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CC and CXC Chemokines Induce Airway Smooth Muscle Proliferation and Survival

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The increase in airway smooth muscle (ASM) mass is a major structural change in asthma. This increase has been attributed to ASM cell (ASMC) hyperplasia and hypertrophy. The distance between ASMC and the epithelium is reduced, suggesting migration of smooth muscle cells toward the epithelium. Recent studies have suggested a role of chemokines in ASMC migration toward the epithelium; however, chemokines have other biological effects. The objective of the current study is to test the hypothesis that chemokines (eotaxin, RANTES, IL-8, and MIP-1α) can directly influence ASMC mass by increasing the rate of proliferation or enhancing the survival of these cells. Human ASMCs were exposed to different concentrations of eotaxin, RANTES, IL-8, or MIP-1α. To test for proliferation, matched control and stimulated ASMC were pulsed with [3H]thymidine, or ASMCs were stained with BrdU and then analyzed with flow cytometry. Apoptosis was measured using Annexin V staining and flow cytometry. Expression of phosphorylated p42/p44 and MAPKs was assessed by Western blot. In a concentration-dependent manner, chemokines stained with BrdU and then analyzed with flow cytometry. Apoptosis was measured using Annexin V staining and flow cytometry.

Asthma is a common chronic inflammatory disease of the airways associated with bronchial hyperresponsiveness and airway remodeling. The increase in airway smooth muscle (ASM) mass is an important phenomenon in asthma, especially in severe cases. Different mechanisms were proposed to explain smooth muscle remodeling including proliferation (hyperplasia), increase in myocyte size (hypertrophy), and smooth muscle migration. Airway myocytes are thought to have a capacity to contribute to remodeling and airway hyperresponsiveness due to their ability for graded and reversible phenotype switching, which confers broad functional capacity (1).

The distance between the smooth muscle mass and the epithelium, especially in tissues obtained from asthmatic subjects, is needed to demonstrate a similar response on ASMC. Moreover, chemokines such as CCR3, CCR1, CCR5, CXCL1, and -2 were identified on the ASM surface (5–7).

Little is known about the role of chemokines in smooth muscle proliferation and survival. Mitogenic activity of RANTES in T lymphocytes was reported when high concentrations were used (8). Recently, RANTES was shown to promote the growth of some tumor cells as was shown in small lung cell carcinoma and breast cancer (9). Antagonizing RANTES reduced tumor growth in a mouse model of breast cancer and in another model of prostate cancer (10). For MIP-1α, the effect seems to differ according to the cell type and species tested. Parkinson et al. (11) identified MIP-1α as an inhibitor of hematopoietic stem cell proliferation. MIP-1α was also shown to inhibit the proliferation of dermal keratinocytes and spermatogonia (11, 12). In rat vascular smooth muscle cells, MIP-1α induces chemotaxis and to a lesser extent stimulates proliferation (13).

This study describes ASM proliferation in response to four different chemokines—IL-8, RANTES, eotaxin, and MIP-1α—that are all upregulated in asthmatic airways. Our data indicated that RANTES and eotaxin induce ASMC proliferation at low concentrations, whereas higher doses of IL-8 and MIP-1α were needed to demonstrate a similar response on ASMC. Moreover,
we hypothesize that chemokines may influence ASM mass through regulating apoptosis. Human ASMCs that were treated with the above chemokines showed better survival compared with the matched control. In addition, the mechanisms behind the proliferative effect of chemokines on ASMCs were investigated. Our data suggest that RANTES and eotaxin induce the proliferation of ASMCs through the activation of p42/p44 MAPK.

Materials and Methods

Cell culture

The ASMCs were grown from primary cultures of human nonasthmatic bronchial ASMCs, which were obtained from surgical specimens. They were identified as smooth muscle cells by positive immunohistochemical staining for smooth muscle-specific α-actin and positive identification of myosin L chain kinase and calponin by Western blot analysis. For the proliferation experiments, we also used ASMCs isolated from four asthmatic patients. Two of them were a kind gift from Dr. Andrew Halyko and Tak Lee (Department of Physiology, University of Manitoba, Winnipeg, MB, Canada) and another two from Lonza. All of the chemokines used in the experiments were recombiant human and purchased from R&D Systems (Minneapolis, MN).

[^3H]Thymidine incorporation

Metabolic incorporation of tritiated [^3H]thymidine into the newly synthesized DNA is a widely used method to detect the rate of DNA synthesis and monitor cell proliferation. ASMCs were plated in 96-well plates at a density of 3000–5000 cells/well in triplicate and grown to 60–70% confluence in complete SmGm2 containing 5% FBS at 37°C in a humified incubator with 5% CO2. Cells were then starved for 48 h in 0.3% FBS SmGm2 (starving media) containing all other additives. Following the starvation period, the starvation medium was replaced with medium supplemented with the chemokine of interest. Cells were then treated with chemokines or the appropriated vehicle for 24, 48, or 72 h. The concentrations for each chemokine tested ranged from 0.1–100 ng/ml, except for IL-8, in which concentrations of 50, 100, 500, and 1000 ng/ml were tested. Eighteen hours before cell harvesting for thymidine study, cells were pulsed with 1 μCi thymidine/well. A Skatron Micro96 cell harvester (Molecular Devices, Sunnyvale, CA) was used to isolate DNA following the manufacturer’s instructions, and then radioactivity was measured by a liquid scintillation and luminescence counter (PerkinElmer).

BrdU incorporation, Ki-67 staining, and flow cytometric analysis

ASMCs were grown in 12-well plates at a density of 40,000 cells/well in complete medium, and then medium was switched to SmGm2 (supplemented with 0.5% FBS) for 48 h to induce quiescence and synchronize the cell cycle. The vehicle or test chemokines were then added to the cells for 24, 48, or 72 h. The thymidine analog BrdU (FITC BrdU Flow Kit; BD Biosciences) was added (10 μl/m1 1 mM BrdU solution) 18 h before cells are processed for flow cytometric analysis. The BrdU flow kit staining protocol provided by the manufacturer was followed to prepare cells for flow cytometric analysis. After removing the culture medium, the treated cells were trypsinized and washed with PBS. Cells were incubated with 1:50 diluted anti-BrdU fluorescent Ab for 20 min at room temperature. Alternatively, stimulated ASMCs were stained with Ki-67 (Ki-67-PE; BD Biosciences) at the end of stimulation time. The stained cells were analyzed immediately with an FACScalibur flow cytometer system using Cell Quest Software (BD Biosciences). Ten thousand events were collected on each sample.

Annexin V and flow cytometric analysis

To test if RANTES, eotaxin, IL-8, and MIP-1α have an antiapoptotic effect, cells were plated in 12-well plates at a density of 40,000 cells/well in a complete medium. The cells were treated exactly the same as in the BrdU test except for the step of BrdU addition to the treated cells. When the stimulation period was over, Annexin V (Annexin V-FITC Apoptosis detection kit; BD Biosciences) was used to quantify the percentage of cells undergoing early apoptosis. The chemokine-treated cells were trypsinized, washed with PBS, and then suspended in 100 ml 1X Annexin V binding buffer (10× is supplied by the manufacturer). Five microliters Annexin V and 5 μl propidium iodide (PI) stains were added to each test tube for 15 min at room temperature and in the dark. Annexin V binds to the cells in the early stage of apoptosis, whereas PI stains the necrotic cells and the cells in late apoptotic phase. Within 1 h, the cells were analyzed using an FACScalibur flow cytometer (BD Biosciences) by counting 10,000 events per sample.

Western blotting

For the Western blotting studies, ASMC were grown in six-well plates at a density of 100,000 cells/well. The cells were starved for 48 h and then stimulated with the chemokine of interest for 1, 5, or 10 min. After chemokine stimulation, the medium was removed, and ASMCs were washed with ice-cold PBS. The cells were then harvested in lysis buffer made of 1% Triton X-100, 50 mM HEPES (pH 8), 150 mM NaCl, 10% glycerol, 2 mM EGTA, 1.5 mM MgCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 1 mM sodium orthovanadate. Following 10 min incubation on ice, the lysates were clarified by centrifugation at 14,000 × g for 10 min, and supernatants were collected. Protein concentrations were determined using Bradford method. To detect activation of p42/p44 MAPKs, equal amounts of whole-cell lysates were solubilized in a boiling Laemmli sample buffer, subjected to a 10% SDS-PAGE, and transferred to nitrocellulose (Bio-Rad Laboratories, Mississauga, ON, Canada). For Western immunoblotting, membranes were blocked for 1 h in TBST (10 mM Tris-Cl [pH 7.4], 2.5 mM EDTA, 150 mM NaCl, and 0.1% Tween-20) containing 1% BSA at room temperature. The p42/p44 phosphorylation was identified using phospho-p42/p44 Ab. The nitrocellulose membranes were incubated overnight at 4°C with the anti-phospho-MAPK polyclonal Ab used at a dilution of 1:1000 in TBS + 0.5% Tween-20. Membranes were washed with TBS + 0.5% Tween-20 five times for 5 min each and incubated with a 1:1500 dilution of anti-rabbit HRP Ab (Amersham Biosciences, Baie d’Urfé, QC, Canada) for 1 h. Immunoreactive bands were detected by ECL (Amersham Biosciences), then visualized and quantified on a FluorChem 8000 Imaging System using AlphaEase software (Alpha Innotech, San Leandro, CA).

Data analysis

Statistical significance was determined using a Student t test. All p values < 0.05 were considered statistically significant.

Results

RANTES, eotaxin, IL-8, and MIP-1α induce proliferation of ASMCs

To investigate the ability of chemokines to induce ASM proliferation, we measured [^3H]thymidine as well as BrdU incorporation into ASMCs following treatment with increasing concentrations of RANTES, eotaxin, IL-8, and MIP-1α. Non-asthmatic ASMCs were plated in 96-well plates and incubated overnight in 5% FBS SmGm2 complete media at 37°C and grown to 60–70% confluency. Cells were then starved for 48 h in 0.3% FBS SmGm2, then treated with different concentrations of chemokines or the appropriate vehicle for 24, 48, or 72 h. The concentrations for each chemokine tested ranged between 0.1 ng/ml and 100 ng/ml, except for IL-8, for which concentrations of 50, 100, 500, and 1000 ng/ml were used. Cells were then pulsed with [^3H]thymidine, and the level of radioactivity incorporation into ASMCs was determined. Treating ASMCs with eotaxin at 0.1 and 1 ng/ml for 48 or 72 h induced a significant increase in ASM proliferation compared with the vehicle, as shown in Fig. 1A. Higher eotaxin concentrations, however, did not trigger significant responses. Similarly, RANTES treatment induced significant proliferation of ASMCs at low (1 and 5 ng/ml) but not high concentrations. This increase, however, was observed at 48 but not 72 h following incubation. In contrast, high concentrations of MIP-1α and IL-8 were required to trigger significant ASM proliferation. Up to 100 ng/ml MIP-1α was required to induce significant ASM proliferation. This increase in proliferation was mostly seen at 48 h of incubation (Fig. 1D). Similarly, a 25.2% increase in ASM number was detected only after the cells were stimulated with 800 ng/ml IL-8 with a significant increase seen following incubation for 48 as well as 72 h (Figs. 1C, 2). These results indicated that chemokines (RANTES, eotaxin, IL-8, and
MIP-1α) trigger a significant increase in human airway ASMC proliferation that is better observed following 48 h of incubation (Fig. 1E).

The proliferative effect of chemokines on nonasthmatic ASMCs was also confirmed using Ki-67 staining and BrdU incorporation assay. Stimulated ASMCs were stained with Ki-67 for 20 min at room temperature and analyzed on an FACS Calibur (BD Biosciences). As shown in Fig. 2, stimulation with chemokines, represented by RANTES and IL-8, induced upregulation of Ki-67 expression, indicating an increase in ASM proliferation. BrdU, however, was introduced to stimulated ASMCs 18 h prior to analysis. On the day of analyses, harvested cells were stained with anti-BrdU fluorescent Ab for 20 min at room temperature. Cells were then passed on an FACS Calibur flow cytometer (BD Biosciences), and data were analyzed using Cell Quest software. PDGF-treated smooth muscle cells were used as a positive control. PDGF is a strong mitogen of ASMCs. A 6-10-fold increase in the number of proliferating cells was seen after PDGF treatment (Fig. 3A). Similar to what has been observed using [3H]thymidine, treatment with chemokines induced a significant increase in BrdU-positive ASMCs. Low concentrations of eotaxin (1–5 ng/ml) were sufficient to trigger significant increase in BrdU incorporation; 29.2% of eotaxin-treated ASMCs incorporated BrdU compared with 19.8% of cells treated only with the vehicle (p < 0.002). Higher concentrations, however, resulted in a negative-feedback response. Fig. 3A shows the percentage of BrdU-positive ASMCs after treatment with different concentrations of eotaxin over 24 h. Incubating ASMCs with eotaxin for longer time intervals did not yield a significant difference. In addition, ASMCs treated with 5 ng/ml RANTES had a significant increase in BrdU-positive ASMCs compared with treating with just the vehicle. In fact, 24.3% of ASMCs were shown to synthesize new DNA compared with the vehicle-only treated cells. Similarly, incubating ASMCs with either MIP-1α or IL-8 (but at higher concentrations, 100 ng/ml and 800 ng/ml for MIP-1α and IL-8, respectively) resulted in a significant increase in BrdU incorporation compared with the vehicle (p < 0.002). Neither higher concentrations of these chemokines nor incubating ASM with them for >48 h had any significant difference in BrdU incorporation. Graphs presented in Fig. 3 represent the mean of four independent experiments. In contrast, treating ASMCs with IP-10 (CXCL-10) did not have any proliferative effects (data not shown). Therefore, using three different approaches to measure the effect of chemokines on human airway ASMCs confirmed the significant role these chemokines play in stimulating ASM proliferation and hence increasing ASM mass within the lung airways.
We have also tested the effect of chemokine stimulation on ASMCs isolated from four asthmatic patients. Those were the only asthmatic ASMCs available to us and were on late passage stages. We have stimulated them with RANTES, eotaxin, MIP-1α, and IL-8 as well as PDGF (positive control) as described in Materials and Methods. The concentrations for each chemokine tested ranged between 0.1 and 100 ng/ml, except for IL-8, in which concentrations of 50, 100, 500, and 1000 ng/ml were used. The proliferation of asthmatic ASMCs in response to chemokine stimulation was comparable to that of nonstimulated cells in all four cell lines (data not shown). This indicated that other in vivo cofactors could be required, in addition to chemokines, to enhance asthmatic ASMC proliferation.

**RANTES and eotaxin induce ASM proliferation via p42/p44 MAPK activation**

Activation of MAPK was shown to regulate the proliferation of different types of cells (14). Hence, to investigate the mechanism behind chemokine-induced ASM proliferation, we tested the ability of these chemokines to activate the MAPK pathway via the phosphorylation of p42/p44 MAPK. Several growth factors, such as cytokines and neuropeptides that induce ASMC proliferatio,
activate this MAPK isoform (15). Chemokine-induced p42/p44 MAPK activation (phosphorylation) occurs as early as 1 min and peaks at 5 min and then goes back to the basal levels. ASMCs were starved for 48 h in serum-free medium then stimulated with increasing concentrations of eotaxin or RANTES for 1, 5, or 10 min. Cells were then lysed in the presence of 1% Triton-X, equal amounts of whole-cell lysates were resolved by Western blot analysis, and the state of p42/p44 phosphorylation was assessed using phospho-p42/p44 Ab. The total level of p42/p44 MAPKs was also determined using polyclonal Abs (Fig. 4). Immunoreactive bands were then visualized and quantified using a densitometer. Integrated density value ratio of phosphorylated to total p42/p44 was calculated, and the relative increase in MAPK phosphorylation was then compared between chemokine and vehicle-stimulated ASMCs. As shown in Fig. 4A, eotaxin treatment induced a concentration-dependent increase in p42/p44 phosphorylation with a maximal response at 1 ng/ml. As expected, treatment with higher concentrations of eotaxin had a negative-feedback effect. Treatment with 5% FBS was used as a positive control. Likewise, treating ASMCs with increasing concentrations of RANTES induced a concentration-dependent increase in MAPK phosphorylation. This increase, however, was only significant when treating ASMCs with 1 or 5 ng/ml RANTES. These results, therefore, suggest that chemokines may induce ASM proliferation through the phosphorylation of p42/p44 MAPKs.

To confirm this observation, the effect of inhibition of MAPK phosphorylation on chemokine-induced ASM proliferation was tested. To do that, ASMC were stimulated, or not, with IL-8 (800 ng/ml), RANTES (5 ng/ml), eotaxin (1 ng/ml), and MIP-1α (100 ng/ml) for 12 h in the presence, or absence, of PD184352 (100 μM). ASMCs were then collected and stained with Ki-67–PE and analyzed with FACS (LSR II; BD Biosciences). Data were then analyzed using Diva software (BD Biosciences). As shown in Fig. 4C, ERK1/2 (p42/p44 MAPK) inhibitor significantly decreased the level of chemokine-induced ASM proliferation. These results confirm that chemokines induce ASM proliferation via the activation of the MAPK pathway.

Chemokines induce an antiapoptotic effect on ASMCs

The increase in ASM mass could be due to an enhanced proliferation rate, decreased apoptosis rate of ASMCs, or both. Hence, we examined the effect of epithelial-derived chemokines on ASMC apoptosis. Apoptosis was determined by staining stimulated cells with both Annexin V and PI. ASMCs were starved for 48 h, then stimulated with an increasing concentration of RANTES, eotaxin, IL-8, and MIP-1α for 48 h. Following stimulation, cells were stained with Annexin V and PI and analyzed using an FACSCalibur flow cytometer (BD Biosciences), and data were evaluated using Cell Quest software (BD Biosciences). Annexin V+PI+ cells are cells undergoing apoptosis, whereas Annexin V+PI− cells are cells undergoing proliferation.

**FIGURE 4.** Chemokines enhance ASM proliferation via the stimulation of MAPKs. Activation of p42/p44 MAPKs in ASMCs treated with eotaxin (A) or RANTES (B) in a concentration-dependent manner. The ASMCs were serum deprived for 48 h before stimulation with eotaxin or RANTES for 1 min. The cell lysates were analyzed using Western blot and anti-phosphorylated (pP42, pP44) or total (p42, p44) MAPK polyclonal Abs. Bands were quantified by a densitometer. Similar results were obtained in three independent experiments. C, ERK phosphorylation inhibitor reduced the level of chemokine-induced ASM proliferation. ASMCs were stimulated, or not, with IL-8 (800 ng/ml), RANTES (5 ng/ml), eotaxin (1 ng/ml), and MIP-1α (100 ng/ml) for 12 h in the presence or absence of PD184352 (100 μM). ASMCs were then collected and stained with Ki-67–PE and then analyzed on FACS (LSR II; BD Biosciences). Data were analyzed using Diva software (BD Biosciences). *p < 0.05, as compared with the vehicle.
Discussion

ASM proliferation and enhancement of ASM survival by reducing apoptosis rate are possible mechanisms responsible for the increase in ASM mass in asthma. To date, many growth factors, proinflammatory cytokines, and contractile agonists such as histamine and endothelin-1 have been shown to promote ASM proliferation (16). Little is known about the effect of chemokines on ASM growth and survival. To our knowledge, this study is the first to demonstrate the mitogenic and antiapoptotic activity of asthma-related chemokines on ASMCs. We have investigated the proliferative as well as antiapoptotic effect of eotaxin, RANTES, IL-8, and MIP-1α on ASMCs. These chemokines are usually upregulated in asthmatic airways, and ASMCs express functional receptors to each one of them. Our results indicated that RANTES and eotaxin induce ASM proliferation at low concentrations, whereas higher doses of IL-8 and MIP-1α are needed to demonstrate a similar response. IL-8, predominantly a neutrophil chemoattractant, also interacts with the structural cells and causes the vascular and ASMCs to migrate (3). Furthermore, IL-8 was shown to be a mitogen for both human and rat aortic smooth muscle cells (17).

Airway epithelium secretes many chemokines including RANTES, IL-8, eotaxin, and MIP-1α. In addition to their classical role in leukocyte recruitment and activation, recent studies have shown that chemokines could also have effects on structural cells. We have recently demonstrated the expression of chemokine receptors CCR1 and CCR3 (receptors of RANTES, MIP-1α [CCR1], and eotaxin [CCR3]) as well as CXCR1 and -2 (receptors of IL-8) on human ASMCs (5, 6). We have also shown that ASMCs migrate toward eotaxin (3). Moreover, work from other groups demonstrated that IL-8 induces the contraction and migration of ASMCs (18). These observations, in addition to the upregulation of these chemokines in asthmatic airways as well as data presented in this study, suggest that chemokines could be implicated in human airway tissue remodeling by promoting the increase in smooth muscle mass (Fig. 6).

RANTES is known to induce the proliferation of cancer cells and enhances their survival. In addition to epithelial cells, this chemokine was shown to be produced by many cells including T cells as well as ASMCs, which also express RANTES receptors (CCR1, -5) (3). The expression of RANTES, IL-8, MIP-1α, and eotaxin by the airway epithelium and smooth muscle cells suggest that these chemokines act in both an autocrine and paracrine manner. As demonstrated in Figs. 1 and 5, RANTES exerted a proliferative as well as antiapoptotic effect on ASMCs at a low concentration ranging between 1 and 5 ng/ml. After 48 h, cells stimulated with RANTES showed more DNA synthesis and decreased uptake of both Annexin V and PI stains compared with the nontreated cells. These results reflect the important role RANTES may play in enhancing the growth and survival of ASMC in the asthmatic airways.

MIP-1α was recently reported to play an important role in muscle repair through the stimulation of myocyte proliferation (7). Treating ASMCs with MIP-1α enhanced their proliferation in a concentration-dependent manner; however, a significant increase in ASMCs proliferation was only seen when high doses of MIP-1α (100 ng/ml) were used. The best results were obtained after incubating ASMCs with MIP-1α for 48 h. Similarly, treating ASMCs with IL-8 did not have any effect except at high doses (800 ng/ml). Although we do not know the exact physiological concentrations of these chemokines in vivo, their long t1/2 could ensure their effectiveness in inducing ASMC proliferation or enhancing their survival. Moreover, using a rabbit model, Frevert
et al. (19) have shown that IL-8 is retained in the lungs to a much higher levels than in other tissues such as the skin, and the dimerization of IL-8 plays a role in this process. Knowing that, one can predict that effective levels of IL-8 within the lung tissue could be reached upon its enhanced production and accumulation with time. In contrast, a higher concentration of these chemokines had an inhibitory effect on proliferation of ASMCs (Figs. 1, 3, 4, 5), indicating a negative-feedback effect. This could be a normal response of nonasthmatic ASMCs in response to chronic chemokines stimulation.

It is well established that activation of MAPK by growth factors and G-protein coupled receptors, including the chemokine receptors, results in the stimulation of DNA synthesis and proliferation of eukaryotic cells (14). Growth factor-dependent myoblast proliferation has been associated with activation of ERKs (p42/p44 [ERK1/2]) (20–22). In fact, stimulation of myoblasts with MIP-1α induced strong activation of the ERK1/2 pathway as indicated by the increased level of phosphorylation of ERK1/2 relative to total ERK (7). Moreover, several growth factors, cytokines, and neuropeptides were shown to induce ASMC proliferation via activating the p42/p44 MAPK isoform (15). Hence, to determine the mechanism behind chemokine induction of ASM proliferation, the ability of RANTES and eotaxin to activate the MAPK pathway was investigated. Treatment of ASMCs with eotaxin as well as RANTES induced a concentration-dependent increase in MAPK phosphorylation; however, this increase did not reach significance except at 1 ng/ml for eotaxin and 1 or 5 ng/ml for RANTES. Interestingly, the same physiological concentrations of eotaxin and RANTES that induced ASM proliferation resulted in triggering MAPK phosphorylation. Higher or lower chemokine concentrations had no significant phosphorylation response. More importantly, blocking the phosphorylation of ERK1/2 MAPK using an ERK inhibitor completely aborted chemokine-induced ASM proliferation (Fig. 4C). These results, therefore, indicated that chemokines may induce ASM proliferation through the phosphorylation of p42/p44 MAPK.

In addition to cell proliferation, ASM mass could be increased by promoting the persistence of ASMCs by lowering their apoptosis rate. IL-8 was previously shown to delay the spontaneous and TNF-α–mediated neutrophil apoptosis in vitro, and blocking the IL-8 receptor (CXCR2) was shown to enhance neutrophils apoptosis (23). Similarly, our data indicated that treating ASMCs with increasing concentrations of eotaxin, RANTES, IL-8, as well as MIP-1α results in a significant proportional decrease in the ASM rate of apoptosis. Treating ASMCs with these chemokines for 48 h reduced the number of Annexin V+ cells compared with the control. Importantly, the decrease in apoptosis rate of ASMCs following treatment with eotaxin or RANTES was observed even when treating the cells with very low concentrations, which did not induce a proliferative effect. This indicates that the observed decrease in apoptosis is not just a result of the enhancement of proliferation but a complementary process. In addition, we have tested the ability of these chemokines to reduce dexamethasone-induced apoptosis of ASMCs. Our preliminary data indicated that treatment of ASMCs with RANTES (1 or 5 ng/ml) significantly reduced the apoptotic effect of dexamethasone (data not shown).

Our experiments were conducted on nonasthmatic ASMCs. It has been shown previously that smooth muscle cells from asthmatic subjects proliferate at twice the rate seen in nonasthmatic cells (24). They also express higher levels of chemokine receptors including CCR3. In fact, Joubert et al. (5) clearly showed that CCR3, receptor of eotaxin and RANTES, is significantly more expressed in asthmatic compared with nonasthmatic ASMCs. However, the proliferation of asthmatic ASMCs that became available to us recently in response to chemokine stimulation was comparable to that of nonstimulated cells. This could be due to the fact that the asthmatic cells that were available to us were in late passages and hence may not respond properly to stimulation. Another possibility is that other in vivo cofactors are required in addition to chemokines to enhance asthmatic ASM proliferation. Recently, an interesting report by Burgess et al. (25) demonstrated that in nonasthmatic ASMCs, both ERK and P38 pathways control ASM proliferation under strong stimulation. In contrast, ERK activation was significantly reduced in asthmatic ASMCs under the same conditions (25). Our results suggest that chemokines in vivo might induce smooth muscle proliferation at early stages of asthma development. However, at late stages and under chronic stimulation with a high concentration of chemokines, ASMCs may develop a negative-feedback mechanism of ERK inhibition such as an upregulation of an endogenous MAPK inhibitor (25). Further experiment might be essential to prove these hypotheses.

The identification of new chemokine functions on the airways could contribute to a better understanding of their role in immunomodulation and remodeling seen in asthma and other chronic inflammatory diseases. Their newly reported effects on ASMCs may implicate them in remodeling process either by increasing the proliferation or delaying the apoptosis of ASMCs. Presently, ASMCs are considered a new target in asthma therapy. The increase in ASM mass might also explain the acute symptoms related to airway hyperresponsiveness (16). Smooth muscle contraction is
the direct reason to airway narrowing, and the increase in smooth muscle mass may cause excess airways narrowing in asthma by increasing the force generated during airway contraction. Glucocorticosteroids, the main drug used for asthma treatment, was shown to inhibit growth factor-induced proliferation of ASMcs (26). Moreover, it was recently reported that corticosteroids decrease epithelial cells’ secretion of IL-8. This may indicate that asthma patients might benefit from corticosteroids to treat inflammation and remodeling if used early in asthma development (27). Therefore, drugs that could intervene against chemokine proliferative and antiapoptotic effects on ASMcs may represent a novel strategy to prevent airway remodeling.

**Disclosures**

The authors have no financial conflicts of interest.

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