

Montelukast regulates eosinophil protease activity through a leukotriene-independent mechanism

Anick Langlois, MSc, Claudine Ferland, BSc, Guy M. Tremblay, PhD,
and Michel Laviolette, MD *Sainte-Foy, Quebec, Canada*

Background: Migration of eosinophils into bronchial mucosa requires proteolysis. Montelukast, a cysteinyl leukotriene (CysLT) 1 receptor antagonist used in asthma treatment, decreases eosinophil infiltration into the asthmatic airways, suggesting that CysLTs modulate eosinophil protease activity. **Objective:** We sought to determine whether CysLTs and montelukast regulate eosinophil protease activity. **Methods:** Purified blood eosinophils were treated with or without montelukast; MK-0591, a 5-lipoxygenase-activating protein inhibitor; or leukotriene (LT) D₄. Migration assays through Matrigel were performed in the presence of 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-E₂E), a potent eosinophil chemotactic factor, or LTD₄. Expression of molecules implicated in plasmin generation and matrix metalloproteinase (MMP) 9 release were also evaluated. **Results:** Montelukast and MK-0591 decreased eosinophil migration promoted by 5-oxo-E₂E, whereas LTD₄ failed to induce eosinophil migration. However, LTD₄ significantly boosted the migration rate obtained with a suboptimal concentration of 5-oxo-E₂E and partially reversed the inhibition obtained with MK-0591. Montelukast significantly reduced the maximal rate of activation of plasminogen into plasmin by eosinophils obtained with 5-oxo-E₂E. 5-Oxo-E₂E increased the number of eosinophils expressing urokinase plasminogen activator receptor and stimulated secretion of MMP-9. Montelukast, but neither MK-0591 nor LTD₄, reduced the expression of urokinase plasminogen activator receptor and the secretion of MMP-9 and increased total cellular activity of urokinase plasminogen activator and the expression of plasminogen activator inhibitor 2 mRNA. **Conclusion:** Montelukast inhibits eosinophil protease activity *in vitro* through a mechanism that might be independent of its antagonist effect on CysLT 1 receptor. **Clinical implications:** This could partially explain montelukast's anti-inflammatory effect in asthma and

eventually amplify to improve its therapeutic efficacy. (J Allergy Clin Immunol 2006;118:113-9.)

Key words: Asthma, eosinophil, leukotrienes, matrix metalloproteinase 9, migration, montelukast, MK-0591, plasminogen activator inhibitor, urokinase plasminogen activator, urokinase plasminogen activator receptor

Asthma is characterized by leukocyte infiltration, notably eosinophils, in the bronchial mucosa. Inflammatory cell recruitment into airway walls is initiated by various chemotactic factors. 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-E₂E) is an arachidonic acid metabolite produced by leukocytes and platelets. It is one of the most potent stimulators of eosinophil migration *in vivo* and *in vitro*, both in Boyden Chambers and through Matrigel (Becton Dickinson, Bedford, Mass), a reconstituted basement membrane.^{1,2} Cell migration in a Boyden Chamber involves adhesion and cell motion, whereas migration through Matrigel also requires protease activation. Eosinophils express matrix metalloproteinase (MMP) 9, urokinase plasminogen activator (uPA), its receptor (uPAR or CD87), and plasminogen activator inhibitor (PAI) 2, which are required to regulate tissue extracellular matrix digestion.³⁻⁵ 5-Oxo-E₂E activates both the MMP-9 and the plasmin-plasminogen system.¹ MMP-9, also known as gelatinase B, cleaves gelatin, collagens, and laminin.⁶ uPAR and uPA promote matrix degradation by generating plasmin, a serine protease, from the abundant zymogen plasminogen. uPA activity is negatively regulated by PAI. Plasmin can digest fibrin, fibrinogen, and laminin and can also convert inactive pro-MMP into active MMP.⁷

Cysteinyl leukotrienes (CysLTs; leukotriene [LT] C₄, LTD₄, and LTE₄) are likely involved in asthma pathophysiology, notably because of their capacity to induce cell recruitment and bronchospasm and to enhance vascular permeability and mucus secretion *in vivo*.⁸ CysLTs are generated through the action of 5-lipoxygenase in concert with 5-lipoxygenase-activating protein (FLAP).⁸

LTD₄ induces eosinophil migration in a Boyden Chamber.^{9,10} LTD₄ has more affinity for the G protein-coupled transmembrane receptor CysLT1R than the other ligands and is the most potent agonist.⁸ Montelukast, a CysLT1R-specific antagonist, is an effective treatment for asthma.¹¹ Both montelukast and other CysLT1R-specific antagonists, such as pranlukast, reduce the number of eosinophils in bronchoalveolar lavage fluid in experimental allergic asthma¹² and in peripheral blood,

From Unité de Recherche en Pneumologie, Centre de Recherche de l'Hôpital Laval, Institut Universitaire de Cardiologie et de Pneumologie de l'Université Laval.

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Reprint requests: Michel Laviolette, MD, Hôpital Laval, 2725 Chemin Sainte-Foy, Québec G1V 4G5, Canada. E-mail: Michel.Laviolette@med.ulaval.ca. 0091-6749/\$32.00

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Abbreviations used

CysLT: Cysteinyl leukotriene
 CysLT1R: Cysteinyl leukotriene 1 receptor
 FLAP: 5-Lipoxygenase-activating protein
 LT: Leukotriene
 MMP: Matrix metalloproteinase
 mOD: Milli-optical density
 5-oxo-EETE: 5-Oxo-6,8,11,14-eicosatetraenoic acid
 PAI: Plasminogen activator inhibitor
 uPA: Urokinase plasminogen activator
 uPAR: Urokinase plasminogen activator receptor
 Vmax: Maximal velocity

bronchial mucosa, and sputum of asthmatic subjects.^{13,14} These observations suggest that CysLTs are involved in eosinophil recruitment. However, the capacity of CysLTs to activate proteases and to initiate migration through extracellular matrix has not been reported. In the present study we evaluated the role of CysLTs and the effect of montelukast in protease activation, which is required during *in vitro* 5-oxo-EETE-induced eosinophil migration.

METHODS**Reagents**

Recombinant human IL-5 was from PeproTech Inc (Rocky Hill, NJ), LTD₄ and 5-oxo-EETE were from Cayman Chemical (Ann Arbor, Mich), and FBS was from Hyclone (Logan, Utah). BSA (fraction V) was purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada); Dextran T-500, Ficoll-Paque, and ECL staining were from Amersham Biosciences (Piscataway, NY); and RPMI 1640 medium, HBSS without calcium/magnesium, and penicillin/streptomycin were from Invitrogen Canada (Burlington, Ontario, Canada). Montelukast and MK-0591 were kindly provided by Jean-Bruno Langdeau from Merck Frosst Canada Ltée (Kirkland, Quebec, Canada).

Selection of subjects

Twenty-eight asthmatic subjects (13 female and 15 male subjects; mean $\pm$ SEM age, 28.1 $\pm$ 5.8 years) meeting the criteria of the American Thoracic Society for the diagnosis of asthma were recruited for this study.¹⁵ The inclusion criteria were stable asthma for more than 3 months and no inhaled corticosteroids or asthma medication other than β_2 -agonists for more than 3 months. Approval from the local ethics committee was obtained, and subjects signed informed consent forms.

Blood cell processing and eosinophil purification

Blood eosinophils were purified as previously described.¹⁶ Briefly, venous blood was centrifuged to remove platelet-rich plasma, and the resulting pellet was sedimented with dextran. Leukocytes were separated on Ficoll-Paque (1.077 g/mL). The granulocyte layer was resuspended, and red cells were lysed. Eosinophils were purified from neutrophils by means of negative selection with magnetic bead-conjugated anti-CD16 mAb and MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of eosinophil preparations

was always greater than 98%. Eosinophil viability, determined by using trypan blue exclusion, was always higher than 99%.

LTC₄ assay

Eosinophils (1×10^6 /mL) in HBSS-CaCl₂ (1.6 mmol/L) were stimulated with 1 μ mol/L 5-oxo-EETE for 10 minutes. The reaction was stopped by putting the cells on ice. LTC₄ levels were measured in the cell-free supernatant by means of quantitative immunoassay, according to the manufacturer's recommendations (Cayman Chemical).

Migration assay

Migration of eosinophils through basement membrane components was assessed in 24-well inserts coated with 125 μ g/cm² Matrigel (Becton Dickinson), which was used as previously described.^{1,17} To verify the role of CysLTs on migration, eosinophils (1×10^6 cells/mL) were incubated in complete RPMI (supplemented with 10% FBS, 0.2% penicillin/streptomycin, and IL-5 [10 ng/mL]) with or without montelukast (0.01–10 μ mol/L), a CysLT1R antagonist, or MK-0591 (10 μ mol/L), a FLAP inhibitor, for 30 minutes at 37°C in 5% CO₂. The cells were then placed in the upper chamber of the Matrigel-coated insert and incubated at 37°C in 5% CO₂ for 18 hours. 5-Oxo-EETE (1 μ mol/L) or LTD₄ (0.01–1 μ mol/L) were used as chemotactic factors and added in the lower chambers. In some experiments cells were preincubated with various concentrations of LTD₄ (0.01–1 μ mol/L) with or without MK-0591 for 30 minutes and then placed in the upper chamber, with 5-oxo-EETE (0.001 or 1 μ mol/L) in the lower chamber. At the end of the incubation, the remaining cells in both the upper and lower chambers were harvested and counted on a hemacytometer. In the absence of chemotactic factors, very few cells moved through the insert. For each condition, the percentage of migration was calculated as previously described.¹ Eosinophil viability measured at the end of these assays was greater than 95%.

Plasmin activity assays

Eosinophils (1×10^6 cells/mL) were incubated in HBSS-BSA 1% with or without montelukast (10 μ mol/L) for 30 minutes and then stimulated with 5-oxo-EETE (1 μ mol/L) for 2 hours at 37°C in 5% CO₂. Plasminogen activation into plasmin was assayed by adding glu-plasminogen (1 μ mol/L) and the synthetic chromogenic substrate Spectrozyme PL (100 μ mol/L; American Diagnostica Inc, Montreal, Quebec, Canada) to the cells. Recombinant human plasmin (0.1 μ mol/L, American Diagnostica Inc) was incubated with montelukast (10 μ mol/L) for 30 minutes and Spectrozyme PL (150 μ mol/L) was added to assess the direct effect of montelukast on plasmin activity. Absorbance was measured at 405 nm by using a Thermomax plate reader (Molecular Devices Corp, Sunnyvale, Calif) previously heated to 37°C and monitored for 30 minutes. The OD is directly proportional to the plasmin activity. Rates of plasmin production were calculated from slopes of milli-optical density (mOD) per minute generated automatically from Thermomax data by using the software SOFTmax PRO (Molecular Devices Corp, Stanford, Conn) and expressed as maximal velocity (Vmax).

uPAR expression measured by mean of flow cytometric analysis

To measure uPAR cell-surface expression, eosinophils (350,000 cells), treated in complete RPMI with or without montelukast (10 μ mol/L) or MK-0591 (10 μ mol/L) for 30 minutes, were stimulated or not with 5-oxo-EETE (1 μ mol/L) for either 2, 4, 6, and 18 hours or 2 and 18 hours, respectively, at 37°C in 5% CO₂. In some experiments cells were incubated for 2 and 18 hours with LTD₄ (0.01–1 μ mol/L) with or without 5-oxo-EETE (0.001 μ mol/L) to clarify the role of CysLTs

in uPAR expression. Cells were then washed once with HBSS-BSA 1%, incubated with 1 μ g of mouse anti-human uPAR (CD87) mAb (clone 3936) (American Diagnostica Inc) or mouse IgG (isotype control antibody; BD Biosciences, Mississauga, Ontario, Canada), and then incubated with 1 μ g of r-phycoerythrin-conjugated goat anti-mouse IgG antibody (BD Biosciences). Flow cytometric analysis was performed as described previously¹⁸ with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, Fla). Results were expressed as a percentage of uPAR-positive eosinophils.

Urokinase activity assays

Eosinophils (3×10^6 cells) were incubated in complete RPMI with or without montelukast (10 μ mol/L) or MK-0591 (10 μ mol/L) for 30 minutes and stimulated or not with 5-oxo-ETE (1 μ mol/L) for 4 hours at 37°C in 5% CO₂. The total cell uPA activity was measured in cell pellets with a urokinase activity assay kit, according to the manufacturer's recommendations (EMD Biosciences Inc, San Diego, Calif).

PAI-2 mRNA quantification by means of RT-PCR

Eosinophils (2×10^6 cells) were treated in complete RPMI, as described above. Total RNA was isolated with TRIzol reagent, according to the manufacturer's instructions (Invitrogen Canada). Both cDNA synthesis and PCR amplification were performed in a single tube by using the SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen Canada). Fifteen nanograms of RNA was used, and cDNA was amplified (PAI-2 forward primer: 5'-GTT ACC CCC ATG ACT CCA GA-3'; reverse primer: 5'-CGC AGA CTT CTC ACC AAA CA-3'; GAPDH forward primer: 5'-ATG CAA CGG ATT TGG TCG TAT-3'; reverse primer: 5'-TCT CGC TCC TGG AAG ATG GTG-3'). RT-PCR reactions were carried out on a Peltier Thermal Cycler (PTC-200; MJ Research, Watertown, Mass) for 35 cycles with the following settings: denaturation at 94°C for 1 minute (2 minutes for the first cycle), annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute (5 minutes for the last cycle). After amplification, the PCR products were resolved by means of electrophoresis on ethidium bromide-stained 2.0% agarose gel and were visualized with ultraviolet illumination. Chemigenius² (Syngene, Frederick, Md) was used to capture images of the gels. Densitometry was performed with the National Institutes of Health Image software.

Measurement of MMP-9 by means of zymography and Western blotting

Eosinophils (1×10^7 cells/mL) were treated for 1 hour in HBSS-BSA 1%, as described above. The level of gelatinolytic enzymes released in cell-free supernatants was measured by means of gelatin zymography, as defined by Wiehler et al.¹⁹ Chemigenius² was used to capture images of the gels. Densitometry of MMP-9 (82 kd) was performed with the National Institutes of Health Image software.

For analysis of MMP-9 expression by means of Western blotting, eosinophils (3×10^6 cells) were treated for 18 hours in complete RPMI as described above. Cell-free supernatants were immunoprecipitated with Sepharose A gel beads coupled to a mouse anti-human MMP-9 (R&D Systems Inc, Minneapolis, Minn). Immunoprecipitates were run on 10% SDS-PAGE. Proteins were transferred by means of Western blotting on a polyvinylidene difluoride membrane, which was incubated with 2 μ g/mL mouse anti-human MMP-9 antibody (R&D Systems Inc) and 0.1 μ g/mL goat anti-mouse antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc, West Grove, Pa). Revelation was made with ECL staining. Chemigenius² was used to capture images of the blot. Densitometry was performed with the National Institutes of Health Image software.

Statistical analysis

Means $\pm$ SEM were used for continuous variables. The effects of different LT modifiers were compared by using a 2-way randomized block design. One factor was linked to subjects (random effect) and the other to LT or LT modifier concentrations. *Post-hoc* comparisons were performed with the Tukey method. The results were considered significant if *P* values were .05 or less. The data were analyzed with the statistical package program SAS version 9.1.3 (SAS Institute Inc, Cary, NC).

RESULTS

CysLTs are involved in 5-oxo-ETE-induced eosinophil migration through Matrigel

5-Oxo-ETE induced blood eosinophil release of LTC₄ (143.3 ± 38.6 ng/10⁶ cells, *n* = 6). Without stimulation, no LTC₄ was detectable. Eosinophils were preincubated with different doses of a CysLT1R antagonist, montelukast (0.01-10 μ mol/L), to investigate the involvement of CysLTs in 5-oxo-ETE-induced migration. Optimal inhibition was obtained at a concentration of 10 μ mol/L (data not shown), and thus this concentration was used in subsequent experiments. Montelukast diminished the 5-oxo-ETE-induced cell migration by 30%, from $71.3\% \pm 2.7\%$ to $49.8\% \pm 4.1\%$ (*n* = 14, *P* < .0001; Fig 1, A). To further confirm the implication of CysLTs in this process, we measured 5-oxo-ETE-induced migration in the presence of a FLAP inhibitor, MK-0591. As observed with montelukast, MK-0591 decreased the eosinophil migration by 28%, from $71.3\% \pm 2.7\%$ to $51.6\% \pm 8.2\%$ (*n* = 9, *P* = .006; Fig 1, A).

Because LTD₄ showed a chemotactic activity toward eosinophils in Boyden Chambers, we verified whether LTD₄ was able to promote eosinophil migration through Matrigel. In contrast to 5-oxo-ETE, LTD₄ alone did not induce any significant migration at the tested concentrations (*n* = 4; Fig 1, B). However, the addition of LTD₄ (0.1 and 1 μ mol/L) to eosinophil suspensions in the upper chambers boosted cell migration initiated by a suboptimal concentration of 5-oxo-ETE (0.001 μ mol/L), from $40.4\% \pm 7.5\%$ to $53.1\% \pm 9.2\%$ and $49.8\% \pm 8.1\%$, respectively (*n* = 6; *P* = .001 and *P* = .04, respectively; Fig 1, C). Moreover, exogenous LTD₄ partially reversed the inhibitory effect of MK-0591 on 5-oxo-ETE-induced migration (*n* = 5, *P* = .0003; Fig 1, D). These data strongly suggest that CysLTs are involved in eosinophil 5-oxo-ETE-induced migration.

Montelukast modulates the activation of the plasmin-plasminogen system

An enzymatic activity assay was performed to establish the effect of montelukast on plasmin generation by eosinophils in the presence of 5-oxo-ETE. Montelukast decreased by 40% the maximal rate of eosinophil plasmin activity compared with 5-oxo-ETE alone (*V*_{max}: 2.69 ± 0.34 and 4.39 ± 0.70 mOD/min, respectively; *n* = 11, *P* = .004; Fig 2, A). Recombinant human plasmin was incubated with montelukast to confirm that montelukast reduced cell plasmin generation without affecting its

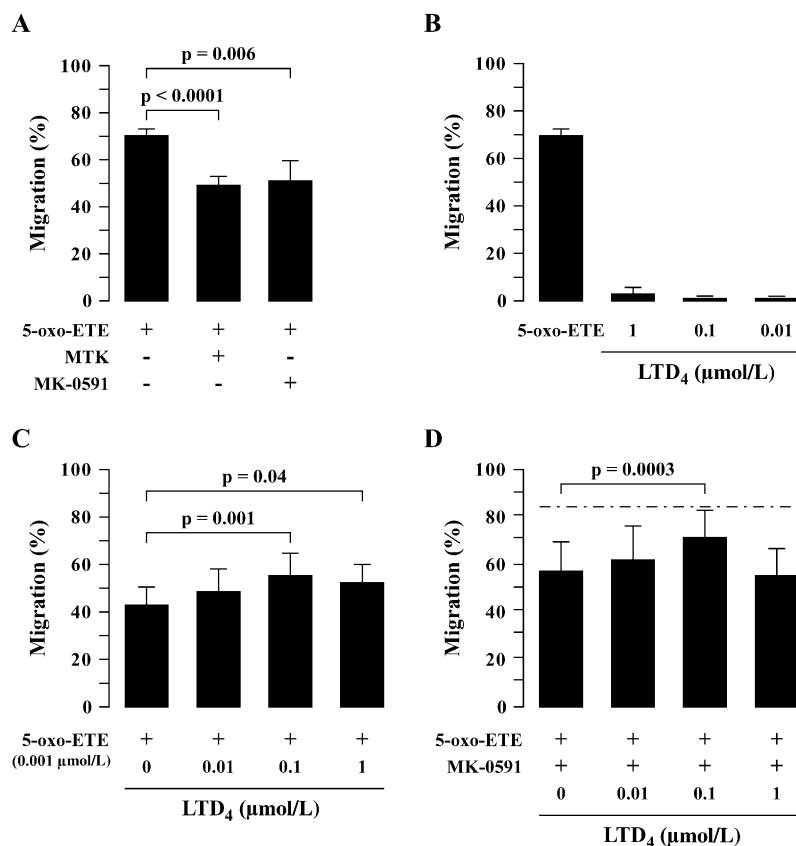


FIG 1. Role of CysLTs in 5-oxo-EETE-induced eosinophil migration through Matrigel basement membrane. 5-Oxo-EETE was used as a chemotactic factor (**A**, **B**, and **D** = 1 μM; **C** = 0.001 μmol/L). The dashed line represents the percentage of optimal migration obtained with 5-oxo-EETE (1 μmol/L). MTK, Montelukast.

activity. Montelukast failed to modify recombinant human plasmin activity ($n = 3$, data not shown). Thereafter, we evaluated the effect of 5-oxo-EETE, montelukast, and MK-0591 on uPAR cell-surface expression, uPA activity, and PAI-2 mRNA level, which are implicated in plasmin generation.

In control conditions the number of uPAR-positive eosinophils did not change significantly in a time-related manner up to 18 hours (data not shown). In 2 sets of experiments, 5-oxo-EETE augmented the percentage of uPAR-positive cells compared with the control condition (values at 2 hours for control and 5-oxo-EETE in the first set: $53.0\% \pm 11.5\%$ and $71.4\% \pm 8.0\%$, respectively [$n = 8$, $P = .007$]; second set: $58.5\% \pm 8.9\%$ and $72.1\% \pm 7.7\%$, respectively [$n = 7$, $P = 0.03$]; Fig 2, B). Montelukast downregulated the 5-oxo-EETE-induced increase of uPAR expression close to the baseline value (value at 2 hours: $60.3\% \pm 10.1\%$ [$n = 8$, $P = .007$]; Fig 2, B). However, no significant effect was observed with MK-0591 (value at 2 hours: $67.7\% \pm 7.6\%$ [$n = 7$, $P = .6$ compared with 5-oxo-EETE]; Fig 2, B). Similar results were obtained for the different times tested concerning the modulation of uPAR-positive cells (in percentages) by 5-oxo-EETE, montelukast, and MK-0591 (data not shown).

LTD₄ alone (0.01–1 μmol/L) did not modify uPAR expression (data not shown). Moreover, the addition of LTD₄ (0.01–1 μmol/L) to eosinophil suspensions did not amplify the number of uPAR-positive cells obtained with a suboptimal concentration of 5-oxo-EETE (0.001 μmol/L; values at 2 hours for 5-oxo-EETE [0.001 μmol/L] and 5-oxo-EETE plus LTD₄ [0.1 μmol/L]: $48.9\% \pm 7.9\%$ and $52.1\% \pm 6.9\%$ [$n = 7$, $P = .3$]). These data show that montelukast, but neither MK-0591 nor LTD₄, downregulates 5-oxo-EETE-increased uPAR expression.

5-Oxo-EETE did not modify uPA activity measured in the cell pellet. Preincubation of cells with montelukast, but not with MK-0591, increased total cellular uPA activity ($n = 8$, $P < .0001$; Fig 2, C). These data suggest that montelukast either prevented the release of uPA from the cells or increased its reuptake. Finally, RT-PCR was performed to determine PAI-2 mRNA expression. 5-Oxo-EETE did not regulate significantly PAI-2 mRNA level compared with the control value. Montelukast, but not MK-0591, significantly increased the PAI-2 mRNA level over the control value ($n = 3$, $P = .025$; Fig 2, D). These data show that montelukast, but not MK-0591, regulates the 5-oxo-EETE induced-activation of the plasminogen-plasmin system at different levels.

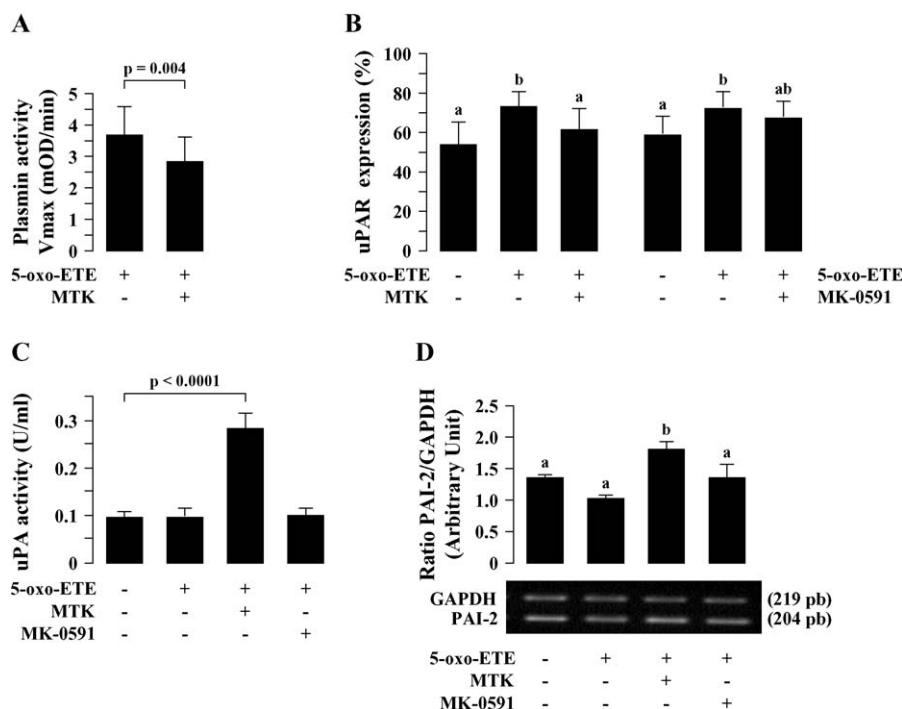


FIG 2. Modulation of eosinophil plasmin-plasminogen system. **A**, Plasmin generation expressed in mOD per minute ($n = 11$). **B**, uPAR cell-surface expression measured by means of flow cytometry ($a < b$: $P = .007$, $n = 8$ and $P = .003$, $n = 7$, respectively). **C**, uPA activity measured in cell pellets ($n = 8$). **D**, mRNA PAI-2 expression ($a < b$: $P = .025$, $n = 3$). MTK, Montelukast.

Montelukast modulates the activation of MMP-9

Eosinophils were stimulated as described for 1 hour. Cell-free supernatants were collected and assayed for the presence of MMP-9 by means of gelatin zymography. In these conditions 5-oxo-ETE provoked a significant release of MMP-9 activity ($n = 4$, $P = .001$; Fig 3, A). In this 1-hour assay, montelukast and MK-0591 did not affect MMP-9 activity. The quantity of pro-MMP-9 remained stable in all conditions. We also evaluated whether montelukast and MK-0591 had an effect on MMP-9 secretion for a long-term period. Absence of serum during long-term periods affects eosinophil viability, limiting zymographic assays to short-term periods. Consequently, Western blotting was performed on cell-free supernatants to assess for the presence of MMP-9 after an incubation of 18 hours. Compared with control conditions, the amount of secreted MMP-9 was significantly increased by 5-oxo-ETE ($n = 5$, $P = .002$; Fig 3, B). Montelukast, but not MK-0591, reduced the 5-oxo-ETE–boosted MMP-9 secretion close to the baseline value ($n = 5$, $P = .003$ compared with 5-oxo-ETE). No pro-MMP-9 was detected at this time point (Fig 3, B).

DISCUSSION

Several teams have demonstrated that CysLTs are involved in eosinophil recruitment *in vivo* and

in vitro.^{9,10,13,14,20,21} However, to our knowledge, no study investigated the effect of CysLTs on protease activation.

Matrigel, a gel-containing basement membrane component, was used as an *in vitro* model of basement membrane to evaluate protease activity implicated in eosinophil migration. Cells were treated with 5-oxo-ETE, a powerful promoter of eosinophil migration *in vitro* through Matrigel^{1,17} and *in vivo* into tissue.²² Moreover, among chemotactic factors, 5-oxo-ETE effectively activates *in vitro* both MMP-9 and the plasminogen-plasmin system, 2 major proteases expressed by eosinophils.^{1,17,18,23} In this study we showed that 5-oxo-ETE induces the release of LTC₄. We also showed that both the CysLT1R antagonist montelukast and the FLAP inhibitor MK-0591 diminished 5-oxo-ETE–mediated migration through Matrigel. Furthermore, because exogenous LTD₄ alone did not initiate significant migration through Matrigel but potentiated the effect of 5-oxo-ETE and partially reversed the inhibitory effect of MK-0591, we concluded that CysLTs were important for eosinophil migration in our model. However, this assay did not permit us to determine which of the actin reorganization, adhesion, or protease activation steps are affected by CysLTs. The great efficiency of 5-oxo-ETE to induce migration is most likely a result of its capacity to promote all these steps.^{1,24,25}

Our data provide more direct evidence of the 5-oxo-ETE actions on both the plasminogen-plasmin system and MMP-9. The effects of montelukast and MK-0591 were

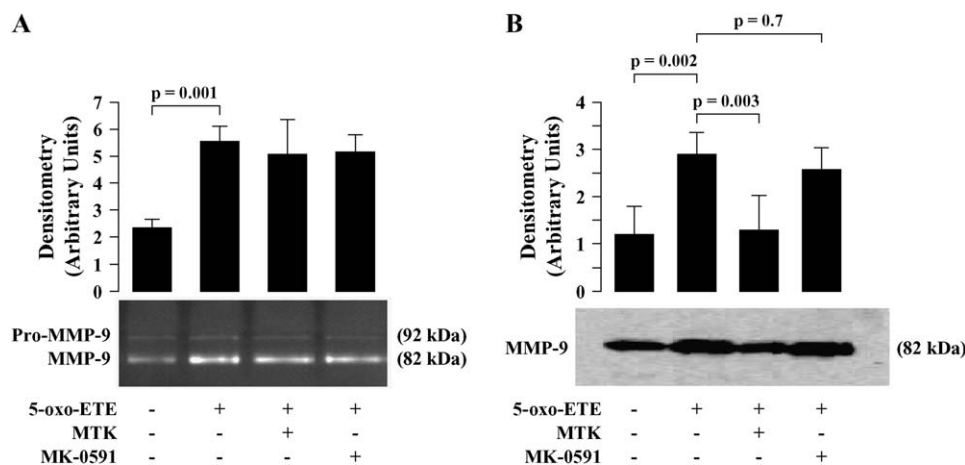


FIG 3. Modulation of MMP-9 activity. **A**, Gelatin zymographic analyses performed on eosinophil-free supernatants collected after 1 hour of incubation. The histogram represents densitometry of MMP-9 (82 kD). The picture is representative of 4 experiments. **B**, Western blot analyses performed on cell-free supernatants collected after 18 hours of incubation. The picture is representative of 5 experiments. *MTK*, Montelukast.

evaluated to further document whether CysLTs were required in the activation of those proteases. We observed that montelukast decreased the rate of plasmin generation induced by 5-oxo-EET. This plasmin generation implies many steps, and the effects of the chemoattractant and LT modifiers on some of them were verified. Cell membrane expression of uPAR is increased by 5-oxo-EET and prevented by montelukast. However, neither MK-0591 nor LTD₄ significantly modified cell membrane expression of uPAR. Moreover, montelukast increased the total amount of uPA and the PAI-2 mRNA levels in the cells. On the other hand, 5-oxo-EET and MK-0591 had no effect on these parameters. A recent study showed that PAI-2 is internalized through a uPA/uPAR-dependent pathway and that this internalization causes its degradation or recycling and affects cell motility.²⁶ Our finding suggests that montelukast either prevents the release of uPA from the cell or promotes the internalization of the uPAR/uPA complex by upregulating PAI-2. Further experiments will be needed to clarify this mechanism.

Finally, measurement of MMP-9 in supernatants of stimulated eosinophils showed that montelukast, but not MK-0591, abrogated the activation of MMP-9 mediated by 5-oxo-EET. MMP activation is plasmin dependent.⁷ However, the experiments that were performed here do not allow us to determine whether MTK directly inhibits MMP-9 activation or indirectly by reducing plasmin activity. Collectively, these observations suggest that montelukast acts not only as a CysLT1R antagonist but also through other undefined mechanisms.

Even if MK-0591 decreased migration, LTD₄ alone was not able to promote significant migration through Matrigel, suggesting that CysLTs had few, if any, effects on protease activation. CysLTs, particularly LTD₄, stimulate actin reorganization and upregulate CD11b/CD18 expression.^{9,10,20,21} In our model CysLTs probably facilitated cell migration through these effects. The priming

effect of LTD₄ on 5-oxo-EET-induced migration and its reversed effect on migration inhibition induced by the FLAP inhibitor are likely mediated through these mechanisms.

Both montelukast and MK-0591 are known to interfere with the CysLT cascade and have an effect on migration. In spite of this, in this study montelukast presented actions that neither MK-0591 nor LTD₄ exhibited, suggesting that montelukast has mechanisms of action that are independent of its antagonist effect on CysLT1R. Moreover, 2 teams observed that pranlukast inhibits eosinophil functions through a mechanism distinct from CysLT1R antagonism.^{21,27} Montelukast and pranlukast could act as inverse agonists and negatively regulate the eosinophil response, explaining why no effects were observed with LTD₄. It has been demonstrated that montelukast, possibly as an inverse agonist, reduces the intracellular level of phosphatidyl inositol triphosphate and diminished the intracellular calcium flux essential for a number of functions in eosinophils.²⁸ Intracellular calcium is essential for cell migration.²⁹⁻³¹ However, further studies are needed to better understand the role of calcium in protease activation or expression.

The maximum plasma concentration obtained after oral administration of a 10-mg tablet of montelukast sodium is around 0.7 $\mu\text{mol/L}$.³² In our *in vitro* model we used a montelukast concentration of 10 $\mu\text{mol/L}$ because this concentration provided the optimal inhibition of migration through Matrigel. Moreover, this concentration is usually used for *in vitro* experiments.³³⁻³⁵ Further experiments should be performed to verify whether a lower concentration of montelukast is effective to diminish protease activity in our *in vitro* model. Moreover, although 5-oxo-EET represents an excellent tool to study activation of eosinophil proteases, other chemotactic factors, such as platelet-activating factor and eotaxin, should be tested to confirm that montelukast also modulates protease

activation mediated by other inducers of eosinophil migration.

In conclusion, this *in vitro* study demonstrated that montelukast, but neither MK-0591 nor LTD₄, had an effect on eosinophil migration through Matrigel by diminishing protease activation. These results suggest that montelukast acts through a mechanism of action that is independent of its antagonist effect on CysLT₁R. These results could, at least in part, explain some of the anti-inflammatory effects of montelukast in asthma.

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