_{84-95}$-induced migration of epithelial cells resulted from chemotaxis, rather than from chemokinesis (data not shown).

Epithelial cell migration and FPRs

We next performed experiments to determine which member of the FPR family mediates fMLP- and $uPAR_{84-95}$-dependent nasal epithelial cell chemotaxis (Fig. 3). We previously demonstrated that cyclosporin H (CsH) is a specific antagonist of FPR (19). Preincubation (15 min at 37°C) of RPMI-2650 cells with CsH (8 x 10^{-7} M) strongly inhibited chemotaxis induced by fMLP (10^{-8} M), whereas it did not affect cell migration induced by $uPAR_{84-95}$ (10^{-10} M) (Fig. 3A). These results indicate that fMLP induced nasal epithelial cell chemotaxis by activating FPR, whereas $uPAR_{84-95}$ used a different receptor.

To evaluate whether FPRL2 are involved in

PROOF

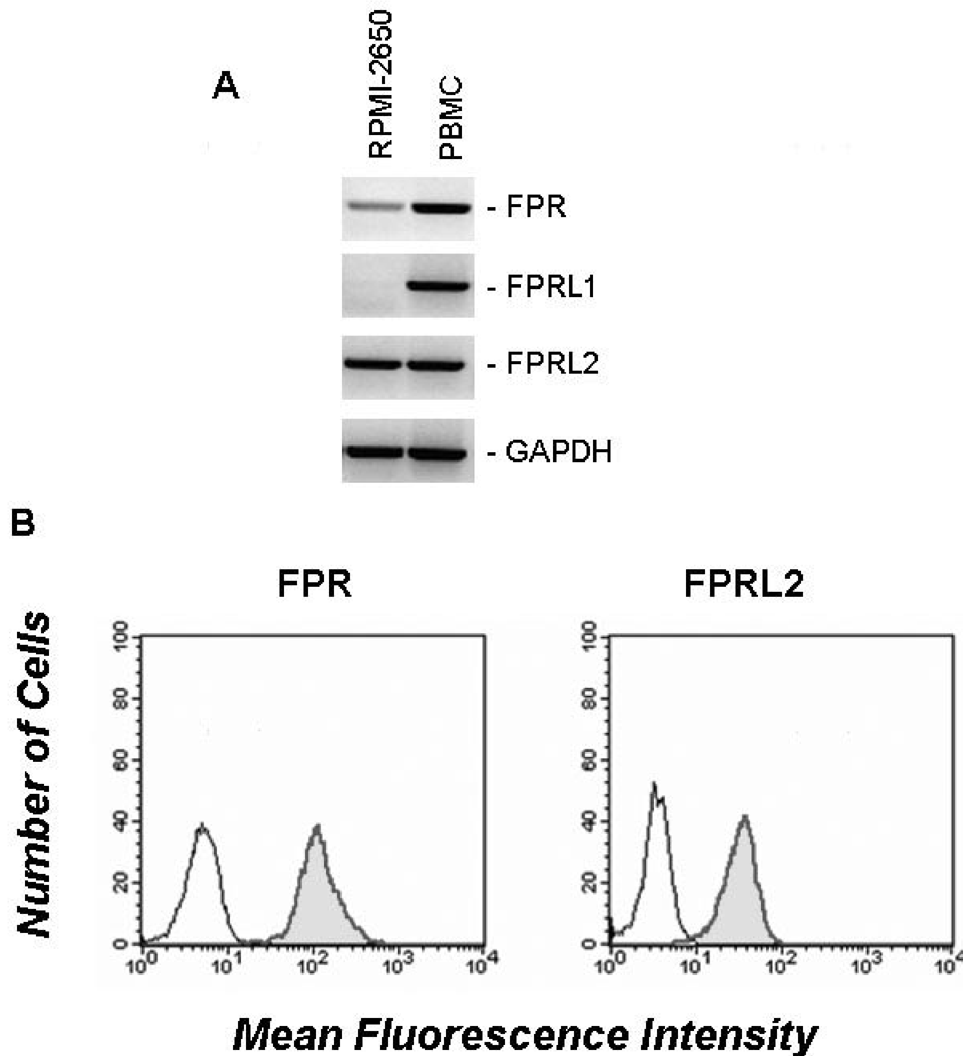

Fig. 1. *Expression of formyl-peptide receptors in nasal epithelial cell lines.* ***A****) RPMI-2650 cells were lysed in TRIzol. Total RNA was prepared, reverse transcribed and amplified by 40 PCR cycles in the presence of FPR, FPRL1 and FPRL2, and GAPDH specific primers. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide.* ***B****) Cytofluorimetric analysis of FPR, and FPRL2 expression in RPMI-2650. After cell membrane permeabilization, cells were incubated for 30 min at 4°C with specific (grey histogram) or isotype (empty histogram) control Abs. For indirect staining, this step was followed by a second incubation for 20 min at 4°C with appropriate anti-isotype FITC-conjugated Abs.*

$uPAR_{84-95}$-induced nasal epithelial cell chemotaxis, we performed desensitization experiments (15). Fig. 3B shows the results of 6 experiments in which the response to $uPAR_{84-95}$ (10^{-10} M) was abolished, as expected, by preincubation with the homologous stimulus. We then investigated the involvement of FPRL2 by preincubating cells with WKYMVm that binds with high affinity to FPRL2 and with a lower affinity also to FPR and FPRL1 (8). Pre-treatment of nasal epithelial cells with WKYMVm (10^{-8} M) strongly reduced $uPAR_{84-95}$-dependent migration (Fig. 3B), thus indicating that $uPAR_{84-95}$-dependent chemotaxis is mediated mainly by activation of FPRL2.

In vitro scratch assay

To complete data regarding the migration of nasal epithelial cells we performed a *scratch motility*

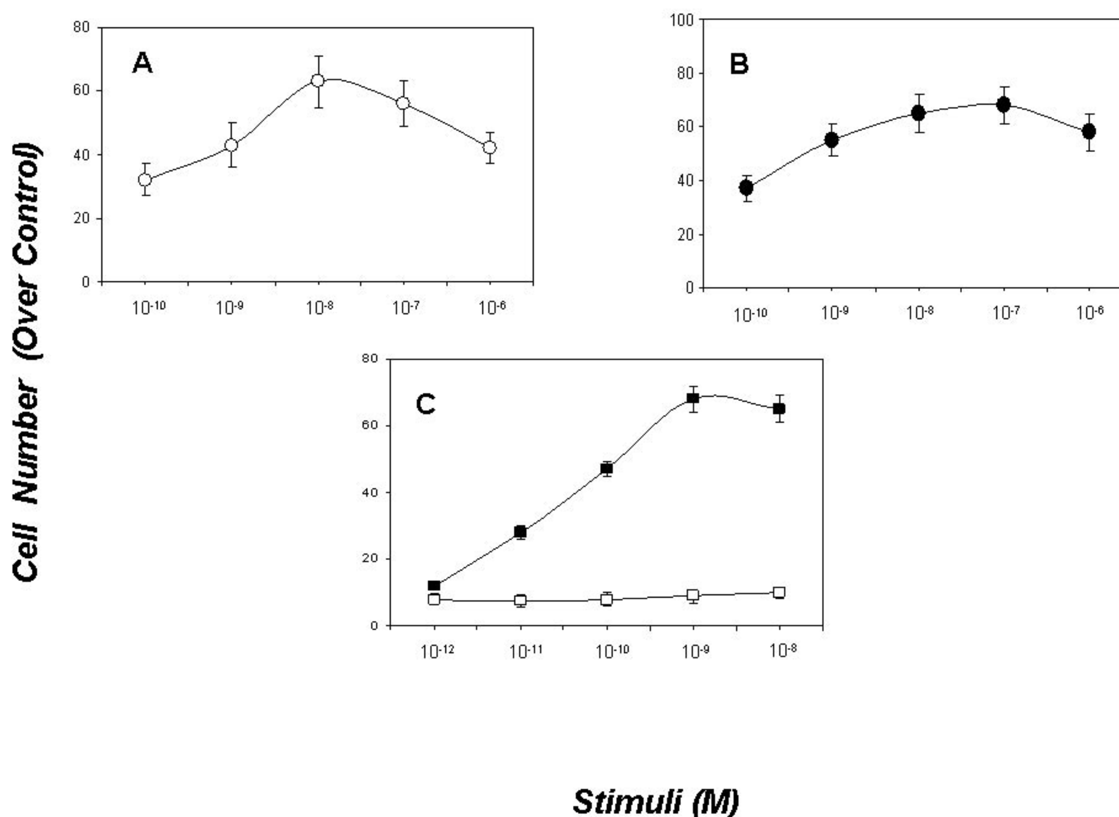

Fig. 2. *Effects of fMLP, WKYMVm and uPAR$_{84-95}$ on nasal epithelial cell chemotaxis. RPMI-2650 cells were allowed to migrate with the indicated concentrations of fMLP (○) (panel **A**) and WKYMVm (●) (panel **B**) or uPAR$_{84-95}$ (■) and its scrambled peptide (□) (panel **C**) for 6 h at 37°C in a humidified (5% CO_2) incubator. Values are the mean ± SEM of six experiments.*

assay, which is an *in vitro* model of wound healing. The *in vitro* scratch assay is a straightforward and economical method to study cell migration *in vitro*. Fig. 4 shows that fMLP (10^{-8} M) induced cell migration, thereby increasing the number of migrated cells by approximately 2-fold compared to the control. Preincubation of cells with CsH (8 x 10^{-7} M) strongly inhibited the migration in response to fMLP. uPAR$_{84-95}$ (10^{-10} M) induced cell migration, thereby increasing the number of migrated cells by approximately 3-fold compared to the control. CsH was not able to inhibit uPAR-induced migration. The stimulatory effect of fMLP and uPAR$_{84-95}$ on cell migration was dose-dependent (data not shown).

Effects of fMLP and uPAR$_{84-95}$ peptides on VEGF-A expression and release from nasal epithelial cells

Nasal epithelial cells have the potential to induce neovascularization. Vascular Endothelial Growth Factor (VEGF) plays an important role in perpetuating primary nasal epithelial cell overgrowth, a key feature of hyperplastic polyps. FPR activation caused by fMLP induced VEGF expression in a model of glioblastoma (20) and in gastric epithelial cells (8). Therefore, we investigated the effects of fMLP (10^{-8} M) and uPAR$_{84-95}$ (10^{-10} M) on VEGF-A mRNA expression in nasal epithelial cell lines. Our experiments demonstrate that fMLP slightly induced VEGF-A mRNA, and that uPAR$_{84-95}$ caused an approximately 1.7-fold increase of VEGF-A mRNA level compared to control (Fig. 5A). We then

PROOF

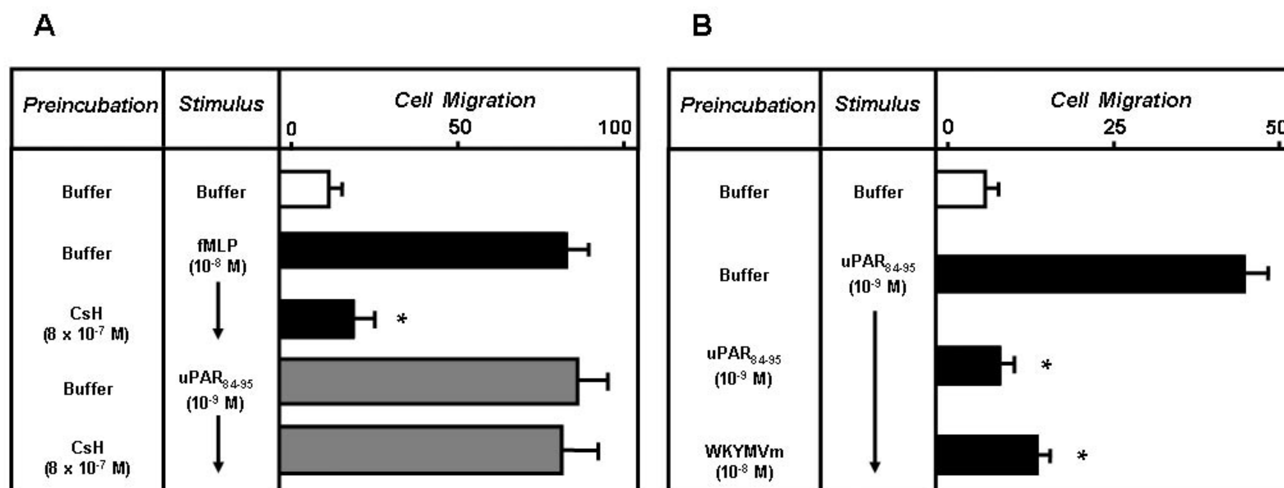

Fig. 3. *Nasal epithelial cell migration and FPRs. **A**) Effects of CsH on RPMI-2650 chemotaxis induced by fMLP and $uPAR_{84-95}$. RPMI-2650 cells were preincubated (15 min at 37°C) with buffer or CsH (8 x 10^{-7} M). Then, cells were allowed to migrate toward fMLP (10^{-8} M) or $uPAR_{84-95}$ (10^{-10} M) for 6 h at 37° C in a humidified incubator with 5% CO_2. Values are the mean ± SEM of six experiments. * p<0.05 when compared with the relative control. **B**) Effects of homologous and heterologous desensitization between $uPAR_{84-95}$, and WKYMVm on RPMI-2650 chemotaxis. RPMI-2650 cells were treated with buffer or with $uPAR_{84-95}$ (10^{-10} M), or WKYMVm peptide (10^{-8} M) in culture medium containing EDTA (4 mM) for 1 h at 37°C. At the end of incubation, cells were washed and resuspended in MEM containing 10% FCS and allow to migrate in response to $uPAR_{84-95}$ (10^{-10} M). Values are the mean ± SEM of six experiments. * p<0.05 when compared with the relative control.*

PROOF

evaluated VEGF-A release in RPMI-2650 cells treated with fMLP (10^{-8} M) or $uPAR_{84-95}$ (10^{-10} M). We found that incubation for 6 h caused a significant ($p<0.05$) increase in the VEGF-A release (Fig. 5B) in response to both fMLP (10^{-8} M) and $uPAR_{84-95}$ (10^{-10} M).

Effects of fMLP and $uPAR_{84-95}$ peptides on TGF-β expression and release from nasal epithelial cells

Overexpression of TGF-β plays a key role in tissue remodeling. Therefore, we investigated the effects of fMLP (10^{-8} M) and $uPAR_{84-95}$ (10^{-10} M) on TGF-β mRNA expression in nasal epithelial cell lines. Our experiments demonstrate that fMLP and $uPAR_{84-95}$ significantly induced TGF-β mRNA level compared to control (Fig. 6A). We then evaluated TGF-β release in RPMI-2650 cells treated with fMLP (10^{-8} M) or $uPAR_{84-95}$ (10^{-10} M). We found that incubation for 6 h caused a significant ($p<0.05$) increase in the release of TGF-β (Fig. 6B) in response to both fMLP (10^{-8} M) and $uPAR_{84-95}$ (10^{-10} M).

DISCUSSION

This study demonstrates that nasal epithelial cells express FPR and FPRL2 at mRNA and protein levels. Moreover, we provide evidence that fMLP and a peptide derived from uPAR, i.e. $uPAR_{84-95}$, stimulate nasal epithelial cell migration and up-regulate VEGF-A and TGF-β expression at both transcriptional and translational levels through the interaction with FPR and FPRL2 receptors, respectively.

FPRs have been identified on different cells and belong to the STM, G protein-coupled rhodopsin superfamily (5). FPR is activated by nanomolar concentrations of fMLP. FPRL1 is a promiscuous receptor activated in response to high concentrations of fMLP, and various synthetic, bacterial and viral

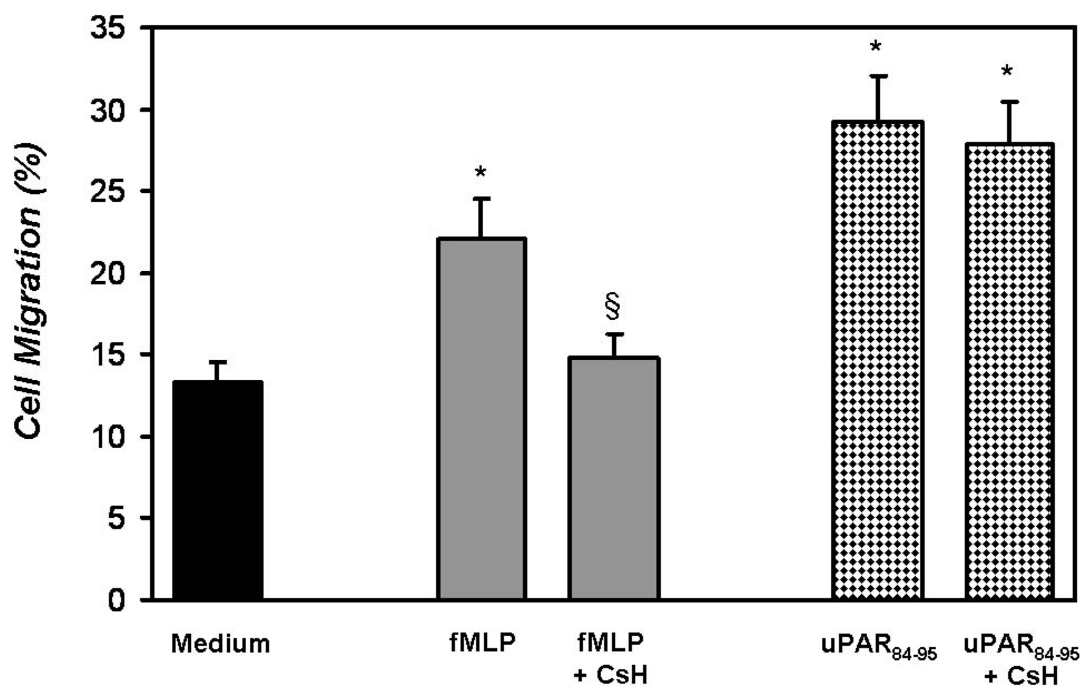

Fig. 4. *$uPAR_{84-95}$ affects cell migration in a scratch motility assay. RPMI-2650 cell monolayers were wounded with a single-edge razor blade. The wounded monolayers were stimulated for 24 h with medium alone as control, fMLP (10^{-8} M) or $uPAR_{84-95}$ (10^{-10} M), preincubated or not with CsH (8 x 10^{-7} M). Results are expressed as percentage of cell migration observed in cells treated with stimuli compared to unstimulated cells. Values are the mean ± SEM of three experiments. * $p<0.05$ when compared with unstimulated cells; § $p<0.05$ when compared with fMLP*

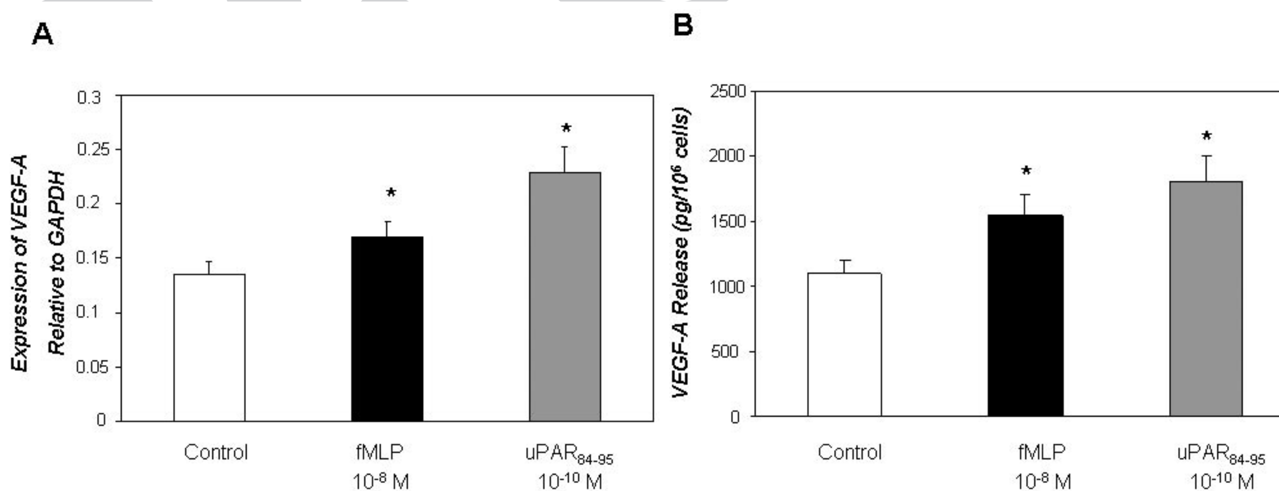

Fig. 5. *FPRs activation induces VEGF-A production in RPMI-2650 cells both mRNA and protein level.* ***A)*** *Real-time quantitative RT-PCR analysis of VEGF-A in RPMI-2650 cells cultured with medium alone, fMLP or $uPAR_{84-95}$ for 2 h. The expression of VEGF-A mRNA was expressed as fold increase vs GAPDH. The values are expressed as the mean ± SEM of three experiments.* ***B)*** *fMLP and $uPAR_{84-95}$-induced release of VEGF-A from RPMI-2650 cells. 10^6 cells/sample were incubated for 6 hour without or with fMLP (10^{-8} M) and $uPAR_{84-95}$ (10^{-10} M). Supernatants were collected at each time-point. VEGF-A was determined by ELISA. Values are expressed as the mean ± SEM of three experiments. * $p<0.05$ when compared with unstimulated cells.*

PROOF

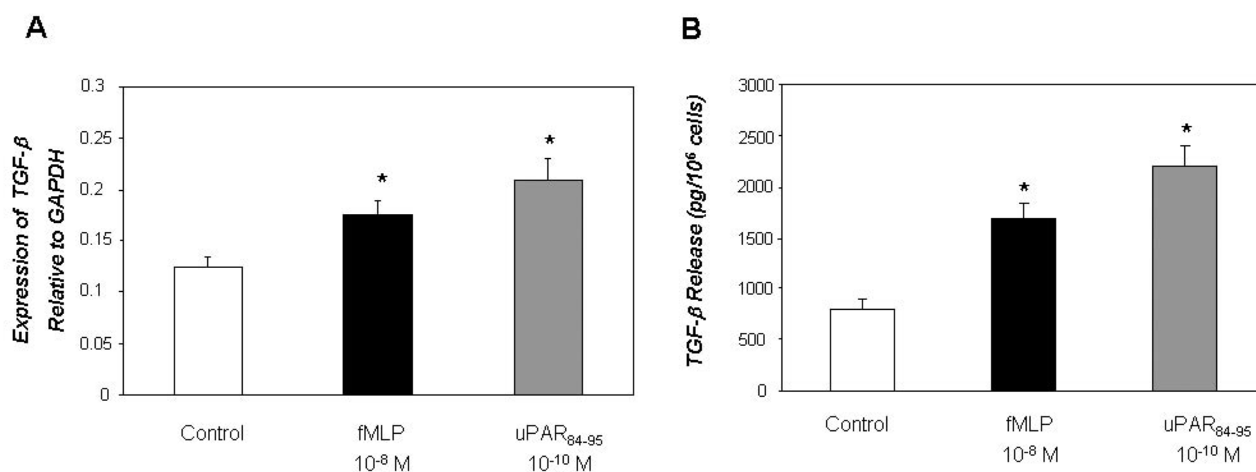

Fig. 6. *FPRs activation induces TGF-β production in RPMI-2650 cells both mRNA and protein levels. **A)** Real-time quantitative RT-PCR analysis of TGF-β in RPMI-2650 cells cultured with medium alone, fMLP or $uPAR_{84-95}$ for 2 h. The expression of TGF-β mRNA was expressed as fold increase vs GAPDH. **B)** fMLP and $uPAR_{84-95}$-induced release of TGF-β from RPMI-2650 cells. 10^6 cells/sample were incubated without or with fMLP (10^{-8} M) and $uPAR_{84-95}$ (10^{-10} M). Supernatants were collected at each time point. TGF-β was determined by ELISA. Values are expressed as the mean ± SEM of three experiments. * $p<0.05$ when compared with unstimulated cells.*

peptides (5). Interestingly, these peptides do not share any sequence homologies, in agreement with the promiscuous nature of FPRL1. Three natural ligands for FPRL2 have been described: Hp(2-20), $uPAR_{84-95}$ peptide and F2L (5). We have demonstrated that human basophils express FPR, FPRL1, and FPRL2 (15). A previous report indicates that epithelial cells express FPR and that its activation enhances intestinal epithelial cell restitution (7). We recently demonstrated that human gastric epithelial cells express FPR, FPRL1, and FPRL2 and that activation of FPRs causes gastric epithelial cell migration (8). In order to evaluate the functions of FPRs in nasal epithelial cells we used two natural agonists: fMLP and $uPAR_{84-95}$.

Proinflammatory peptides are produced by aerobic and anaerobic bacteria. The NH_2 terminal *formyl* peptides have potent chemotactic activity with fMLP being the major peptide chemotactic factor produced by *Escherichia coli* (21). Bacteria producing fMLP has been used to induce animal model of mucosal inflammation (22).

The uPA-uPAR system is an important and complex cellular recognition system that mediates fibrinolysis, cell adhesion and migration, and tissue remodeling. uPAR can be cleaved by several enzymes, including uPA, exposing a chemotactic epitope on uPAR anchored on the cell surface (23). This chemotactic epitope (residues 84-95), in a soluble form, also acts as a potent chemoattractant for monocytes by activating FPRL1 (24) and for basophils by activating FPRL2 (15). Soluble cleaved forms of uPAR, containing the chemotactic epitope, have been found in tissues and biological fluids (11). The involvement of uPA and uPAR in inflammation, regardless of plasmin activity, has been demonstrated *in vitro* and *in vivo*. uPA is a potent chemoattractant for neutrophils, basophils and monocytes and primes neutrophils for superoxide anion release (23). Several intriguing observations support a role also for uPAR in cell locomotion (25) and for neutrophil degranulation (26). Finally, $uPA^{-/-}$ and $uPAR^{-/-}$ mice show impaired neutrophil recruitment and susceptibility to bacterial infections (27).

Remodeling is defined as a process leading to transient or permanent changes in tissue architecture, which involves breakdown of tissue structures (e.g., basement membranes and interstitial stroma) as well as repair. The nasal and paranasal sinuses are lined with a stratified columnar epithelium comprising

PROOF

ciliated and secretory cells and supported by basal cells. In CRS there is damage to the respiratory epithelium, with squamous metaplasia, ciliary destruction, increase of microvillus cells, and mucous gland and goblet cell hyperplasia (28).

In this study we demonstrate that FPR agonists are able to induce nasal epithelial cell migration in two different models (Boyden chamber and *Scratch Motility Assay*). CsH blocks FPR-evoked responses (19). Accordingly, CsH blocked the chemotactic activity of fMLP on nasal epithelial cells, but had no effect on the response mediated by $uPAR_{84-95}$. We performed desensitization experiments to verify the specificity of the activation route for $uPAR_{84-95}$. The results of these experiments suggest that $uPAR_{84-95}$ acts through FPRL2.

Although angiogenesis appears to be an important event in chronic rhinosinusitis pathogenesis, little is known about the mechanisms of vascular remodeling. Numerous factors are involved in vessel remodeling. Vascular Endothelial Growth Factor (VEGF) could play a key role, exhibiting pro-oedematous and angiogenic properties targeted to endothelial cells. In the present study, the expression of VEGF-A was investigated and, for the first time, it was shown that it is intensely expressed in nasal epithelial cells following innate immune receptor activation.

The TGF-β family is one of the largest families of secreted multifunctional peptides and exerts an array of biological effects in many cell types. TGF-β is involved in the regulation of processes crucial to the initiation, maintenance, and resolution of inflammatory responses (29). TGF-β also plays a role in epithelial cell regeneration, inflammation, and tissue repair (30). Many reports have suggested that TGF-β promotes formation and growth of nasal polyps (2). TGF-β may promote eosinophil infiltration into nasal polyps. Myofibroblast accumulation and activation induced by TGF-β is involved in the pathogenesis of nasal polyps (30). In this study, we show that epithelial cells are a source of TGF-β in response to FPR activation.

The present study raises the intriguing possibility that FPRs are involved in nasal mucosa remodeling. Nevertheless, further studies are required to demonstrate unequivocally that the activation of FPRs observed *in vitro* in transformed cells, sustains the physiology of the remodeling response that occurs *in vivo*.

In conclusion, this study provides further evidence of the complex role of FPRs. In fact FPRs, while playing a central role in counteracting bacterial infections, modulate a number of relevant pathophysiological processes. Our observation on nasal epithelial cells supports and extends our findings on gastric epithelial cells. The observation that activation of FPRs is associated with increased migration and release of VEGF-A and TGF-β in different epithelial cell systems suggest that FPRs could positively influence tissue remodeling.

Finally, the results described in this study may have practical implications in chronic rhinosinusitis. In fact, FPRs may provide a target for novel therapies, highlighting the need for better understanding of their functions in chronic rhinosinusitis.

REFERENCES

1. Fokkens W, Lund V, Mullol J. European position paper on rhinosinusitis and nasal polyps 2007. Rhinol Suppl 2007; 1-136.
2. Otto BA, Wenzel SE. The role of cytokines in chronic rhinosinusitis with nasal polyps. Curr Opin Otolaryngol Head Neck Surg 2008; 16:270-74.
3. Bachert C, Gevaert P, Holtappels G, van Cauwenberge P. Mediators in nasal polyposis. Curr Allergy Asthma Rep 2002; 2:481-87.
4. Ramanathan M, Jr., Lee WK, Dubin MG, Lin S, Spannhake EW, Lane AP. Sinonasal epithelial cell expression of toll-like receptor 9 is decreased in chronic rhinosinusitis with polyps. Am J Rhinol 2007; 21:110-16.
5. Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. Trends Immunol 2002; 23:541-48.
6. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN, Murphy PM. International union of basic and clinical pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev 2009; 61:119-61.
7. Babbin BA, Jesaitis AJ, Ivanov AI, Kelly D, Laukoetter M, Nava P, Parkos CA, Nusrat A. Formyl peptide receptor-1 activation enhances intestinal epithelial cell restitution through phosphatidylinositol

PROOF

3-kinase-dependent activation of rac1 and cdc42. J Immunol 2007; 179:8112-21.
8. de Paulis A, Prevete N, Rossi FW, et al. Helicobacter pylori Hp(2-20) promotes migration and proliferation of gastric epithelial cells by interacting with formyl peptide receptors in vitro and accelerates gastric mucosal healing in vivo. J Immunol 2009; 183:3761-69.
9. Li J, Zhang YP, Kirsner RS. Angiogenesis in wound repair: Angiogenic growth factors and the extracellular matrix. Microsc Res Tech 2003; 60: 107-14.
10. Tarnawski AS. Cellular and molecular mechanisms of gastrointestinal ulcer healing. Dig Dis Sci 2005; 50(S):S24-33.
11. Sidenius N, Sier CF, Blasi F. Shedding and cleavage of the urokinase receptor (uPAR): Identification and characterisation of uPAR fragments *in vitro* and *in vivo*. FEBS Lett 2000; 475:52-56.
12. De Fraissinette A, Brun R, Felix H, Vonderscher J, Rummelt A. Evaluation of the human cell line RPMI-2650 as an *in vitro* nasal model. Rhinology 1995; 33: 194-98.
13. de Paulis A, Prevete N, Fiorentino I, et al. Expression and functions of the vascular endothelial growth factors and their receptors in human basophils. J Immunol 2006; 177:7322-31.
14. de Paulis A, Montuori N, Prevete N, Fiorentino I, Rossi FW, Visconte V, Rossi G, Marone G, Ragno P. Urokinase induces basophil chemotaxis through a urokinase receptor epitope that is an endogenous ligand for formyl peptide receptor-like 1 and -like 2. J Immunol 2004; 173:5739-48.
15. de Paulis A, Prevete N, Fiorentino I, et al. Basophils infiltrate human gastric mucosa at sites of Helicobacter pylori infection, and exhibit chemotaxis in response to H. Pylori-derived peptide Hp(2-20). J Immunol 2004; 172:7734-43.
16. Snedecor GW, Cochran WG. Statistical methods, ED. 7th. Ames, Iowa, Iowa State University Press, 1980.
17. Fenteany G, Janmey PA, Stossel TP. Signaling pathways and cell mechanics involved in wound closure by epithelial cell sheets. Curr Biol 2000; 10: 831-38.
18. Thomson DM. Various authentic chemoattractants mediating leukocyte adherence inhibition. J Natl Cancer Inst 1984; 73:595-605.
19. de Paulis A, Ciccarelli A, de Crescenzo G, Cirillo R, Patella V, Marone G. Cyclosporin H is a potent and selective competitive antagonist of human basophil activation by N-formyl-methionyl-leucyl-phenylalanine. J Allergy Clin Immunol 1996; 98:152-64.
20. Zhou Y, Bian X, Le Y, et al. Formylpeptide receptor FPR and the rapid growth of malignant human gliomas. J Natl Cancer Inst 2005; 97:823-35.
21. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, Becker EL, Ward PA. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli*. J Biol Chem 1984; 259:5430-39.
22. Nast CC, LeDuc LE. Chemotactic peptides. Mechanisms, functions, and possible role in inflammatory bowel disease. Dig Dis Sci 1988; 33: 50S-57S.
23. Hoyer-Hansen G, Ronne E, Solberg H, Behrendt N, Ploug M, Lund LR, Ellis V, Dano K. Urokinase plasminogen activator cleaves its cell surface receptor releasing the ligand-binding domain. J Biol Chem 1992; 267:18224-29.
24. Resnati M, Pallavicini I, Wang JM, Oppenheim J, Serhan CN, Romano M, Blasi F. The fibrinolytic receptor for urokinase activates the G protein-coupled chemotactic receptor FPRL1/LXA4R. Proc Natl Acad Sci USA 2002; 99:1359-64.
25. Cao D, Mizukami IF, Garni-Wagner BA, Kindzelskii AL, Todd RF 3rd, Boxer LA, Petty HR. Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. Possible roles of complement receptor type 3 and calcium. J Immunol 1995; 154:1817-29.
26. Lanza F, Castoldi GL, Castagnari B, et al. Expression and functional role of urokinase-type plasminogen activator receptor in normal and acute leukaemic cells. Brit J Haematol 1998; 103:110-23.
27. Gyetko MR, Sud S, Kendall T, Fuller JA, Newstead MW, Standiford TJ. Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary pseudomonas aeruginosa infection. J Immunol 2000; 165:1513-19.
28. Pawankar R, Nonaka M. Inflammatory mechanisms

PROOF

and remodeling in chronic rhinosinusitis and nasal polyps. Curr Allergy Asthma Rep 2007; 7:202-08.
29. Sporn MB, Roberts AB. Transforming growth factor-beta: Recent progress and new challenges. J Cell Biol 1992; 119:1017-21.
30. Wang QP, Escudier E, Roudot-Thoraval F, Abd-Al Samad I, Peynegre R, Coste A. Myofibroblast accumulation induced by transforming growth factor-beta is involved in the pathogenesis of nasal polyps. Laryngoscope 1997; 107:926-31.

PROOF