Mechanisms of serum potentiation of GM-CSF production by human airway smooth muscle cells

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Lalor, D. J., B. Truong, S. Henness, A. E. Blake, Q. Ge, A. J. Ammit, C. L. Armour, and J. M. Hughes. Mechanisms of serum potentiation of GM-CSF production by human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 287: L1007–L1016, 2004; doi:10.1152/ajplung.00126.2004.—Inflammation and vascular leakage are prevalent in asthma. This study aimed to elucidate the mechanisms involved in serum potentiation of cytokine-induced granulocyte macrophage colony stimulating factor (GM-CSF) production by human airway smooth muscle cells and to identify possible factors responsible. Serum-deprived cells at low density were stimulated with TNF-α and IL-1β for 24 h. Human AB serum (10%), inhibitors of RNA and protein synthesis or specific signaling molecules, or known smooth muscle mitogens were then added for 24 h. Culture supernatants were analyzed for GM-CSF levels, and cells were harvested to assess viability, cell cycle progression, GM-CSF-specific mRNA content, and p38 phosphorylation. Serum potentiated GM-CSF release when added before, together with (maximal), or after the cytokines. The potentiation involved both new GM-CSF-specific mRNA production and protein synthesis. The mitogens IGF, PDGF, and thrombin all potentiated GM-CSF release, and neutralizing antibodies for EGF, IGF, and PDGF reduced the serum potentiation. Inhibitor studies ruled as unlikely the involvement of p70S6kinase and the MAPK JNK, while establishing roles for p38 and NF-κB in the potentiation of GM-CSF release. Detection of significant p38 phosphorylation in response to serum stimulation, through Western blotting, further demonstrated the involvement of p38. These studies have provided evidence to support p38 being targeted to interrupt the cycle of inflammation, vascular leakage and cytokine production in asthma.

Vascular leakage also occurs in the airways of asthmatics (19). Agents found within human serum/plasma such as insulin-like growth factor (IGF) (14), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and thrombin have been shown to cause the proliferation of airway smooth muscle cells (ASMCS) (17). Their effects on other airway smooth muscle functions, such as chemokine/cytokine production, are largely undetermined.

We have previously reported that exposure of human ASMCS to human serum potentiates the amount of granulocyte macrophage colony stimulating factor (GM-CSF) released by these cells in response to stimulation with tumour necrosis factor (TNF)-α and interleukin (IL)-1β in vitro (20). This potentiation of GM-CSF may be important in acute episodes of asthma as GM-CSF enhances the survival and activation of a number of key inflammatory cells, including eosinophils (21), stimulates the release of other cytokines, and changes the contraction profiles of airway smooth muscle (8). Recently, a potential role for GM-CSF in airway wall remodeling has also emerged. In airway smooth muscle it has been shown to induce synthesis of collagen and fibronectin and, while not affecting the amount of transforming growth factor (TGF)-β secreted by the smooth muscle, to elevate the expression of TGF-β receptors, thereby making the muscle more sensitive to the fibrogenic effects of TGF-β (5). Elucidation of the mechanisms by which serum potentiates GM-CSF release may lead to the development of a novel treatment for acute asthmatic episodes and identification of the agent(s) within serum exerting this effect may also provide a new target for asthma treatment.

TNF-α and IL-1β-stimulated GM-CSF release comes as a result of activation of the transcription factor nuclear factor (NF)-κB (21). NF-κB can be activated by the mitogen-activated protein kinases (MAPKs) p38 and p42/p44 (2), which also play a role in the release of cytokines (13). These MAPKs, particularly p42/p44, are also involved in the transduction of signals from mitogens found in serum/plasma resulting in cell proliferation (17). Recently, it has been demonstrated that the cytokine-induced release of GM-CSF by human ASMCS can be reduced by inhibition of the MAPK JNK, clearly indicating a role for JNK in the regulation of cytokine-induced GM-CSF release (16). To date, p70S6kinase in airway smooth muscle has been found to play a role in proliferation (reviewed in Ref. 1) but not synthetic activity of the cells.

The aims of the current study were to elucidate some of the mechanisms by which serum potentiates human ASMCS release.

Asthma is an inflammatory disease of the airways. The chronic inflammation in asthmatic airways has been long known to cause changes in the airways. These changes have been shown to be both physical changes (11) and changes in the types of inflammatory cells and agents present in the airways (7).

Airway smooth muscle plays an important role in these changes. Increased airway smooth muscle bulk, due to both increased size and proliferation of the smooth muscle cells, is a major component of the documented airway remodeling (11). Moreover, airway smooth muscle can influence the local inflammatory environment by synthesis of chemokines and cytokines that modulate subsequent inflammatory cell recruitment and activation.

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of GM-CSF in response to stimulation with TNF-α and IL-1β and to identify possible factors responsible. To do this, we examined the effect of serum/plasma-derived airway smooth muscle mitogens on cytokine-primed airway smooth muscle GM-CSF release and cell cycle progression. We also treated serum with neutralizing antibodies for EGF, IGF, and PDGF and explored their effects on GM-CSF release. Furthermore, we investigated whether new gene transcription as well as new protein synthesis are involved in the serum potentiation. Finally, we determined whether the new protein synthesis involved comes as a result of stimulation of synthetic or proliferative pathways by exploring the role of NF-κB, p38, p42/p44, JNK, and p70S6k in the cell signaling leading to the observed serum potentiation of GM-CSF release.

MATERIALS

Recombinant human TNF-α, IL-1β, neutralizing antibodies to EGF (AF236), IGF-I (AF-291-NA), and PDGF (AB-23-NA), and an isotype control (AB-108-C) were all obtained from R&D Systems (Minneapolis, MN). Cycloheximide, rapamycin, and PDGF were purchased from Sigma Australia. MAPK inhibitors SB-203580 and PD-98059 and the negative congener SB-202474 were provided by Calbiochem (San Diego, CA). Monoclonal antibodies used for the Western blotting of p38 and phospho-p38 (Thr180/Tyr182) were supplied by Cell Signaling Technology (Beverly, MA). EGF and IGF were obtained from Invitrogen Life Technologies. Thrombin was obtained from Pfizer (Sydney, Australia). All reagents were reconstituted and stored according to the suppliers’ instructions.

The Access RT-PCR kit, Random-Primed DNA labeling kit, and RNA Gel Extraction kit were obtained from Promega, Roche Diagnostics, and QIAGEN Australia, respectively. TRIzol was purchased from Sigma Australia. MAPK inhibitors SB-203580 and PD-98059 and the negative congener SB-202474 were provided by Calbiochem (San Diego, CA). Monoclonal antibodies used for the Western blotting of p38 and phospho-p38 (Thr180/Tyr182) were supplied by Cell Signaling Technology (Beverly, MA). EGF and IGF were obtained from Invitrogen Life Technologies. Thrombin was obtained from Pfizer (Sydney, Australia). All reagents were reconstituted and stored according to the suppliers’ instructions.

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METHODS

Human ASM

Human lung was obtained from patients undergoing either lung transplant or resection. Ethics approval for the use of human lung tissue was supplied by the Central Area Health Service and for this study by the Human Ethics Committee of the University of Sydney. Airway smooth muscle bundles were dissected free from surrounding tissues, and the cells were grown in culture at 37°C in a humidified 5% CO₂ in air atmosphere, as previously described (10). Cells established in culture from each lung donor were checked for the presence of the airway smooth muscle contractile proteins α-smooth muscle actin and h-calponin by immunohistochemistry (12). At the time of plating, the average viability of each cell line was 96.2 ± 1.1% (average ± SE, n = 11) as tested by trypsin blue dye exclusion testing.

Potentiation of Airway Smooth Muscle GM-CSF Release by Human Serum

The experimental protocol closely followed that used by Sukkar et al. (20). Briefly, ASMC from three to seven lung donors were plated down at a density of 5 × 10⁵ cells/well in six-well plates (5.2 × 10⁵ cells/cm²) in the presence of 2 ml of DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B. To study the effect of serum later in the protocol, after 24 h the medium was removed and replaced with serum-free medium (phenol red-free DMEM supplemented with 2 mM glutamine, 100 units/ml penicillin G, 100 µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B, 1% vol/vol NEAA, and ITS solution with a final concentration of 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) for a further 24 h. At this point, the cells were stimulated with TNF-α and IL-1β (both at 10 ng/ml) in fresh serum-free medium. After a further 24 h, the cytokine-containing culture medium was removed, and the cells were stimulated with 10% vol/vol human AB serum (in serum-free medium). After a further 24 h, culture supernatants were harvested and stored at −20°C for GM-CSF quantification, and cells were harvested with trypsin-EDTA for estimation of total cell number using Kimura Light stain and for cell viability using trypan blue dye exclusion.

In an additional series of experiments the above protocol was extended to include extra treatments where human serum (10% vol/vol in serum-free medium) was added first for 24 h, followed by the cytokines in serum-free medium for the final 24 h, or together with the cytokines for 24 h followed by serum-free medium for the final 24 h. In these experiments, supernatants were collected and stored after each 24-h treatment period for GM-CSF quantification.

Inhibition of New RNA and Protein Synthesis

To determine whether new RNA synthesis and new protein synthesis were involved in serum potentiation of GM-CSF release, specific inhibitors were added concurrently with human serum to ASMC from three to seven lung donors in the protocol described above. The inhibitor treatments included actinomycin D (0.01, 0.1, 0.5, 1.0, and 5.0 µg/ml), the highest concentration of its vehicle (DMSO 0.05% vol/vol), or cycloheximide (0.1, 0.5, and 1.0 µg/ml). The inhibitors were added to duplicate wells, and 24 h later, the culture medium was harvested and stored at −20°C for quantification of GM-CSF. Cells were then counted for total number and viability.

GM-CSF Gene Transcription

The role of new GM-CSF gene transcription in serum potentiation of GM-CSF release was examined in ASMC from three lung donors. The cells were seeded into 75-cm² flasks at the same density of 5.2 × 10⁵ cells/cm² as used above. The protocol used to examine serum potentiation of ASMC GM-CSF release was followed. However, in this case, the cells were harvested at 4 h postexposure to serum using TRIzol at 0.1 ml/cm², and total RNA was extracted according to the manufacturer’s instructions. The RNA was then run on a 1% agarose formaldehyde gel, and the GM-CSF mRNA was quantified by Northern blot using GAPDH as a housekeeping gene (as described in Ref. 6). Northern blotting was performed using 5 µg of RNA. A probe for GM-CSF was prepared from human ASMC maximally stimulated with cytokines with the Access RT-PCR kit. The probe used was a region of the human GM-CSF gene (sequence accession number E02287) spanning 195 base pairs from 348 to 542. It was constructed using the forward primer 5′-CTTCCTCTGCAACCCAGATT-3′ and the reverse primer 5′-CTTGTTCCCTCAAGATGAC-3′. The probe was then used in conjunction with a Random Primed DNA labeling kit and ³²PdCTP to quantify the amount of GM-CSF-specific mRNA present. All kits were used according to manufacturers’ instructions.
Inhibition of Signaling Pathways

Experiments to explore the signaling pathways involved were conducted in ASMC from three to five lung donors. ASMC were plated into 24-well plates but at the same cell density (1 × 10^5 cells in 400 μl per well) as previously. Inhibitors were used on triplicate wells at: 30 nM rapamycin (an inhibitor of p70S6kinase) (18), 10 and 100 μM DMF (an inhibitor of NF-κB translocation) (23), 30 μM PD-98059 (an inhibitor of p42/p44 MAPK) (2), 10 μM SB-203580 (an inhibitor of p38 MAPK) (2), and 10 μM SP-600125 (a JNK inhibitor) (16) as previously reported. The negative control for MAPK inhibition studies, SB-202474, was also used at 10 μM (2). The inhibitors or their vehicle (0.1% DMSO) was added to the cells 30 or 60 min (DMF only) before the serum. For the studies using DMF, cell counts by trypan blue dye exclusion were conducted to assess cell viability.

Phosphorylation of p38 MAPK

Experiments were conducted as for the inhibitor studies with the following exceptions: cells were grown in 10-cm petri dishes. The studies with SB-203580 were conducted in duplicate and following 30-min incubation with SB-203580 or serum-free medium, cells were stimulated with 10% human serum for 15 min. Cells were lysed and analyzed by Western blotting using specific monoclonal antibodies against p38 and phospho-p38 (Thr180/Tyr182) performed as previously described (17).

Potentiation of GM-CSF Release by ASMC Mitogens

To establish the effect of ASMC mitogens on GM-CSF production a protocol mirroring that used to potentiate GM-CSF release by human serum was used, except that in place of adding serum, we added the mitogens PDGF, EGF, IGF, and thrombin (or their vehicles where appropriate) to ASMC from five to nine lung donors. The mitogen stocks were freshly diluted in serum free medium and added to wells of untreated or cytokine-primed ASMC at concentrations that had previously been shown to induce human ASMC proliferation: 40 ng/ml PDGF (10), 100 ng/ml EGF (17), 100 ng/ml IGF (14), and 1.0, 1.0, and 10 U/ml thrombin (22). A vehicle control of 1.0 μM acetic acid in serum-free medium was included for PDGF-AB and EGF. Culture supernatants were collected 24 h later for GM-CSF quantification, and the cells were harvested with trypsin-EDTA for cell cycle analysis.

Cell Cycle Analysis

To examine the cell cycle progression of mitogen-treated ASMC, the protocol previously published by Johnson et al. (12) was followed. Briefly, harvested ASMC were permeabilized and stained using a solution of 0.5% wt/vol saponin and 0.1% wt/vol bovine serum albumin in PBS containing 50 μg/ml propidium iodide and 50 μg/ml ribonuclease A. A FACScalibur Sort (Becton Dickinson, Sydney, Australia) in conjunction with Cell Quest software (Becton Dickinson, Sydney, Australia) was used to acquire the data from the stained cells. The resulting DNA profiles were analyzed using FL2 peak area, FL2 peak width, and Modfit software (Verity Software House, Topsham, ME) to determine the percentage of cells in each phase of the cell cycle.

Studies with Neutralizing Antibodies to Growth Factors

To determine the contribution of EGF, IGF, and PDGF to the serum potentiation of GM-CSF release from cytokine-primed cells, we used neutralizing antibodies to the growth factors. The protocol was as for the initial experiments examining the potentiating effects of serum except that the medium containing 10% human antibody serum was preincubated with a neutralizing antibody to EGF (2.5, 5, and 10 μg/ml), IGF (1.25, 2.5, 5, and 10 μg/ml), or PDGF (5, 10, and 20 μg/ml) for 15–30 min at 37°C and then added to triplicate well cultures of ASMC from three to five lung donors. AB serum treated with an irrelevant antibody of the same isotype was used as a control. The cells were left to incubate for 24 h, and then the supernatants were collected for GM-CSF quantification.

GM-CSF Quantification

The GM-CSF concentration of the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Capture and detection antibodies for GM-CSF were used at a concentration of 1 and 0.25 μg/ml, respectively. ELISA was conducted according to the protocol supplied with these antibodies. The limit of detection for the ELISA studies was 15.6 pg/ml.

Data Analysis

Data were analyzed using the StatView statistical package. Analysis of variance was used together with Fisher’s paired least significant difference to compare each treatment on the outcome measures (GM-CSF release, cell number, cell viability, mRNA/GAPDH, p38 phosphorylation, and %S/G2+M). A value of P < 0.05 was considered significant. Potentiation of GM-CSF secretion by serum or the growth factors was defined as a significant increase in GM-CSF secretion by cytokine-primed ASMC over their baseline secretion of GM-CSF in the same period.

RESULTS

Potentiation of GM-CSF Release by Human Serum

In this study we have extended our previously reported findings that human serum potentiated GM-CSF release by cytokine-primed human ASMC when added after the cytokine treatment period (20). Here we demonstrate that a 24-h treatment with serum potentiated total GM-CSF release over a 48-h period, irrespective of whether the serum was added the day before, together with, or the day after the ASMC were primed with the cytokines TNF-α and IL-1ß for 24 h. Changes in GM-CSF release in response to the different serum treatments are summarized in Fig. 1. The GM-CSF release induced by the cytokines alone was 750.4 ± 231.2 pg/ml (mean ± SE) over the 48-h period. Serum caused the greatest potentiation of this release if it was added together with the cytokines (ninefold) and increases of 3- and 5.4-fold when added before and after the cytokine treatment period, respectively (Fig. 1).

Inhibition of RNA and Protein Synthesis

Inhibition of new RNA synthesis. Actinomycin D added over a range of concentrations (0.5, 1.0, and 5.0 μg/ml) to cytokine-primed cells to inhibit RNA synthesis significantly reduced, in a concentration-related manner, the amount of GM-CSF produced by 60.00 ± 11.44, 78.00 ± 5.57, and 81.93 ± 4.17%, respectively (n = 4, P < 0.001; Fig. 2A). At two lower concentrations, 0.01 and 0.001 μg/ml, actinomycin D reduced the expression of GM-CSF by 20.09 ± 6.03 and 35.38 ± 12.41%, neither of which was significant compared with the positive control (n = 3, P = 0.316 and P = 0.090). The vehicle for actinomycin D, DMSO, at a concentration of 0.05% (the highest concentration at which it was present), reduced the amount of GM-CSF produced by the ASMC by 30.27 ± 7.65% (n = 4, P = 0.001). The effect of the actinomycin D, at the two highest concentrations, was significantly greater (>50%) than that of the vehicle (P <
Addition of actinomycin D did not have a significant effect on the amount of GM-CSF produced by cytokine-primed cells treated with serum for 24 h when compared with the positive control ($P = 0.258$, $n = 3$; see Fig. 4A).

Similarly, 10 μM of SP-600125, an inhibitor of the MAPK JNK caused no significant decrease in GM-CSF produced by cytokine-primed cells treated with serum for 24 h when compared with the positive control ($P = 0.887$, $n = 3$; see Fig. 4B).

Inhibition of the p42/p44 MAPK by PD-98059 at 30 μM resulted in a significant decrease of 32.36 ± 11.99% in the amount of GM-CSF produced by cytokine-primed cells treated with serum for 24 h when compared with the positive control ($P = 0.0092$, $n = 3$; Fig. 4B). However, a similar decrease (36.22 ± 4.50%; $P = 0.0042$, $n = 3$) in GM-CSF was achieved by treating the cells with 0.1% vol/vol DMSO, the vehicle in which PD-98059 was dissolved (Fig. 4B). This was not different from the reduction caused by the PD-98059 ($P = 0.737$, $n = 3$).

In contrast to these observations, addition of the p38 MAPK inhibitor SB-203580 to the cell cultures caused a 62.08 ± 4.42%
DMSO, 

production was greater than that of its vehicle, 0.1% vol/vol H9262 positive control and its vehicle. In fact, 100 P was signifi

antly different from those detected in the unstimulated cells (2.6 ± 1.0% positive control). All growth factors (EGF, IGF, PDGF, and thrombin) caused increases in GM-CSF release by ASMC that had been pretreated with cytokines (Fig. 6, A and B). GM-CSF release from cytokine-primed cells in the presence of these growth factors was 60–80% of that with 10% human serum (positive control). IGF (P = 0.0133, n = 5) significantly increased GM-CSF release by 2.8-fold compared with the cytokine control (Fig. 6A). Although EGF caused a significant increase in release compared with the cytokine control (P = 0.0043, n = 5), it was not significantly different (P = 0.079, n = 5) from release in the presence of its vehicle (1.0 µM acetic acid). However, PDGF did significantly increase (P = 0.0133, n = 5) GM-CSF release twofold over the vehicle control (1.0 µM acetic acid, Fig. 6A). Thrombin at 0.1, 1.0, and 10 units/ml also caused significant (P < 0.05, n = 5–9) increases in GM-CSF release from cytokine-treated cells. The levels released in the presence of thrombin at 10 units/ml were 80% of those in the presence of human serum (Fig. 6B).

Effects of Serum and Growth Factors on Cell Cycle Progression

Treatment of cytokine-primed ASMC with 10% human serum for 24 h induced a twofold increase in the number of ASMC in the S/G2/M phases of the cell cycle (Table 1). The growth factors had no significant effect on unstimulated or cytokine-stimulated ASM cell cycle progression over the 24-h period. However, although the growth factors did not significantly alter the DNA profiles of the ASMC, a trend was evident in cytokine-pretreated cells that received PDGF or thrombin, with increases of 6.4 and 6.3%, respectively, in cells progressing into S/G2/M, compared with cytokine-primed cells receiving no growth factors (Table 1).

Effect of Neutralizing Antibodies for Growth Factors on Serum Potentiation

To assess the role of the individual growth factors in the human serum potentiation of ASMC GM-CSF release, medium
containing 10% human serum was treated with a range of concentrations of neutralizing antibodies for EGF, IGF, or PDGF before its addition to the ASMC. Interestingly, all three neutralizing antibodies significantly reduced human serum potentiation of GM-CSF release (Fig. 7), whereas an irrelevant antibody of the same isotype had no significant effect over the same concentration ranges (Fig. 7A). The EGF neutralizing antibody caused significant concentration-related reductions in the serum potentiation, with GM-CSF release being only 36.6 ± 2.5% of the serum control (P < 0.0001, n = 4) in the presence of the highest concentration (10 μg/ml) of antibody used (Fig. 7B). The IGF neutralizing antibody caused a significant but similar reduction to ~43% of control at concentrations of 5 μg/ml or higher (P < 0.001, n = 4; Fig. 7C). The PDGF neutralizing antibody also caused significant concentration-related reductions in the serum potentiation, reducing GM-CSF release to 46.8 ± 5.63% of control at 20 μg/ml (P < 0.0001, n = 5; Fig. 7D).

**DISCUSSION**

In this study we have extended our previous findings (20) and demonstrated for the first time some of the mechanisms by which serum potentiates cytokine-induced GM-CSF release from human ASMC. We have shown that both new protein production and new gene transcription are involved. Further findings specifically demonstrate that the increase in GM-CSF
secretion due to serum stimulation is regulated at the transcriptional level. Interestingly, we have provided evidence that signaling via the MAPK p42/p44 or p70S6 kinase molecules, important in ASMC proliferation (reviewed in Ref. 1), or via the MAPK JNK, which has a role in cytokine induced GM-CSF release (16), is not implicated, but that the MAPK p38 and the transcription factor NF-κB are involved. Furthermore, we have also demonstrated, for the first time, that the mitogens IGF, PDGF, and thrombin have a significant potentiating effect on IL-1β- and TNF-α-induced GM-CSF release from human ASMC before having a significant effect on cell cycle progression. Finally, we have shown that the growth factors EGF, IGF, and PDGF were significant contributors to the potentiating effect of serum.

Inflammation and vascular leakage are both important processes in acute asthmatic attacks (19). Previous research conducted by our group showed that serum potentiates the release of the cytokine GM-CSF by human ASMC in response to TNF-α and IL-1β (20). This was a finding confirmed and extended by the present study. It may be hypothesized that this serum potentiation is responsible for some of the inflammation seen in vivo during an acute attack of asthma. The determination of the mechanism through which serum is acting and the particular components of serum exerting this effect may provide a potential treatment target in an acute exacerbation of asthma.

Under our experimental conditions, a 24-h serum treatment caused both increased synthesis of GM-CSF and the initiation of proliferation by the ASMC. The latter was demonstrated by an increase in the number of cells moving out of the resting or G0/G1 phase of the cell cycle into the S/G2/M phases, although a significant increase in cell number was not yet

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<th>Treatment (ng/mL)</th>
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<tr>
<td>Serum (control)</td>
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<td>IGF 100 ng/ml</td>
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<td>Vehicle 1.0 μM acetic acid</td>
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Table 1. Effect of a 24-h exposure to mitogens or human serum on cell cycle progression of unstimulated and cytokine-primed ASMC

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<th>Treatment (ng/mL)</th>
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<tr>
<td>Serum (control)</td>
<td>0</td>
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Values are means ± SE; n = 5. *% in S/G2/M. †P < 0.05 vs. unstimulated and cytokine controls. ASMC, airway smooth muscle cells.
Exploring the effects of known proliferative agents contained within serum on GM-CSF production was therefore interesting. This was especially so in light of a recent report showing that, with a much longer exposure time, some mitogens have the ability to both induce GM-CSF secretion by and cause cell cycle progression of ASMC (3).

The effects of a number of mitogens, EGF, IGF, PDGF, and thrombin, on ASMC function were investigated in this study. IGF, PDGF, and thrombin were shown to increase the amount of GM-CSF produced by cytokine-primed ASMC, whereas EGF, PDGF, and IGF were shown to contribute to the potentiating action of serum. The mitogens alone induced little release of GM-CSF over a 24-h period. Additionally, the mitogens failed to cause any significant progression through the cell cycle in that period under the same conditions.

Fig. 7. Serum potentiation of GM-CSF production by cytokine pretreated ASMC is not affected by an irrelevant antibody with the same isotype (n = 3–8, A) but is reduced by neutralizing antibodies for EGF (*P < 0.02 vs. the positive control of 10% human AB serum, n = 3–4; B), IGF (*P < 0.001 vs. positive control, n = 3–5; C), and PDGF (*P < 0.001 vs. positive control, n = 5; D). Values are means ± SE.

EGF, PDGF, and IGF were shown to contribute to the potentiating action of serum. The mitogens alone induced little release of GM-CSF over a 24-h period. Additionally, the mitogens failed to cause any significant progression through the cell cycle in that period under the same conditions. This demonstrates a separation of the proliferative and synthetic functions of ASMC and suggests that the activity of an ASMC may depend on the environmental conditions that the cell is exposed to. That is, the same stimulus, for example the mitogens used in these experiments, may have different effects on
ASMC depending on the preexisting condition of the cells as well as the period of time for which these conditions prevail.

Rapamycin has previously been shown to inhibit growth factor-induced p70S6kinase activity and proliferation in ASMC at the concentration used in this study (18). Our results showing that rapamycin had no effect on serum potentiation of GM-CSF production are consistent with serum also exerting its action through synthetic rather than proliferative pathways. Although the p70S6kinase pathway is not the only proliferative pathway, this finding lends more weight to the above suggestion that ASMC have a synthetic and a proliferative nature and that the two need not coincide.

The potentiating effects of the ASMC mitogens may be important physiologically. All the mitogens are present in the airways (4, 15, 24). However, their levels in the airways of asthmatics are likely to vary depending on the degree of inflammatory cell activation and vascular leakage occurring locally. The growth factors studied and thrombin are of particular interest as, under inflammatory conditions, a wide range of concentrations of thrombin potentiated ASMC GM-CSF production and all three growth factors contributed significantly to serum potentiation of its production. Thus the findings reported here are consistent with these ASMC mitogens playing an important role in amplifying the locally sustained inflammation occurring in the airways of asthmatics, particularly during periods of vascular leakage.

Examination of the effects of the ASMC mitogens on the release of GM-CSF by cytokine-primed cells provided evidence that the stimulatory effect of serum comes as a result of a concerted effect of these mitogens. Each of the mitogens was shown to potentiate GM-CSF release from cytokine-stimulated cells. In the case of EGF, however, this effect was not deemed significant compared with its vehicle. It is possible that at higher concentrations the effects of EGF may become significant. In addition, antibodies to PDGF, IGF, and EGF all showed an ability to inhibit the potentiating effects of serum. It is of note that the inhibitory effect of no one antibody alone was sufficient to completely ablate the potentiation caused by serum. These data suggest a concerted, and perhaps synergistic, effect of the mitogens. It is not obvious from the data whether this concerted action comes as a result of stimulating one common pathway or whether an array of mitogen-activated pathways may be involved in the mitogen potentiation of GM-CSF. What is clear from the data is that inhibition of NF-κB translocation is sufficient to negate the effects of serum. It is apparent then, that if these agents are causing the effects of serum by stimulating different pathways, these messages must combine to cause an increase in NF-κB activation. Additionally, a large proportion of the potentiating effect of serum is inhibited by blocking p38 signaling. This indicates that even if individual mitogens are signaling via different pathways, the effects of the physiologically important stimulus, serum, can be reduced by p38 inhibition.

After initially demonstrating that the observed increase in GM-CSF release following serum exposure required production of both new protein and mRNA, we extended these findings to show that there was also a significant increase in production of GM-CSF-specific mRNA. These findings indicate that the observed serum potentiation of GM-CSF release comes as a result of an increase in the signal to transcribe the GM-CSF gene.

The MAPKs p38, p42/p44, and JNK have been shown to be involved in a number of processes in human airway smooth muscle, including proliferation and cytokine release. ASMC proliferation in response to known mitogens such as PDGF and EGF is dependent on activation of the p42/p44 MAPK (17). Results from other studies indicate that the regulation of cytokine-mediated cytokine release also involves the MAPKs. The release of several cytokines, GM-CSF, regulated on activation normal T cells expressed and secreted (RANTES), and eotaxin, is dependent on the activation of the MAPKs in a complex fashion (9). Hallsworth et al. (9) showed that GM-CSF release was dependent on the activation of p42/p44 but was suppressed by the activation of p38, and more recently Oltmanns et al. (16) demonstrated the involvement of JNK in the TNF-α- and IL-1β-induced expression of GM-CSF, IL-8, and RANTES. These few examples demonstrate the complexity of cell responses to activation of the MAPKs.

In light of the various signals that are conducted through the MAPK families p38, p42/p44, and JNK, it was of interest to determine whether they were involved in the cell signaling leading to serum potentiation of GM-CSF release. We used pharmacological agents at concentrations previously demonstrated to specifically inhibit these MAPKs in human ASM (2, 16) to do this. It is intriguing that we have found evidence for p38, but not p42/p44 or JNK, to be involved in the cell signaling leading to serum potentiation of GM-CSF release. The exact effect of this signaling through p38 remains unclear. The role of p38, therefore, will become an interesting area of study.

NF-κB activation has been shown to be of importance in the production of GM-CSF (21). Through the use of DMF, which has been used on different cell types such as normal dermal fibroblasts to inhibit the translocation of NF-κB (23), we have been able to significantly decrease the amount of GM-CSF released by cytokine-primed ASMC in response to human serum. This finding, combined with the observed increases in GM-CSF message and protein, lends weight to the suggestion that components found within human serum are causing the potentiation of ASMC GM-CSF through increasing the transcription of the GM-CSF gene.

Previously it has been suggested that p38 and p42/p44 can exert their actions either via NF-κB-dependent or independent pathways (2). We have demonstrated that both p38 and NF-κB are involved in the cell signaling leading to the serum potentiation of GM-CSF release and that preventing NF-κB translocation markedly reduces the serum potentiation. Our findings are consistent with p38 exerting its action via an NF-κB-mediated mechanism. This observation also helps to explain the lack of involvement of JNK in these signaling processes, since JNK activation results in downstream phosphorylation of c-Jun and subsequent activation of the transcription factor activator protein-1 (16).

We have provided a picture of the mitogens involved in and the mechanisms leading to the serum potentiation of GM-CSF release by airway smooth muscle. Serum was shown to advance the cell cycle of the ASMC, whereas the known mitogens (EGF, IGF, PDGF, and thrombin) failed to do so under the above experimental conditions yet still caused a potentiation of GM-CSF release by cytokine-primed ASM. EGF, IGF, and PDGF were shown to contribute significantly to the potentiating effect of human serum. An increase in both gene
transcription and protein production was required for the serum potentiation of GM-CSF release. Findings from studies analyzing the mRNA produced by the cells showed that GM-CSF-specific mRNA levels were increased by stimulation with serum and that these same cells also showed increased GM-CSF protein secretion, suggesting that the effect of serum was at the transcriptional level. The involvement of the proliferative pathway p70S6kinase and the MAPKs p42/p44 and JNK were ruled unlikely, whereas the importance of p38 and NF-κB were highlighted. Given that the p38 pathway is involved in the serum potentiation of GM-CSF release, further studies should investigate ways in which it might be targeted to interrupt the cycle of inflammation, vascular leakage, and cytokine production in asthma.

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