GM-CSF increases airway smooth muscle cell connective tissue expression by inducing TGF-β receptors

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ABSTRACT

Fibrosis around the smooth muscle of asthmatic airway walls leads to irreversible airway obstruction. Bronchial epithelial cells release GM-CSF in asthmatics and are in close proximity to airway smooth muscle cells (ASMC). The findings in this study demonstrate that GM-CSF induces confluent prolonged serum deprived cultures of ASMC to increase expression of collagen I and fibronectin. GM-CSF also induced ASMC to increase the expression of TGF-β receptors type I, II and III (TβR-I, TβR-II, TβR-III), but had no detectable effect on the release of TGF-β1 by the same ASMC. The presence of GM-CSF also induced the association of TGF-β1 with TβR-III, which enhances binding of TGF-β1 to TβR-II. The induction of TβRs was parallel to the increased induction of phosphorylated Smad-2 (p-Smad-2) and CTGF, indicative of TGF-β mediated connective tissue synthesis. Dexamethasone decreased GM-CSF induced TβR-I, TβR-II, TβR-III, p-Smad-2, CTGF, collagen I and fibronectin. In conclusion, GM-CSF increases the responsiveness of ASMC to TGF-β1 mediated connective tissue expression by induction of TβRs, which is inhibited by corticosteroids.

Keywords: airway remodeling, corticosteroids, irreversible airway obstruction, phosphorylated Smad-2, connective tissue growth factor.
INTRODUCTION

A functional abnormality in asthma is obstruction to airflow as a consequence of recruitment of inflammatory cells, mucus plugging and airway wall thickening (17, 30). The airways are thickened by inflammatory cell infiltrate consisting of eosinophils, lymphocytes and mast cells, mucosal edema and vasodilation (17, 30). Although many of these changes are reversible (30), airway obstruction in asthmatics may also be irreversible due to airway remodeling (30). Remodeling of airways occurs when myofibroblasts just beneath the bronchial epithelium proliferate and synthesize increased amounts of collagens I, III, V and fibronectin (30). In addition, airway remodeling occurs from increases in collagens, elastins, fibronectin, laminin, hyaluronan and versican around airway smooth muscle cells (ASMC) (27). ASMC may become hyperplastic and hypertrophied which also contribute to structural changes, airway narrowing and obstruction (27). Based on observations made on the pathogenesis of fibrosis at other sites, it is speculated that a number of cytokines and growth factors may be important in regulating the recruitment and proliferation of fibroblasts and connective tissue synthesis by fibroblasts, myofibroblasts and ASMC (24, 33). The source of cytokines that regulate fibroblasts could be the structural cells themselves such as bronchial epithelial cells and ASMC (3). Alternatively, the inflammatory cells recruited to the airways or serum protein and platelets that leak into the airways could be additional sources of fibrogenic cytokines (3, 17).

Two cytokines of particular interest in the context of airway remodeling are granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor-beta (TGF-β). GM-CSF is a 22-25 kDa homodimeric glycoprotein originally
described to induce proliferation and differentiation of bone marrow cells (13, 15). GM-CSF is produced and released by a number of cells (13, 35), such as fibroblasts, keratinocytes, bronchial epithelial cells, vascular and airway smooth muscle cells (6, 7, 13, 25, 26, 35). Using immunohistochemistry and Northern analysis, the expression of GM-CSF is increased in the bronchial epithelial cells (BEC) of asthmatics compared to non-asthmatics (26). Isolated BEC from asthmatic airways release more GM-CSF than BEC from non-asthmatic controls (26). It is speculated that in asthma the increased presence of GM-CSF in the airways leads to survival of eosinophils, which have been demonstrated to have an important role in regulating a number of events in asthma (6, 7, 25, 26). Recently, an additional effect of GM-CSF has been described demonstrating the induction of connective tissue synthesis (8, 31, 36). For example, in a rat model, subcutaneous instillation of GM-CSF led to fibroblast accumulation and stimulation of alpha-smooth muscle actin synthesis in myofibroblasts (31). In another rat model, intratracheal administration of an adenovirus containing the GM-CSF gene led to irreversible pulmonary fibrosis (36). The mechanism by which GM-CSF induces collagen synthesis is not well understood but could be due to induction of TGF-β, a regulator of connective tissue synthesis (4, 24, 33). GM-CSF induced mRNA of TGF-β1 by vascular smooth muscle cells (29) and leiomyoma cells (10). GM-CSF also caused myometrial smooth muscle cells to release TGF-β1 (10).

TGF-β is a multifunctional polypeptide (4, 24, 33) that is present as 3 isoforms in mammals but TGF-β1 is the isoform most commonly associated with disorders characterized by inflammation and fibrosis (4). Signal transduction of TGF-β1 is mediated when TGF-β1 associates with TGF-β receptor type II (TβR-II), a 73 kDa serine
threonine receptor that is constitutively phosphorylated (5, 14). Upon binding TGF-β1, TβR-II phosphorylates TGF-β receptor type I (TβR-I), a 53 kDa protein, leading to signal transduction mediated by a number of intracellular proteins that include the Smads (5, 14, 28). TGF-β receptor type III (TβR-III), or betaglycan, is a 250-350 kDa proteoglycan that also binds TGF-β1 but has no definite signaling capability (5, 14). However, the association of TGF-β with TβR-III enhances the interaction of TGF-β with TβR-II and subsequent signal transduction (5, 14). Using immunohistochemistry we had previously demonstrated that all isoforms of TGF-β and TβR-I, TβR-II were expressed by ASMC of human and rat lungs (21, 23). We had also demonstrated that subconfluent bovine ASMC or mechanically wounded monolayers of ASMC release biologically active TGF-β1, that in an autocrine fashion induced collagen I synthesis (12). Furthermore, the addition of TGF-β1 to ASMC also led to collagen synthesis in a dose dependent fashion (12). Despite the fibrogenic effects of TGF-β1 on ASMC, previous studies using Northern blot analysis and immunohistochemistry have not demonstrated evidence of increased expression of TGF-β1 in ASMC of patients with asthma compared to non-asthmatic airways (2, 11, 32). Furthermore, there has been no correlation of expression of TGF-β1 with the severity of asthma and airway remodeling (11). Unlike our observations, these findings do not demonstrate the release of biologically active TGF-β1 in a model simulating ASMC injury (12). The release of active TGF-β1 is important in autocrine regulation of collagen synthesis by ASMC (12). In addition, the aforementioned studies do not provide any evidence on the regulation of TβRs, which may be equally important in TGF-β1 mediated effects.
Bronchial epithelial cells, which express and release GM-CSF during episodes of asthma, are in close proximity to ASMC. It is then conceivable that GM-CSF previously demonstrated to induce TGF-β1 (10, 29) might regulate ASMC to synthesize and release TGF-β1, which in turn, would induce collagen synthesis in the ASMC layer of bronchi. In this study, we demonstrate that GM-CSF induces collagen I and fibronectin expression by bovine confluent prolonged serum deprived cultures of ASMC in a dose-dependent fashion. The presence of GM-CSF does not lead to detectable release of TGF-β1 by ASMC. However, the presence of GM-CSF in confluent prolonged serum deprived cultures of ASMC increases the density of TGF-β1 on the ASMC and expression of TβR-I, TβR-II and TβR-III, as well as the association of TGF-β1 with TβR-III. The presence of GM-CSF also induces TGF-β mediated signal transduction necessary for connective tissue synthesis. These findings for the first time demonstrate that regulation of TβRs on ASMC without evidence of release of TGF-β1 can lead to enhanced connective tissue expression that is mediated by TGF-β1.

MATERIALS AND METHODS

Materials: Alpha-Modified Eagle's Medium (αMEM), fetal calf serum (FCS) and other cell culture reagents were purchased from GIBCO BRL (Burlington, ON, Canada). Anti-TβR-I and TβR-II antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To block the interaction of TGF-β1 with TβR-II, anti-TβR-II antibody from R&D Systems (Minneapolis, MN) was used. Anti-collagen type I and anti-fibronectin antibody was from Cedarlane Laboratories (Hornby, ON, Canada). Anti-phosphorylated Smad-2 (p-Smad-2) antibody and anti-TβR-III antibody were
purchased from Upstate Biotechnology (Lake Placid, NY). Connective tissue growth factor (CTGF) was obtained from Dr. Grotendorst (University of Miami School of Medicine, Miami, FL).

**Cell culture:** Bovine tracheas were obtained from the local slaughterhouse. An explanted culture of the smooth muscle tissue was established as described previously with some modification (12). Briefly, the associated fat and connective tissues were removed in cold Krebs-Ringer solution (NaCl 118, KCl 4.8, CaCl₂·2H₂O 2.5, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.15, dextrose 5.6 and HEPES 12.6, in mM) with antibiotic-antimycotic reagents containing penicillin G 100 units/ml, streptomycin 100 µg/ml, amphotericin B 0.25 µg /ml and sodium desoxycholate 0.25 µg /ml (GIBCO BRL). Then the smooth muscle was isolated, cut into 1-2mm cubic size and placed on culture dishes with a minimal volume of αMEM supplemented with 10% FCS and antibiotic-antimycotic reagents. In an incubator at 37°C in a humidified atmosphere (5% CO₂-balanced air), ASMC migrated from the tissue explants the following day. When cells were approaching confluence in some parts of the dish at 6-10 days after explanting, the explants were removed, and the ASMC were passaged with 0.05% trypsin/0.53 mM EDTA. For the experiments, the ASMC in passage 1-3 were grown in αMEM with 10% FCS and antibiotic-antimycotic reagents to confluence, and then changed to serum-free αMEM containing antibiotics and ITS (10 µg/ml of insulin, 5.5 µg/ml of transferring and 5 ng/ml of sodium selenite, from Sigma) for 10 days prior to the addition of GM-CSF, with or without 10⁻⁴ M dexamethasone (SABEX, Boucherville, QC, Canada). It is of note that we had previously demonstrated that when ASMC are subconfluent in culture, they release biologically active TGF-β1 (12). In these conditions, the release of TGF-β1
results in the synthesis of collagen I (12). However, when ASMC were grown to confluence and kept in serum free conditions for 10 days there was no active TGF-β1 present in the CM (12). Such conditions also had minimal or no collagen I synthesis (12) and were considered suitable to evaluate the effects of GM-CSF on the release of TGF-β1 or connective tissue synthesis by GM-CSF. Henceforth when we refer to confluent monolayers of ASMC, the term refers to prolonged serum deprived confluent ASMC.

**Collection of conditioned media:** At various times after incubation of cells, the overlying conditioned media (CM) was removed, stored in sterile siliconized eppendorf tubes in the presence of the protease inhibitors including leupeptin 5µg/ml (Boehringer Mannheim), aprotinin 5µg/ml (Sigma), pepstatin A 5µg/ml (Sigma) and 1mM PMSF (Sigma) and frozen at -86°C until ready for use.

**TGF-β1 receptor assay by flow cytometry:** Twenty-four hours after treatment with GM-CSF or normal saline, ASMC were removed from the culture dishes using a non-enzymatic cell disassociation solution (Sigma), and then stained with Fluorokine Cytokine Flow Cytometry Reagents (R & D Systems) as instructed by the manufacturer. Briefly, each sample in both control and the GM-CSF group was divided into two aliquots of 1 x 10^5 cells. The first aliquot was incubated at 4°C for 1 hour with biotinylated rh TGF-β1, which binds to the cell predominantly via specific cell surface receptors. The cells were then incubated with avidin-fluorescein, which attaches to the cell-bound biotinylated TGF-β1, for 30 minutes at 4°C in the dark. The other aliquot, which served as the negative staining control was stained exactly as the first aliquot except biotinylated negative control reagent was used instead of biotinylated rh TGF-β1. The cells were washed twice with RDF1 buffer to remove unreacted avidin-fluorescin
and resuspended in RDF1 buffer for final flow cytometric analysis using Coulter Epics XL-MCL Flow Cytometer and Expo 32 Software. Each sample was normalized by its individual negative staining control prior to obtaining a mean, SEM and applying statistical analysis. The specificity of the reaction was confirmed using anti–TGF-β1 antibody.

**TGF-β1 assay by ELISA:** TGF-β1 is secreted in a biologically latent form due to a non-covalent association of the latency associated peptide-1 (LAP-1) with the N-terminal 25 kDa portion of the protein. In this form TGF-β1 is called latent TGF-β1 (L-TGF-β1). To convert L-TGF-β1 to a biologically active TGF-β1 the LAP-1 must be removed which can be achieved by acidification of the CM. The current assay only detects TGF-β1 in its active conformation. Each sample was divided into two aliquots where one aliquot of CM had a neutral pH containing biologically active TGF-β1. To detect total TGF-β1 in each sample, the CM was acidified by 1N HCL for 10 minutes, neutralized with 1.2N NaOH/0.5 M HEPES, and used in the assay as described. DuoSet TGF-β1 ELISA kit (R&D Systems) was used to determine TGF-β1 in neutral CM (representing active TGF-β1) or CM that was acidified and subsequently neutralized (representing total TGF-β1), according to the manufacturers instructions. Briefly, anti-TGF-β1 capture antibody was coated onto a 96-well microplate (Costar) overnight at room temperature. The antibody was diluted to a working concentration of 2 ug/ml and 100 ul of the preparation was added to each well. After washing with wash buffer and blocking with block buffer, 100 µl of sample or the standard, rh TGF-β1 was added and incubated for 2 hours at room temperature. The plate was washed prior to adding 100 ul of the detection antibody (biotinylated chicken anti-human TGF-β1). The TGF-β1
binding was colored by streptavidin-HRP and the optical density was read using a microplate reader at 450 nm with correction at 540 nm.

**Western blotting and immune detection:** After collection of the CM, ASMC were washed with phosphate buffered saline (PBS) and then detached by trypsinization. Cell numbers were determined using a hemocytometer. Whole cell protein was extracted on ice with triple-detergent lysis buffer (50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1% TritonX-100, 0.1% SDS, 5mg/ml sodium deoxycholate) in the presence of the protease inhibitors (as above). In addition to these inhibitors, the samples used for detection of p-Smad-2 were extracted in the presence of NaF (1 mM) and Na3VO4 (1 mM), potent phosphatase inhibitors. Protein concentration was calculated using the Bradford method with a BioRad Protein Assay Reagent (BioRad; Hercules, CA). Protein extracts were separated by SDS-PAGE on polyacrylamide SDS gels (gel concentrations were: 8% for collagen I, fibronectin and TβR-III, 10% for TβR-II and p-Smad-2, and 12% for TβR-I and CTGF) as per Laemmli’s method and then transferred onto a PVDF membrane (BioRad) using CAPS transfer buffer (25 mM CAPS, pH 10, 20% methanol) (12). The equal loading of proteins was confirmed prior to immunoblotting, using Ponceau S Staining Solution (Sigma). Briefly, after transfer of the protein from SDS-PAGE, the PVDF membrane was immersed in Ponceau S Staining Solution for 5 minutes, rinsed, and the protein bands visualized. Once equal loading was established, the membrane was prepared for immunoblotting. After blocking with 5% milk in Tris-buffered saline containing 0.05% Tween-20 overnight at 4°C, the membrane was incubated with primary antibody at various dilutions. The dilution for collagen I was 1:3000, fibronectin 1:3000, TβR-I 1:500, TβR-II 1:500, TβR-III 1:600, CTGF 1:500, p-Smad-2 1:500 for 1 hour at
room temperature or overnight at 4°C. This was followed by incubating the blots with a secondary antibody (Santa Cruz) at a dilution of 1:8000 for 1 hour at room temperature. The proteins on the membrane were then immunodetected by the ECL system (Amersham, Arlington Heights, IL) according to the manufacturer’s instruction. Relative absorbance of the resultant bands was determined using the Quantity One imaging system (BioRad, Hercules, CA), normalized with data of untreated control and expressed as fold of control.

Immunoprecipitation of TGF-β1 associated proteins: One hundred µg of total cell lysate prepared as described above was precleared for 1 hour at 4°C with 0.25 µg of normal rabbit IgG and 20 µl of Protein A/G plus-Agarose (Santa Cruz). The precleared extract was incubated with 1µg of rabbit anti-human TGF-β1 polyclonal IgG (Santa Cruz) at 4°C for 1 hour, then 20 µl of Protein A/G plus-Agarose was added and incubated at 4°C on a rocker platform overnight. The pellet was washed with triple-detergent lysis buffer 4 times and then resuspended in 40 µl of non-reducing sample buffer. After boiling for 3 minutes, 20 µl of the sample was loaded on 8% polyacrylamide gel and electrophoresis and immunoblotting done as described above. Beads, both unused and precleared, were mixed with the sample buffer and served as negative control.

Statistical analysis: The results were expressed as mean ± standard error of the mean (SEM). All p-values (2-tailed) were based on the Wilcoxon signed rank test. Results were considered statistically significant when p<0.05.

RESULTS
GM-CSF regulation of connective tissue expression and release of TGF-β1 by ASMC: We had previously demonstrated that addition of TGF-β1 to monolayers of ASMC induced collagen I synthesis in a dose dependent fashion (12). Similar to TGF-β1, the presence of GM-CSF also induced collagen I synthesis in a dose dependent manner (Figures 1 A, B). In addition, TGF-β1 increased the expression of fibronectin by a greater magnitude than GM-CSF at equivalent amount of 0.05 and 0.1 ng/ml. We next determined if the increase in connective tissue protein by ASMC treated with GM-CSF was associated with an increased release of TGF-β1 by the ASMC. The presence of GM-CSF at a number of concentrations and for a number of lengths of incubation time had no effect on the secretion of TGF-β1 in the active or latent form by confluent ASMC in culture (Figure 2A-C).

GM-CSF regulation of TβRs, p-Smad 2 and CTGF: To study the changes in TGF-β signal transduction, we first determined the expression of TβRs using flow cytometry and Western analysis. Flow cytometry revealed that the presence of GM-CSF in cultures of ASMC significantly increased the intensity of fluorescence from labeled TGF-β1, reflecting an increased density of TGF-β1 receptor on the ASMC compared to untreated ASMC (Figure 3A, p<0.05). In addition, the presence of GM-CSF increased the expression of TβR-I, TβR-II and TβR-III compared to untreated control ASMC as detected by Western analysis (Figure 3B). Next we determined if there was an increase in TβR-III associated to TGF-β1 by immunoprecipitating ASMC proteins using TGF-β1 antibody followed by Western analysis using anti-TβR-III or anti-TGF-β1 antibody. ASMC protein extracts immunoprecipitated with TGF-β1 all expressed the same quantity of TGF-β1 (Figure 3C, lower blots). ASMC cultured alone had a minor quantity of TβR-
III when immunoblotted with anti-TβR-III antibody (Figure 3C, upper blots). However, in the presence of GM-CSF, the quantity of TβR-III was markedly increased (Figure 3C, upper blots), indicating that in the presence of GM-CSF, there is an increase in the number of TβR-III associated with TGF-β1. We next determined if GM-CSF also increased TGF-β mediated downstream signal transduction. An index of target cell response to TGF-β is the phosphorylation of the c-terminal SSXS motif of Smad-2 and 3 that mediate intracellular signals of the TGF-β superfamily (28). ASMC cultured with GM-CSF had induction of p-Smad-2 compared to untreated controls (Figure 3D). Induction of connective tissue proteins by TGF-β is mediated by CTGF, a 33-38 kDa cysteine rich protein (16). To confirm that connective tissue synthesis was mediated by TGF-β1, the protein from ASMC was immunoblotted with anti-CTGF antibodies. ASMC cultured with GM-CSF had an induction of CTGF (Figure 3D). To further confirm that GM-CSF induced above effects are via TβRs, GM-CSF treated ASMC were cultured with or without TβR-II antibody, which interrupts the TGF-β1/TβR-II interaction. The increased p-Smad-2 by GM-CSF was inhibited in the presence of 20 ug/ml of TβR-II antibody (Figure 3E). It is of note that in the presence of GM-CSF, the magnitude of increases in the expression of TβRs, collagen I, fibronectin, p-Smad-2 and CTGF is modest. However, the results are highly reproducible. Furthermore, there is a statistically significant difference in the expression of all these proteins when ASMC treated with GM-CSF are compared to ASMC receiving no treatment.

Effects of dexamethasone on GM-CSF induced expression of TβRs, p-Smad-2, CTGF and connective tissue proteins: Corticosteroids are used as standard therapy in treatment of asthma and have been demonstrated to inhibit collagen I synthesis (18, 31,
There are not significant differences in the expression of collagen I, fibronectin, p-Smad-2, CTGF, TβR-I, TβR-II, and TβR-III between the extracts from ASMC treated with dexamethasone (10⁻⁴ M) and with normal saline (Table 1). However, the presence of dexamethasone inhibited collagen I and fibronectin expression induced by GM-CSF (Fig. 4A, B). Since the induction of connective tissue synthesis by GM-CSF was demonstrated to be on the basis of increase in TβRs, it was next determined if the presence of dexamethasone altered TβRs. The presence of dexamethasone inhibited the increased expression of TβR-I, TβR-I and TβR-III (Figure 3B) as well as p-Smad-2 and CTGF (Figure 3D). These findings demonstrate that dexamethasone inhibits the expression of GM-CSF mediated induction of TβRs and TGF-β1 mediated signal transduction important for collagen synthesis. The presence of dexamethasone did not have an effect on GM-CSF mediated increase of association of TGF-β1 with TβR-III (Figure 3C, upper bands). The use of corticosteroids in asthmatics can vary from being used chronically or at various time intervals after the onset of an exacerbation of asthma (9). To simulate these possibilities, in some instances, ASMC were pre-treated with dexamethasone by 1.5 hours prior to the addition of GM-CSF. In other instances, dexamethasone was added concomitantly or at a number of intervals after the addition of GM-CSF (Figure 4A, B). The GM-CSF induced increases in collagen I and fibronectin were inhibited by dexamethasone added at all time intervals (Figure 4A, B).

**DISCUSSION**

Although a vast spectrum of cytokines are induced and released in asthma, some cytokines have been reported to be fibrogenic such as TGF-β and GM-CSF (3, 8, 17, 25,
However, this is the first observation to demonstrate that GM-CSF increased induction of TGF-β receptors, TβR-I, TβR-II and TβR-III, as well as downstream signal transduction as evidenced by increases in p-Smad-2 and CTGF. The connective tissue effects of GM-CSF mediated by TGF-β1 occurred without a detectable release of biologically active TGF-β1. These findings are highly significant to our understanding of the biology of TGF-β1. Since TGF-β1 is secreted as a biologically latent protein, the most important mechanism in the regulation of the effects of TGF-β1 is the conversion of latent TGF-β to its biologically active form (19, 37). However, the current findings demonstrate that an increase in the receptors to TGF-β can be equally important in regulating the effects of TGF-β1 in the pathogenesis of fibrosis (22, 23). In the context of airway remodeling, previous observations did not report differences in expression of TGF-β1 in airways of asthmatics and normal controls (2, 11, 32). However, the findings of others do not take into account that biologically active TGF-β1 may be released during ASMC injury (12), or that there may be induction of TβRs by cytokines present in asthmatic airways (11, 32). A cytokine reported to be released by BEC during an episode of asthma is GM-CSF (26). Based on the current findings, the release of GM-CSF by bronchial epithelium, which is in direct contact with ASMC, could then induce expression of TβR-I, TβR-II and TβR-III. Of equal importance is the observation that GM-CSF increased the association of TGF-β1 with Tβ-III. When TGF-β1 is associated with TβR-III, the TβR-III presents TGF-β1 to the signal transducing complex composed of TβR-I and TβR-II (5). It has been demonstrated that binding of TGF-β to TβR-III enhances Smad-2 phosphorylation, an indication of TGF-β mediated signaling (14). Collectively, the findings demonstrate enhanced association of TGF-β1 to
ASMC leads to induction of connective tissue synthesis. This then suggests that in patients with asthma, the presence of GM-CSF can induce fibrotic effects via a TGF-β1 pathway by increasing the expression of TβRs despite the lack of apparent increase in TGF-β1 expression (2). Alternatively, it is possible in the current model that GM-CSF induced the release of active TGF-β1 but the quantity was so low that it was not detectable by the assay used. However, since there is an increase in TβR-I, TβR-II and TβR-III on ASMC, these cells are likely to be more responsive to the effects of TGF-β1 in the conditioned media even if the increase in the quantity is not detectable.

The use of corticosteroids in many asthmatics has been demonstrated to relieve acute airway obstruction and when used chronically, corticosteroids inhibit irreversible airway obstruction attributed to increased connective tissue deposition (30, 32). The findings in the current study demonstrate that when dexamethasone is present concomitantly with GM-CSF, there is a reduction of TβR-I, TβR-II, TβR-III, collagen I and fibronectin expression. Dexamethasone did not affect the increase in the association of TGF-β1 with TβR-III induced by GM-CSF. However, in the same culture conditions, p-Smad-2 and CTGF, both indicators of TGF-β mediated effects were decreased. The later findings strongly suggest that the effect of corticosteroids on connective tissue synthesis is the result of inhibiting the GM-CSF regulation of TβRs-I and II despite a lack of effect on the association of TGF-β1 with TβR-III.

The current findings demonstrate a unique interaction between two totally different cytokines, GM-CSF and TGF-β1, commonly found at sites of injury and fibrosis (8, 13, 19, 20, 21, 23, 25, 29, 31, 33, 36, 37). For example, in models of pulmonary fibrosis induced by the anti-neoplastic antibiotic bleomycin, there is increase of both
GM-CSF (1, 36) and TGF-β1 (20, 37). Furthermore, after intratracheal administration of an adenovirus carrying the gene for GM-CSF to rats, there was an increase in TGF-β1 in the bronchoalveolar lavage fluid (BALF) (12). In those instances where both GM-CSF and TGF-β1 are increased, the induction of the TβRs by GM-CSF could lead to a synergistic effect of GM-CSF and TGF-β1 on cells for connective tissue synthesis.

In conclusion, we have demonstrated that GM-CSF, a prevalent cytokine released by BEC in asthma stimulates confluent prolonged serum-deprived culture of ASMC to synthesize connective tissue proteins by induction of the TβRs type I, II and III, and association of TGF-β1 with TβR-III. This effect of GM-CSF on ASMC is reversible by corticosteroids confirming the importance of using corticosteroids in asthma to prevent irreversible airway obstruction.
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FIGURE LEGENDS

Figure 1: Induction of collagen I and fibronectin by GM-CSF and TGF-β1.
Confluent prolonged serum-deprived cultures of ASMC were cultured in the presence of various concentrations of GM-CSF or TGF-β1 for 24 hours prior to protein extraction, electrophoresis, and immunoblotting followed by measuring the relative absorbance of the resultant bands. A. Collagen I and fibronectin increased in a dose dependent fashion after the addition of GM-CSF. B. Collagen I and Fibronectin had a similar tendency to increase in a dose dependent fashion after the addition of TGF-β1. * p< 0.05, ** p<0.01 compared to ASMC cultured in the absence of GM-CSF for A and TGF-β1 for B. The data presented is from 6 (for A) and 3 (for B) different experiments.

Figure 2: Release of TGF-β1 from confluent ASMC. A. Prolonged serum-deprived cultures of confluent ASMC were incubated with various concentrations of GM-CSF for 24 hours, the CM collected and used to measure the active and total TGF-β1 activity in each sample using ELISA. The quantities of active and total TGF-β1 were not significantly different compared to control conditions in which no GM-CSF was present. B. ASMC in the absence or presence of GM-CSF generated small quantities of active TGF-β1 that increased slightly with increase in time interval. ASMC in the absence or presence of GM-CSF generated increasing quantities of total TGF-β1 with time in culture detected after acidification and neutralization of CM. There was not significant difference in active TGF-β1 or total TGF-β1 at each interval between the untreated and GM-CSF treated ASMC. C. Percentage of active TGF-β1 in the absence of GM-CSF was not
statistically different compared to that in the presence of GM-CSF at each time interval. The data presented is from 9 (for A) and 4 (for B) different experiments.

**Figure 3: GM-CSF regulation of TGF-β receptors, p-Smad 2 and CTGF.** A. After 24-hour treatment of prolonged serum-deprived cultures of confluent ASMC with normal saline (Line a, b) or 5 ng/ml of GM-CSF (Line c, d), the cells were stained with biotinylated rh TGF-β1 (Line b, d) or biotinylated negative control reagent (Line a, c), followed by incubation with avidin-FITC and flow cytometric analysis. The insert: the geometric mean of the fluorescence intensity from each sample was normalized by its individual negative staining control. The normalized values representing relative density of TGF-β receptor were calculated for a mean, SEM and statistical significance using the Wilcoxon signed rank test. The density of TGF-β receptor increased significantly after treatment with GM-CSF compared to control receiving no GM-CSF (p<0.05). The data is from 6 separate experiments. B. Proteins from confluent cultures of ASMC without treatment (□) are the control conditions. ASMC treated with 5ng/ml of GM-CSF in the absence (■) or presence (■) of dexamethasone (10⁻⁴ M) (Dex) were used for separation by SDS-PAGE prior to immunoblotting with antibodies to TβR-I, TβR-II, or TβR-III. All culture conditions were maintained for 24 hours prior to collection of cells for Western analysis. There was induction of TβR-I, TβR-II and TβR-III in the presence of GM-CSF. Treatment with GM-CSF plus dexamethasone demonstrated a decrease in expression of TβR-I, TβR-II and TβR-III compared to GM-CSF treatment alone. The data is from 3-6 separate experiments. C. Protein from ASMC lysate immunoprecipitated with TGF-β1 antibody was electrophoresed and immunoblotted with anti-TβR-III
antibody or anti-TGF-β1 antibody. For each condition the quantity of TGF-β1 detected by Western analysis was the same (lower blots). ASMC cultured in the absence of GM-CSF, which were the control conditions, had barely detectable TβR-III. ASMC incubated with GM-CSF had a marked increase in TβR-III associated with TGF-β1. The presence of dexamethasone had no effect on the increased association of TβR-III with TGF-β1. The data is from 5 separate experiments. D. Cells used for controls received no treatment. There was induction of p-Smad-2 and CTGF in the presence of GM-CSF, which was inhibited by the presence of dexamethasone. The data is from 6-8 separate experiments. E. ASMC that received no treatment were used as conditions for controls. ASMC cultured in the presence of GM-CSF and an antibody to TβR-II that interrupts association of TGF-β1 with TβR-II had decreased p-Smad-2 expression compared to ASMC cultured with GM-CSF alone. * p< 0.05, ** p<0.01 compared to control. ♦ p<0.05 compared to GM-CSF treatment.

Figure 4: Dexamethasone regulation of collagen I and fibronectin by GM-CSF stimulated ASMC. Using prolonged serum-deprived confluent cultures of ASMC, GM-CSF induced increases in expression of collagen I (A) and fibronectin (B). Dexamethasone (Dex) was added 1.5 hours before GM-CSF (-1.5) (lane3), concomitantly with GM-CSF (0) (lane 4) or 2 (lane5), 4 (lane 6) and 6 (lane7) hours after the addition of GM-CSF. Each culture condition was maintained for 24 hours prior to collection of protein for Western analysis. The data presented is from 9 (for A) and 4 (for B) different experiments. * p< 0.05 compared to control. ♦ p<0.05, ♦♦ p<0.01 compared to GM-CSF treatment.
### Table 1

Effects of dexamethasone on expression of collagen I, fibronectin, p-Smad2, CTGF, TβRI, II and III

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold of Control* (mean ±SEM)</th>
<th>n</th>
<th>p value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>0.875 ± 0.100</td>
<td>16</td>
<td>0.220</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.083 ± 0.267</td>
<td>16</td>
<td>0.757</td>
</tr>
<tr>
<td>p-Smad-2</td>
<td>0.992 ± 0.098</td>
<td>9</td>
<td>0.933</td>
</tr>
<tr>
<td>CTGF</td>
<td>1.162 ± 0.237</td>
<td>7</td>
<td>0.509</td>
</tr>
<tr>
<td>TβR I</td>
<td>0.839 ± 0.177</td>
<td>4</td>
<td>0.391</td>
</tr>
<tr>
<td>TβR II</td>
<td>1.054 ± 0.255</td>
<td>6</td>
<td>0.836</td>
</tr>
<tr>
<td>TβR III</td>
<td>1.051 ± 0.132</td>
<td>6</td>
<td>0.713</td>
</tr>
</tbody>
</table>

*Denotes readings from Western blots.

**Comparison of proteins from prolonged serum deprived confluent cultures of ASMC receiving no treatment or cultured with dexamethasone ($10^{-4}$M). The ASMC were treated with or without dexamethasone for 24 hours prior to extracting proteins for Western analysis.
Fig. 1

A
- Relative Absorbance (fold of control)
- Collagen I
- Fibronectin

0 0.05 0.1 0.5 2.5 5 10
GM-CSF (ng/ml)

B
- Collagen I
- Fibronectin

0 0.01 0.05 0.1 0.5 1
TGF-β1 (ng/ml)
Fig. 2
Fig. 4