

Proliferative aspects of airway smooth muscle

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Increased airway smooth muscle (ASM) mass is perhaps the most important component of the airway wall remodeling process in asthma. Known mediators of ASM proliferation in cell culture models fall into 2 categories: those that activate receptors with intrinsic receptor tyrosine kinase activity and those that have their effects through receptors linked to heterotrimeric guanosine triphosphate-binding proteins. The major candidate signaling pathways activated by ASM mitogens are those dependent on extracellular signal-regulated kinase and phosphoinositide 3'-kinase. Increases in ASM mass may also involve ASM migration, and in culture, the key signaling mechanisms have been identified as the p38 mitogen-activated protein kinase and the p21-activated kinase 1 pathways. New evidence from an *in vivo* rat model indicates that primed CD4⁺ T cells are sufficient to trigger ASM and epithelial remodeling after allergen challenge. Hyperplasia has been observed in an equine model of asthma and may account for the increase in ASM mass. Reduction in the rate of apoptosis may also play a role. β_2 -Adrenergic receptor agonists and glucocorticoids have antiproliferative activity against a broad spectrum of mitogens, although it has become apparent that mitogens are differentially sensitive. Culture of ASM on collagen type I has been shown to enhance proliferative activity and prevent the inhibitory effect of glucocorticoids, whereas β_2 -agonists are minimally affected. There is no evidence that long-acting β_2 -agonists are more effective than short-acting

agonists, but persistent stimulation of the β_2 -adrenergic receptor probably helps suppress growth responses. The maximum response of fluticasone propionate against thrombin-induced proliferation is increased when it is combined with salmeterol. (J Allergy Clin Immunol 2004;114:S2-17.)

Key words: Asthma, airway smooth muscle, airway remodeling, hyperplasia, hypertrophy, apoptosis, cellular migration, β_2 -adrenergic receptor agonists, glucocorticoids

The importance of airway smooth muscle (ASM) in asthma was realized almost 150 years ago.¹ The consensus that has prevailed since is that ASM is an end-response effector cell that regulates regional differences in ventilation by contracting in response to various neurotransmitters, proinflammatory mediators, and exogenous substances released under homeostatic or pathologic conditions such as asthma. However, in the past 10 to 15 years, a new era in ASM biology has begun that is eroding this traditional notion. Exciting new evidence, mainly from *in vitro* studies, supports the concept that in addition to contraction and relaxation in asthma, this structural cell fulfills other diverse functions, encompassing an increased capacity for growth, the secretion of immunomodulatory cytokines and chemokines, expression of key surface receptors involved in cell adhesion and leukocyte activation, and a newly recognized function involving cellular migration.²⁻⁴ As our understanding of this remarkable functional adaptivity develops, it seems that the likely role played by ASM in regulating airway caliber in asthma is far more integrative than previously recognized.

The first section of this article focuses on the processes that drive cellular proliferation and migration, two aspects of this adaptivity that potentially lead to overall increases in ASM content in asthma. This article also examines potential mechanisms of allergen-induced airway remodeling in asthma and highlights pharmacologic approaches that may limit ASM proliferative and migratory activity in remodeling.

MECHANISMS AND MEDIATORS DRIVING GROWTH AND MIGRATION OF ASM IN ASTHMA

The first report documenting increases in ASM content in asthma appeared >75 years ago.⁵ More than 50 years later, increased ASM content was recognized as a

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Abbreviations used

ASM:	Airway smooth muscle
β_2 AR:	β_2 -Adrenergic receptor
BAL:	Bronchoalveolar lavage
cAMP:	Cyclic adenosine monophosphate
cdk:	Cyclin-dependent kinase
EGF:	Epidermal growth factor
ERK:	Extracellular signal-regulated kinase
FGF-2:	Fibroblast growth factor 2
GPCR:	G protein-coupled receptor
GTPase:	Guanosine triphosphatase
HSP27:	Heat shock protein 27
JAK:	Janus kinase
LABA:	Long-acting β_2 -agonist
MAPK:	Mitogen-activated protein kinase
MEK:	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
NADPH:	Nicotinamide adenine dinucleotide phosphate
PAK1:	p21-activated kinase 1
PCNA:	Proliferating cell nuclear antigen
PDGF:	Platelet-derived growth factor
PGE ₂ :	Prostaglandin E ₂
PI3K:	Phosphoinositide 3'-kinase
Rb:	Retinoblastoma restriction protein
ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinase
SABA:	Short-acting β_2 -agonist
STAT:	Signal transducer and activator of transcription
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP: Deoxy uridine triphosphate nick end labeling
uPA:	Urokinase plasminogen activator
uPAR:	Urokinase plasminogen activator receptor

prominent pathological feature and major component of the structural changes that result in airway luminal narrowing in fatal⁶⁻¹³ and nonfatal¹³⁻¹⁵ asthma. The mechanisms underlying such increases involve multiple processes that likely include both hyperplastic and hypertrophic changes. Their anatomical distribution and relative importance to the overall muscle accumulation remain controversial (Table I).

Mechanisms of hyperplasia

There is little doubt that the increase in ASM mass in the airways of asthmatic patients is comprised in part by hyperplasia.^{8,12,16,17} The increases in ASM cell number could occur through increased rates of division or decreased rates of apoptosis or, more speculatively, through migration of mesenchymal cells to the ASM bundles and/or differentiation. Much attention has been focused on increased rates of proliferation, because such responses are readily measured *in vitro* by using ASM cultured from biopsies, airway resections, or postmortem specimens. As noted in more detail below, diverse influences on proliferative rate have been described, including growth factors, G protein-coupled receptor (GPCR) stimulants, cytokines, reactive oxygen species, enzymes, extracellular matrix components, and increased stretch.¹⁸ Plausible arguments link these diverse stimuli to

the environment of the asthmatic airway. However, there are no data to identify definitively any 1 or more of these influences as major contributors to the hyperplasia in asthma.

Several biopsy-based investigations have failed to reveal the expected increases in the prevalence of markers of proliferation in smooth muscle cells,¹⁹ despite confirmation of hyperplasia in airways accessible by biopsy.²⁰ These findings are not necessarily incompatible with the observed hyperplasia in the airway wall, because biopsy fails to sample the ASM completely, and there may be some compartmentalization of the proliferative response. Alternatively, ASM could accumulate through reduced rates of apoptosis, as has been described in the Brown Norway rat model of chronic inflammation and remodeling.²¹ Previous investigations of apoptosis in human airway biopsy studies have not revealed the presence of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)—positive ASM cells, but the possibility of apoptosis of fibroblasts was not excluded.²² Cultured ASM appears to be relatively resistant to apoptosis,²³ but ASM can undergo apoptosis when challenged with a protein synthesis inhibitor. This apoptotic response is inhibited by extracellular matrix components interacting via β 1-integrins.²⁴

The failure to observe an increase in the prevalence of proliferation markers in the ASM captured in airway biopsies may also be explained by the possibility that mesenchymal cells migrate to the muscle to form the additional muscle bulk,¹⁸ as explained in more detail below.

Mechanisms of hypertrophy

Less is known about the stimuli that are able to induce hypertrophy of ASM. Autopsy and airway biopsy studies have led to variable conclusions regarding the existence of hypertrophy in larger airways, with cell diameters shown to be increased,^{12,25} but stereologic approaches do not suggest the existence of ASM hypertrophy in biopsies.²⁰ Increased levels of stretch and TGF- β have been identified as potential hypertrophic influences. In addition, IL-1 β has a hypertrophic action in rabbit cultured ASM. The cytokine cardiotrophin has also been recently characterized as a hypertrophic factor for human cultured ASM.²⁶

MEDIATORS DRIVING ASM GROWTH

The basis of excessive ASM growth is presumed to lie in stimulation of ASM by mitogenic or inflammatory stimuli. However, few reports have examined either mitogenic indices for ASM cells in human asthmatic airways or the presence of ASM mitogenic activity in the airways. Benayoun et al¹⁴ recently examined ASM proliferation by immunodetection of the nuclear antigen Ki67, which was distributed in the airway epithelium and bronchial submucosa of control volunteers or patients with varying severities of asthma but was absent in the ASM layers across all subject groups. In contrast, bronchoalveolar lavage (BAL) fluid collected from atopic

TABLE I. Mechanisms underlying ASM accumulation in fatal and nonfatal asthma

Asthma severity	Increased ASM content in asthma	Proposed mechanism of ASM accumulation	Reference
Fatal	Yes*	ND	Huber and Koessler ⁵
Fatal	Yes*	ND	Dunnill et al ⁶
Fatal	Yes†	ND	Takizawa and Thurlbeck ⁷
Fatal	Yes	Hyperplasia*	Heard and Hossain ⁸
Fatal	Yes†‡	ND	Bai et al ⁹
Fatal	Yes*†	ND	James et al ¹⁰
Fatal	Yes†	ND	Saetta et al ¹¹
Fatal	Yes*†	Hyperplasia*/hypertrophy†	Ebina et al ¹²
Fatal	Yes*†	ND	Carroll et al ¹³
Severe, nonfatal	Yes	Hypertrophy*	Benayoun et al ¹⁴
Moderate, nonfatal	No*, yes†	ND	Carroll et al ¹³
Moderate, nonfatal	Yes	Hypertrophy*	Benayoun et al ¹⁴
Mild, nonfatal	Yes	Hyperplasia*	Woodruff et al ¹⁵

ND, Not determined.

*Large airways (cartilaginous).

†Small airways (noncartilaginous).

‡ASM accumulation present in both adults and children.

asthmatic patients 48 hours after segmental allergen challenge, and then applied to human ASM cells cultured from normal subjects, caused marked increases in DNA synthesis, cell number, and cyclin D1 protein abundance (expressed in the S phase of the cell cycle and required for ASM proliferation²⁷), compared with either preallergen challenge or BAL fluid from healthy controls.²⁸ Cell culture-based studies in the past 10 years have identified numerous putative mitogens for ASM in human beings and other species, some of which are increased in BAL fluid from asthmatic airways. To date, mitogenic stimuli include polypeptide growth factors such as epidermal growth factor (EGF),²⁹ insulin-like growth factors,³⁰ platelet-derived growth factor (PDGF) isoforms,³¹ and fibroblast growth factor 2 (FGF-2)³²; plasma-derived or inflammatory cell-derived mediators such as lysosomal hydrolases (β -hexosaminidases and β -glucuronidase),³³ α -thrombin,³⁴ tryptase,³⁵ and sphingosine 1-phosphate³⁶; and contractile agonists such as endothelin-1,³⁷ substance P,³⁸ phenylephrine,³⁹ serotonin,⁴⁰ thromboxane A₂,⁴¹ and leukotriene D₄.⁴²

Although proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α are also increased in BAL fluid from asthmatic patients, their effects on proliferation of ASM are modest and variable.⁴³⁻⁴⁶ This result may relate to the species selected for study as well as the presence of autocrine cyclooxygenase products such as prostaglandin E₂ (PGE₂), which inhibits DNA synthesis.⁴⁷ In a recent article by Chambers et al,⁴⁸ autocrine PGE₂ release was found to be reduced in ASM cells cultured from patients with mild asthma compared with those from persons without asthma. The importance of autocrine pathways is demonstrated in another recent study in which TNF- α markedly upregulated DNA synthesis after treatment of human ASM cells with a neutralizing antibody to IFN- β .⁴⁹ Thus, the importance of proinflammatory cytokines in driving accumulation of ASM, several of which are produced by the ASM itself,³ requires re-evaluation in light of these findings.

Other trophic factors, such as mechanical stress⁵⁰ and reactive oxygen species (ROS),⁵¹ have been identified. Components of the extracellular matrix that are increased in asthma may affect ASM accumulation as well (see the article by Howarth et al in this supplement).

Signaling pathways driving ASM growth

Airway smooth muscle cells proliferate in response to at least 2 major groups of mitogens: polypeptide growth factors that activate receptors with intrinsic receptor tyrosine kinase (RTK) activity (eg, PDGF, EGF, FGF-2, insulin-like growth factor) and contractile agonists that ligate receptors linked to heterotrimeric guanosine triphosphate-binding proteins (G proteins; eg, α -thrombin, serotonin, thromboxanes, endothelin-1, leukotriene D₄).^{2,52} A third group of mitogens is emerging: the proinflammatory cytokines (IL-1 β , TNF- α).⁴⁹ These cytokines signal through surface glycoprotein complexes, made up of 2 to 4 receptor chains coupled to multiple non-RTKs such as Src family members and components of the mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) systems.

p21^{ras}, a 21-kd guanosine triphosphatase (GTPase), may act as a point of convergence for the mitogenic signal activated by these varying receptor-operated mechanisms (Fig 1).^{53,54} When it is active (ie, guanosine triphosphate-bound), p21^{ras} binds downstream effectors such as phosphoinositide 3'-kinase (PI3K) and the 74-kd cytoplasmic serine/threonine kinase, Raf-1. Recruitment of Raf-1 to the plasma membrane activates p21^{ras} and the extracellular signal-regulated kinase (ERK) pathway, although Raf-1-independent signaling to ERK has also been demonstrated in bovine ASM.⁵⁵ PI3K can be directly activated by an allosteric interaction with p21^{ras}.⁵⁶ The majority of *in vitro* reports support PI3K and ERK activation as the major signal transduction pathways for RTK-stimulated, GPCR-stimulated, or cytokine-stimulated proliferation of ASM (Fig 1).

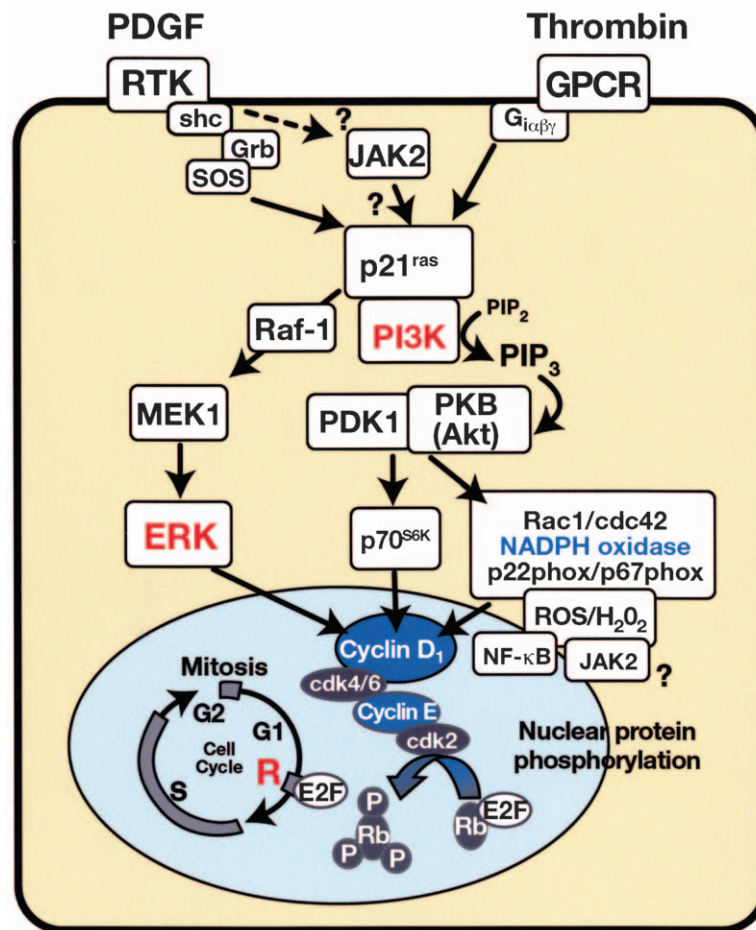


FIG 1. Key signal transduction mechanisms that operate in ASM to regulate cell cycle entry and DNA synthesis. Mitogens acting predominantly through RTK (eg, PDGF) or GPCR (eg, thrombin) activate small GTPase p21^{ras} proteins, which interact with various downstream effectors. These include Raf-1 and PI3K. Raf-1 activates MEK1, which then phosphorylates ERK. PI3K, via 3-phosphorylated phosphoinositides such as 3,4,5-trisphosphate (PIP₃), recruits phosphoinositide-dependent kinase 1 (PDK1), which in turn leads to maximal protein kinase B (Akt) activation and downstream activation of p70^{S6K} and members of the Rho family GTPases, Rac1 and Cdc42. These form part of the NADPH oxidase complex that generates ROSs such as H₂O₂, which may lead to cyclin D1 activation via nuclear factor-κB (NF-κB) or JAK2. Thus, the ERK and PI3K pathways are considered to be 2 major, independent signaling pathways regulating ASM cell proliferation. PIP₂, Phosphoinositide 3,4-bisphosphate. SOS, Son of sevenless.

Ras-dependent ERK pathways driving proliferation. Page et al,⁵⁷ by using expression of a dominant-negative form of p21^{ras} in bovine tracheal smooth muscle cells, have shown that unlike Raf-1, activation of ERK by PDGF is critically dependent on p21^{ras}. Expression of a constitutively active p21^{ras} induced not only activation of ERK and c-jun N-terminal kinase but also transcriptional activation of the downstream cyclin D1 promoter.⁵⁷ The critical importance of p21^{ras} proteins in EGF-dependent cell cycle traversal to the S phase was confirmed by microinjection of an antipan p21^{ras} neutralizing antibody in human ASM cells.⁵³ Likewise, microinjection with a neutralizing antibody against the cyclin D1 protein, a D-type cyclin regulating G1 cell cycle progression in mammalian cells, has demonstrated that it, too, is required

for S-phase traversal.²⁷ In bovine tracheal myocytes, induction of transcriptional activation of the cyclin D1 promoter by a constitutively active MAPK/ERK kinase (MEK)–1 is prevented by the MEK inhibitor PD98059, implying that in these cells, the pathway from p21^{ras} to cyclin D1 is MEK1-dependent.⁵⁷ These observations may extend to other species, in which chemical or dominant-negative inhibition of MEK-1 attenuates mitogen-induced ERK activation, cyclin D1 promoter activity or cyclin D1 protein expression, and DNA synthesis.⁵⁸⁻⁶¹ One study with human ASM has reported no effect of chemical inhibitors on cyclin D1 mRNA, despite reduced cyclin D1 protein levels. This finding suggests that in contrast with bovine ASM, cyclin D1 activation by ERK in human beings may be more dependent on posttranscriptional

mechanisms.⁶² Nevertheless, for effective transmission of the mitogenic signal, ERK activation must be sustained. Orsini et al⁶⁰ showed that both RTK and GPCR mitogens produced robust and sustained activation of ERK, which correlated with proliferation and was prevented by MEK inhibition. Chemical inhibition of ERK activation as long as 6 hours after the addition of mitogen reduced DNA synthesis in these cells, further supporting a requirement of sustained ERK activity.⁶³ Collectively, these findings support a role for sustained ERK activation in ASM that drives the transcriptional activation of the cyclin D1 promoter or activation of cyclin D1 protein, and they suggest that ras proteins, MEK1, and ERK activation constitute a discrete pathway to cyclin D1 protein expression (Fig 1).⁵⁷

As noted recently by Zhou and Hershenov,⁵² however, ERK activation and induction of cyclin D1 expression alone may not be sufficient for progression from the G1 phase to the S phase. Additional events are required in other cell systems, such as maximal phosphorylation of the retinoblastoma restriction protein (Rb). Activation of the cyclin E/cyclin-dependent kinase (cdk)—2 complex and Rb hyperphosphorylation, after binding of cyclin D1 protein to cdk4 (or cdk6), reduces the affinity of Rb for the nuclear elongation factor E2F (Fig 1). This results in derepression of E2F, activation of DNA polymerase, and transcriptional activation of specific S-phase-dependent genes. Other key events include induction of cyclin A expression and degradation of the cdk inhibitor p27^{Kip1}. Thus, the apparently paradoxical observation that p21^{ras} proteins are critically important for cell cycle traversal into the S phase,⁵³ and that ERK activation of cyclin D1 expression is necessary^{27,57} but not sufficient for a full mitogenic response,⁶⁴ reiterates the possibility that p21^{ras} proteins can coordinate S-phase entry via cyclin D1 by regulating mitogenic signaling through both ERK-dependent and ERK-independent pathways. A requirement for ERK-dependent pathways may also relate to the concentration of agonist eliciting a mitogenic response. In human ASM cells, ERK activity increases in cyclin D1 protein levels, and DNA synthesis stimulated by low, but not by higher, concentrations of thrombin was sensitive to MEK inhibition.⁶²

PI3K: Ras-dependent, ERK-independent pathways driving proliferation. A major additional or alternative signaling pathway driving ASM proliferation involves activation of PI3K. Three distinct classes of PI3K isoforms exist on the basis of structure and substrate specificity, as reviewed elsewhere.⁶⁵ Common to all class IA and IB PI3Ks, the catalytic subunit is composed of a phosphoinositide kinase domain, a protein kinase domain, and a p21^{ras}-binding domain. Class II PI3K is composed of a catalytic subunit only, whereas class III PI3K binds a binding/regulatory subunit and, in human beings, is postulated to play a role in vesicle trafficking.⁶⁵ Human ASM cells express class IA, II, and III PI3K, but surprisingly not the class IB isoform.⁶⁶ Both RTK and GPCR mitogens appear to require class IA PI3K.⁶⁶ PI3Ks regulate cell functions by phosphorylating membrane

phosphoinositides on the 3-hydroxyl of the inositol ring to form 3-phosphorylated phosphoinositides (phosphoinositide 3-phosphate, phosphoinositide 3,4-bisphosphate, and phosphoinositide 3,4,5-trisphosphate).⁶⁵ This recruits 3-phosphorylated phosphoinositide-binding proteins to the plasma membrane, including phosphoinositide-dependent kinase 1, which in turn leads to maximal protein kinase B activation and downstream activation of p70^{S6K} (Fig 1).^{67,68} Other PI3K targets in ASM include members of the Rho family GTPases (Rac 1⁶⁹ and Cdc42, but not RhoA⁷⁰) important in cell cycle progression and regulation of the actin cytoskeleton.⁷¹

Inhibitors of PI3K (eg, LY294002) have greatly facilitated understanding of the importance of PI3K and its downstream intermediates in defining ERK-independent cell cycle progression in ASM. For example, LY294002 prevents mitogen-stimulated cyclin D1 promoter activation, as well as cyclin D1 protein abundance⁷² and DNA synthesis,^{67,68,73} without affecting ERK activation.⁶⁸ Likewise, overexpression of the catalytic subdomain of PI3K in ASM was sufficient for cyclin D1 promoter activation without inducing ERK activation,⁷³ and introduction of a constitutively active class IA PI3K was sufficient to stimulate DNA synthesis.⁶⁶ Studies with constitutively active class IA PI3K have also demonstrated that induction of DNA synthesis is submaximal compared with that observed when receptor-mediated pathways are activated.⁶⁶ Walker et al⁶⁴ compared the extent to which the ERK or PI3K cascades contributed to PDGF-stimulated or α -thrombin-stimulated proliferation of bovine tracheal smooth muscle. They showed that although the PI3K pathway was essential, activation of ERK was required for a full mitogenic response. Such findings suggest that although active PI3K is sufficient to stimulate ASM DNA synthesis, either by RTK-coupled or G-protein-coupled pathways, ERK-dependent parallel signaling events are required for maximal proliferation.

The downstream events by which PI3K induces cyclin D1 abundance in ASM remain poorly defined. In other cell types, phosphorylation by glycogen synthase kinase 3 β continuously degrades cyclin D1 by targeting it to the proteasome. Glycogen synthase kinase 3 β is inhibited by phosphorylation by protein kinase B from PI3K, resulting in increased cyclin D1 levels. Protein kinase B activation also represses transcription of cdk inhibitors such as p27^{Kip1} to permit cell cycle entry.⁶⁵ Conversely, rapamycin, an inhibitor of p70^{S6K}, decreases mitogen-stimulated cyclin D1 mRNA and protein levels.⁷⁴ In bovine⁶⁷ and human ASM,⁶⁸ rapamycin also attenuates growth factor-induced DNA synthesis, supporting p70^{S6K} as an essential step in the pathway for proliferation in ASM.

Increasing evidence also supports PI3K-dependent activation of Rho family GTPases (Rac 1⁶⁹ and Cdc42⁷⁰) in cyclin D1 upregulation and cell cycle progression. Overexpression of Cdc42, but not overexpression of RhoA, induced cyclin D1 promoter activation in an ERK-independent manner.⁷⁰ Similarly, Rac1-induced transcription from the cyclin D1 promoter was unaffected by MEK inhibition,⁶⁹ suggesting that Rac1-mediated cell cycle

progression, like that after activation of PI3K, is ERK-independent (Fig 1). In the same study, it was found that active PI3K, Rac1, and Cdc42 each induce cyclin D1 promoter activity via the cyclic adenosine monophosphate (cAMP) response element binding protein/activating transcription factor 2 binding site,⁶⁹ indicating that these intermediates may lie on the same signaling pathway. Overexpression of the catalytically active subunit of PI3K and activation of the cyclin D1 promoter were found to be attenuated by inhibitors of Rac1 signaling,⁷³ implying that Rac1 likely lies downstream of PI3K activation (Fig 1).^{70,73}

ROS driving proliferation. Rac1 constitutes part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that generates ROSs such as H₂O₂. Recent studies suggest that ROS intermediates contribute to mitogen-stimulated growth of ASM (Fig 1).^{69,75,76} In cells cultured from fetal human trachealis, oxygen or exogenous H₂O₂ upregulated growth factor–induced proliferation, which was prevented by chemical antioxidants.⁷⁷ Elsewhere, treatment with chemical antioxidants attenuated both mitogen-induced cyclin D1 expression and DNA synthesis.^{69,75,76} Likewise, inhibition of p22phox or p67phox (components of human and bovine NADPH oxidase, respectively) attenuated mitogen-induced cyclin D1 promoter activity⁶⁹ and cell proliferation.⁷⁶ Although these observations implicate ROS and NADPH oxidase components in ASM mitogenesis, the relevant downstream effectors are less well characterized. It is known, however, that several transcription factors are involved. Rac1 induces transactivation of the cyclin D1 promoter cAMP response element binding protein/activating transcription factor 2 binding site, which is prevented by antioxidants.⁷³ Antioxidants also prevent activation of nuclear factor- κ B, implying it acts as a downstream target of NADPH oxidase–generated ROS (Fig 1).⁷⁶ In addition, a recent report implicates JAK2 and STAT3 activation in PDGF-stimulated ASM mitogenesis.⁷⁸ Inhibition of JAK2 with AG490 attenuated JAK2 and STAT3 phosphorylation as well as PDGF-dependent cyclin D1 protein expression and DNA synthesis.⁷⁸ Intriguingly, overexpression of catalase attenuated STAT3 phosphorylation, suggesting that JAK/STAT activation is redox-dependent and that JAK/STAT-dependent signaling constitutes an alternative downstream target of ROS in regulating ASM proliferation.

MIGRATION CONTRIBUTING TO ASM ACCUMULATION

Cellular migration is a process characterized by substantial cytoskeletal remodeling with consequent spatially directed filopodia and lamellipodia to allow increased nondirected movement (chemokinesis) or directed movement along a concentration gradient (chemotaxis). Cellular migration is a newly recognized function of ASM, identified only in the past 3 to 4 years. Although vascular smooth muscle cell migration from the media to

the neointima is widely accepted as key in the pathogenesis of vascular wall smooth muscle accumulation in atherosclerosis and restenosis,⁷⁹ no direct evidence is available to support an analogous process in remodeled airways in asthma. Indeed, no consensus exists on the likely direction in which ASM might be expected to migrate!

The hypothesis addressed here is that ASM muscle accumulation in asthma results from excessive growth of the existing smooth muscle population or subpopulation induced by the coordinated action of various growth factors, cytokines, and other inflammatory mediators. Inflammation has been hypothesized to result in reversible modulation of smooth muscle toward a less differentiated or immature phenotype in which additional secretory or immunomodulatory functions of ASM are manifest.^{3,80} However, on the basis of the existence of migratory responses occurring *in vitro* (reviewed elsewhere⁴), there is growing interest in the possibility that such cells, as in the systemic vasculature, might migrate out of the muscle bundles toward the lumen and contribute to the appearance of myofibroblasts in the submucosal space in asthma. These myofibroblasts are considered to have ultrastructural features between those of fibroblasts and those of mature smooth muscle.^{81,82} There is yet another possibility that, along with growth factor and cytokine exposure, myofibroblasts or other cells outside the muscle bundles could undergo directed migration toward the bundles. This migration of myofibroblasts would precipitate proliferation or change of their phenotype, once they were in the bundles, to adopt the differentiated characteristics of mature smooth muscle and so add to its overall content. Which of these possibilities is most likely remains an open question, but clearly the concept of ASM migration is relevant to the pathology of asthma and offers new therapeutic potential. An important goal for the immediate future is to identify the occurrence and direction of muscle cell migration as it contributes to ASM accumulation in asthma.

Mediators driving ASM migration

A substantial literature exists regarding the factors that influence vascular smooth muscle migration.⁸³ To date, relatively few growth factors and inflammatory cytokines have been shown to promote ASM migration, but the list is rapidly expanding.^{4,84} ASM migratory stimuli include polypeptide growth factors such as PDGF-A–chain and PDGF-B–chain isoforms,^{72,85–87} FGF-2,⁸⁴ TGF- β ,⁸⁸ and TGF- α /EGF;⁸⁴ and plasma-derived or inflammatory cell–derived mediators such as the plasminogen activators, tissue plasminogen activator⁸⁷ and urokinase,⁸⁹ and IL-1 β .⁸⁸ In addition, contractile agonists such as leukotriene E₄ increase nondirectional movement (chemokinesis) and can potentiate ASM migratory responses to PDGF.⁷² Most, if not all, of these migratory stimuli are mitogens for ASM. However, Goncharova et al⁸⁴ recently reported that stimuli for ASM proliferation and migration do not necessarily overlap in regulation by

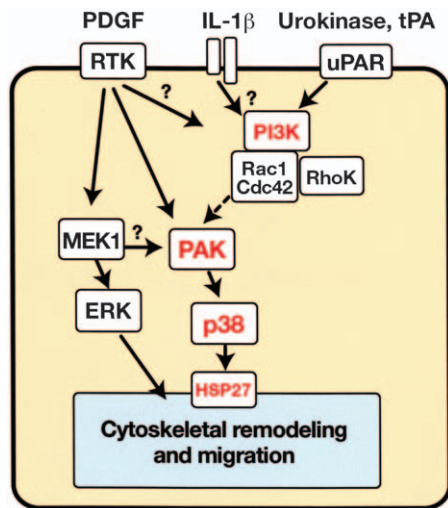


FIG 2. Key signal transduction mechanisms that operate in ASM to regulate migration. Migration is associated with activation of glycolipid-anchored receptor proteins (eg, uPAR, urokinase receptor) and cytokine receptors. The major pathway so far identified for ASM migration signals to p38 MAPK via PAK to culminate in HSP27 phosphorylation, which favors F-actin formation and cytoskeletal remodeling. Other events recently implicated in ASM migration include PI3K activation, possibly acting via Rho kinase (RhoK) and Rac1/Cdc42, and activation of ERK-dependent pathways. *tPA*, Tissue plasminogen activator.

growth factors, because the GPCR agonist α -thrombin failed to increase migration.

Urokinase is a well-established migratory stimulus for many cell types. It acts directly by high-affinity binding to the urokinase plasminogen activator receptor (uPAR) and by low-affinity binding of the kringle domain of urokinase to an unidentified cell surface receptor.^{86,89} Examination of urokinase plasminogen activator (uPA) mutants suggests that the protease activity domain, growth factor–like domain, and kringle domain of uPA differentially contribute to urokinase-stimulated migration of human ASM cells.⁸⁶ Recombinant forms of uPA lacking proteolytic activity and the uPAR-binding growth factor–like domain, as well as recombinant uPA-kringle polypeptides, each induce cell migration. Deletion of the growth factor–like domain from uPA abolished its normal ability to bind to uPAR with high affinity but did not abolish its chemotactic properties on human ASM. This result suggests that the major mechanism responsible for uPA-induced muscle cell motility involves the kringle domain of uPA, which was both necessary and sufficient.⁸⁶ Likewise, treatment with a monoclonal antibody against the kringle region or with a monoclonal antibody that prevented interaction between uPA and uPAR inhibited uPA-induced migration.⁸⁹ In contrast with these initial studies, Carlin et al⁸⁷ report that urokinase is not itself a migratory stimulus in these cells, but instead potentiates chemotaxis toward PDGF. Whether urokinase initiates or facilitates migration of human ASM remains an open question. Undoubtedly, the next few years will bring an explosion of information characterizing the

diverse stimuli that directed migration of ASM cells in culture.

Signaling pathways driving ASM migration

Much less is known of the key intracellular events that mediate nondirected and directed migration of ASM compared with those driving increased proliferation. Although significant progress has been made in elucidating candidate pathways for migration (Fig 2), much of our current knowledge relies on selectivity of chemical inhibitors, often at a single concentration, without support from overexpression studies using catalytically active or inactive signaling intermediates.

Initial studies examining urokinase kringle domain signaling requirements for migration demonstrate that uPA mutants containing the kringle domain specifically activate the p38 MAPK pathway and actomyosin by increasing phosphorylation of the myosin regulatory light chain and MAPK sites of the actin-associated regulatory protein caldesmon.⁸⁶ Inhibitors of p38 MAPK activation did not affect myosin light chain phosphorylation, but they did block the increase in nonmuscle caldesmon phosphorylation and uPA-stimulated migration of human ASM, which suggests that these events are required for urokinase-stimulated smooth muscle cell migration.⁸⁶

Other studies suggest that a requirement for p38 MAPK in ASM migration extends beyond uPA as a stimulus. Hedges et al⁸⁸ found that migration of canine tracheal smooth muscle induced by PDGF, IL-1 β , or TGF- β was sensitive to inhibition of p38 MAPK. Activation of p38 MAPK and phosphorylation of heat shock protein 27 (HSP27), whose phosphorylation promotes F-actin polymerization required for cell motility, was prevented by the p38 MAPK inhibitor SB 203580. Overexpression of an upstream activator for p38 MAPK increased migration, whereas overexpression of a p38 α MAPK dominant-negative mutant and an HSP27 phosphorylation mutant abolished migration, consistent with the hypothesis that activation of the p38 MAPK pathway by growth factors and proinflammatory cytokines is essential for ASM cell migration (Fig 2).⁸⁸ In a subsequent study, p21-activated kinase 1 (PAK1) was identified as a regulator of p38 MAPK because overexpression of a catalytically inactive PAK1 mutant blocked chemotactic migration to PDGF and reduced p38 MAPK phosphorylation.⁸⁵ As noted recently by Madison,⁴ this is a significant finding because the PAK family of molecules interacts with several Rho GTPases (RhoA, Rac, Cdc42). These GTPases are recognized as key regulators of actin cytoskeletal dynamics, including the actin polymerization-dependent appearance of filopodia and lamellipodia and membrane ruffles during cell migration, and as key regulators of cell polarity during movement.^{90,91}

Other candidate signaling pathways are emerging as regulators of ASM migration, including PI3K and Rho kinase (Fig 2). The mechanism of PI3K-mediated cell motility is not well understood in ASM, but it may involve activation of various downstream effectors such as Cdc42

and Rac1, PAK1, and PAK-interacting exchange factor.⁹² Treatment of ASM cells with a PI3K inhibitor prevented migration in response to PDGF^{87,92} as well as its augmentation by leukotriene E₄.⁷² Likewise, treatment with a Rho-kinase inhibitor (Y27632) prevented PDGF-stimulated migration, even after potentiation of migration by urokinase.⁸⁷ Finally, a role for ERK-dependent signaling in migration of ASM is also suggested. In a preliminary report, Carlin et al⁸⁷ demonstrated that the MEK inhibitor U0126 prevents PDGF-BB-dependent or PDGF-AB-dependent migration of human ASM. PD98059, another MEK inhibitor, had only a modest inhibitory effect on urokinase-stimulated migration in these cells⁸⁶ and on PDGF-dependent migration of canine tracheal smooth muscle cells.⁸⁸

ALLERGEN-INDUCED AIRWAY REMODELING

Modeling of ASM growth

A variety of small animal models of asthma have been used to study ASM growth. Most studies have been performed in the Brown Norway rat, whose immune system is T_H2-biased. It exhibits exuberant IgE production on active sensitization⁹³ and typical eosinophilic airway inflammation after allergen challenge.^{94,95} Repeated exposure to a sensitizing antigen such as ovalbumin results in an increase in ASM mass, assessed by morphometric analysis.⁹⁶ The increase in ASM mass in the large airways correlates with the change in airway responsiveness to methacholine observed after repeated allergen exposures.⁹⁶ Several investigators have shown that hyperplasia occurs by demonstrating the incorporation into ASM of bromodeoxyuridine, a marker of the S phase.⁹⁷ Cysteinyl leukotrienes and endothelin are both involved in the hyperplastic response of the ASM cells *in vivo*,⁹⁸⁻¹⁰⁰ but the cell sources of these mediators are as yet uncertain. These mediators are also present in elevated amounts in the airways in human asthma. Activated macrophages, mast cells, and the airway epithelium are potentially of importance as sites of synthesis. Indeed, mast cells are found in high numbers interspersed among the ASM fibers in subjects with asthma.¹⁰¹

Theoretically, an increase in ASM mass could result from a reduction in the normal turnover of the tissues, more specifically through the inhibition of apoptosis. Even the normal rat shows a considerable turnover of the tissues of the airway, not only the epithelium (which would not be surprising, because epithelial surfaces are in general in a more rapid state of renewal than other tissues), but also mesenchymal cells such as ASM. Fig 3 shows such proliferation and apoptosis in the airway of normal animals. Cells proliferating and undergoing apoptosis are not rare, suggesting a rapid and dynamic turnover of tissues. Notably, the balance between these 2 phenomena appears to be finely controlled, because the mass of muscle is a constant in airways of different sizes and is similar from animal to animal. How these processes are coupled has not been established, but one is tempted to speculate that the promitogenic signals for the growth of new ASM originate

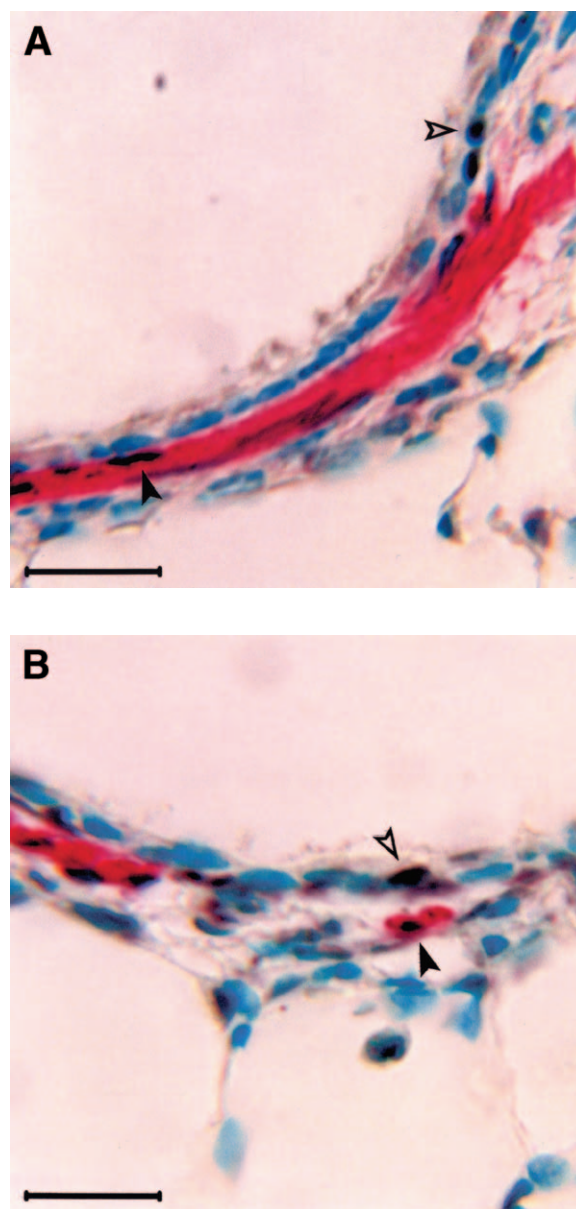


FIG 3. Airway epithelial and smooth muscle cell proliferation (**A**) and apoptosis (**B**) *in vivo*. PCNA immunoreactivity, and apoptosis detected by the TUNEL technique, were colocalized, respectively, with smooth muscle α -actin in illustrative airway sections of control rats. Note the dark gray to black stained nuclei indicating PCNA⁺ or apoptotic cells in the bundles of smooth muscle (closed arrows) and in the epithelium (open arrows). Scale bars, 20 μ m.

from the same cells that remove apoptotic cells, such as the tissue macrophages. These cells are certainly a source of cysteinyl leukotrienes and growth factors.^{102,103}

T cells and ASM growth *in vivo*

CD4⁺ T cells of the T_H2 phenotype play a pivotal role in the inflammatory response associated with asthma. Therefore, it is important to the understanding of airway remodeling to determine whether these cells participate in airway remodeling. T cells can exert mitogenic effects on

ASM cells directly *in vitro*,¹⁰⁴ but they may also mediate effects on ASM and other airway structures through their cytokines or by driving other cells of the immune system. Lazaar et al¹⁰⁴ found that activated T cells adhered to smooth muscle cells *in vitro* via CD44, hyaluronic acid, and integrins. Significantly, direct contact between activated T cells and smooth muscle also evoked a proliferative response of the muscle.

One technique for examining the role of T cells in allergic airway responses *in vivo* is adoptive transfer. Cells harvested from sensitized donors can transfer allergen sensitivity to naive recipient rats such that these animals develop late allergic airway responses, eosinophilic inflammation, and a typical T_H2 profile of cytokines after challenge.¹⁰⁵⁻¹⁰⁷ These responses are generated independently of IgE, which is not present in these animals.¹⁰⁵ Actively sensitized rats that undergo repeated allergen challenge have airway remodeling that includes a hyperplastic response of both the epithelium and the ASM.¹⁰⁸ When CD4⁺ T cells were purified from ovalbumin-sensitized rats and transferred intraperitoneally to unsensitized recipients that underwent 3 ovalbumin challenges at 3-day intervals, both ASM and epithelium showed proliferative responses by proliferating cell nuclear antigen (PCNA) immunoreactivity (Ramos-Barbón, unpublished data, October 2003). The intensity of the proliferative response varied from airway to airway but was similar for the epithelium and ASM in individual airways, suggesting that common mediators might be involved in the responses of these 2 tissues. In addition to proliferation, there was evidence that the mass of ASM is regulated through changes in the rates of apoptosis. Animals that received CD4⁺ T cells from ovalbumin-sensitized donors and that underwent ovalbumin challenges showed a reduction of the density of apoptotic cells in both the epithelium and ASM as assessed by the TUNEL technique. A detailed morphometric analysis of smooth muscle α -actin immunofluorescence confirmed that the changes in these signals were associated with an increase in ASM mass. It appears that CD4⁺ T cells are sufficient for the triggering of ASM and epithelial remodeling after allergen challenge. The relative importance of the inhibition of apoptosis and stimulation of hyperplasia as mechanisms for altering tissue mass remains to be established.

An issue that has been hard to resolve is the site of action of T cells used in adoptive transfer experiments. Studies of T-cell homing have shown that memory T cells home to mucosal surfaces,¹⁰⁹ so one would expect to find some of the transferred cells in the airway wall. However, because the numbers of cells administered are in general quite small, it is hard to find these cells subsequently in the tissues, even when they are labeled intensely with fluorophores. Ramos-Barbón et al^{109a} have now generated populations of antigen-specific CD4⁺ T cells that are transduced with recombinant retroviruses encoding enhanced green fluorescent protein (Ramos-Barbón, unpublished data, 2003). After adoptive transfer, these enhanced green fluorescent protein-expressing cells have been identified in the airways of rats challenged with ovalbumin, some in apparent contact with ASM cells.

These observations support the hypothesis that CD4⁺ T cells can interact directly with ASM or that T-cell-derived cytokines such as IL-13 and IL-4 can have direct effects on ASM (Fig 4). IL-13 enhances mitogenic responses to cysteinyl leukotriene 1 receptor agonists by increasing cysteinyl leukotriene 1 receptor expression on the ASM, but IL-4 appears to be antiproliferative.¹¹⁰ Notably, IL-4 has recently been reported to induce apoptosis of lipopolysaccharide-stimulated monocytes by activating the caspase cascade.¹¹¹ Whether IL-4 has effects of this sort on ASM has not been reported. The plausibility of direct interactions of CD4⁺ T cells with ASM to an extent that could have a significant effect on airway remodeling may require that these cells be present in the ASM layer in sufficient numbers. However, to date, there appear to be no quantitative studies of the density of T cells in the ASM layer. Anecdotal observations suggest that they are relatively sparse in this location.

ASM remodeling in an equine model of asthma

The validity of the mechanisms of tissue remodeling in small animals for human disease needs to be determined, because it is conceivable that the rat and other small animals may have a higher intrinsic rate of tissue turnover. The results of studies of tissue remodeling in larger mammals are of interest in this regard. The horse has a condition called *heaves*, which is a spontaneous form of reversible airway obstruction and airway hyperresponsiveness caused by sensitization to constituents in moldy hay.¹¹² The disease behaves clinically like asthma and is responsive to bronchodilators and corticosteroids. It can result in persistent airflow obstruction, similar to chronic asthma. BAL demonstrates a neutrophilic inflammation, but there is a T_H2 pattern of cytokines expressed in the airways.¹¹³ Therefore, by several criteria, heaves is a pertinent model for human asthma.

Herszberg et al¹¹⁴ used standard morphometric techniques to examine lung tissues from horses with heaves for evidence of remodeling. Both the mass of ASM, as defined by smooth muscle α -actin immunoreactivity, and the density of PCNA⁺ cells within the smooth muscle layer of the airways were determined. The ASM mass was markedly increased (approximately 3-fold) compared with that in control horses, and the PCNA⁺ cells were almost 9 times as frequent. These findings confirmed that even in large mammals, hyperplasia of ASM can occur and may account for the increase in mass.¹¹⁴ The investigators did not attempt to address the issue of hypertrophy in this model.

ACTIONS OF β_2 -AGONISTS AND GLUCOCORTICOIDS ON GROWTH, PROLIFERATION, AND MIGRATION OF ASM

Regulation of cell cycle progression by β_2 -agonists

As described, the regulation of cell cycle progression by growth factors occurs through a complex set of signals that

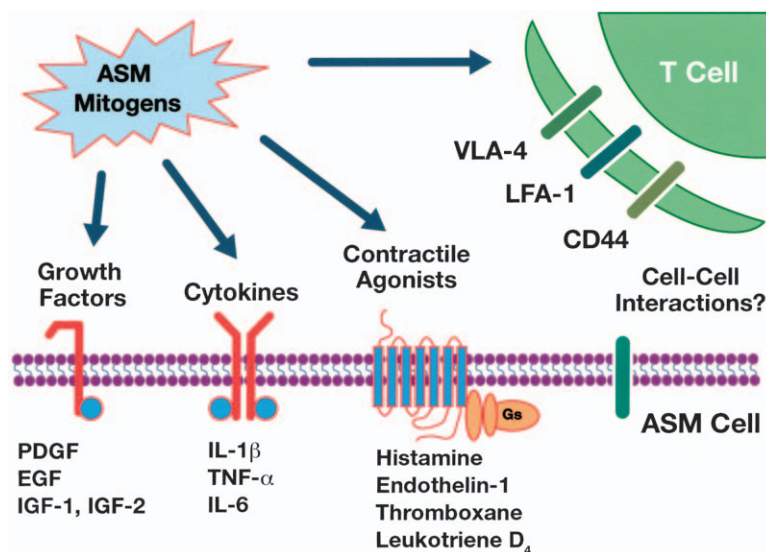


FIG 4. Hypothetical schema of the regulation of the ASM mass in asthmatic airways. *LFA*, Leukocyte function–associated antigen; *VLA*, very late activation antigen.

are closely regulated temporally and possibly spatially, including activation of ERK and PI3K and degradation of cdk inhibitors. Each of these key signaling cascades has been proposed as a target for regulation by asthma drugs (Fig 5).

β_2 -Agonists attenuate the DNA synthesis and increases in ASM cell number in response to a diverse range of mitogenic stimuli.¹¹⁵⁻¹¹⁹ This effect appears to be mediated via the β_2 -adrenergic receptor (β_2 AR), because it is blocked by both propranolol and the β_2 -selective antagonist ICI 118551 and is mimicked by several other β_2 -agonists.¹¹⁵ An increase in cAMP and hence in protein kinase A activity has been implicated in the antimitogenic action: cell-permeant cAMP analogues mimic the action of β_2 -agonists,¹¹⁶ and unrelated agents that activate GPCR coupled to adenylate cyclase through G_s , such as PGE₂¹¹⁶ and vasoactive intestinal peptide,¹²⁰ also reduce proliferative responses. The cAMP antagonist Rp-8-bromo-adenosine-3', 5'-cyclic monophosphate partially inhibits the action of salbutamol (albuterol), and the receptor-independent activation of adenylate cyclase with forskolin mimics the action of the β_2 -agonists.

It has been suggested that β_2 -agonists can activate CCAAT–enhancer binding protein- α , resulting in p21^{cip1} production, as do glucocorticoids.¹²¹ This cdk inhibitor reduces the phosphorylation of Rb by decreasing cyclin D1/cdk4 activity. The β_2 -agonists are able to inhibit ASM proliferation when added late in the G1 phase (to 18 hours). This suggests that one of the mechanisms is rapidly activated and therefore less likely to be explained by an increase in gene transcription.¹¹⁸

Thus, there are multiple points for regulation of ASM proliferation by β_2 -agonists. To date, there is no evidence that the effects of long-acting β_2 -agonists (LABAs) are different from those of short-acting β_2 -agonists (SABAs),

but it appears likely that the persistent stimulation of β_2 ARs would be important in the inflamed airway in contributing to suppression of growth responses, because stimuli for growth may be active for periods longer than the periods of perceptible airway obstruction. Therefore, such signals may not be subject to control by SABAs used as required.

Antiproliferative activity of glucocorticoids

Glucocorticoids, like β_2 -agonists, have antiproliferative activity against a broad spectrum of mitogens.¹²² The antiproliferative action occurs through binding to the glucocorticoid receptor, because it is attenuated by the transactivation antagonist RU486²³ and is mimicked by glucocorticoids but not mineralocorticoids.¹²² The glucocorticoids decrease the transcription and the translation of cyclin D1 and, consequently, reduce Rb phosphorylation, with the capacity to arrest the cell cycle progression late in the G1 phase.²³ Unlike the β_2 -agonists, the glucocorticoids have no effect on ERK activity, but they share a stimulatory effect on CCAAT–enhancer binding protein- α and increase p21^{cip1} under some conditions.¹²¹ Findings by Vlahos et al¹²³ suggest that p21^{cip1} is unlikely to be involved in the antimitogenic action of glucocorticoids when either thrombin or epidermal growth factor is used as a stimulus.

Influence of glucocorticoids on hypertrophy

Difficulties in assessing cellular growth as opposed to proliferation may have limited the interest in investigating the influence of β_2 -agonists and glucocorticoids on hypertrophy. Recent *in vitro* studies by Harris et al¹²⁴ using fluorescence-activated cell sorting revealed that glucocorticoids increased ASM cell size, whereas LABAs

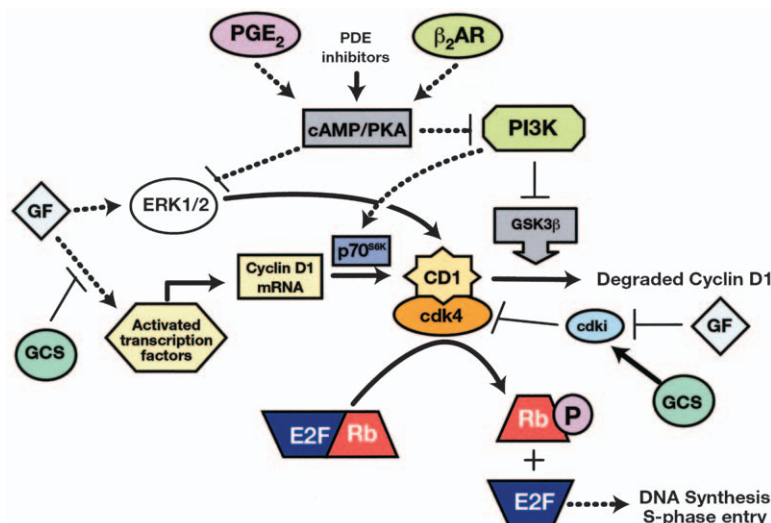


FIG 5. β_2 -Agonists decrease activation of PI3K and ERK, whereas glucocorticoids (GCS) interfere with pathways leading to an increase in cyclin D1 message levels and prevent the GF-induced decline in cdk inhibitor (cdki) levels. *GF*, Growth factor; *GSK*, glycogen synthase kinase; *PDE*, phosphodiesterase; *PKA*, protein kinase A.

had no effect alone, but prevented the trophic action of the glucocorticoids. More detailed studies on the mechanism underlying these changes are required to establish whether the increase in size is accompanied by other changes in cell phenotype. The unexpected observation of an increase in cell size in response to glucocorticoids raises issues about the extrapolation of results obtained in cell culture to the human asthmatic airway. It should be appreciated that the medium in which the cells are incubated is devoid of any glucocorticoid in the absence of fetal bovine serum. Furthermore, given that cells are cultured in no more than 10% fetal bovine serum, the amount of glucocorticoid would be well below physiological levels. Thus, when the effects of exogenous natural or synthetic glucocorticoids are investigated, the effects observed may relate to physiological replacement and could be considered trophic, rather than relating to pharmacologic actions that would be considered hypertrophic.

FACTORS LIMITING THE ANTIPROLIFERATIVE ACTIONS OF β_2 -AGONISTS AND GLUCOCORTICOIDS

It has become apparent that different mitogens are differentially sensitive to the action of the β_2 -agonists and glucocorticoids. In general, thrombin and other GPCR stimuli are more sensitive to the antiproliferative actions of either glucocorticoids or β_2 -agonists.¹²³ The effects of the glucocorticoids are influenced by concurrent exposure of the ASM to cytokines, such as IL-1, that induce PGE₂ synthesis via increasing the expression of COX-2.¹²⁵ Concurrent treatment of cells with cytokines can diminish the activity of glucocorticoids through competition between the antiproliferative effects of glucocorticoids

and the indirect consequences of the loss of induction of COX-2 and, therefore, the loss of the antiproliferative feedback of PGE₂. There is evidence that IL-1 β also reduces the actions of β_2 -agonists, because it reduces coupling between β_2 ARs and adenylate cyclase to reduce the amount of cAMP produced in response to β_2 -agonists¹²⁶ (Fig 6). PGE₂ has been implicated in the effect of cytokines on β_2 AR coupling to adenylate cyclase, and treatment with glucocorticoids would reverse this adverse consequence of cytokine exposure. It is impossible to predict whether the restoration of effectiveness of β_2 ARs outweighs the loss of the antiproliferative effect of PGE₂.

Because ASM proliferation may take place in the interstitium in a rigid and collagen-rich setting, Bonacci et al¹²⁷ have used a collagen-rich environment to re-examine the influence of both glucocorticoids and β_2 -agonists on proliferation. These experiments were performed on Flexcell plates that had a flexible silastic base precoated by the manufacturer with a variety of extracellular matrix components, including collagen and laminin. Stretch of ASM can be achieved by applying subatmospheric pressure to the underside of the 6-well tissue culture plate mounted in a vacuum manifold. Although some studies simulating the asthmatic airway have explored the activity of very high levels of stretch based on the smooth muscle cells contracting to generate strain,¹²⁸ Bonacci et al¹²⁷ reasoned that the fibrosis in the airway would likely reduce the level of stretch applied to the ASM. Indeed, an investigation of airway distensibility measuring the lung volume-dependent increase in anatomic dead space by using a nitrogen washout technique revealed that there was a progressive loss of distensibility with increasing asthma severity. The investigation also revealed that in some patients with severe asthma, there was no

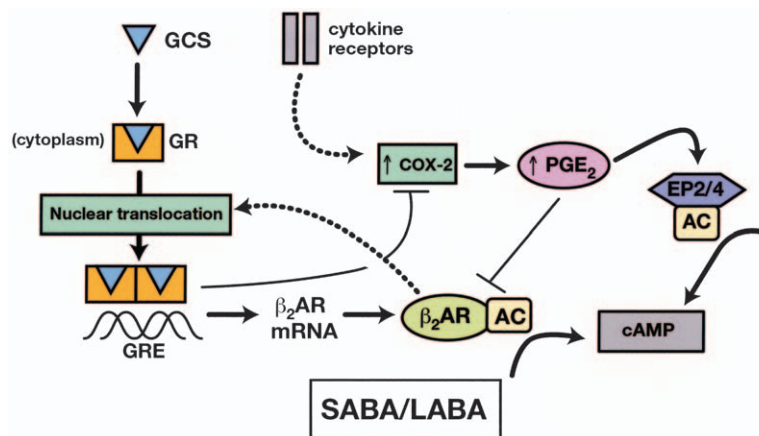


FIG 6. Interactions between glucocorticoids (GCS) and β_2 -agonists in ASM. GCS increase transcription of β_2 AR mRNA and diminish COX-2 induction, limiting the production of PGE₂, which has opposing effects on cAMP: direct stimulation through specific prostaglandin EP2/EP4 receptors and indirect suppression through uncoupling of β_2 AR from adenylate cyclase (AC). GR, Glucocorticoid receptor; GRE, glucocorticoid response element.

measurable distensibility.¹²⁹ Thus, a healthy airway was simulated by culture on laminin, and an asthmatic airway was simulated by culture of ASM on collagen I in the absence of any stretch. Collagen I increased the rate of proliferation and rendered the cells resistant to the antiproliferative action of glucocorticoids¹²⁷ but not β_2 -agonists (Bonacci et al, unpublished data, July 2003). The impairment of glucocorticoid action is not a result of a defect in the glucocorticoid receptor, because it translocates to the nucleus in response to dexamethasone. Moreover, under the same conditions, glucocorticoids maintain the capacity to reduce the production of granulocyte macrophage colony-stimulating factor, suggesting that at least some of the biochemical responses to the glucocorticoids are intact under these conditions. The impairment of glucocorticoid actions is reversed by blocking $\alpha 2\beta 1$ integrin function,¹³⁰ raising the possibility that focal adhesion kinase or integrin-linked kinase mediates the effects of the collagen extracellular matrix, because these kinases are known to link integrin receptor activation to cellular responses.¹³¹

Interactions in regulation of antiproliferative actions of β_2 -agonists and glucocorticoids

The theoretical basis of synergy between β_2 -agonists and glucocorticoids (Fig 6) rests on observations that the latter class of agents increases expression of β_2 ARs, whereas the β_2 -agonists facilitate nuclear translocation of the glucocorticoid receptor.¹³² In practice, such interactions may become important in 2 different situations.

First, it would be expected that each type of interaction would increase the potency of the other agent, because a smaller fractional receptor occupation should be required to achieve the same levels of receptor stimulation and response. This kind of interaction would be detected as a mutual increase in potency. The laborious nature of many of the cell biology assays that examine the effects of the β_2 -agonists and glucocorticoids *in vitro* by using cultured human ASM has often precluded extensive

examination of the concentration-response relationship. Analyses of interactions are based on 1 concentration or just a few discrete concentrations of each agent, alone and in combination. Nevertheless, there is evidence of increases in potency. For example, subthreshold concentrations of the LABA formoterol are rendered antiproliferative after concurrent exposure to threshold concentrations of budesonide, although the effect size appears to be small.¹²¹ Whether such interactions are important in the clinical setting depends on the relationship between the concentrations used *in vitro* and those likely to be attained after coadministration by a metered dose inhaler. Local airway concentrations are difficult to estimate, but they can be presumed to be higher than those determined in plasma. It would appear that interactions between budesonide and formoterol occur at concentrations that are as much as 2 orders of magnitude lower than those achieved in plasma¹²¹ and are, therefore, unlikely to be of clinical significance in compliant patients. On the other hand, when doses are missed and concentrations decline, such interactions may sustain beneficial actions until the next dose is administered.

Investigations by Stewart et al¹³³ have revealed that the maximum response of fluticasone propionate against thrombin-induced proliferation is increased when it is combined with the LABA salmeterol. This second kind of interaction may be important in achieving more complete control of the remodeling process. A biochemical basis for this action has not been elucidated, but the increase in maximum effect is not surprising given that several actions are not shared between these 2 classes of agents and summation of the consequences of these distinct actions should result in an increased effect.

Effects of asthma drugs on ASM migration

Agents that elevate cAMP, including salmeterol, reduce spontaneous migration.⁸⁴ Glucocorticoids also regulate migration induced by PDGF, which is inhibited to a greater

extent by combinations of glucocorticoid and β_2 -agonist.⁸⁴ Urokinase-enhanced PDGF-induced migration is also reported to be sensitive to inhibition by agents that increase cAMP levels, including PGE₂.⁸⁷ Other studies have shown that PDGF-induced migration can be enhanced by cysteinyl leukotrienes and that cysteinyl leukotriene 1 receptor antagonists can reduce the component of the enhanced response caused by cysteinyl leukotrienes, but not the response to PDGF.⁷²

SUMMARY AND IMPLICATIONS FOR FUTURE RESEARCH

Significant overlap exists between the groups of mediators and candidate intracellular signaling intermediates that drive both the proliferation and migration of cultured ASM cells. In recent years, substantial progress has been made, and key signaling events regulating ASM growth have been elucidated. PI3K and ERK have emerged as major, but independent, signaling pathways required for ASM cell proliferation induced by a wide range of mitogens and appear to be well conserved across species. The transduction mechanisms for migration may turn out to be similarly conserved, but our current knowledge of the sequence of events important for ASM migration is rudimentary in comparison, although activation of the PAK1/p38 MAPK/HSP27-dependent pathway appears to be critical.

The majority of available studies have used cell culture-based systems to characterize the growth and migratory responses of ASM. Much needs to be learned concerning the extent to which either of these processes contributes to the overall accumulation of smooth muscle content in the intact remodeled airway in asthma. Understanding the molecular signaling events controlling proliferation and migration represents an essential goal for identifying target molecules that can be exploited to limit remodeling of ASM in asthma.

It is unknown whether intimate contact between the CD4⁺ T cell and ASM is necessary, but it is possible that cell to cell contact occurs and leads to ASM proliferation, as has been shown *in vitro*.¹³² It is also possible that cytokines such as IL-13 facilitate growth by enhancing the responsiveness of the ASM to the actions of mediators such as cysteinyl leukotrienes.¹³⁴ Somewhat problematic is the fact that few studies have shown that ASM proliferation occurs *in situ* in asthma.¹⁴ However, there are several possible reasons for failure to make such observations. Subjects may have been adequately treated with antiasthmatic medications before biopsy, or ASM may be recruited by migration of myofibroblasts in the subepithelial region. Stem cells in the blood might also be a source of new muscle.¹³⁵ Perhaps the rate of tissue turnover in asthmatic subjects is relatively slow, and therefore, any increases in the rate of proliferation may be small and hard to detect. Reduction in the rate of apoptosis is an alternative mechanism for an increase in ASM mass. All of these possibilities will need to be explored to resolve

the issue of whether ASM grows from existing bundles of muscle or is added from other sites. The therapeutic strategies that might ultimately be used to prevent airway remodeling will depend on the elucidation of the biology of the process.

Because PI3K is clearly involved in migration and proliferation, it might be a useful target for antiremodeling agents. However, the obvious problem with targeting key intracellular signaling kinases such as PI3K is the breadth of potential adverse effects. The need for novel agents that effectively control remodeling is evident in patients with severe asthma, who are almost invariably treated with high doses of oral and inhaled glucocorticoids. It is not yet clear whether the relatively recent addition of LABA to the therapeutic regimen for moderate to severe asthma will have a greater influence on the level of chronic remodeling in these patients than use of glucocorticoids and SABA.

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