Role of Chemokines in Airway Remodeling and Effects on Smooth Muscle Proliferation and Survival

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Abbreviations

ASMC	Airway smooth muscle cells
BrdU	Bromodeoxyuridine
CCL/R	CC chemokine ligand/ receptors
CXCL/R	CXC chemokine ligand/ receptors
ECM	Extra cellular matrix
ET-1	Endothelin-1
ERK	Extracellular signal-regulated Kinases
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptors
IFN-γ	Interferon-gamma
IL-	Interleukin
MAPK	Mitogen activated protein kinases
MIP-1α	Macrophage inflammatory protein- 1α
RANTES	Regulated upon activation, normal T cell expressed and secreted
PDGF	Platelet derived growth factor
PGE	Prostaglandin E
Th1, 2	T helper cells type 1, 2
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-α

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Abstract

The increase in ASMC mass is a major structural change described in airway remodeling in asthma. This increase has been attributed to ASMC hyperplasia and hypertrophy. The distance between ASMC and the epithelium is reduced suggesting expansion of the muscle bundle towards the epithelium. Recent studies have suggested a role of epithelial derived chemokines in ASMC migration toward the epithelium. We hypothesized that chemokines (Eotaxin, RANTES, MIP-1α and IL-8) can directly influence ASMC mass by increasing the rate of proliferation or enhancing survival. ASMCs were exposed to different concentrations of eotaxin, RANTES, IL-8 or MIP-1α. To test for proliferation, stimulated ASMC were pulsed with ^{3}H-thymidine or stained with BrdU and then analyzed with flow cytometry. Apoptosis was measured using Annexin V and flow cytometry. Expression of phosphorylated p42/p44 and MAPKinases was assessed by Western analysis. In a concentration-dependent manner, chemokines such as Eotaxin, RANTES, IL-8 and MIP-1α increased ASMCs ^{3}H-thymidine incorporation and DNA synthesis. Eotaxin, RANTES and IL-8 decreased the number of apoptotic ASMCs compared to the matched controls. A significant increase in phosphorylated p42/p44 MAPKs was seen after treating ASMCs with RANTES and eotaxin. We conclude that chemokines might contribute to airway remodeling by increasing the number of ASMCs.

Résumé

L'augmentation de la masse musculaire lisse constitue un des changements majeurs associés au remodelage des voies respiratoires dans l'asthme. Cette augmentation est attribuée à l'hyperplasie et à l'hypertrophie des cellules musculaires lisses (ASMC; *airway smooth muscle cells*). La distance entre les ASMC et l'épithélium est réduite, suggérant une expansion des faisceaux musculaires lisses en direction de l'épithélium. Des études récentes suggèrent le rôle de chimiokines dans la migration des ASMC vers l'épithélium. Nous supposons que MIP-1α, RANTES, Eotaxin, IL-8, peuvent influencer directement l'augmentation de la masse de ASMC en augmentant leur degré de prolifération et/ou en diminuant leur degré d'apoptose. Les ASMC furent exposées à des concentrations croissantes de MIP-1α, RANTES, Eotaxin, IL-8. Pour mesurer la prolifération, des ASMC stimulées ont été exposées à de la thymidine tritiée ou marquées avec du Bromodeoxyuridine (BrdU), et analysées par cytométrie de flux. L'apoptose a été mesurée par Annexin V et cytométrie de flux. L'expression de la p42/p44 MAP kinase phosphorylée a été déterminée par immunoblot / buvardage de western. D'une manière dose-dépendante, MIP-1α, RANTES, Eotaxin, IL-8 ont augmenté la prolifération des ASMC et la synthèse d'ADN. L'IL-8, l'Eotaxine et RANTES ont diminué le nombre de cellules apoptotiques comparativement aux cellules contrôle. Une augmentation marquée fut observée au niveau de la phosphorylation des MAP kinases p42/p44 après stimulation des ASMC avec RANTES et Eotaxin.

Nous concluons que les chimiokines peuvent contribuer au remodelage des voies respiratoires observé dans l'asthme en augmentant la prolifération des ASMC.

Chapter1. Introduction

1.1 Asthma

Asthma is a common chronic inflammatory disease of the airways associated with bronchial hyperresponsiveness and airway remodeling. The Global Initiative for Asthma (GINA) reports that as many as 300 million people worldwide are affected with asthma. The prevalence of clinically important asthma is increasing globally and, if the current trend continues, it is predicted that as many as 400 million people will have asthma by the year 2025 (GINA/Masoli 2004). Asthma is also the leading cause of childhood morbidity as measured by hospitalization and school absence (Asher 1995). The importance of childhood asthma is reflected in the fact that the pattern of asthma establishes during childhood and persists into adulthood. Many pathological studies support this data by showing airway remodeling occurring early in childhood (Payne 2003). The chronic nature of asthma represents a significant economic burden and has an impact on the patient's life, his family, and the society (Masoli 2004).

Despite intensive research in recent years, the pathogenetic mechanisms and phenotypical characterization of asthma are still poorly defined. It is widely accepted that asthma is a heterogeneous disorder and it is considered as a type 2 cytokines-induced inflammatory response (Robinson 1992).

1.1.2 Th2 Type Inflammation, cells and mediators

Type 2 inflammatory responses are characterized by differentiated CD4+ Th2 cells that secrete a specific panel of cytokines, including IL-4, IL-5, IL-9, IL-13, and other inflammatory mediators. The cytokines recruit other multiple effector cells including B cells, mast cells, eosinophils, and basophils (Azzawi 1990). Increased numbers of these cells within the airway lumen and submucosa have been shown in asthmatic airways (Bradley 1991). The eosinophils, mast cells, and basophils unique and separate roles play a major effector function in the perpetuation of asthmatic inflammation. In addition, Th2 cytokines act on their gene expression and phenotype to induce and orchestrate the allergic inflammation (Romagnani 2002). For example, mast cells releasing histamine induce airway hyperresponsiveness and eosinophils are believed to be responsible for tissue damage and remodeling.

It is widely accepted that Th2 cytokines account for the major pathophysiological manifestations of allergy and asthma. IL-4 and IL-13 were found to be absolutely required for allergic inflammation to occur. IL-4 plays a major role in naïve T cell differentiation into Th2 cells (Geginat 2002) and IgE switching in B-cells (Rush 2002). IL-4 induces the expression of adhesion molecules by the endothelial cells, facilitating the trafficking of inflammatory cells to the targeted tissue (Hirata 1998). IL-13 increases IgE production but also increases the contractile response of airway smooth muscle cells to the contractile agonists (Shore 2002). Together, IL-4 and IL-13 are involved in the development of airway hyperactivity by directly affecting airway smooth muscle cells.

Another Th2 cytokine that is involved in eosinophil activation, maturation, survival (Sanderson 1988), and recruitment (Wang 1989) is IL-5. Moreover, IL-5 is also implicated in AHR. IL-9 is involved in mast cell growth and activation; it induces mast cells to express more FcεRI and produce more inflammatory mediators. IL-9 upregulates mucus production and promotes goblet cell hyperplasia (Louahed 2000). All together, Th2 cytokines initiate, modulate, and maintain the allergic inflammation; therefore, understanding asthma and other allergic diseases requires a comprehensive knowledge of interactions among cytokines, infiltrating inflammatory cells, and resident structural cells. Chemokines are produced by many structural, epithelial cells in particular, and by inflammatory cells especially the macrophages. In return, many inflammatory and structural cells express receptors for different chemokines. Many studies of human asthma show chemokines in relation to asthmatic inflammation and airway hyperreactivity (Stellato 1997). Chemokines recruit and activate leukocytes and induce degranulation in mast cells, basophils and eosinophils, initiating the early response in asthma. Careful examination in the animal models of allergic airway responses reveals that the expression and function of these chemokines occur at a specific stage of the disease (Lukacs1999). Moreover, the chemokines production is regulated by Th2 cytokines (Marfaing-K 1995).

Th2 cells in asthma highly express CC chemokine receptors such as CCR4, CCR8 (Bonecchi 1998), and CCR3 (Sallusto 1997). These receptors may preferentially recruit lymphocytes that would specifically create the allergic milieu leading to the altered airway physiology in such diseases. CCR4 and CCR8 are known to be upregulated selectively upon activation of polarized human Th2 cells (D'Ambrosio 1998). The Th2 cells will induce selectively the production of chemokines that act on Th2 chemokine

receptors. These chemokines act on other leukocytes, recruiting and activating them at the site of inflammation.

1.2 Airway remodeling in Asthma

Th2 cytokines induce airway structural cells and the infiltrating inflammatory cells to express inflammatory and remodeling related cytokines, chemokines, growth factors, and adhesion molecules. Therefore, Th2 cytokines are central in many aspects of airway remodeling where remodeling can explain part of the disease development. Animal models continuous exposures to allergen lead to epithelial thickening, goblet cell hyperplasia, and subepithelial fibrosis (Blyth 1996) and elevated levels of IL-4, IL-5, and IL-13 were found in these models (Johnson 2004). IL-4 induces collagen production by bronchial fibroblast (Bergeron 2003) and increases the production of several remodeling associated factors, such as TGF-β, PDGF, and others. IL-13 is a mitogen for ASMC (Shore 2004); therefore, this cytokine might be involved in the increased smooth muscle mass in asthma. IL-13 and IL-4 cause goblet cell hyperplasia while IL-5 transgenic mice showed epithelial hypertrophy, goblet cell hyperplasia and focal collagen deposition. IL-5 deficient mice had significantly less peribronchial fibrosis and significantly less peribronchial smooth muscle compared with wild type mice (Jae Youn Cho 2003). In addition to mucus cell hyperplasia, IL-9 indirectly increases IL-8 and eotaxin production (Baraldo 2003) from ASMC, where both chemokines cause ASMC migration (Govindaraju 2006; Joubert 2006).

Th-2 cytokines promote the production of leukotrienes that are involved in airway remodeling. Cysteinyl-leukotrienes augment growth factor-induced ASM mitogenesis through activation of CysLT receptors (Panettieri 1998). Few studies showed Th2 cells

adhering to ASMC by CD44 and integrins can induce ASMC to proliferate (Lazaar 1994).

Remodeling refers collectively to a group of structural changes seen in asthmatic airways. The changes include subepithelial fibrosis which contributes to the thickening of airway walls due to the deposition of extracellular proteins such as collagens I and III, laminin, and tenascin (Roche 1989), (Robinson 2007). Mucus gland hyperplasia leads to excessive mucus secretion and can, in severe cases, lead to occlusion of the airways. Bronchial blood vessels increasing in number and size is another remodeling feature studied in asthma (Li 1997). This change in airway vascularity could be the reason behind the airway wall edema by increasing the production of VEGF (Hoshino 2001). Another remarkable change is the increase in the bronchial smooth muscle mass (Ebina 1990); the inner border of the smooth muscle bundles is closer to the epithelium, suggesting the new muscle is preferentially added to the luminal side of the muscle (Benayoun 2003). It seems these structural changes are accompanied by changes in cellular phenotypes at the molecular level that are of equal importance (Fixman 2007).

It has been postulated that airway remodeling is responsible for the gradual decline in lung function; however, remodeling was detected in mild asthma and other studies failed to relate asthma severity to the degree of airway remodeling (Bousquet 1998).

Many Th2 cytokines, chemokines, and mediators are involved in initiation and perpetuation of the inflammatory process and remodeling. Consequently, treatments that suppress this Th2 inflammatory response have been developed for therapeutic benefits; however, some individuals are left with residual obstruction after optimal treatment with corticosteroids and this obstruction is possibly attributed to airway remodeling (Jeffery

2005). Although airway remodeling occurs in response to acute injury or inflammation, no effective treatment to prevent or to reduce remodeling is available. A few studies have shown decreased remodeling after blocking some of the Th2 cytokines.

1.2.1 Epithelial Cells Remodeling in Asthmatic Airways

The epithelium under normal circumstances forms a highly regulated and almost impermeable barrier. It is widely reported that the asthmatic epithelium undergoes several pathological changes and these changes could lead to alterations in host defense and in the response of the epithelium to exogenous stimuli (Fahy 2001). Increased epithelial shedding or desquamation is a characteristic feature of asthma, although this fact has been long debated. Areas with desquamated epithelial cells where only basal cells are resting on the basement membrane are often seen in post partum and bronchial biopsies specimens from asthmatic subjects (Laitinen 1985). Whenever the usually fragile in appearance epithelium is seen in these specimens it is non-ciliated and vacuolized (Montefort 1992). It is common to find epithelial clumps in the sputum and an increased numbers of epithelial cells in bronchoalveolar lavage of patients with asthma (Kirby 1987). However, epithelial shedding can be due to artifacts of the biopsy procedure (Fahy 2000).

Epithelial damage characterized by an increase in intracellular space and epithelial shedding have been correlated with bronchial hyperreactivity (Jeffrey 1989). Removal of the epithelium from human isolated bronchial smooth muscle appears to enhance the responsiveness to acetylcholine and histamine (Knight 1990). Enhanced responsiveness consequent to epithelium loss may prove important with respect to the development of

worsening asthma. Epithelial loss also causes exposure of the nerves and mast cells, increasing the sensitivity of the isolated human airways to methacholine (Jongejan 1991).

The bronchial epithelial cells are activated in asthma and these can be activated through different mechanisms. It has been recently reported that epithelial cells of asthma patients but not of healthy subjects express the FceRI and FceRII functional receptors and are capable of fixing IgE (Campbell 1998). It is therefore possible that these cells may be able to interact directly with inhaled allergens. Other studies showed that pollutants such as NO_2 and ozone can activate the bronchial epithelial cells (Davies 1993); histamine, Platelet Activating Factor (PAF) (Vignola 1993; Salary 1991), and cytokines were shown to activate the epithelial cells as well.

Repair of bronchial epithelium in asthma is important in maintaining the integrity of the epithelial barrier. The repair process is a series of complex interactions to re-epithelize the luminal surface (Zahm 1991). There is increasing evidence to suggest that aberrant repair signals in the epithelium occur early in the pathogenesis of asthma. An aberrant repair process occurring at the mucosal surface may trigger a cascade of events deeper within the sub-mucosa, leading to direct effects on the amount and behavior of airway smooth muscle, as well as impacting on sub-mucosal glands and the deposition of extracellular matrix (Knight 2007).

Asthmatic epithelium exhibits abnormal expression of several pro-inflammatory transcription factors, signal transducers, and activators of transcription (STAT1 and STAT6) (Mullings 2001). Using epithelial cells obtained by bronchoscopic sampling, significant intrinsic biochemical and functional differences were reported between healthy and asthmatic epithelium (Knight 2007). Asthmatic epithelia produce substantially greater amounts of IL-6, prostaglandin E2, and EGF (Kicic 2006). Not only

the biochemical function of airway epithelium is altered but structurally these cells are disturbed with separation of these columnar cells from their basal attachments and increased epithelial permeability (Chetta 1996)

1.2.2 Role of Epithelium in Airway Remodeling

Epithelial shedding and hypertrophy, particularly of goblet cells, are indicative of remodeled airways (Lloyd 2007). Increasing evidence shows that epithelial cells play a crucial role in the development of airway remodeling and it has been suggested that these may act independently of inflammatory events (Holgate 2000). Asthmatic airway epithelium contributes to the airway remodeling by producing different inflammatory mediators and growth factors that induce remodeling of other airway tissues. Emerging theory suggests that not only the intact epithelial cells damage seen in asthma contributes to remodeling but even the damaged cells do so (Holgate 2001).

The airway epithelium is a source of a range of proinflammatory molecules, including those derived from arachidonic acid (15-HETE, PGE2, and lipoxins), nitric oxide (NO), endothelin-1, a range of cytokines (IL-1β, IL-5, IL-6, IL-11, GM-CSF, IL-16, IL-18), chemokines (IL-8, GROα, MCP-1, MCP-3, RANTES, MIP1α, MIP2), eotaxin, and proliferative growth factors (TGFβ1, TGFβ2), platelet-derived growth factor (PDGF), insulin like growth factor (IGF-1), and basic fibroblast growth factor (b-FGF) (Holgate 2002).

Several reports show that epithelial supernatant induces a proliferative response in fibroblasts and myofibroblasts. The supernatant contains several growth factors, such as

PDGF, bFGF, and TGF-β and blocking these growth factors partially inhibits the proliferative response of the myofibroblasts (Zhang 1999).

Recent evidence suggest that epithelial cells are capable of expressing receptors in a manner that suggests a regulatory role in controlling local inflammatory and remodeling events (Lukacs 2001). It has been previously shown that, upon appropriate stimulation, airway epithelial cells have the ability to release a number of cytokines. For example, TNF-alpha stimulates the epithelial cells to synthesize IL-8 (CXCL8), RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), and MCP-1 (Macrophage chemoattractant Protein) (Cromwell 1992). Furthermore, epithelial cells produce eotaxin and the production is upregulated by IL-4 and IL-13, which are Th2 cytokines involved in remodeling, as well as by TNF-alpha (Fujisawa 2000). IL-8, RANTES, eotaxin, and MCPs have been shown to be upregulated in asthmatic airways thereby highlighting the importance of these molecules in the pathogenesis of asthma (Holgate 1997).

1.2.3 Smooth Muscle Cells (ASMC) in Asthma

Airway smooth muscle (ASM) is an important tissue involved in the regulation of bronchomotor tone and exists in the trachea and in the bronchial tree up to the terminal bronchioles. Evidence suggests that ASM undergoes marked phenotypic modulation in lung development and in disease states such as asthma, chronic bronchitis, and emphysema; however, the precise function of ASMC in healthy bronchioles remains unclear (Panettieri 2003).

ASMC is a key determinant of airway hyperresponsiveness and remodeling in asthma. Airway myocytes are thought to have capacity to contribute to remodeling due to their ability for graded and reversible phenotype switching which confers broad functional capacity (Halayko 2001). Airway myocytes exist in an immature phenotype that is characterized by a high tendency for proliferation, expression, and secretion of ECM proteins and by synthesis of inflammatory mediators in response to a number of environmental cues (Halayko 2003).

ASMC also serve as a rich source for many cytokines, chemokines, growth factors, adhesion molecules, and even lipid mediators. They express and release eotaxin in response to Th2 cytokines. Interleukin-13 can act directly on airway smooth muscle cells leading to changes in the ability of smooth muscle cells to generate chemokines such as eotaxin and TARC (Shore 2004). ASMC also produce monocyte chemoattractant protein MCP-1, MCP-2, MCP-3, IL-8, and RANTES when stimulated with proinflammatory cytokines, such as IL-1b and TNF-α (Hamid 2005). ASMC have been shown to release a number of extracellular matrix proteins, including collagen I, collagen III, lumican, and versican. These proteins were shown to be upregulated in the asthmatic airways (Black JL 2001). By the expression of the above mentioned molecules and others, ASMC regulate and contribute to the ongoing inflammatory process seen in asthma.

In addition to contraction, ASMC contribute to the remodeling in asthma by increased proliferation leading to an increase in smooth muscle mass. Such increase in ASMC could contribute to the exaggerated airway narrowing observed in asthma. Data obtained from in vivo studies suggest that ASMC can exhibit heterogeneity, i.e., exhibit proliferative, synthetic, and contractile phenotypes. It has been shown that under

stimulation with proinflammatory and inflammatory cytokines, ASMC is capable of expressing a wide range of molecules (Chung 2000). Some reports suggest that the ASMC immature phenotype is one characterized by high tendency for proliferation and mediators synthesis (Halayko 2006). In contrast, myocytes of mature phenotype are of contractile ASMC but are still able to produce some ECM, suggesting that some ASMC might exist in intermediate phenotype (Merrilees 1990). Interestingly, ASMC serum deprivation in vitro can switch or modulate proliferative and synthetic myocytes to a contractile phenotype (Stephens 1998).

The increase in ASMC content in asthma was first reported more than 78 years ago (Dunnill 1969). The mechanisms underlying such an increase could be an increased rate of division or decreased rate of apoptosis. It could also be due to the migration of ASMC towards the luminal side of the airway through the migration of mesenchymal cells to the ASMC bundle or by differentiation (Hirst SJ 2004). Proven to a lesser extent, some morphometric studies reported ASMC hypertrophy as an additive explanation of ASMC mass increase.

Several stimuli have been identified as mitogens for ASMC. Contractile agonists such as histamine and LTD4 (Panettieri 1998), cytokines such as TNF-α (Amrani 1996) and IL-1β (De S 1993), growth factors, extracellular matrix, and increased stretch (Hasaneen 2005) were described to influence the proliferation rate of airways smooth muscle. Fibronectin and collagen I enhance proliferation and encourage expression of the nuclear proliferation marker Ki67 (Black 2002), whereas cells grown on laminin divide more slowly but express contractile proteins. This points to the fact that cell–matrix interactions, in addition to growth factors and cytokines, have important effects on myocytes phenotype and cell cycle control. Adding fibronectin, collagen I, and laminin

to the cultured ASMC provides a strong survival signal (Freyer 2001). Mitogens are expressed by ASMC and other structural or inflammatory cells, they may modify the proliferation in a paracrine or autocrine manner. Although a number of mitogens for ASMC were identified *in vitro*, it is still not clear which plays the most important role *in vivo* (Knox et al 2000). It seems that the chronicity of the inflammation in asthma primes the myocytes by changing the ECM and exposing the cells to many different types of mediators profiles associated with different types of inflammation.

Asthmatic airway smooth muscle cells grow at approximately twice the rate of cells from subjects without asthma (Johnson 2001). Accumulated evidence shows that ERK, one of the MAP Kinases, is the reason behind this difference. ERK activation is necessary and involved in human ASMC proliferation but activation of ERK is altered in asthmatic smooth muscle cells. Peak ERK activation in response to a low concentration of mitogens is greater than what is seen in non asthmatic ASMC (Lee JH 2001).

ASMC proliferation is a complex process and is regulated by different cell growth modulators; some modulators are enhancing cell growth and proliferation while others inhibit it. Activation of G-protein coupled receptors that increase cyclic AMP levels reduce ASMC proliferation and activation of nuclear hormone receptors by sex hormones (Hughes 2002), glucocorticoids (Hirst 2004), and prostaglandin E2. In addition, short and long acting β2- selective agonists, such as salmeterol and formoterol respectively, reduce proliferative response by reducing cyclin D1 levels which are required for the cell entry to S-phase. Recent identification of other regulators described some enzymatic regulation activity. For example, chymase of the mast cells was shown to modulate ASMC proliferation and this may indicate the importance and contribution of the mast cells found in the bundles of smooth muscle (Lazaar 2002). More recently, activated (antigen-

specific) CD4+ T cells were shown to regulate myocyte turnover and proliferation in a direct cell-cell interaction manner (Ramos-Barbon et al 2005).

Data drawn from bronchial biopsies has shown that interstitial region beneath the epithelium is rich in collagen, particularly type I and III that are enhancing ASMC proliferation. In contrast, ECM in the smooth muscle bundle is rich in the antiproliferative proteoglycans (Black 2002). Therefore, smooth muscles might migrate from the bundle toward the epithelium under the influence of epithelial chemoattractants, such as PDGF, eotaxin, and others and migrate away from the antiproliferative media.

In vitro, ASMC shows a little evidence of undergoing apoptosis. ASMC apoptosis occurs under extreme conditions such protein synthesis inhibition or prolonged incubation in protein deficient media (Freyer 2001). It has been suggested that regulation of apoptosis may be a primary process by which ASM cell number is determined; the threshold for change in rates of apoptosis may be lower than for proliferation and reduction in rates of apoptosis may contribute to hypertrophy (Halayko 2006). Endothelin-1 (ET-1) induces hypertrophy and reduces apoptosis in ASMC (Halayko 2007). Cardiotrophin (CT-1) enhances airway smooth muscle survival under conditions of serum deprivation and markedly inhibits TNF-α/Fas-induced apoptosis (Zhou 2003).

The ASMC survival depends strongly on the integrity of the surrounding tissue. Many proteinases such as neutrophil elastase drive ASMC to apoptosis because they simply degrade the matrix molecules and cause detachment induced apoptosis (Oltmanns et al. 2005).

1.2.4 Interaction between ASMC and epithelium

Morphometric studies done on biopsies from asthmatic subjects strongly indicate that the increase in ASM mass is a remarkable phenomenon in asthma, especially in severe cases of this disease. The increase in ASM area and augmented cell size are selective structural changes that distinguish severe asthmatic patients from the mild (Benayoun L 2003). In addition, the distance between the smooth muscle mass and the epithelium, especially in tissues obtained from asthmatic subjects, is reduced (Pepe 2005). This phenomenon might reflect the migration of smooth muscle cell toward the epithelium or it could be the attraction of circulating precursor stem cell populations. The close proximity could be also due to the ASMC mitogens produced from the epithelial layer that induce the myocytes growth toward the luminal side of the airway. Epithelium is a potent source of different mediators that act as mitogens for ASMC, such PDGF, EGF, FGF, IGF , TGF-β, TNF-α and IL-1β, and this proliferative medium provided by the epithelium could be also the reason for the ASMC to move away from the antiproliferative medium found in the smooth muscle bundle it self (Black 2003).

ASMC possess receptors for different epithelial derived products but more important to the study of epithelial-ASM interaction is that receptors for epithelial derived CC and CXC chemokine were identified. These chemokines may be important in inducing airway smooth muscle cells to grow on the epithelial side of the existing bundle resulting in close proximity of the ASMC to the epithelium. Some of these chemokines are eotaxin, RANTES, IL-8, and MIP-1α and they act through specific receptors that have been shown to be expressed on ASMC.

ASMC react to IL-8 by expressing CXCR1 and CXCR2 constitutively at the mRNA and protein level but the level of the expression of both receptors is relatively low compared with neutrophils (Govindaraju 2006). Upon stimulation, these receptors increase the intracellular Ca2+ and cause the smooth muscle to contract and migrate. Therefore, IL-8 might contribute to the ASM remodeling in asthma.

CCR3 is the most extensively studied receptor among the chemokine receptors. It interacts with a number of ligands such as eotaxin and RANTES. In addition, CCR3 is upregulated in bronchial smooth muscle cells of asthmatics. ASMC express functional CCR3 that upon binding to eotaxin induces smooth muscle migration (Joubert 2005). The same group and others demonstrated the expression of CCR1 (Amin 2005), which binds RANTES and MIP-1α, by ASMC.

1.3 Role of Chemokines in Asthma

1.3.1 Chemokines in Allergic Inflammation

The chemokines are a subgroup of chemotactic cytokines (8–10 kD) which have been subdivided into four subfamilies on the basis of the position of either one or two cysteine residues located near the amino terminus of the protein (CXC, CC, C, and CX_3C subfamilies) (Zlotnik 2000; Table.1/ Appendix)

A number of chemokines have been identified in relation to asthma and some are correlated to the inflammation severity and airway hyperreactivity. In addition to the chemokines main function of recruiting leukocytes to the site of the inflammation, some members also have the capacity to direct T helper cells differentiation towards Th1 or Th2 differentiation (Karpus 1997). MCP-1 can drive undifferentiated T helper cells toward IL-4 producing lymphocytes while MIP-1α promotes the Th1 differentiation (Karpus 1997). MCP-1 plays a significant role at the early stages of the diseases development. This chemokine induces mast cell activation as well and LTC4 release into the airways, which directly induces AHR (Campbell 1999). Other members of the CC subfamily recruit eosinophils in the early stage of asthma through the vessels to the lung interstitium, such as RANTES and MIP-1α (Campbell 1998), whereas eotaxin is needed at the chronic stage of asthma for eosinophils accumulation (Campbell 1997). Thus the coordinated expression and ligation of these chemokines appears to be differentially regulated at specific stages of asthma (Lukacs 1999).

IL-8 is a member of the CXCL subfamily while RANTES, eotaxin and MIP-1α belongs to the CC subfamily. A lot of initial work on chemokines has focused on those that have chemotactic activity for the inflammatory cells in the inflammatory lung

diseases. In addition to eotaxin, which is one of the most potent chemokines for eosinophils, MIP-1α and RANTES also lead to recruitment and degranulation of these cells. Eotaxin and other chemokines have been previously shown to selectively attract Th2 lymphocytes (Gutierrez-Ramos 2000). Th2 cells are the cornerstone of asthma pathogenesis and the importance of chemokines in the inflammatory process mediated by lymphocytes is likely to be crucial for the initiation and perpetuation of the disease.

The main stimuli for secretion of chemokines are proinflammatory cytokines such as IL-1 and tumor necrosis factor (TNF-α), bacterial products such as lipopolysaccharide (LPS), and viral infection. In addition, products of both Th1 and Th2 cells, Interferon-γ (IFN-γ) and IL-4, respectively, can also induce the production of these chemokines independently and in synergy with IL-1 and TNF-α.

The regulation of chemokine production and the expression specifically during an active inflammatory process was demonstrated by several reports. It seems that chemokine production is controlled by different inflammatory cytokines as well as other chemokines; IL-4, IL-13, IL-1β and TNF-alpha upregulate the expression of the above mentioned chemokines in asthma models (Rothenberg 2003 and others). More recently, the potentials of chemokines to regulate the immune response were demonstrated where down-regulation of IL-8 could be achieved by eotaxin introduction in human endothelial cells (Kunkel 2002).

1.3.2 Chemokines receptors

Chemokines mediate their actions by binding to specific receptors. Chemokine receptors belong to class A of the G protein–coupled receptor (GPCR) superfamily. They are rhodopsin-like receptors that span the membrane seven times and are coupled to heterotrimeric $G\alpha\beta\gamma$ proteins. These receptors are unique among cytokine receptors because they are seven spanners and coupled to G proteins that in turn activated another second messenger signals. When the receptors are termed seven spanners it indicates that they have a serpentine configuration and snake in and out of the cell membrane seven times. Some other cytokines have soluble receptors that represent fragments of the membrane-bound receptor that have been proteolytically cleaved; with the seven-spanner chemokine receptors, this is not likely to occur (Kunkel 1991).Chemokines interact with their receptors at two main sites; one site is the N-terminal region and the other one is located within an exposed loop that extends between the second and the third cysteine residue. The N-terminal binding is the part that triggers the receptor (Clark-Lewis 1995).

To date, eight CCR receptors and seven CXCR receptors have been identified. Upon ligand–receptor interaction, different intracellular signaling pathways are activated, ultimately leading to cell mobilization and activation. These receptors are expressed by different inflammatory cells such T cells, B cells and dendritic cells, and by structural cells such smooth muscle, fibroblasts and endothelial cells. A feature of the chemokine system is the complexity of the ligand–receptor interactions. Thus, a particular chemokine (i.e., CCL5) may bind several receptors (CCR1, CCR3, and CCR5), and different chemokines (i.e., CXCL9, CXCL10, and CXCL11) may bind a single receptor (CXCR3).

Evidence suggests that, in some cases, ligand redundancy does not mean duplicity of functions (Bardi 2001).

Several studies show that some CCR and CXCR receptors are upregulated in airways of asthma patients (Pillete 2004). During migration, leukocytes, eosinophils, and neutrophils express a wide range of chemokines receptors on the cell surface. Differences in the expressed chemokine receptors were seen between cells from the peripheral blood and the cells at the inflamed tissue (Lukacs 1999). These differences must be necessary to interact with the inflammatory environment; for example, CCR3 is highly expressed by Th2 effector cells, eosinophils, mast cells, and basophils in the lung specimens of asthmatics. Also, the CCR3 ligands include the major chemokines expressed in the allergic lungs such as RANTES, eotaxin, MCP-4 and MCP-3 (Ying 1999). As well CCR4 and 8 are highly expressed by Th2 in allergic conditions (Panina-Bordignon 2001)

Upon binding to their G-protein coupled receptors, chemokines induce conformational changes in the α and β subunits that can further activate various effector enzymes such as MAPKinases. The activation includes a rise in the intracellular Ca^{2+} concentrations, degranulation of the intra-plasmic granules, and increased production of oxygen radicals and lipid mediators (Lukacs 2001).

Chemokine receptors are not only expressed by leukocytes because many airways structural cells express functional receptors for the major chemokines in asthma. For example, CCR was detected on epithelial cells, endothelial cells (Ying 1997), and ASMC (Joubert 2006). The epithelial expression of CCR3 is upregulated by the proinflammatory cytokines such as TNF-α and INF-γ (Stellato 2001). On the other hand, eotaxin binding to CCR3 increases the eotaxin and IL-8 production by the structural cells via several MAPKinase pathways (Cui 2002).

1.3.3 Activation of MAPKinases by Chemokines

It is well established that activation of MAPK by growth factors and G protein-coupled receptors results in the stimulation of DNA synthesis and cell proliferation (Marshall 1995). Previous studies have shown that PDGF, EGF, and Substance P (SP) activate MAPK isoforms upon binding to their G-protein coupled receptors in tracheal smooth muscle cells (Pouyssegur 1992). Once activated, these MAPKs in turn activate their specific substrates on downstream targets. It is suggested that the binding of the growth factor to the GPCR induces the activation of inositol phospholipids hydrolysis pathway and leads to the release of intracellular Ca^{2+} and then phosphorylation of Protein Kinase C (PKC) (Reggoli 1994). PKC is a predominant component in the kinase cascade that is initiated by ligand attachment to both G-protein coupled receptors and receptors containing intrinsic tyrosine kinase activity (Otsuka 1993).

Upon activation, PKC mediates intermediate kinase activation. In growth factors binding it is suggested that Raf-1/MEKinases are subsequently phosphorylated in this cascade leading to sequential phosphorylation of p42/p44 MAPK which enhances DNA synthesis and cell proliferation (Yang 2002). Chemokines are expected to follow a similar signaling pathway to induce cellular proliferation.

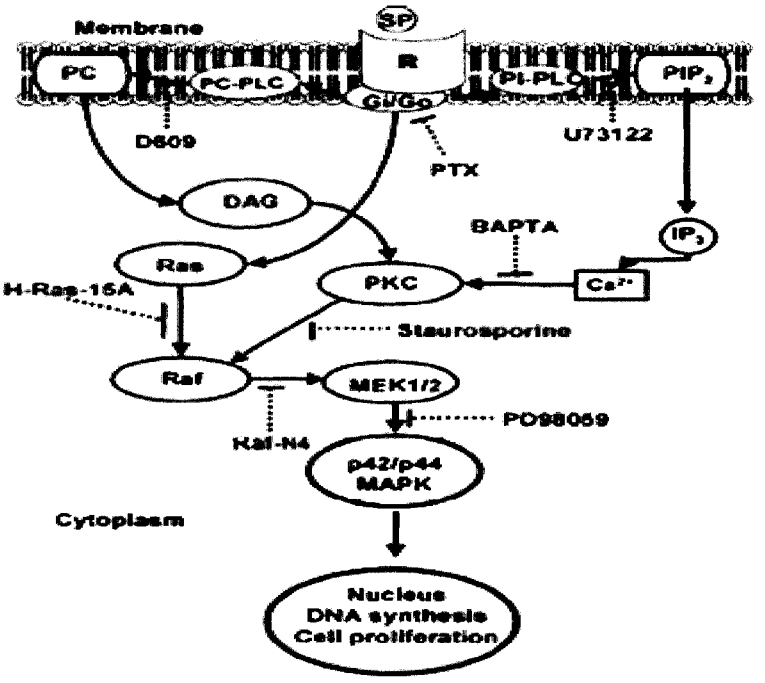


Fig 1. Schematic pathway for growth factors signaling of cellular proliferation. Chemokines are expected to follow the same pathway (Adapted from Yang 2002)

1.3.4 Epithelial Derived Chemokines

1.3.4.1 CCL11 (eotaxin-1) is a member of CC chemokine subfamily. Eotaxin is widely recognized to mediate eosinophil chemotaxis and activation in both mice and humans and is thought to play a central role in eosinophil recruitment to the airways of Ag-challenged animals (Griffiths 1993). CCL11 is also recognized to induce chemotaxis of basophils (Yamada 1997), Th2 lymphocytes (Sallusto 1997), mast cells (Oshi 1999), and airway smooth muscle cells (Joubert 2005). In humans, CCL11 can be detected in the sputum of patients with moderate and severe asthma and 50% of the total eosinophil chemotactic activity in such samples is predicted to be due to eotaxin. Recently, an anti- apoptotic

effect of eotaxin was reported in the pulmonary artery endothelial cells (Farahi 2007). Eotaxin is produced by a variety of inflammatory cells including T lymphocytes (Loetscher 1996), macrophages, eosinophils, and structural cells such bronchial epithelial cells (Ponath 1996), fibroblasts (Teran 1999), smooth muscle cells, and, most recently, airway parasympathetic neurons (Fryer 2006) and the pulmonary artery endothelial cells (Farahi 2007). In the lungs, the epithelium is the major producer of eotaxin (Ying 1997).

1.3.4.2 RANTES/ CCL5 is a member of the CC chemokines and was identified early in 1986 (Yokota 1986). RANTES is recognized as a powerful chemoattractant of eosinophils, T lymphocytes, neutrophils, monocytes, and basophils (Schall 1990). This chemokine activates these immune cells and induces the exocytosis of bronchoconstrictive mediators, such as histamine and cysteinyl leukotrienes, from basophils and eosinophilic cationic protein from eosinophils (Chung 1999). RANTES is involved in inflammatory cell recruitment and in the induction of bronchoconstrictive mediators from cells which result in airflow limitation (Matsunaga 2006). RANTES initiates several other proinflammatory events, such as integrin activation and lipid mediator biosynthesis (Taub 1996). RANTES is generated predominantly by T cells, epithelial cells (Matsukura 1995), fibroblasts, and platelets (Kameoshi 1992). RANTES is constitutively expressed in the lungs of patients with asthma, where increased levels are detected in the broncoalveolar lavage (BAL) fluid of these patients (Folkard 1997).

RANTES also contributes to Th2 type of inflammation by upregulating the production of IL-5 and IL-6 (Lillard 2001). This particular contribution was detected shortly after the allergen challenge in the animal models; however, RANTES role shifts

toward Th1 augmentation (increases IL-12 and INF-γ levels) in the sensitized animals after several challenges (Koya 2006).

1.3.4.3 Macrophage inflammatory protein (MIP)-1α was identified 17 years ago as the first of four members of the MIP-1 CC chemokine subfamily (Baixeras 1990). These proteins were termed CCL3 (MIP-1α), CCL4 (MIP-1β), CCL9/10 (MIP-1γ), and CCL15 (MIP-1δ) (Orlofsky 1991). MIP-1α has been shown to contribute to monocyte/macrophage, mast cells (Alam 1992) and neutrophil chemotaxis and activation. It has been demonstrated that MIP-1α plays an important role in eosinophilic accumulation and activation in humans (Rot 1992) and in vivo after airway challenge (Lukacs 1996). The epithelial cells are the main source for MIP-1α in the lungs (Stellato 1997); MIP-1α/ CCL3 is also produced by macrophages (Wolpe 1989), dendritic cells (Mohamadzadeh 1996), and lymphocytes (Conlon 1995).

MIP-1 proteins mediate their own biological effects by binding to cell surface CC chemokine receptors which belong to the G-protein-coupled receptor superfamily (CCL3 binds to CCR1 and CCR5) (Gao 1993). Receptor binding involves high affinity interactions and a subsequent cascade of intracellular events that lead rapidly to a wide range of target cell functions, including chemotaxis, degranulation, phagocytosis, and mediator synthesis (Maurer 2004).

1.3.4.4 IL-8/ CXCL8 is the major neutrophil chemoattractant and activating factor in the lungs (Kunkel 1991). IL-8 induces neutrophils exocytosis of stored proteins as well as release of hydrogen peroxide and superoxide anions. In addition, this CXC chemokine evokes eosinophil (Shute 1994) and monocyte migration to the inflamed site (Remick 2005). IL-8 is also involved in a wide variety of physiological and pathological

processes, including host defense against bacterial infection, angiogenesis, arteriosclerosis, and autoimmune disorders of skin, bones, and joints (Harada 1996). Elevated concentrations of IL-8 are found in sputum, bronchoalveolar lavage fluid, and bronchial tissues of subjects with pulmonary diseases such as allergic (Smith 1991) and severe asthma, occupational asthma (Gibson 2001), cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) (Nocker 1996), bronchitis, acute respiratory distress syndrome, and idiopathic pulmonary fibrosis (Richman 1993).

Bronchial epithelial cells, neutrophils, macrophages, mast cells, and fibroblasts are the major producers of CXCL8. IL-8 acts through the specific receptors CXCR1 and CXCR2 which are located on the surface of neutrophils, monocytes, airway epithelial cells, and endothelial cells. Recently it has been shown that airway smooth muscle constitutively expresses mRNA and protein for both CXCR1 and CXCR2 receptors (Govindaraju 2006). All of these inflammatory and structural cells produce IL-8 and express functional receptors of this chemokine; thus, an auto-regulatory pathway for chemokines within the airways could contribute to the amplification of the allergic inflammatory response (Eddlestone 2003).

In asthma, airway smooth muscle migration has been postulated as an explanation for the increased mass of smooth muscle. IL-8 has been shown to be a potent chemoattractant of human airway smooth muscle cells when compared to PDGF, which is a strong ASMC chemotactic agent (Govindaraju 2006). The cellular recruitment of ASMC or inflammatory cells occurs through the development of the chemotactic gradient so the cell moves toward an area of increased chemokine concentration. *In vivo* this gradient could be generated by IL-8 or other chemoattractant molecules, such as PDGF, binding to basement membrane proteins.

The physiological concentration of IL-8 is not known. IL-8 is known to have a long half life which means it may be accumulated in concentrations high enough to induce chemoattraction for neutrophils, ASMC, and eosinophils (Remick 2005). Based on role of IL-8 on airway hyperreactivity and airway remodeling, it is considered as a potential therapeutic target especially in severe asthma. Strategies designed to target IL-8, such as anti–IL-8 antibodies or receptor blockade of TLR-4 to limit the release of IL-8, are still under investigation (Barnes 2007). Another reason to target IL-8 especially in lung diseases is the link between the excessive release of IL-8 after airway damage and the damage to the IL-8 receptor on airway neutrophils which might disregulate cytokine production and lead to excessive neutrophil accumulation (Koehler 2004).

1.4 Rationale

It is not surprising that the altered asthmatic epithelium participates in airway remodeling in asthma by the production of different mediators that induce remodeling in other airway tissues. Asthmatic epithelium expresses molecules that have receptors on inflammatory cells therefore recruiting more cells to the airway. For example, ASMC express receptors for most of the growth factors produced by the epithelium while smooth muscle expresses functional receptors for the epithelial-derived chemokines, such as RANTES, eotaxin, IL-8 and MIP-1α. These molecules are released by the ASMC as well and their effect could be paracrine autocrine.

ASMC mass increase is a well documented phenomenon of asthmatic airways, especially in severe asthma. The distance between the epithelium and smooth muscle bundle is reduced and this could be attributed to epithelial-derived migratory and proliferative signals that induce ASMC remodeling. It has been shown that IL-8 and eotaxin cause migration of ASMC; these cells express receptors for both chemokines, CCR3, CXCR1, and CXCR2 respectively. It has been recently demonstrated that ASMC migrate towards concentration gradients of eotaxin and IL-8 but the effect of these chemokines on smooth muscle proliferation and survival is not yet examined. Those two chemokines and others, such as MIP-1α and RANTES, could be mitogens for ASMC. If so, it could be an additive explanation for why myocytes grow and proliferate toward the epithelium, the source of these chemokines, and other mitogens.

1.5 Hypothesis

We hypothesize that airway epithelial derived chemokines such as eotaxin, RANTES, IL-8, and MIP-1α, induce proliferation of airway smooth muscle cells and promote enhanced survival of airway smooth muscle cells by inhibiting apoptosis.

1.6 Specific Objectives

Firstly, to investigate the effect of the chemokines, IL-8, Eotaxin, RANTES, and MIP-1α, on ASMC remodeling and to examine the effect on ASMC proliferation in vitro. Secondly, to examine the possible effect of the above mentioned chemokines on ASMC survival. We set out to examine the possible effect of chemokines on the rate of apoptosis in ASMC.

Chapter 2. Materials and Methods

2.1 Cell culture and Reagents

The ASMC were obtained from two sources. Bronchial/tracheal smooth muscle cells (B/TSMC) were purchased from Cambrex and were positively stained for α-smooth muscle actin and negatively for factor VIII, CD45, and CD3, as indicated by the manufacturer. B/TSMC were grown in their optimal medium (SmGM-2; Cambrex) containing 5% FBS at 37°C in a humidified incubator with 5% CO2, as recommended by the supplier. The second source were primary cultures of human non- asthmatic bronchial ASMC which were obtained from surgical specimens. Segments of lobar or main bronchus measuring 5 x 2 mm were incubated for 90 min at 37°C in 10 ml Hanks' balanced salt solution buffer (in mM: 5 KCl, 0.3 KH2PO4, 138 NaCl, 4 NaHCO3, and 5.6 Na2HPO4) to which 640 units of collagenase (type IV), 10 mg of soybean trypsin inhibitor, and 100 units of elastase (type IV) had been added. The digested tissue was then filtered through a 125− m Nytex mesh and the resulting cell suspension was centrifuged. The pellet was then reconstituted in growth medium (Cambrex, SmGm-2) and plated in 25-cm2 flasks. Complete SmGm2 media were purchased from Cambrex Bio Science, Walkersville, USA. Confluent cells were detached with 0.025% trypsin and 0.02% EDTA and counted using a hemacytometer. They were identified as smooth muscle cells by positive immunohistochemical staining for smooth muscle specific α-actin and positive identification of myosin light chain kinase and calponin by Western blot analysis. All the chemokines used in the experiments were recombinant human and purchased from R&D Systems (Minneapolis, MI). All ASMC used were at passages 2-5.

2.2 (3H)-thymidine Incorporation

Metabolic incorporation of tritiated (^{3}H)-thymidine into the newly synthesized DNA is a widely used method to detect the rate of DNA synthesis and monitor cell proliferation. ASMC were plated in 96 well plates at a density of 3000-5000 cell/well in triplicates and grown to 60-70% confluence in complete SmGm2 containing 5% FBS at 37°C in a humidified incubator with 5% CO2. Cells were then starved for 48 hours 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 hours. The chemokines of interest were RANTES, eotaxin, IL-8, and MIP-1α. The concentrations for each chemokine tested ranged from 0.1ng/ml up to 100ng/ml, except for IL-8 where concentrations of 50, 100, 500, and 1000 ng/ml were tested. The vehicle used as a control was 0.1% BSA in PBS; the same vehicle used to dilute the chemokines. All the results were compared to the vehicle and to the positive control of PDGF 10ng/ml.

Eighteen hours before cell harvesting for thymidine study, cells were pulsed with 1 μCi of thymidine/ well. Cells then were washed with PBS and trypsenized prior to DNA isolation. 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 (Perkin Elmer).

2.3 BrdU incorporation and Flow Cytometric Analysis

Cell proliferation was also determined using colorimetric assay based on the measurement of a synthetic thymidine analog bromodeoxyuridine (BrdU) incorporation during DNA synthesis (FITC BrdU Flow Kit, BD Biosciences, CA). The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies and the levels of cell-associated BrdU are then measured with flow cytometry. ASMC were grown in 12 wells plate at a density of 40,000 cell/well in complete and then medium was switched to SmGm-2 (supplemented with 0.5% FBS) for 48 hours to induce quiescence and synchronize the cell cycle. The vehicle or test chemokines were then added to the cells for 24, 48, or 72 hours. The thymidine analog BrdU was added (10μl/ml of 1mM BrdU solution) 18 hours 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 media, the treated cells were trypsenized and washed with PBS. Cells were fixed and permeabilized using the manufacturer reagents (BD Cytofix/ Cytoperm buffer). The cells were then treated with DNase to expose the incorporated BrdU for one hour at 37°C. Next, cells were incubated with 1:50 of diluted anti-BrdU fluorescent antibody for 20 minutes at room temperature. The stained cells were analyzed immediately with a FACS Calibur flow cytometer system using Cell Quest Software (BD Biosciences). Ten thousand events were collected on each sample.

The experiment controls were as follows: cells treated with PDGF as appositive control and cells treated only with vehicle as a negative control.

The staining controls were the following: cells stained with BrdU only, cells stained with BrdU and anti-BrdU antibody, cells stained only for anti-BrdU antibody, and totally unstained cells.

2.4 Annexin V and Flow Cytometric Analysis

One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V. Annexin V is a 35-36 kD Ca 2+-dependent phospholipid binding protein with a high affinity for PS. The externalization of PS precedes other apoptotic processes, such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation, that come at the end stages of cell death. For these reasons Annexin V staining is typically used in conjunction with a vital dye such as Propidium Iodide (PI) to allow the identification of early apoptotic cells (Annexin V-FITC positive, PI negative). As such, Annexin V can be conjugated to a fluorochrome such as FITC and used for flow cytometric identification of cells in the early phase of apoptosis.

To test if RANTES, eotaxin, IL-8, and MIP-1α have an anti-apoptotic effect, cells were plated in 12 well plates at a density of 40,000 cell/well in a complete media. The cells were treated exactly the same as in the BrdU test except for the addition of BrdU 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 and washed with PBS and then suspended in 100 ml of 1X Annexin V binding buffer (10X is supplied by the manufacturer). Five µl of Annexin V and 5 µl of PI stains were added to each test

tube for a duration of 15 minutes at room temperature and in the dark. Within one hour, the cells were analyzed using FACS Calibur flow cytometer by counting ten thousand events per sample. The following controls were used: totally unstained cells, cells stained only with Annexin V, and cells stained only with PI.

2.5 Protein extraction, Immunoprecipitation and Western Blotting

Western blotting is used to detect the presence of select protein. We used Western blotting to detect the expression of p42/p44 MAPKinases in a whole cell extract after stimulation with the chemokines of interest. These MAPKinases are downstream products expressed upon the stimulation of G-Protein Coupled Receptors (GPCR) which in this case were the chemokines receptors. P42/p44 activation is essential for subsequent DNA synthesis and cell proliferation. The specificity of the Western blotting in this case will depend on the specific binding of the antibody to the epitope. Anti p42/p44 total and phosphorylated antibodies were obtained from Santa Cruz Biotechnology, CA.

For the Western blotting studies, ASMC were grown in 6 well plates at a density of 100,000 cells/well. The cells were starved for 48 hours and then stimulated with the chemokine of interest for one, five, or ten minutes. Chemokine induced P42/p44 MAPK activation occurs as early as 1 minute and peaks at 5 minutes and then goes back to the basal levels.

After the chemokine stimulation, the medium was removed and ASMC were washed with ice-cold PBS. The cells were then harvested in lysis buffer made of 1% Triton X-100, 50 mM HEPES (pH 8.0), 150 mM NaCl, 10% glycerol, 2mM EGTA, 1.5 mM MgCl2, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 1 mM sodium orthovanadate. Following 10-minute incubation on ice, the

lysates were clarified by centrifugation at 14,000 x g for 10 minutes and supernatants were collected. Protein concentrations were determined using Bradford method. To detect activation of p42/p44 MAPK, equal amounts of whole cell lysates were solubilized into a boiling Laemmli sample buffer, subjected to a 10% SDS polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose (Bio-Rad Laboratories Ltd., Mississauga, Ontario). For Western immunoblotting, membranes were blocked for one hour in TBST (10 mM Tris-Cl pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 0.1% Tween-20) containing 1% BSA at room temperature. The p42/p44 phosphorylation was identified using phosphor-p42/p44 antibody. The nitrocellulose membranes were incubated overnight at 4°C with the anti-phospho-MAPK polyclonal antibody used at a dilution of 1:1000 in TTBS. Membranes were washed with TTBS five times for 5 minutes each and incubated with a 1:1500 dilution of anti-rabbit horseradish peroxidase antibody (Amersham Biosciences, Inc., Baie d'Urfe, QC) for one hour. Immunoreactive bands were detected by enhanced chemiluminescence or ECL (Amersham Biosciences, Inc., Baie d'Urfe,QC) and then visualized and quantified on a FluorChem 8000 Imaging System using AlphaEase software (Alpha Innotech, San Leandro, CA).

2.6 Data Analysis

Data are represented as mean ± SEM. Statistical significance was determined using a Student's *t* test. Values of $p<0.05$ were considered statistically significant.

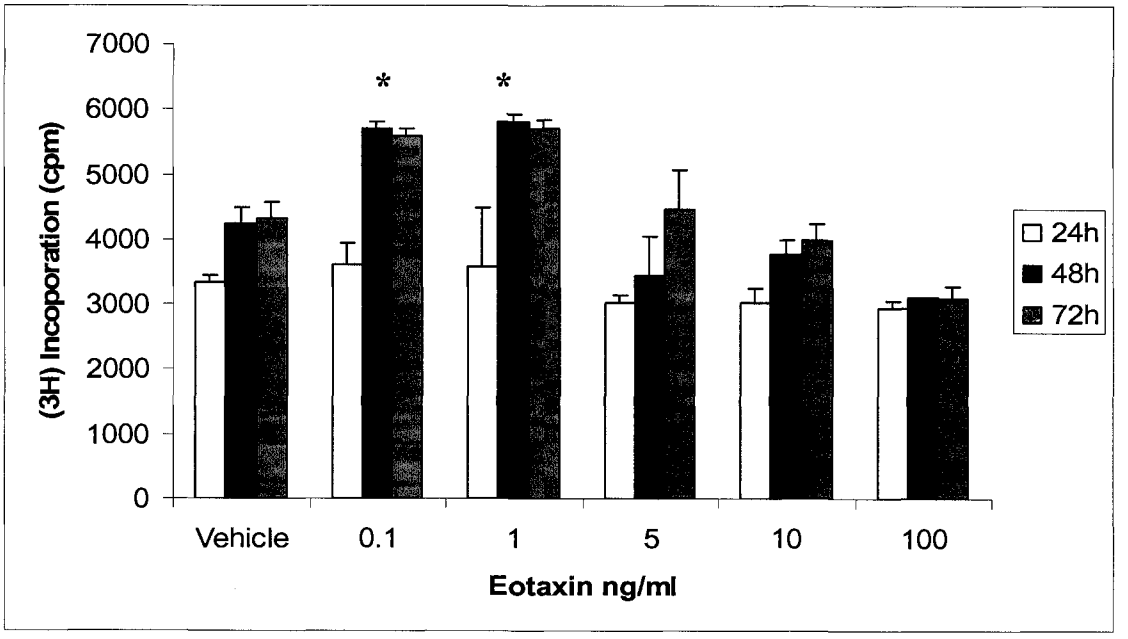
Chapter 3. Results

3.1 Effects of RANTES, eotaxin, IL-8 and MIP-1α on (^{3}H)-thymidine Incorporation by ASMC

To investigate the effect of chemokines on 3H-thymidine incorporation, ASMC were treated with different concentrations of RANTES, eotaxin, IL-8, and MIP-1α over different periods of incubation. No increase in ^{3}H-thymidine incorporation was detected before 12 hours of incubation with any of the chemokines mentioned above. A significant increase was detected after incubating ASMC with 0.1 and 1 ng/ml of eotaxin compared to the vehicle as shown in fig 2.*A*. Incubation of cells with eotaxin for 48 hours induced more proliferation than 24 hours incubation. There was a 1.2 fold increase in ASMC numbers after treatment with either 1 or 5ng/ml of RANTES. In contrast, higher concentrations of IL-8 were tested to see a similar effect; a 25.2% increase in ASMC number was detected after the cells were stimulated with 800ng/ml of IL-8 with a significant increase seen after incubation for 48 and 72 hours, see Fig. 2.*B*. MIP-1α induced significant 3H-thymidine incorporation after 72 hours of incubation, compared to the vehicle. The best results were obtained at a concentration of 100ng/ml of MIP-1α.

PDGF treated smooth muscle cells were used as positive control. PDGF is a strong mitogen of ASMC. A six to ten fold increase in the number of proliferating cells was seen after PDGF treatment. These results are consistent with other studies that tested PDGF mitogenesis. Whenever we used a combination of PDGF and a chemokine, a synergistic effect could be seen.

A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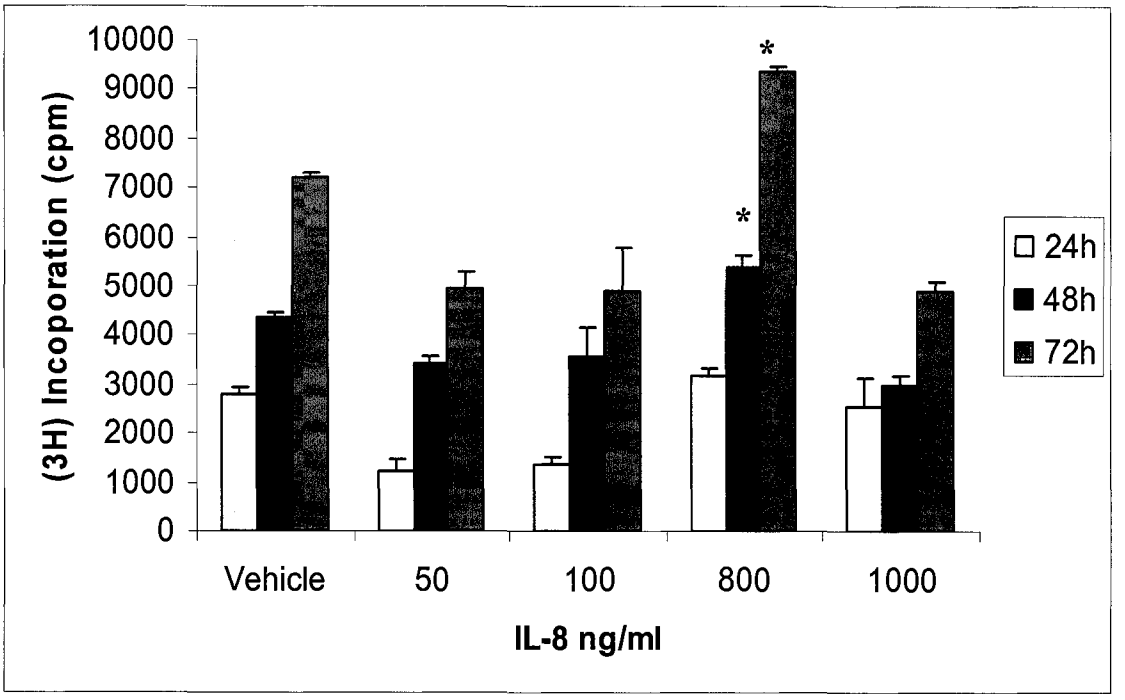
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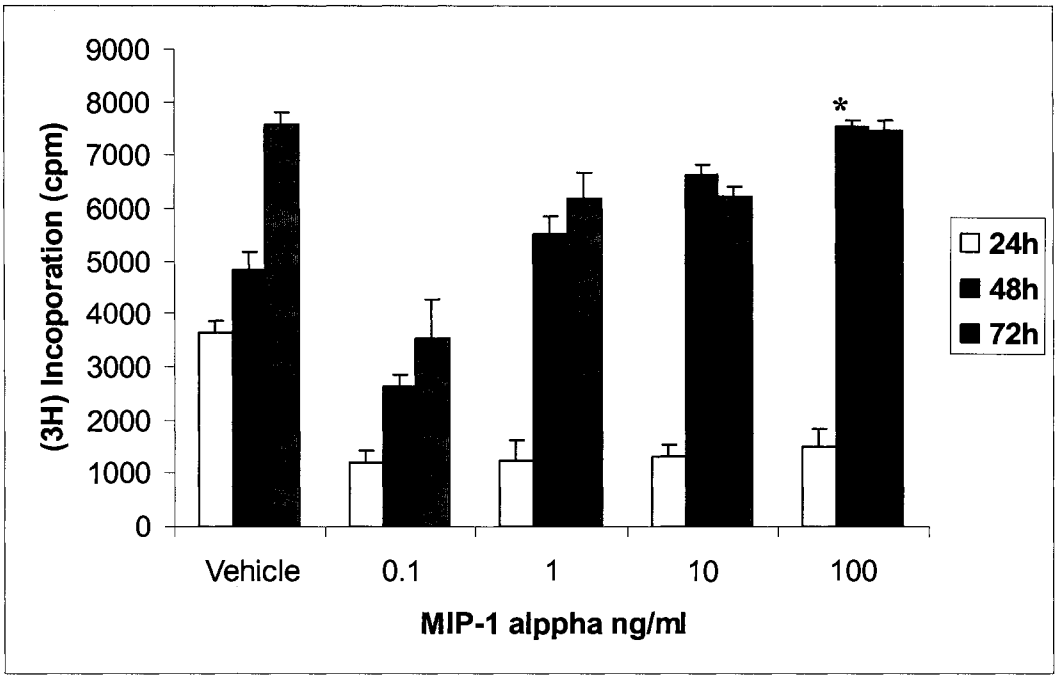

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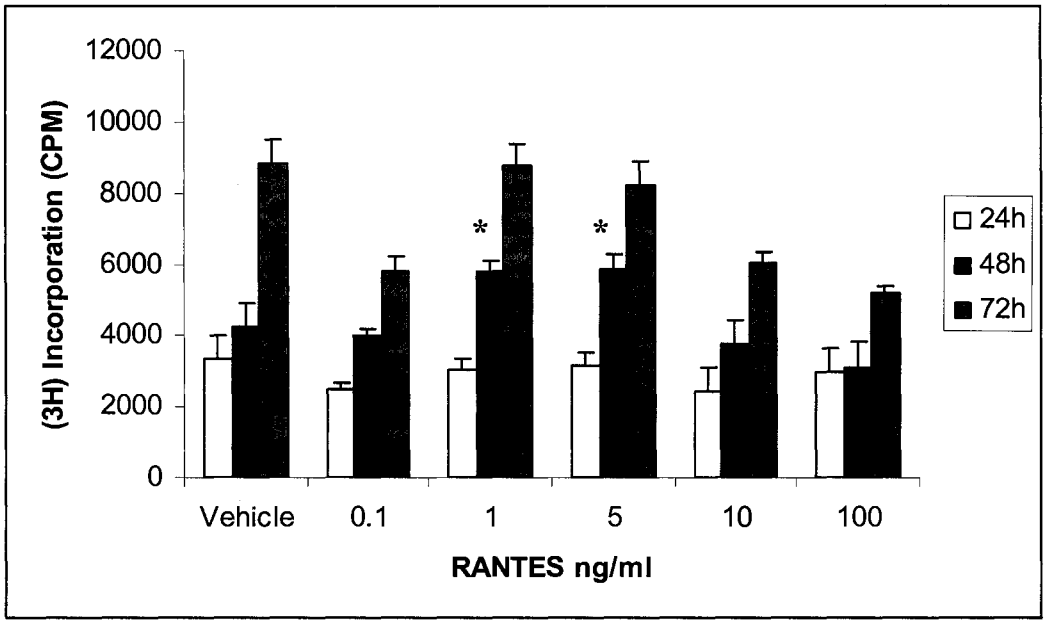


Figure 2. Serum deprived ASMC were stimulated with increasing dose of eotaxin (***A***), IL-8 (***B***), MIP-1α (**C**) and RANTES (**D**). 3H-thymidine incorporation then quantified. * $p<0.05$ for chemokines vs. vehicle (control). Significant increase of DNA synthesis in treated ASMC was seen at 1-5ng/ml of eotaxin as well for RANTES and at 800ng/ml of IL-8.

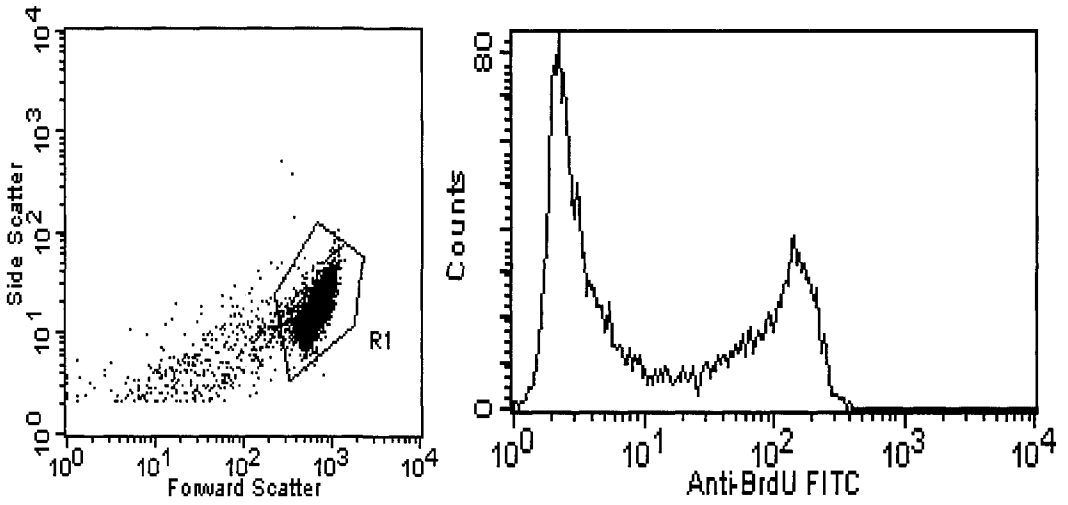
3.2 BrdU Incorporation by Proliferating ASMC

To further examine the effect of chemokines on ASMC proliferation and increase in mass, the treated cells were stained with BrdU and anti-BrdU antibody. To quantify the percentage of the proliferating and newly synthesizing DNA ASMC, we analyzed by flow cytometer to measure the BrdU positive cells in each sample after different chemokines treatment. The percentage of BrdU positive cells is compared to the vehicle (negative control). PDGF treated cells were used as a positive control.

Our results show that eotaxin induces ASMC proliferation. ASMC that are treated with low concentrations (1-5 ng/ml) of eotaxin showed increased BrdU incorporation; 29.2 % of eotaxin treated ASMC incorporated BrdU compared to 19.8% of ASMC treated only with vehicle ($P<0.002$). Fig.3 shows the percentage of BrdU positive ASMC after treatment with different concentrations of eotaxin over 24 hours. Incubating the ASMC with eotaxin in excess of 24 hours did not yield significant results. The graph represents the mean of 4 independent experiments. The error bars represent the standard error of mean (SEM).

After 24 hours of incubation with RANTES, 24.3% of ASMC started to synthesize new DNA compared to the vehicle only treated cells. In a concentration dependent manner, MIP-1α increased BrdU incorporation by ASMC after 24 hours of treatment.

A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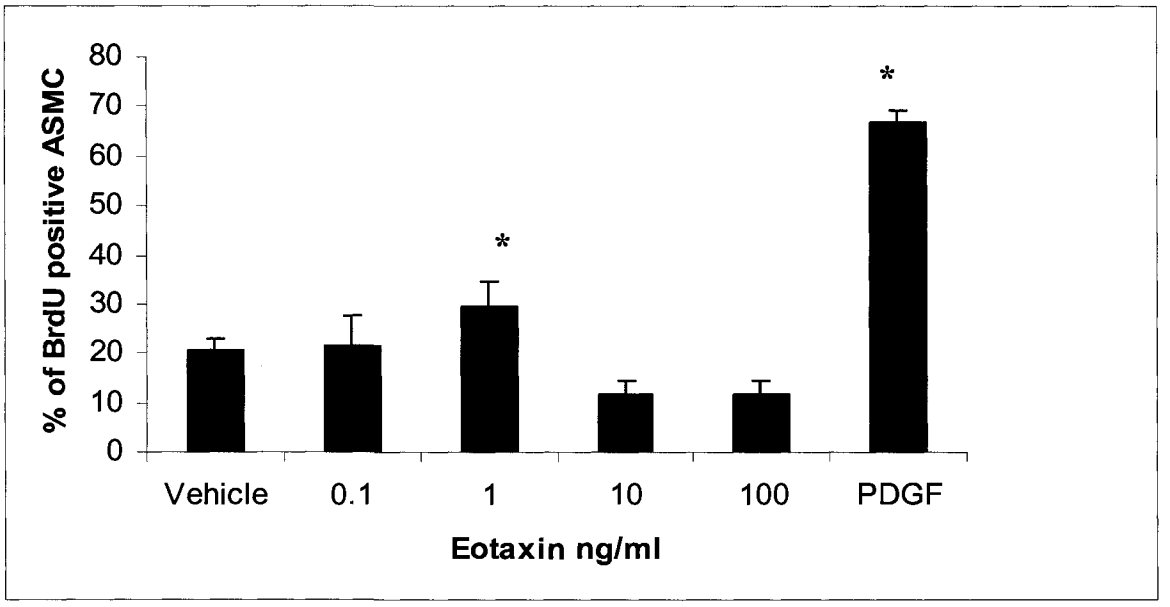
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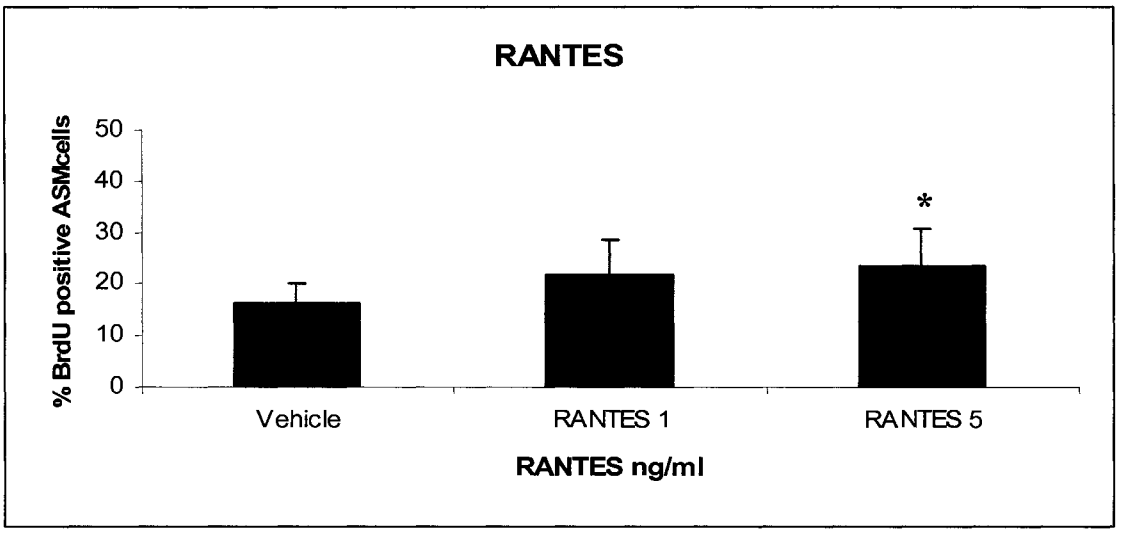

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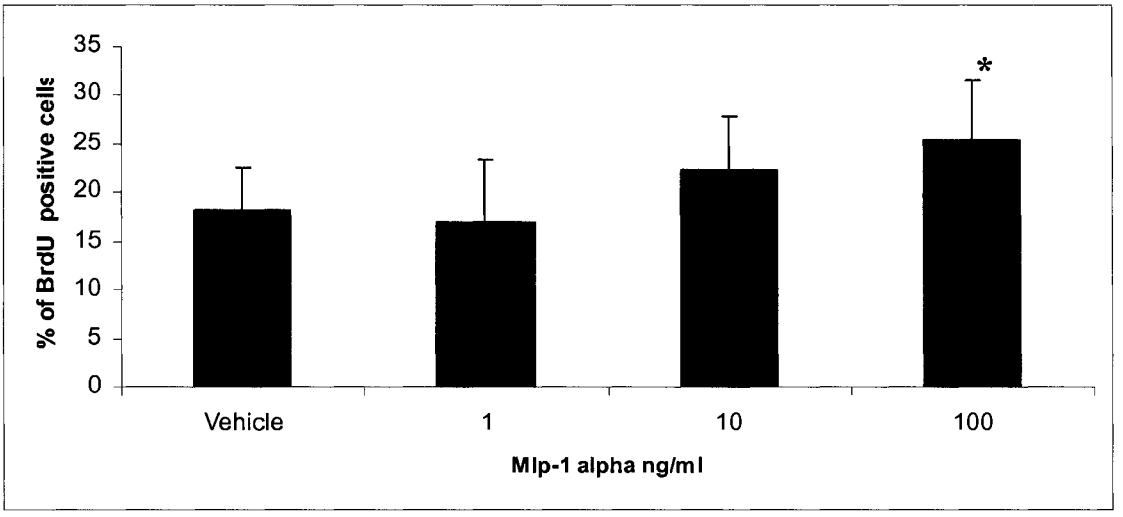
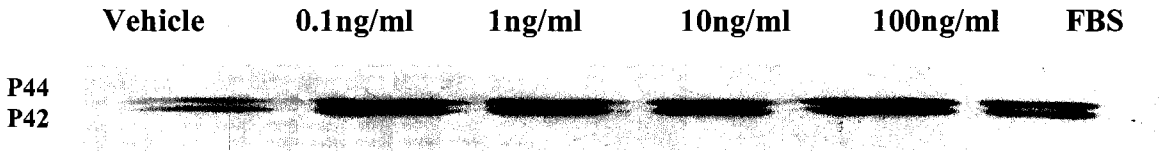


Figure 3.*A* Chemokines induced DNA synthesis in airways SMC, determination of BrdU positive ASMC using flow cytometry. BrdU incorporation was measured after 24 hours of chemokine treatment. ***A:*** Flow cytometry plots of side scatter versus BrdU positive ASMC. ***B.*** Quantification of BrdU positive ASMC cells after treatment with different concentrations of eotaxin. At low concentrations, eotaxin induce the ASMC to proliferate. Data represent means ± SEM from four independent experiments. Significant difference for eotaxin treated cells vs. vehicle treated, $P<0.002$ by t test. ***C:*** BrdU incorporation in RANTES treated airway Sacs. In comparison to the vehicle, RANTES treated cells had significant increase in BrdU positive ASM cells ($P<0.001$) by t test. ***D:*** An increase in DNA synthesis with the increase in MIP-1 alpha concentrations added to ASMC. A significant difference seen after stimulation with 10 and 100 ng/ml of MIP-1 alpha compared to the vehicle.

3.3 RANTES and eotaxin induce p42/p44 MAPKinases activation

MAPKs activation leads to the proliferation of different types of cells. ASMC were cultured in serum-free medium and the state of p42/p44 phosphorylation was assessed using Western Blot analysis. ASMC were stimulated with increasing concentrations of eotaxin and RANTES for one minute. Chemokine induced a concentration-dependent p42/p44 phosphorylation with a maximal response at 1 ng/ml of eotaxin and 1-5 ng/ml of RANTES as shown in Fig.3. The phosphorylation was quantified using a densitometer where the Integrated Density Value (IDV) ratio of phosphorylated to total p42/p44 was calculated.

A



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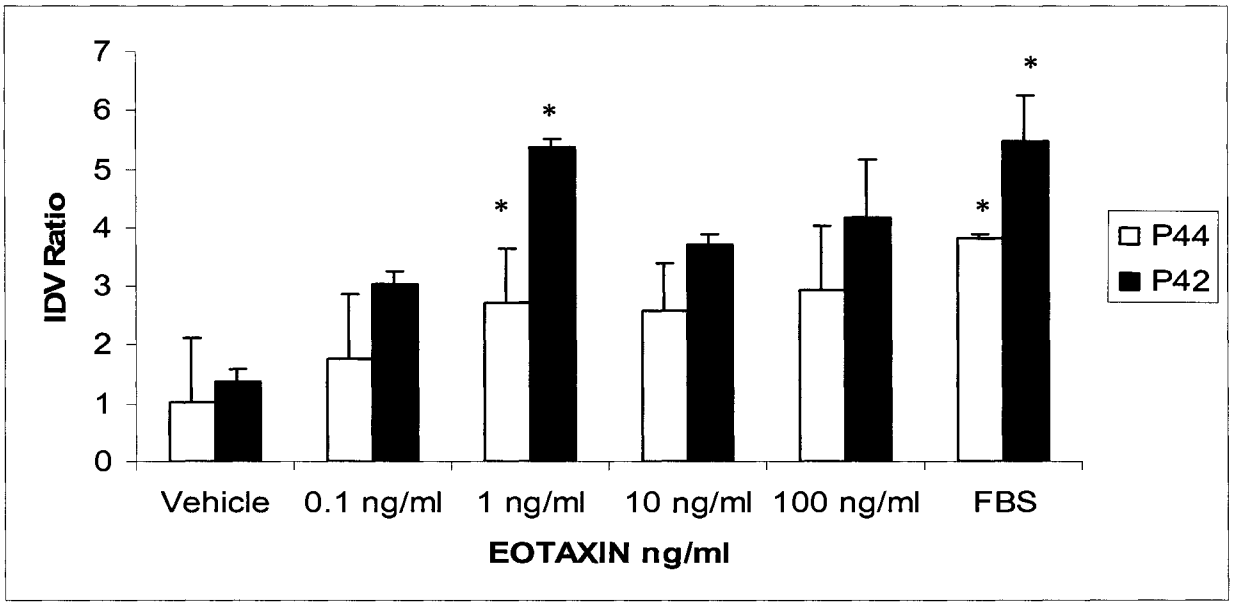

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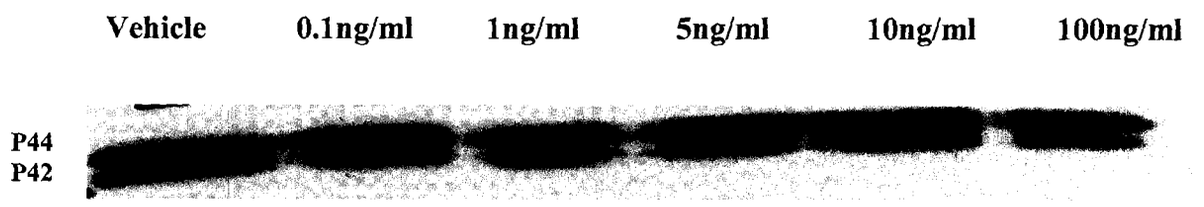

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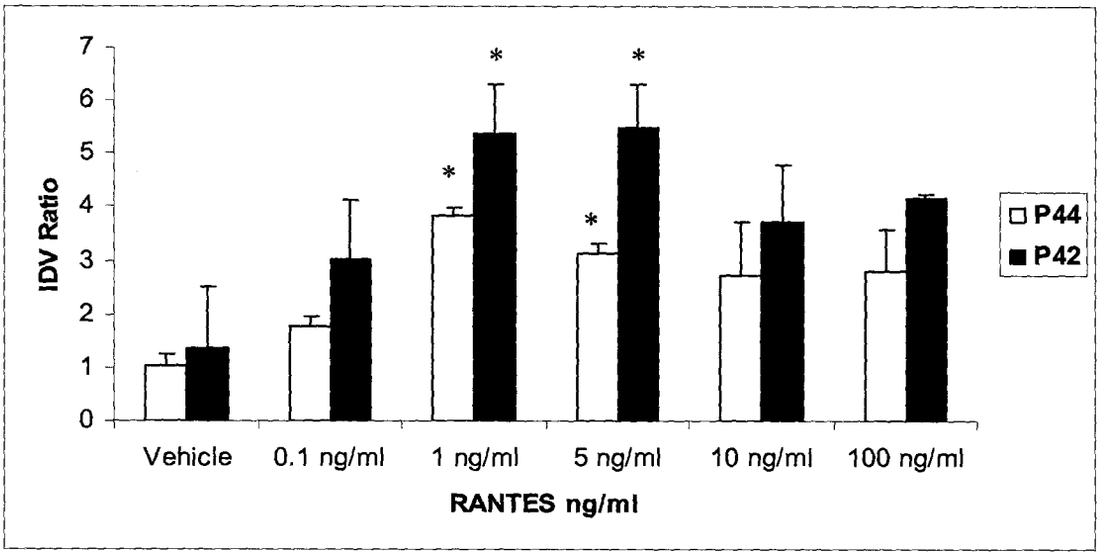
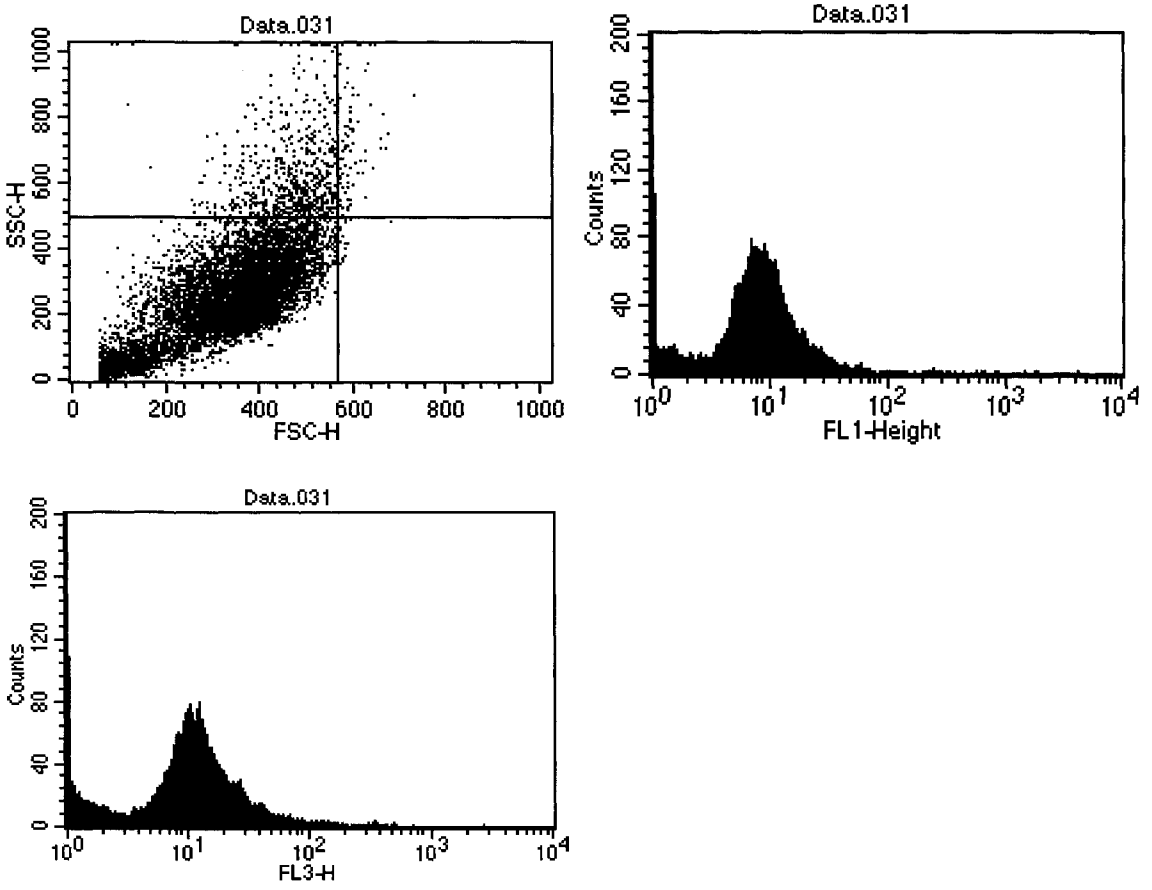


Figure 4. ***(A)*** Activation of p42/p44 MAPKs in ASMC treated with eotaxin or **(B)** RANTES in a concentration dependent manner. The ASMC were serum deprived for 48h before stimulation with eotaxin or RANTES for 1 minute. The cell lysates were analyzed using Western blot and anti-phospho-p42/p44 or total p42 MAPK polyclonal antibody. Bands were quantified by a densitometer. Similar results were obtained in three independent experiments. *$P<0.05$, as compared to the vehicle.

3.4 Anti-apoptotic effects of chemokines on ASMC

Since the increase in airway smooth muscle mass could be due to imbalanced cell proliferation and rate of apoptosis, we examined the effect of epithelial derived chemokines on ASMC apoptosis. Cell apoptosis was examined by Annexin V, propidium iodide (PI), and flow cytometry after cells were treated with the chemokines of interest. Compared with the appropriate control, IL-8, RANTES, and eotaxin could significantly reduce the apoptotic rate from 1.2% to an average of 0.5% (Fig 4 ***A*** and ***B***, $P< 0.01$). A significant difference was seen after 72 hours of treatment with chemokines. These findings suggest that chemokines, especially RANTES, eotaxin and IL-8, could have an anti-apoptotic effect on ASMC and therefore enhance the survival of these cells.

A



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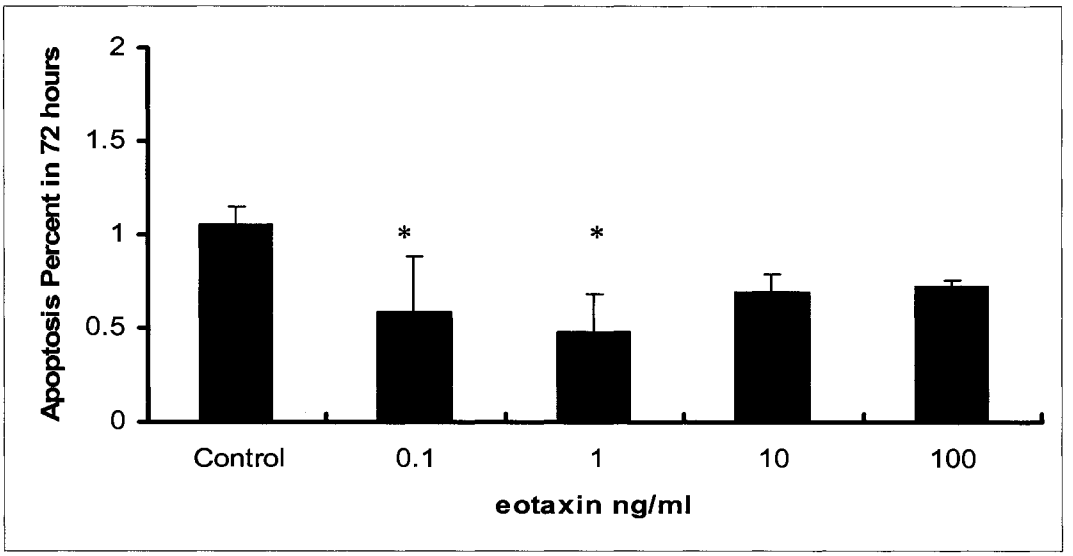

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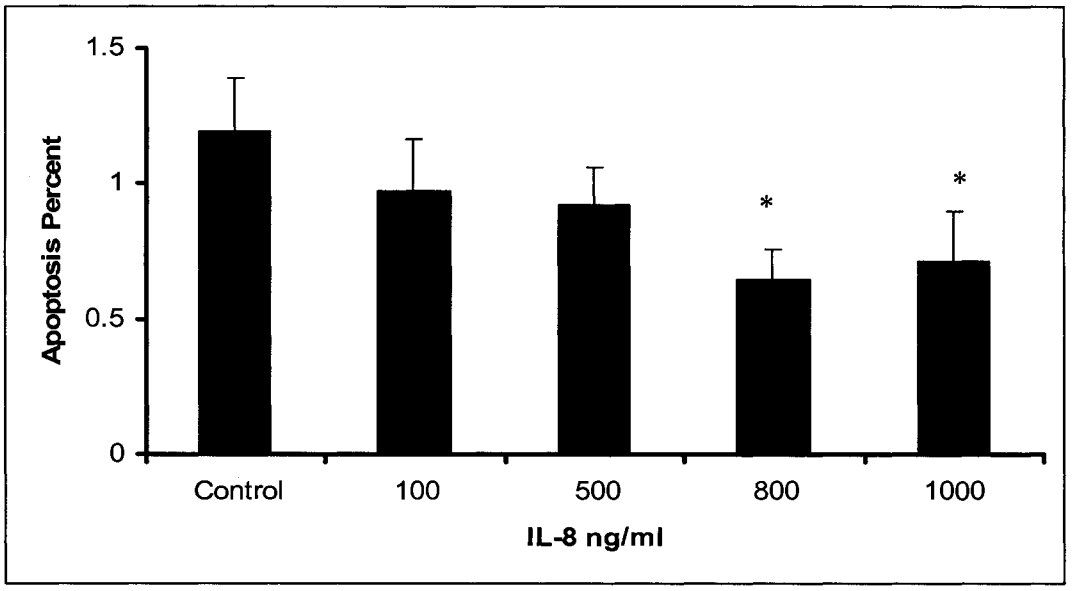

Figure 5. The apoptotic smooth muscle cells were quantified by flow cytometry using Annexin V and PI ***(A):*** Flow cytometry plots of side scatter versus Annexin V positive ASMC. FL1 represents Annexin V and FL3 represents PI. ASMC were seeded in 12 well plates, serum starved for 48 hours and then stimulated for 72 hours with eotaxin **(*B*)** or IL-8 in figure 4***(C)***. Values are represented as mean ± SEM of three independent experiments. * $p< 0.05$

Chapter 4. Discussion

The aims of this study were to show a specific interaction between the airway epithelium and the airway smooth muscle cells. The epithelium is a potent source of different mediators and a significant number of them contribute to the remodeling seen in the airway smooth muscle mass. The increase in smooth muscle mass in asthmatic airways is a well documented structural change, especially in severe asthma. The imbalance between the ASMC proliferation and the rate of apoptosis could be one explanation for such increase in mass. Many studies on ASMC challenged the old concept of treating ASMC as pure structural cells. It has been shown that ASMC play an immunoregulatory role in asthmatic airways through their ability to react to many inflammatory mediators and secrete many more. Part of this interaction could be between the ASMC and the epithelial derived mediators, specifically the chemokines.

Many recent studies show that chemokines could also have effects on structural cells in addition to their classical role of leukocyte recruitment and activation. Airway epithelium secretes RANTES, IL-8, eotaxin, MIP-1α and many others. The receptors for these chemokines have been recently identified on the ASMC. We have demonstrated the expression of CCR1 and CCR3 (Joubert 2005, Joubert 2008), as well as CXCR1 and 2 (Govindaraju 2006) by human ASMC. In previous studies we also showed the migration of ASMC toward eotaxin (Joubert 2005). Another study showed that IL-8 causes the ASMC to contract and migrate (Govindaraju 2006). These observations, in addition to the upregualtion of these chemokines in asthmatic airways, suggest that epithelial derived chemokines could be implicated in the smooth muscle remodeling. Furthermore, they

could be implicated in the distance reduction between the airway epithelium and ASMC mass seen in asthma. Evidence already exists implicating chemokine involvement in angiogenesis and in cancer cell growth (Vaday 2006, Robinson SC 2003).

This study is not the first to show ASMC proliferation *in vitro*. ASMC proliferation is considered as one possible mechanism responsible for the increase in ASMC mass in asthma. To date, many growth factors, pro-inflammatory cytokines, and contractile agonists such as histamine and endothelin-1 have been shown to promote ASMC proliferation. The other possible mechanism is the decreased rates of apoptosis. To our knowledge, this study is the first show the anti-apoptotic and mitogenic activity of some epithelial derived chemokines on ASMC.

This study describes airway smooth muscle proliferation in response to four different chemokines. IL-8, RANTES, eotaxin, and MIP-1α are all up-regulated in asthmatic airways. ASMC react to these chemokines by expressing functional receptors to each one of them. RANTES and eotaxin induce ASMC proliferation at low concentrations while higher doses of IL-8 were needed to demonstrate a similar response. IL-8, predominantly a neutrophils chemoattractant, also interacts with the structural cells. IL-8 causes the vascular and airway smooth muscle cells to migrate. Furthermore, it has been shown that IL-8 is a mitogen for both human and rat aortic smooth muscle cells (Yue TL 1994).

RANTES/ CCL5 is implicated in tumor growth; high levels of RANTES correlate with poor prognosis in breast cancer and small cell lung carcinoma. RANTES is a known

pro-malignant factor because it induces the proliferation of cancer cells and enhances their survival. However, introducing a CCL5 antagonist (TAK-799) reduced the tumor growth in a mouse model of breast cancer (Robinson et al 2003) and in a model of prostate cancer (Vaday et al 2006). The studies on ASMC showed that RANTES is expressed by this type of cell and they express functional receptors for RANTES (CCR1, 5). RANTES is also produced by the epithelial and T cells. Therefore, it could be available in sufficient amounts to enhance the growth and survival of ASMC in the asthmatic airways. Our data shows the mitogenic and the anti-apoptotic effects of RANTES on ASMC. After 48 hours, the cells stimulated with RANTES were showing more DNA synthesis and decreased cell death compared to the non-treated cells.

Our results show that MIP-1α increases the number of ASMC in a concentration dependent manner. A significant increase was seen when higher doses of MIP-1α were used to treat the myocytes (a dose range of 0.1-100 ng/ml was used). The best results were obtained after incubating the ASMC with MIP-1α for 72 hours. It seems the effect of MIP-1α differs according to the cell type and the species tested. Parkinson et al 1993. identified MIP-1α as an inhibitor for hematopoietic stem cell proliferation. MIP-1α also inhibits the proliferation of dermal keratinocytes and spermatogonia (Hakovirta 1994). In rat smooth muscle cells, MIP-1α induces chemotaxis and stimulates proliferation (Luo Y 1996).

PDGF is a potent mitogen for ASMC (Hirst 1996) and was used as a positive control for the proliferation experiments. Epithelial derived chemokines bind to G protein-coupled receptors (GPCR) and activate MAPKinases which leads to DNA

synthesis. In comparison to PDGF, it seems that chemokines are a weaker mitogen for ASMC. The percentages of cells entering the S phase after stimulation with PDGF were higher than those stimulated with chemokines only.

The expression of RANTES, IL-8, MIP-1α, and eotaxin by the airway epithelium and smooth muscle cells may indicate that these chemokines act in both autocrine and paracrine manner. Though we do not know the exact physiological concentrations of these chemokines *in vivo*, their long half life could provide the ASMC with sufficient concentrations to induce proliferation or enhance their survival.

The mitogenic effects seen in ASMC could be mediated through the activation of p42/p44 MAPKinases. Several growth factors, such as cytokines and neuropeptides that induce ASMC proliferation activate this MAPK isoform (Yang et al 2002). In the present study, we demonstrate the involvement of the epithelial derived chemokines in phosphorylation of p42/p44 MAPK which enhances the DNA synthesis and cell proliferation.

Proliferating ASMC are believed to be distinctive phenotype from the fully differentiated mature non-proliferating smooth muscle cells (Halayko 1994). The proliferating ASMC are mitotically active cells that are also able to synthesize different molecules due to the high number of organelles they contain. The mature contractile ASMC possess a high-volume fraction of myofilaments. In adult tissue both phenotypes exist and, under certain stresses, mature ASMC can modulate their phenotype. The plasticity of smooth muscle cells produce divergent smooth muscle populations within

and between tissues (Halayko 1997). This may explain the small number of ASMC that undergo proliferation in regard to the total number of cells used in a single test; another reason could be the use of the primary cell cultures. Although using such cultures has the advantage of functional and structural similarities to the in vivo environment, these cultures are heterogeneous in nature and could be one source of variability in response (Adler 2001). In the current study, both cell lines and primary cell cultures were examined. The advantage of cell lines lies in the homogeneity of the cell population; however, some phenotypic modulation seems unavoidable and using early passages seems to matter in the studies of proliferation.

Apoptosis is the programmed cell death that regulates tissue integrity and development. We investigated the ability of the epithelial derived chemokines to decrease the rate of ASMC apoptosis. It had already been reported that IL-8 delays the spontaneous and TNF-α mediated neutrophils apoptosis in vitro (Kettritz et al 1998). Blocking the CXCR2 receptor diminishes the effect partially. It is believed that IL-8 effect did not diminish completely because CXCR1 was not blocked (Glynn et al 2002). Our results show similar effects on ASMC. Treating the cells with IL-8 reduces the number of Annexin V positive cells compared to the control. After 24 hours of treatment, no significant difference in the number of apoptotic ASMC was seen but extending the incubation to 72 hours yielded a difference between the two. Adding to the IL-8 ability to cause ASMC migration, we speculate that this CXC chemokines does play a significant role in the ASMC increased mass in asthmatic airways. Keeping in mind that our experiments were conducted on non-asthmatic ASMC and that smooth muscle cells from

asthmatic subjects proliferate at twice the rate seen in non asthmatic cells; then the IL-8 effect on asthmatic cells could be even greater.

The identification of new chemokine functions on the airways will contribute to a better understanding of their role in immunomodulation and remodeling seen in asthma and the other chronic inflammatory diseases. Their newly reported effects on ASMC may involve them in the irreversible airway remodeling by enhancing the number of ASMC, either by increasing the proliferation and/or delaying the apoptosis. Presently, ASMC are considered a new target in asthma therapy and even though targeting ASMC may not be the cure to asthma, it might be useful in relieving the acute symptoms related to airway hyperreactivity. Smooth muscle contraction is the direct cause of airway narrowing and, in addition, the increase in smooth muscle mass may cause excess airways narrowing in asthma by increasing the force generated during airway contraction.

Controlling the airway smooth muscle remodeling has been studied intensively and shown that glucocorticosteroids inhibit some but not all growth factor induced proliferation of ASMC (Fernandes et al 1999). One study showed that corticosteroids decrease the IL-8 production by epithelial cells IL-6 and TNF-α. These cytokines are mitogens for ASMC; therefore, asthma patients might benefit from corticosteroids to treat inflammation and to a lesser extent remodeling (Bergeron et al 2006). Combining salbutamol to glucocorticosteroids might result in better reducing airway remodeling (Kamachi et al 2001). Anti-IgE treatment could be used as an anti inflammatory but no anti-remodeling benefits were reported. The same case applies for the anti-cytokines treatments.

Our observations showed that RANTES, eotaxin, MIP-1α and IL-8 increase the number of ASMC. Those CC and CXC chemokines are also enhancing the survival of ASMC. The treated cells are showing a decreased rate of apoptosis compared to the none treated cells. These effects are mediated in part by MAPK activation. A significant increase in phosphorylated p42/p44 MAPKs was seen after treating ASMC with the chemokines of interest. On the basis of these findings, we speculate that chemokines can influence ASMC mass by direct mechanisms.

Chapter 5. Future Directions

This study examined the interaction between the airway epithelium and the smooth muscle cells in terms of remodeling. The focus was to reveal the role of epithelial derived chemokines in smooth muscle layer thickness reported in asthmatic airways and to examine if these chemokines influence the distance shortening between the two layers. The identification of epithelial derived chemokines', specifically IL-8, RANTES, eotaxin, and MIP-1α, mitogenic and anti-apoptotic effect on ASMC emphasize the involvement of these small molecules in the remodeling seen in asthma in addition to their major role as chemoattractant for the inflammatory cells. The study was performed on non-asthmatic ASM cells; therefore, exploring the role of these chemokines on asthmatic cells will be crucial to complete the picture and may expand this study by exploring the mechanisms by which these chemokines induce proliferation and delay apoptosis in ASMC.

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APPENDIX 1.

Table 1. Chemokines Subfamilies and Receptors

Chemokine Subfamily	**Ligands**	**Receptors**
C chemokines	XCL1, XCL2	XCR1
CC chemokines	CCL1-CCL28	CCR1, 2, 3, 4, 5,6,7 and 8
CXC chemokines	CXCL1-CXCL16	CXCR1,2,3,4,5,6 and 7
CX_3C chemokines	CX_3CL1	CX_3CR1

APPENDIX 2.

Ethical Approval for the use of human cells in the project. Documents attached.