Published in final edited form as:

Immunobiology. 2019 July ; 224(4): 490–496. doi:10.1016/j.imbio.2019.05.006.

Airway smooth muscle cells are insensitive to the anti-proliferative effects of corticosteroids: The Novel Role of Insulin Growth Factor binding Protein-1 in Asthma

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Abstract

Airway remodeling in asthma manifests, in part, as enhanced airway smooth muscle (ASM) mass, due to myocyte proliferation. While the anti-proliferative effects of glucocorticoid (GC) were investigated in normal ASM cells (NASMC), little is known about such effects in ASM cells derived from asthma subjects (AASMC). We posit that GC differentially modulates mitogen-induced proliferation of AASMC and NASMC. Cells were cultured, starved, then treated with Epidermal growth factor (EGF) (10 ng/ml) and Platelet-derived growth factor (PDGF) (10 ng/ml) for 24 hr and/or fluticasone propionate (FP) (100 nM) added 2 hr before. Cell counts and flow cytometry analyses showed that FP failed to decrease the cell number of and DNA synthesis in AASMC irrespective of mitogens used. We also examine the ability of Insulin Growth Factor Binding Protein-1 (IGFBP-1), a steroid-inducible gene that deters cell growth in other cell types, to inhibit proliferation of AASMC where FP failed. We found that FP increased IGFBP1 mRNA and protein levels. Interestingly, the addition of IGFBP1 (1 µg/ml) to FP completely inhibited the proliferation of AASMC irrespective to the mitogens used. Further investigation of different signaling molecules involved in ASM growth and GC receptor functions (Protein kinase B (PKB)/AKT), Mitogen-activated protein kinases (MAPKs), Focal Adhesion Kinase (FAK)) showed that IGFBP-1 selectively decreased mitogen-induced p38 phosphorylation in AASMC. Collectively, our results show the insensitivity of AASMC to the anti-proliferative effects of GC, and

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demonstrate the ability of IGFBP1 to modulate AASMC growth representing, hence, a promising strategy to control ASM growth in subjects with GC insensitive asthma.

Keywords

asthma; glucocorticoid insensitivity; airway smooth muscle; proliferation; growth; airway remodeling

Introduction

Asthma is a chronic airway inflammatory disease characterized by shortness of breath, chest tightness, coughing, and wheezing [1]. While inhaled glucocorticoids (GC) are the most commonly used treatment in asthma, some patients with severe asthma are insensitive to GC therapy [2]. Consequently, such patients contribute disproportionately to the health care costs and morbidity associated with asthma [3]. Hence, significant research efforts aiming at defining the mechanism(s) underlying GC insensitivity, more importantly, strategies that reestablish GC responsiveness are needed.

Studies showed that patients with severe asthma benefited from bronchial thermoplasty, a therapy that attenuates bronchoconstriction by reducing airway smooth muscle (ASM) mass; this finding and that of increased ASM mass in bronchial biopsies obtained from patients with asthma [4, 5] supports the notion that ASM is a critical player in the pathogenesis of asthma [6, 7]. Mitogens such as Platelet-Derived Growth Factor (PDGF) and Epidermal Growth Factor (EGF) modulate ASM growth [8, 9]. The molecular mechanisms modulating ASM cell proliferation involve numerous signaling pathways. For example, to promote cell growth, PDGF and EGF bind to receptors with intrinsic tyrosine kinase activity (RTK) (reviewed in [10]) and promote the activation of serine-threonine kinases AKT and mitogen-activated protein kinases (MAPKs). Ultimately, via the phosphorylation of other intermediate molecules, the upregulation of cyclin D1 facilitates ASM cells transition through the cell cycle [10]. Interestingly, the ability of some mitogens to promote growth of normal ASM cells is sensitive to the anti-proliferative effects of GC, although differentially with thrombin being more sensitive to GC than EGF [9, 11, 12]. Yet, little is known about the anti-proliferative effects of GC on ASM cells derived from asthma subjects.

GC acts through different molecular mechanisms of action. The two most well-described are transrepression and transactivation [13]. When GC binds to its receptor in the cytoplasm, the GC receptor (GR) is released from heat shock protein (HSP)-90 complex to translocate to the nuclear compartment of the cell. Transrepression occurs when the GR binds to the cis-element of inflammatory transcription factors such as activating protein-1 (AP-1) and nuclear factor κ B (NF- κ B) to inhibit the expression of pro-inflammatory genes. Transactivation occurs when the GR binds to the GC response element (GRE) on the promoter of GC inducible genes, such as MAPK phosphatase 1 (MKP-1) [14], glucocorticoid-induced leucine zipper (GILZ) [15], and insulin-like growth factor binding protein-1 (IGFBP-1) [16-19] to induce their transcription. Importantly, while the role of MKP-1 and GILZ in mediating GC effects has been extensively investigated, the role of IGFBP-1 in mediating GC effects has been poorly investigated.

IGFBP1 has IGF1-dependent and -independent activities. IGF1-dependent activity of IGFBP1 involves its binding to IGF1 and subsequently modulates IGF1 cellular actions [20]. IGF1-independent activities involve IGFBP-1's RGD (Arg-Gly-Asp) motif present in the C-terminal domain. RGD is a ligand of integrin $\alpha 5 \beta 1$ that acts independently of IGF receptors [21]. Several studies investigated the role of IGFBP1 in modulating cell growth. For instance, IGFBP-1 decreases the growth of breast cancer cells in response to estrogen and serum [22] and the thymidine uptake in rat aortic smooth muscle cells [23]. Evidence suggests that IGFBP-1 downregulates the expression of key proteins associated with cell growth such as ERK and AKT in fibroblasts [24]. However, the expression and the role of IGFBP-1 in modulating ASM cell responses remains unknown.

Because increased ASM mass in asthma is a critical component of asthma pathogenesis, therapeutic strategies aiming at reducing ASM cell growth will likely improve the therapeutic outcome. The current study demonstrates that anti-proliferative effects of GC in ASM cells is impaired in cells derived from asthma subjects but restored in the presence of IGFBP1, a steroid inducible gene. Thus, targeting IGFBP1 represents a promising strategy to deter GC-insensitive features in asthma.

Materials and Methods

Cell Culture

Primary human bronchial ASM cells derived from asthma (AASMC) and non-asthma (NASMC) subjects were bought from Lonza (Walkersville, MD). Briefly, Human Bronchial Smooth Muscle Cells (BMSC) were isolated from the major bronchial tissue of normal or mild asthmatic donors and used from passage 2 onward. All cells were tested negative for mycoplasma, bacteria, yeast, and fungi. HIV-1, hepatitis B and hepatitis C were not detected in all donors. Cells were then cultured as we previously described [25, 26]. Cells between passages 3 and 7 were used.

Cell Count

ASM cells growth-arrested in serum free media for 48 hr were treated with EGF 10 ng/ml and PDGF 10 ng/ml for 24 hr in the presence or absence of Fluticasone Propionate (FP, 100 nM) added two hours prior treatments with growth factors. IGFBP-1 (1 μ g/ml) was administered one hour before FP to assess its potential modulatory effect on the anti-proliferative effects of FP. After that, cells were trypsinized, pelleted by centrifugation, and suspended in a 15 ml conical tubes. Next, 10 μ l of suspended cells were mixed with 10 μ l of Trypan Blue stain (Life Technologies, Frederick, MD) and loaded onto chamber slides. The Countess II automated cell counter (Life Technologies) was used to perform cell count of viable cells. Of note, IGFBP-1 used at 1 μ g/ml did not affect the viability of ASM cell viability as assessed by the MTT assay (data not shown).

Bromodeoxyuridine (BrdU) Incorporation

ASM cells were treated as above. To measure DNA synthesis, BrdU (GE Healthcare, Mickleton, NJ) was added to culture media for 18 hr before cells were trypsinized and pelleted by centrifugation. To denature DNA, cells were exposed to 2N Hydrochloric Acid

(HCl) for 30 min in 37°C water bath. Next, cells were neutralized with 0.1 M Sodium Tetraborate and washed with Immunofluorescence Antibody Assay (IFA) buffer supplemented with HEPES Saline, Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, Utah), and Sodium Azide (Sigma-Aldrich, St. Louis, MO). After that, cells were incubated with anti-BrdU fluorescence conjugated secondary antibody (BD Biosciences, San Jose, CA). Tween 20/IFA buffer at 0.5% was then used to wash the cells before they were pelleted by centrifugation and re-suspended in solution of Propidium Iodide and RNase (Sigma) for Flow Cytometric analysis on FACScalibur (BD Biosciences).

Immunoblot Analysis

Growth-arrested ASM cells were exposed to FP (100 nM), then total proteins were extracted. Immunoblot analyses were conducted as we reported previously [25, 26] using IGFBP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For normalization purposes, an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) was used to stain the corresponding stripped nitrocellulose membrane.

In parallel experiments, growth-arrested AASMC were treated with PDGF 10 ng/ml for 24 hr in and/or FP (100 nM) and/or of IGFBP-1 (1 µg/ml) added 2 and 3 hr before, respectively, then total proteins were extracted. Immunoblot analyses were conducted as we reported previously [25, 26] using several phosphorylated antibodies against focal adhesion kinase (FAK), AKT, p38, ERK, c-Jun N-terminal kinase (JNK) (Cell signaling Technologies, Danvers, MA). For normalization purposes, anti-total FAK, AKT, p38, ERK1/2, or JNK (Santa Cruz Biotechnology) antibodies were used to stain the corresponding stripped nitrocellulose membrane. A quantification of the intensity of the western blot band signals was then conducted as we previously reported using ImageJ image processing software (National Institutes of Health, Bethesda, MD) [25, 26].

Real-time PCR

Growth-arrested ASM cells were exposed to FP (100 nM) for different time points, then mRNA was extracted. Real-time RT-PCR experiments were conducted as we formerly reported [25, 26] using predesigned primers for IGFBP-1 (Forward sequence CCAAGGCACAGGAGAGACATCA and Reverse AGGGTAGACGCACCAGCAGA) (Sigma) and previously published primers for β -actin (Sigma) [25, 26]. The data were analyzed using the comparative cycle threshold method as we previously reported [25, 26].

Materials and Reagents

Tissue culture reagents were purchased from Lonza. Human recombinant IGFBP-1 protein was obtained from R&D systems (Minneapolis, MN). IGFBP-1 antibody, EGF, and FP were provided by Sigma. PDGF was obtained from Millipore (Billerica, MA).

Statistics Analysis

Intragroup differences were analyzed using ANOVA (Bonferroni-Dunn test) or t test. A P value < 0.05 was considered significant. Each set of experiments was performed in triplicate with a minimum of three different human ASM cell lines with “n” corresponding to the number of donors.

Results

Mitogens and FP differentially modulate ASM proliferation

First, we sought to examine GC effects on the ability of mitogens to increase NASMC number. As shown in Fig. 1A, PDGF and EGF significantly increased ASM cell number by $35\% \pm 3.2\%$ and $32\% \pm 2.9\%$ over basal, respectively. Interestingly, while FP significantly decreased by 50% PDGF-induced increase in ASM cell number, EGF-induced mitogenesis was unaffected. In AASMC (Fig. 1B), PDGF and EGF significantly increased ASM cell number by $25\% \pm 2.1\%$ and $20\% \pm 1.8\%$ over basal, respectively. Surprisingly, FP had little effect on AASMC number irrespective of the mitogens used. Collectively, these findings indicate that *mitogens and GC differentially modulate ASM proliferation and that the ability of PDGF to promote ASM hyperplasia is insensitive to GC in cells obtained from patients with asthma.*

Mitogens and FP differentially modulate ASM DNA synthesis

Next, we sought to examine GC effects on the ability of different mitogens to increase DNA synthesis in NASMC using BrdU incorporation assays. Flow cytometry analysis showed that PDGF and EGF significantly increased BrdU incorporation (Fig. 2A) by $28\% \pm 2.5\%$ and by $25\% \pm 2.1\%$ over basal, respectively. While the addition of FP significantly decreased by 44% PDGF ability to increase DNA synthesis, it had no significant effect on EGF-induced DNA synthesis. We also examined the effect of GC on the ability of different mitogens to increase DNA synthesis in AASMC. As shown in Fig. 2B, the addition of mitogens significantly increased DNA synthesis in AASMC (PDGF by $37\% \pm 3.1\%$ and EGF by $32\% \pm 2.7\%$). Surprisingly, not only FP did not decrease but rather significantly increased DNA synthesis in AASMC irrespective of the mitogen used (PDGF by +30% and EGF by +35%). Collectively, these findings indicate that the failure of FP to suppress PDGF-induced increase cell number (Fig. 1B) may derive from its ability to increase DNA synthesis (Fig. 2B).

IGFBP1 is induced by GC in ASM cells

Since ASM growth is insensitive to GCs in AASMC (Fig. 1B and 2B), we sought to explore strategies to reduce ASM cell growth. Previous studies reported that IGFBP-1, a GC-inducible gene in other cell types [16-19], modulates cell proliferation in a cell-specific manner [13, 20, 21, 23]. As shown in Fig. 3A and 3B, FP significantly increased, in a time-dependent manner, the proteins and mRNA expression levels of IGFBP-1. Together, these findings indicate that IGFBP-1 is also a GC-inducible gene in ASM cells.

In combination, IGFBP1 and FP inhibit the proliferation of GC-resistant AASMC

In the following experiments, IGFBP1 was used at 1 $\mu\text{g/ml}$, a concentration shown to significantly interfere with the growth of aortic smooth muscle cells [23]. Interestingly, while FP or IGFBP1 alone had no effect on a mitogens-induced increase in AASMC number, combining both IGFBP-1 to FP significantly decreased PDGF and EGF ability to increase ASM cell number by 67% and 53%, respectively (Fig 4A). Similarly, when DNA synthesis was measured by BrdU incorporation assay, while FP or IGFBP1 alone had no

effect on a mitogens-induced increase in DNA synthesis, the combination of IGFBP-1 with FP abrogated mitogens' ability to increase DNA synthesis in AASMC (Fig. 4B). Collectively, these findings demonstrate the ability of exogenous IGFBP-1 to restore the inhibitory effect of fluticasone in AASMC.

IGFBP1 does not affect FAK phosphorylation in AASMC.

Since IGFBP1 modulation of cancer cells' growth involves FAK inhibition [27, 28], we next assessed the phosphorylation of FAK in AASMC by immunoblot analysis. As shown in Fig. 5, no basal phosphorylation of FAK was detected in AASMC. While the addition of PDGF significantly increased FAK phosphorylation, the addition of FP and IGFBP1 either alone or in combination had no effect on FAK phosphorylation. These findings strongly suggest that IGFBP1 modulates AASMC growth in FAK-independent manner.

IGFBP1 had little effect on the phosphorylation of kinases commonly associated with AASMC growth

Different kinases have been associated with ASM growth including ERK1/2 and PI3K/AKT pathways [29-32]. As shown in Fig. 6A, phosphorylation of AKT was not detected in untreated AASMC but significantly increased by PDGF, a response not affected by the addition of FP and IGFBP1 either alone or in combination. Similarly, low basal phosphorylation of ERK1/2 was detected in AASMC (Fig. 6B). While the addition of PDGF significantly increased ERK1/2 phosphorylation, the addition of FP and IGFBP1 either alone or in combination did not affect ERK1/2 phosphorylation (Fig. 6B). Together, these data suggest that IGFBP1 modulates AASMC growth in AKT/PI3K and ERK1/2-independent manner.

IGFBP1 differentially affects the phosphorylation of kinases commonly associated with GR function in AASMC

Since IGFBP1 did not affect the phosphorylation of kinases commonly associated with ASM growth, we next sought to determine whether IGFBP1 enhances GC actions by inhibiting kinases known to negatively regulate GR functions such as JNK [33, 34] and p38 [25]. As shown in Fig. 7A, no basal phosphorylation of JNK was detected in AASMC. While the addition of PDGF significantly increased JNK phosphorylation, the addition of FP and IGFBP1 either alone or in combination did not affect JNK phosphorylation. Interestingly, while low phosphorylation of p38 was detected in AASMC, the addition of IGFBP1 abrogated basal p38 phosphorylation irrespective of FP treatment (Fig. 7B, lane 3 and 4 versus lane 1). Similarly, IGFBP1 either alone (Fig. 7B, lane 7 versus lane 5) or in combination with FP (Fig. 7B, lane 8 versus lane 5), significantly decreased PDGF-induced p38 phosphorylation (by 76% and 94%, respectively). Collectively, these findings indicate that IGFBP1 differentially affects the phosphorylation of kinases commonly associated with GR function in AASMC.

Discussion

Evidence suggests ASM growth as a critical contributing factor in airway remodeling and narrowing in asthma [4, 5]. In the present study, we showed that mitogen-induced cell

growth in AASMC was insensitive to GC actions. Interestingly, the addition of exogenous IGFBP1 to fluticasone restored its ability to inhibit cell growth suggesting a new novel therapeutic strategy to treat the main GC insensitive feature contributing to airway remodeling.

In our study, we used both normal and asthmatic cells to introduce the new concept that specific differences with regard to response to growth factors are dependent on health status of the subjects. Indeed, when EGF was used as a stimulus, responses of ASM cells were insensitive to GC effects irrespective of ASM cells origin. However, when PDGF was used as a stimulus, the responses of ASM cells were sensitive to GC only in ASM from non-asthmatic subjects but not in ASM cells from asthmatic subjects. Hence, PDGF stimulation was used in the rest of study as a model to examine the differences in steroid responsiveness between ASM cells from healthy (sensitive) versus asthmatics (insensitive) and to explore ways to restore steroid responsiveness in PDGF-treated cells from asthmatics. In the case of EGF, we believe that EGF is interfering somehow with GR signaling thus explaining why GC failed to modulate responses of ASM cells from both asthmatic and non-asthmatic subjects. Whether this is because the cells from nonasthmatic subjects are abnormal or because EGF is naturally insensitive to GC warrants further investigation.

Steroid responsiveness in asthma varies according to the stage of the disease. For instance, mild persistent asthma are typically sensitive to the steroid anti-inflammatory actions unlike severe persistent asthma that is often insensitive [35]. Steroid responsiveness in asthma is usually assessed by examining changes in immunological/inflammatory markers [35] but does not apply to non-immunological parameters such as remodeling features which are often insensitive to steroid therapy even in mild asthmatics. We here show that the proliferation of ASM cells derived from mild asthmatics were insensitive to fluticasone when compared to cells derived from healthy subjects. Perry and colleagues (2014) also showed cell growth induced by another mitogen (TGF β) was also insensitive to dexamethasone in ASM cells derived from mild asthmatics [36]. Interestingly, using the same cells, the authors showed TGF β -induced cytokine secretion was still sensitive to steroid actions [36].

Interestingly, we found that GC enhanced PDGF-induced DNA synthesis in AASMC (Fig. 2B). Misior and colleagues (2008) also found that GC dramatically increase growth of healthy ASM cells treated with EGF in the presence of cytokines (IL1 β and/or TNF α) [39] or treated with bFGF and cytokine combination [40]. The underlying mechanisms affecting the anti-mitogenic action of GC action in ASM cells remain unknown, but could be due to a decrease expression of CCAAT/enhancer binding protein (C/EBP) alpha previously suggested by others [37]. Evidence from cancer cell lines suggest the implications of kinases such as GSK3 β which can phosphorylate GR at key residues and directly impairs GC actions [38]. Whether the activation of such kinases in AASMC interferes with GC anti-proliferative functions merits further investigation.

Several earlier reports showed IGFBP1 is a GC-target/inducible gene in different cell types, in part due to several GRE sites present in the IGFBP1 promoter [16-19]. In addition to being increased *in vitro* by GC in U2OS/A549 cell lines [43] or hepatocytes [16], increased

systemic levels can also be detected in the bloodstream of infants [41] or in the serum of rats following oral dexamethasone treatment [42]. Our report is the first to show that GC rapidly enhanced the expression of IGFBP1 in ASM cells (within 4 hours), as a result of increased gene transcription (Fig. 3). We also showed that while exogenous IGFBP1 alone had little effect on AASMC proliferation, treating cells with both IGFBP1 and FP restored the anti-mitogenic action of FP as evidenced by the profound inhibition of both cell number and BrDU incorporation (Fig. 4).

Although previous reports showed IGFBP1 presented anti-proliferative or mitogenic actions, these effects were highly dependent on the cellular context [45]. In endometrial stromal cells and chick embryo fibroblasts, IGFBP1 inhibited EGF and serum-induced DNA synthesis [46, 47] while in breast cancer cell lines and arterial smooth muscle cells, IGFBP1 suppressed cell proliferation [22, 23]. Conversely, IGFBP1 also demonstrated pro-mitogenic actions as IGFBP1 knockdown was associated with a reduced prostatic cell proliferation [48]. Similarly, hepatic IGFBP1 acts as a pro-survival factor by protecting the liver from apoptosis [49]. Together these findings suggest that IGFBP1 effects on growth is cell-specific and exhibits strong anti-proliferative effects in steroid-insensitive ASM cells when combined with GC via mechanisms that remain to be investigated.

Evidence from cancer cell lines suggest that IGFBP-1 binds directly to the cell surface $\alpha 5 \beta 1$ integrins [27, 28] and inhibit the phosphorylation of FAK and cell growth [50]. Interestingly, in ASM cells, fibronectin has been show to increase PDGF effects on cell growth through $\alpha 5 \beta 1$ integrins [51]. However, we here show that IGFBP1 did not affect PDGF-induced FAK phosphorylation in AASMC irrespective of GC treatment (Fig. 5) suggesting that IGFBP1 modulates ASM growth in a FAK-independent manner.

We therefore focused on other signaling pathways essential in the control of human ASM cell growth such as ERK1/2 [32]. In contrast to what reported in skin fibroblasts where IGFBP1 clearly inhibited ERK1/2 phosphorylation [24], no effect of IGFBP1 on ERK1/2 phosphorylation was seen in PDGF-treated AASMC in the presence or absence of GC (Fig. 6B). Interestingly, the mechanisms underlying the effects of mitogens on ASM growth differ in AASMC versus NASMC [30]. While in NASMC, both PI3K/AKT and ERK1/2 kinases equally mediate mitogens effects on growth [29, 31], in AASMC, however, it was shown that PI3K/AKT predominated over ERK1/2 pathways in mediating mitogenic signals [30]. Despite a report showing that IGFBP1 can inhibit AKT phosphorylation in fibroblasts [24], we failed to see any inhibitory effect of IGFBP1 on AKT activation (downstream of PI3K) irrespective of GC treatment (Fig. 6A) supporting the novel observation that IGFBP1 can enhance the inhibitory action of GC in ASM by involving both ERK1/2- and AKT-independent mechanisms.

Different kinases negatively regulate GR function. The activation of JNK, for instance, interferes with GR nuclear translocation [34] and high levels of JNK phosphorylation occurs in steroid resistant inflammatory cells [33]. Similarly, the activation of p38 also impairs GR function and promotes steroid insensitivity not only in immune cells [53, 54] but also in ASM cells [25]. We previously showed that p38 inhibition enhanced GR-mediated transactivation activities in ASM cells [25]. While the addition of IGFBP1 did not affect

mitogen-induced JNK phosphorylation irrespective of GC treatment (Fig. 7A), it dramatically inhibited mitogen-induced p38 phosphorylation in the presence of GC (Fig. 7B). Since the role of p38 in PDGF and EGF-mediated ASM cells growth is minimal [32] and p38 negatively regulates GR function in ASM cells [25], our result suggests that IGFBP1 indirectly increases GR function by inhibiting p38 activation; consequently the enhanced anti-proliferative effects of GC in the presence of IGFBP1 (Fig. 4) may require the inhibition of p38 activation (Fig. 7B), a hypothesis we are currently exploring.

In conclusion, our study demonstrates that ASM cells obtained from mild asthmatics are insensitive to the anti-proliferative actions of GC. Our study also demonstrates the antiproliferative action of GC can be restored in the presence of IGFBP1 via FAK/AKT/ERK-independent but p38-dependent mechanisms. Further understanding of IGFBP1 signaling may prompt novel insights into the design of novel therapies aimed at treating GC resistant remodeling features in asthma.

Acknowledgments

Funding

This research study was funded by NIH grants R01HL111541 (O. Tliba) and HL2 P01HL114471 (R.A.P.).

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Fig. 1: Mitogen-induced increases in ASM cell number is differentially modulated by GC.

(A) NASMC and (B) AASMC were exposed to PDGF (10 ng/ml) or EGF (10 ng/ml) for 24 hr and/or FP (100 nM) added 2 hr before. Cell count was measured as described in material and methods section. Results are presented as % of increase over basal. * $P < 0.05$ when compared to cells treated with diluent, δ $P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens. Each set of experiments was performed in triplicate with a minimum of three different human ASM cell lines.

Fig. 2: Fluticasone failed to inhibit mitogen-induced increase in BrdU incorporation in AASMC. (A) NASMC and (B) AASMC were exposed to PDGF (10 ng/ml) or EGF (10 ng/ml) for 24 hr and/or FP (100 nM) added 2 hr before. BrdU was added for 18 hr and its incorporation was measured as described in the material and methods section. Results are presented as % of increase over basal. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, δ $P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens. Each set of experiments was performed in triplicate with a minimum of three different human ASM cell lines.

Fig. 3: Fluticasone increased the expression of IGFBP1 in ASM cells.

Growth-arrested ASM cells were exposed to FP (100 nM) for different time points. (A) Total mRNA was extracted and IGFBP1 mRNA expression was assessed by real-time PCR.

IGFBP1 mRNA levels were then normalized to that of GAPDH and are presented as fold increase over basal. Data are representative of three separate experiments. (B) Whole cell lysates were extracted and immunoblotted with anti-IGFBP1 and GAPDH specific antibodies. Results are representative of three separate blots (*top*). Scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding GAPDH content. The results are expressed as the fold increase over basal values (*bottom*). * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent.

Fig. 4: The addition of IGFBP1 to glucocorticoid inhibited mitogen-induced proliferation of AASMC.

(A) AASMC were exposed to PDGF (10 ng/ml) or EGF (10 ng/ml) for 24 hr and/or FP (100 nM) and/or IGFBP1 (1 µg/ml) added 2 hr and 3 hr before, respectively. Cell count was assessed as described in material and methods section. Cell count was compared with that of obtained from cells treated with diluent alone (basal). Results are presented as % of increase over basal. (B) AASMC were treated as above and BrdU was added for 18 hr and its incorporation was measured as described in the material and methods section. DNA synthesis was compared with that of obtained from cells treated with diluent alone (basal). Results are presented as % of increase over basal where basal. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, $\delta P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens.

Fig. 5: The addition of IGFBP1 to glucocorticoid did not affect mitogen-induced FAK phosphorylation in AASMC.

Top, AASMC were exposed to PDGF (10 ng/ml) for 24 hr and/or FP (100 nM) and/or IGFBP1 (1 µg/ml) added 2 hr and 3 hr before, respectively. The whole cell lysate was then extracted and immunoblotted with anti-phospho-FAK and total FAK antibodies. Results are representative of three separate blots. *Bottom*, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding total FAK content. The results are expressed as the fold increase over basal values. ** $P < 0.01$ when compared to cells treated with diluent, NS: not significant when compared to cells exposed to mitogens.

Fig. 6: The addition of IGFBP1 to fluticasone did not affect mitogen-induced AKT and ERK1/2 phosphorylation in AASMC.

Top, AASMC were exposed to PDGF (10 ng/ml) for 24 hr and/or FP (100 nM) and/or IGFBP1 (1 µg/ml) added 2 hr and 3 hr before, respectively. Whole cell lysate was then extracted and immunoblotted with antibodies against phospho-AKT and total AKT (A) or phospho-ERK1/2 and total ERK1/2 (B). Results are representative of three separate blots.

Bottom, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding total AKT (A) or ERK1/2 (B) content. The results are expressed as the fold increase over basal values. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, NS: not significant when compared to cells exposed to mitogens.

Fig. 7: The addition of IGFBP1 to glucocorticoid differentially modulate mitogen-induced JNK and p38 phosphorylation in AASMC.

Top, AASMC were exposed to PDGF (10 ng/ml) for 24 hr and/or FP (100 nM) and/or IGFBP1 (1 µg/ml) added 2 hr and 3 hr before, respectively. Whole cell lysate was then extracted and immunoblotted with antibodies against phospho-JNK and total JNK (A) or phospho-p38 and total p38 (B). Results are representative of three separate blots. *Bottom*, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding total JNK (A) or p38 (B) content. The results are expressed as the fold increase over basal values. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, $\delta P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens.