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Pro-inflammatory cytokines induce c-fos expression followed by IL-6 release in human airway smooth muscle cells

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***Background*: Airway smooth muscle (ASM) is considered to be a target for mediators released during airway inflammation.**

***Aims*: To investigate the expression of c-fos, a constituent of the transcription factor activator protein-1, in human ASM cells. In addition, to measure the release of interleukin (IL)-6 into the conditioned medium of stimulated ASM cells, as well as DNA biosynthesis and changes in cell number.**

***Methods*: Serum-deprived human ASM cells in the G_0/G_1 phase were stimulated with the pro-inflammatory cytokines; tumour necrosis factor-α, IL-1β, IL-5 and IL-6. The expression of mRNA encoding the proto-oncogene c-fos was measured by Northern blot analysis. Cell proliferation was assessed by {^{3}H}-thymidine incorporation assays and cell counting, and IL-6 levels in cell-conditioned medium were measured by enzyme-linked immunosorbent assay.**

***Results*: All of the cytokines investigated induced a rapid (within 1 h) and transient increase in the expression of mRNA encoding c-fos, followed by the expression and enhanced release of IL-6. Cell proliferation remained unchanged in cytokine-stimulated cells.**

***Conclusions*: Cytokine-induced c-fos expression in human ASM cells could be described as a marker of cell 'activation'. The possible association of these results with airway inflammation, through secondary intracellular mechanisms such as cytokine production, is discussed.**

Key words: Airway smooth muscle, Cytokine, c-fos, IL-6, TNF-α, IL-1β, IL-5

Introduction

Asthma is a chronic disease of the airways characterised by reversible airway obstruction, airway hyper-responsiveness and airway wall inflammation. Important pathological features of asthmatic airways include inflammatory cell infiltration, epithelial shedding, basement membrane thickening and increased mass of airway smooth muscle (ASM). Both hyperplasia (an increase in cell number) and hypertrophy (an increase in cell size) of ASM cells contribute to the increased smooth muscle content of the airway wall.[1–3] The release of mediators from the infiltrating inflammatory cells has been proposed to contribute to changes in airway structure. The resulting changes form the basis for airway wall remodelling, a phenomenon believed to have profound consequences on airway function. Human ASM cells in culture provide a useful model for investigating physiological responses, and studies of human ASM cell responses to mediators of asthma are important for understanding the mechanisms involved in airway inflammation and remodelling.

Bronchoalveolar lavage fluid and serum from symptomatic asthma patients contains significantly increased levels of pro-inflammatory cytokines, growth factors and other inflammatory mediators, suggesting that these mediators may also be in the vicinity of the airway mucosa.[4–11] Moreover, pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 have been reported to stimulate DNA synthesis in cultured ASM cells derived from different species.[12–14] IL-6 has pro-inflammatory effects that may be relevant to airway wall inflammation, including mucus hypersecretion, the terminal differentiation of B cells into antibody producing cells, upregulation of IL-4-dependent immunoglobulin (Ig)E production, and differentiation of immature mast cells.[15–18] Possible anti-inflammatory properties of IL-6 include the inhibition of macrophage cytokine production, stimulation of the production of anti-inflammatory molecules such as IL-1 receptor antagonist and soluble TNF receptor p55, and reduced airway responsiveness.[19–24]

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The nuclear proto-oncogene c-fos is a member of the leucine zipper family and it heterodimerises with c-jun to constitute the AP-1 transcription factor.[25] Induction of c-fos, and subsequent cell proliferation and/or differentiation, has previously suggested that c-fos can be considered a proliferation marker.[25,26] The purpose of the present study was to investigate the effects of a range of cytokines (TNF-α, IL-1β, IL-5 and IL-6) on the expression of the immediate early response gene, c-fos, as well as the release of the pleiotropic cytokine IL-6 in cultured human ASM cells. Our results demonstrate that all of the cytokines investigated induced a rapid and transient expression of mRNA encoding c-fos. Moreover, they induced the release of IL-6 protein into the conditioned medium. However, none of the cytokines stimulated DNA biosynthesis or ASM cell mitogenesis. These results suggest that it may be preferable to describe c-fos as a marker of 'activation' in ASM cells – leading to the expression of cytokines, such as IL-6, rather than as a marker of proliferation.

Materials and methods

Materials

The following solutions and reagents were obtained from Life Technologies BV (Breda, The Netherlands): sodium pyruvate, non-essential amino acid mixture, gentamicin, penicillin/streptomycin, amphotericin B, Dulbecco's modified Eagle's medium (DMEM), and 0.5% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA). Insulin, transferrin and ascorbate, IL-1β and IL-5 were purchased from Sigma BV (Zwijndrecht, The Netherlands). We procured foetal bovine serum (FBS) from Bio-Whitaker BV (Verviers, Belgium), TNF-α from Knoll AG (Ludwigshaven, Germany), *methyl*-[^{3}H]-thymidine from Amersham Nederland BV ('s-Hertogenbosch, The Netherlands) and recombinant human IL-6 from Promega (Leiden, The Netherlands). Human specific antibodies for the IL-6 enzyme-linked immunosorbent assay (ELISA assay) were procured from Medgenix (Breda, The Netherlands).

Human ASM cell culture

Human bronchial smooth muscle cells were isolated as described previously.[27,28] Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained immediately following surgery of patients with lung carcinoma. The medical ethical and research committee of Erasmus University Medical Center approved this study (MEC 150.321/1996/45). After removal of the epithelium, pieces of smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle fragments were incubated in HBSS containing bovine serum albumin (fraction V, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (type IV, 3.3 U/ml) at 37°C in a humidified incubator (ASSAB, model T154; Clean Air Techniek BV, Woerden, The Netherlands) containing 5% CO_2 in air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in DMEM containing 10% (v/v) FBS supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml). Cells were subsequently seeded in a 35 mm dish and maintained in culture by replacing the medium every 72 h. After 10–14 days in culture, ASM cells grew to confluence and were then removed from the plastic base of each dish using 0.5% trypsin/0.02% EDTA, and sub-cultured into 25 cm^2 tissue culture flasks. At confluence, cells were further passaged using trypsin/EDTA solution into 75 cm^2 tissue culture flasks. Confluent cells in the fourth to sixth passages were used for experiments.

Stimulation of human ASM cells

The ASM cell growth was synchronised prior to treatment by washing the cell monolayers twice in phosphate-buffered saline (PBS) (140 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$; pH 7.4) and then replacing the medium with serum-free DMEM supplemented with 1 μM insulin, 5 μg/ml transferrin and 100 μM ascorbate for 72 h. Using flow cytometric analysis of human ASM cells, we have found that 72 h of serum deprivation resulted in approximately 85–90% of the cells remaining in the G_0/G_1 phase. Growth-arrested cell monolayers were treated with either TNF-α (10 ng/ml), IL-1β (0.5 ng/ml), IL-5 (2 ng/ml) or IL-6 (5 ng/ml) in fresh FBS-free DMEM in a time-dependent manner. Cell-conditioned medium was collected and stored at –20°C until assayed for IL-6 levels by ELISA. Stimulation of human ASM cells with cytokines can induce the release of prostaglandins, which may then inhibit the proliferation of ASM cells.[29] Therefore, we also examined the effects of the aforementioned cytokines on human ASM cell proliferation in the presence of indomethacin (1 μM), which is a non-specific cyclo-oxygenase inhibitor.

Isolation of total cellular RNA and Northern blot analysis

Treated and untreated human ASM cells were washed in PBS and directly lysed in guanidinium thiocyanate buffer. The lysate was repeatedly passed through a 23-gauge needle to shear the genomic DNA. Total cellular RNA was isolated using the method as described earlier.[27] The RNA concentration was estimated by optical density measurements and a DNA/protein ratio of ≥1.8 was accepted. Samples of total

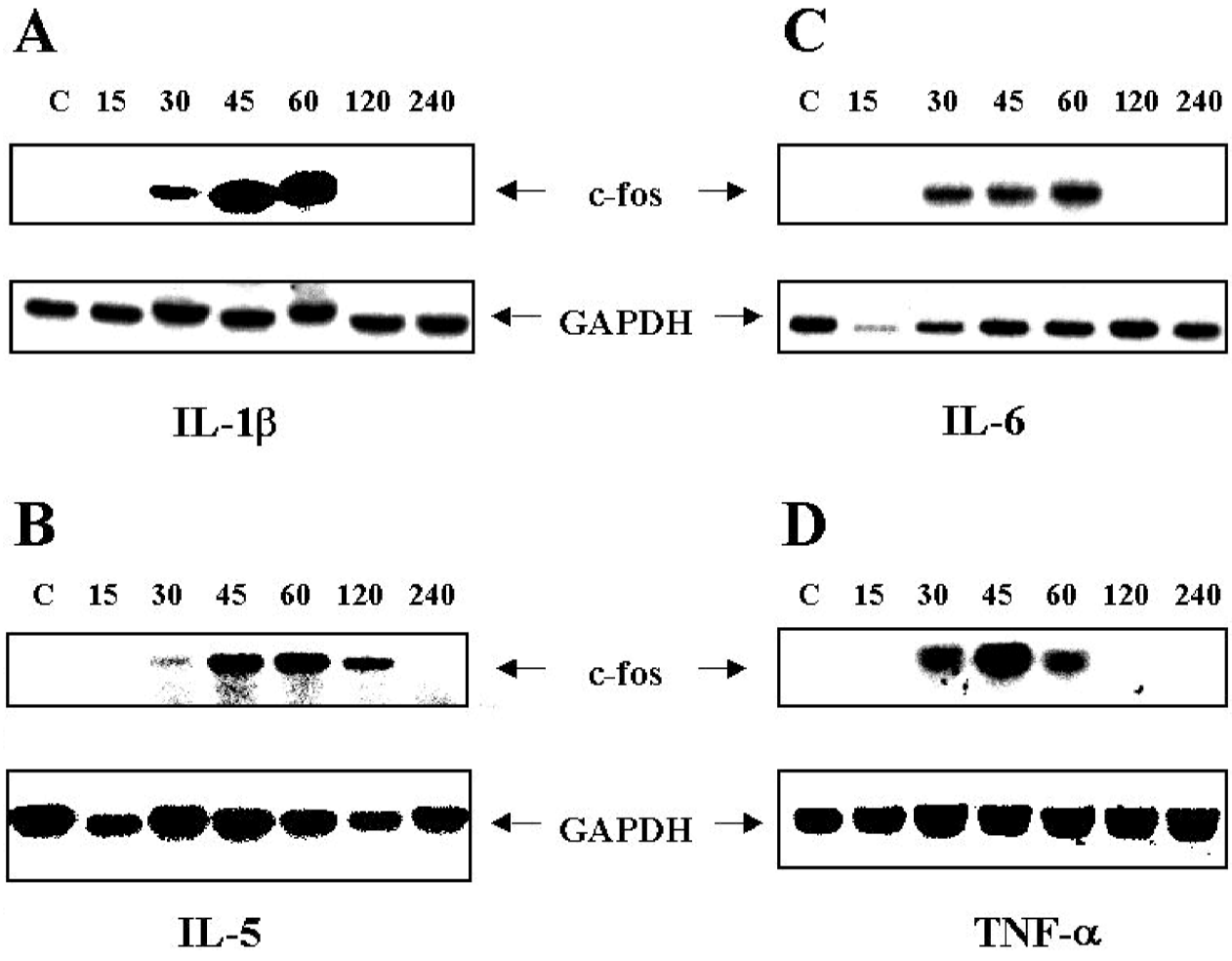

FIG. 1. Representative Northern blots of c-fos expression in human airway smooth muscle (ASM) cells treated with: (A) IL-1β (0.5 ng/ml), (B) IL-5 (2 ng/ml), (C) IL-6 (5 ng/ml), or (D) TNF-α (10 ng/ml). Total RNA from control C (cultured in serum-free DMEM) and treated cells was subjected to Northern hybridisation with a radiolabelled c-fos cDNA probe. Rehybridisation of each filter with a GAPDH cDNA probe was performed for reference purposes. These Northern blots are representative of blots from three independent experiments.

RNA (10 μg) were denatured at 65°C in a formaldehyde-containing loading buffer and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide stained gels were photographed and RNA was transferred onto hybond-N membrane (Amersham Nederland BV) by the alkaline downward capillary transfer method also described previously.[27] The filters were air-dried and ultraviolet cross-linked in a gene linker (Biorad Laboratories B.V., Veenendaal, The Netherlands). Blots were hybridised with radiolabelled mouse c-fos (2.1 kb fragment) or a reference house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (American Type Culture Collection, Rockville, MD, USA).[27] Hybridisation signals were quantified by scanning laser densitometry using the Ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden). Signals were normalised with respect to GAPDH mRNA values and expressed as relative optical density in stimulated cells versus controls.

Measurement of IL-6 protein levels by ELISA

IL-6 protein levels in ASM cell-conditioned medium, after 24 h of stimulation, were determined using a solid-phase sandwich ELISA as described previously.[28] The increase in IL-6 secretion was expressed as nanograms per millilitre, and data are shown as the fold increase in IL-6 secretion relative to controls. The detection limit of the IL-6 ELISA method was 50 pg of IL-6/ml. No correction for cell number variation was made because the cells were serum deprived 16–24 h after plating out, allowing insufficient time for proliferation.

[^{3}H]-Thymidine incorporation assay

Effects of the different cytokines on DNA biosynthesis was evaluated by incorporation of *methyl*-[^{3}H]-thymidine. Cells were transferred into 24-well plates at a seeding density of 3×10^4 cells/well. After

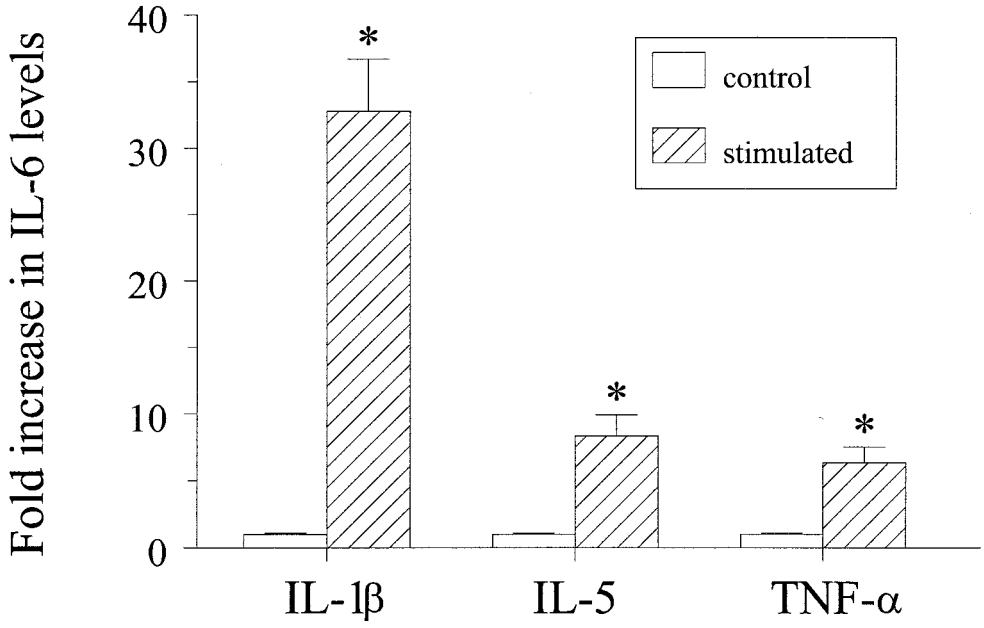

FIG. 2. Production and release of IL-6 by human airway smooth muscle (ASM) cells. Growth-arrested ASM cells were stimulated in the absence (control) or presence of different cytokines for 24 h. Data represent the mean ± SEM of triplicate values from three or four independent experiments using conditioned medium from ASM cells cultured from different patients. Data are expressed as the fold increase in IL-6 secretion compared with respective controls.

24 h in culture, the sub-confluent cell monolayers were growth arrested as already described. Cells were incubated with *methyl*-[^{3}H]-thymidine (1 μCi/well) in either fresh FBS-free DMEM (control), DMEM containing the various cytokines, or 10% serum for 1, 2, 3, 4 or 5 days. After stimulation, the cells were washed in PBS, fixed with ice-cold methanol and exposed to ice-cold trichloroacetic acid (5% w/v). The acid-insoluble fraction was lysed in 0.3 M NaOH and the incorporated radioactivity was determined in a liquid-scintillation counter (Packard 1500 Tri-carb liquid scintillation analyser; Packard-Becker BV, Delft, The Netherlands). The data are expressed as the fold increase in thymidine incorporation relative to controls.

Cell counting

To assess changes in cell number in relation to pro-inflammatory cytokines, we also performed direct cell counting. Cells were incubated with the aforementioned stimuli for 7 days. Following stimulation, the cell-conditioned medium was removed and the cells were washed twice in PBS and then detached in 50 μl of trypsin/EDTA by incubating at room temperature for 10 min. We then added 50 μl PBS to each well and the cells were dispersed by gentle pipetting. The resulting cell suspension was added to 10 ml of isotonic counting solution (sodium chloride, 6.38 g/l; sodium tetraborate, 0.2 g/l; boric acid, 1.0 g/l; EDTA 0.2 g/l) and the cells were counted in a CASY-1 Coulter counter (Schärfe system, Reutlingen, Germany).

Statistical analysis

All data in the figures and tables are presented as mean ± SEM. Statistical analysis was performed using a two-tailed, independent sample *t*-test. Significance was accepted at $p < 0.05$.

Results

Expression of the c-fos proto-oncogene

Expression of the proto-oncogene c-fos, a member of the leucine zipper family, was examined in cultured human ASM cells treated with different cytokines. Representative Northern blots showing the expression pattern of c-fos in relation to IL-1β (0.5 ng/ml), IL-5 (2 ng/ml), IL-6 (5 ng/ml) or TNF-α (10 ng/ml) are shown in Figure 1. IL-1β and IL-5, IL-6 and TNF-α induced the expression of mRNA encoding c-fos in cultured human ASM cells as early as 30 min, and the expression levels reached a maximum between 45 and 60 min. Rapidly and transiently induced mRNA levels declined abruptly to non-detectable levels at 120 min, except in the case of IL-5 where c-fos mRNA levels remained elevated up to 120 min (Fig. 1B). To compare the expression pattern and to verify the integrity of total RNA samples, GAPDH, a housekeeping gene was used to re-hybridise the membranes. A strong dark band at 1.4 kb was expressed in each RNA preparation (Fig. 1 A–D).

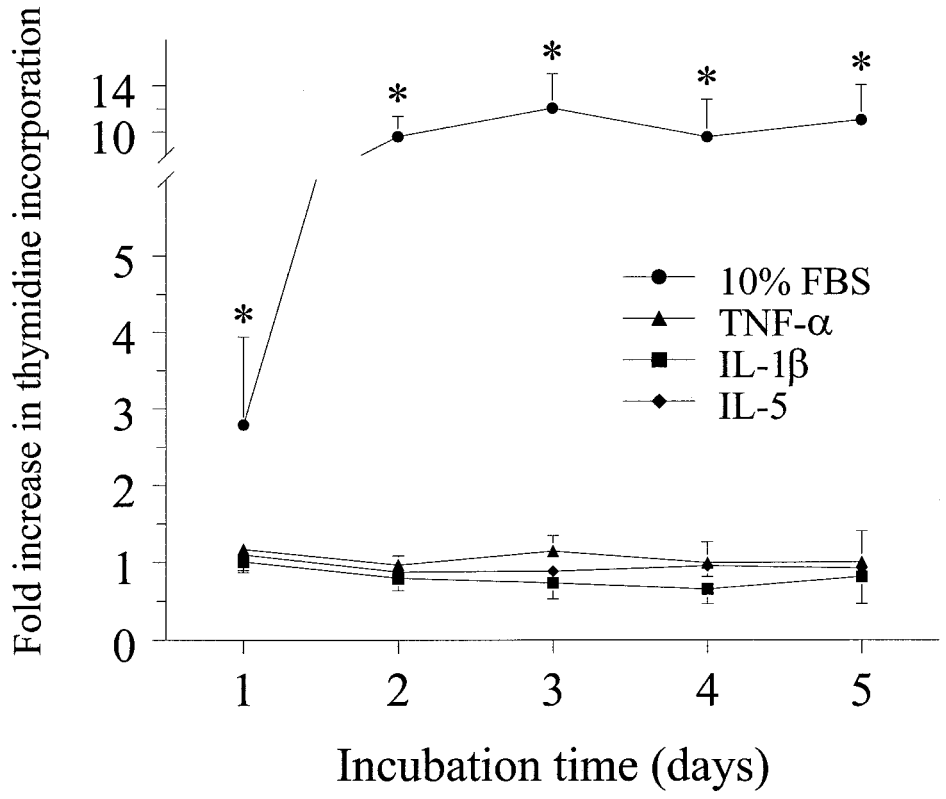

FIG. 3. Thymidine incorporation measured after 5 days in semi-confluent, growth arrested, human airway smooth muscle (ASM) cells stimulated with 10% serum (circles), 10 ng/ml TNF-α (triangles), 0.5 ng/ml IL-1β (squares) or 2 ng/ml IL-5 (diamonds). Values are calculated from three or four independent experiments of four determinations each, and expressed as the fold increase in thymidine incorporation relative to controls. FBS, foetal bovine serum.

Release of IL-6 protein into the conditioned medium

The concentration of IL-6 in the conditioned medium of cultured human ASM cells following 24 h of stimulation with IL-1β, IL-5 and TNF-α was 6.56 ± 0.78, 1.10 ± 0.08 and 3.17 ± 0.64 ng/ml, respectively. Conditioned medium derived from controls cells showed only a negligible amount (0.14 ± 0.02 ng/ml) of IL-6. The fold increase in IL-6 concentration in relation to different cytokines after normalising with their respective controls is shown in Figure 2. IL-1β was the most potent cytokine for cultured human ASM cells in producing IL-6 protein, where the levels rose over 30-fold as compared with the respective control (Fig. 2). To verify that the measured amounts of IL-6 in the conditioned medium were due to the release of *de novo* synthesised IL-6, we also assessed the concentration of IL-6 in the cell lysates of unstimulated human ASM cells. These IL-6 levels were found to be negligible (0.10 ± 0.01 ng/ml).

[^{3}H]-Thymidine incorporation

Figure 3 shows the DNA biosynthesis data assessed by [^{3}H]-thymidine incorporation into DNA during a period of 5 days. We found that 10% FBS increased thymidine incorporation up to 10-fold as compared with cells cultured in medium deprived of FBS (controls). Surprisingly, none of the cytokines studied here could result in the induction of DNA biosynthesis measured by [^{3}H]-thymidine incorporation assay in this cultured human ASM cell model. Even in the presence of the cyclo-oxygenase inhibitor indomethacin, the aforementioned cytokines failed to elicit a proliferative response in cultured human ASM cells even up to 3 days of stimulation (Table 1).

Cell counting

Cell counts did not change after 7 days of incubation of serum-deprived human ASM cells with the different cytokines (TNF-α, IL-1β, IL-5 and IL-6) at the concentrations mentioned earlier. The cell counts data depicted in Table 2 once again verify the non-mitogenic response of the cytokines studied here, and this data is in agreement with the [^{3}H]-thymidine incorporation results.

Discussion

The purpose of the present study was to investigate the relative contributions of a number of pro-inflammatory cytokines, on responses of cultured

Table 1. [^{3}H]-Thymidine incorporation into DNA, measured after 3 days in semi-confluent, growth-arrested human airway smooth muscle cells stimulated with TNF-α (10 ng/ml), IL-1β (0.5 ng/ml), IL-5 (2 ng/ml) and IL-6 (5 ng/ml) in the presence or absence (control) of 1 μM indomethacin

Treatment	TNF-α	IL-1β	IL-5	IL-6
– 1 μM indomethacin	1.0 ± 0.11	1.0 ± 0.11	1.0 ± 0.11	1.08 ± 0.16
+ 1 μM indomethacin	0.88 ± 0.12	1.08 ± 0.20	0.97 ± 0.20	0.88 ± 0.12

Values are calculated from one to three independent experiments of four determinations each, and are expressed as the fold increase in thymidine incorporation relative to controls ± SEM. Baseline thymidine incorporation at 72 h of stimulation was 5500 ± 1000 disintegration per minute (dpm)/well.

human ASM cells, by evaluating their individual ability to induce the expression of the immediate early response gene c-fos, as well as the release of the pleiotropic cytokine IL-6. Also, the ability of the pro-inflammatory cytokines to induce cell mitogenesis, under identical experimental conditions, was evaluated. Our results demonstrate that a rapid and transient expression of mRNA encoding c-fos was induced by all of the cytokines investigated. However, none of the cytokines induced DNA biosynthesis or an increase in cell numbers in this human ASM cell model. Moreover, all of the cytokines investigated induced the release of *de novo* synthesised IL-6 protein into the cell-conditioned medium.

The rapid and transient induction of the immediate early response gene c-fos, and subsequent cell proliferation and/or differentiation, has previously suggested that c-fos can be considered a proliferation marker in ASM cells.[16,30] However, it has also been shown that c-fos induction alone is insufficient for cell proliferation in a number of cells of mesenchymal origin.[31,32] Histamine and ET-1 have previously been shown to increase levels of c-fos mRNA, peaking at 30 min of stimulation, in canine and ovine ASM cells, respectively.[16,30] Both of these studies associated c-fos expression with ASM cell mitogenesis. However, we could not demonstrate that c-fos expression was directly associated with a proliferative response in our human ASM cell model. These findings are also supported by one of our previous studies, where we showed that angiotensin II induced the expression of c-fos without subsequent cell proliferation.[27]

Table 2. Human airway smooth muscle cell numbers after 7 days of stimulation with different cytokines

Treatment	Cell number (fold increase relative to control)
TNF-α	0.97 ± 0.10
IL-1β	0.77 ± 0.12
IL-5	1.30 ± 0.39
IL-6	1.06 ± 0.13

Values are calculated from three or four independent experiments of four determinations each, and are expressed as the fold increase in cell number relative to controls ± SEM.

Stimulation of human ASM cells with either TNF-α (10 ng/ml) or IL-1β (0.5 ng/ml), in serum-free medium, induced the expression of c-fos but did not result in an increase in thymidine incorporation or cell counts in our study. Although work from other groups has previously shown a mitogenic response, it is important to emphasise that the culture media, TNF-α (10 ng/ml) in the study from Amrani *et al.*[14] and IL-1β (0.1 ng/ml) in the study from De *et al.*,[13] contained serum.[12–14] TNF-α, IL-1β and growth factors (present in serum) can activate mitogen-activated protein (MAP) kinases in smooth muscle cells.[33–35] MAP kinases are key transducers of extracellular signals in pathways leading to cell proliferation and differentiation. Signals activated by TNF-α or IL-1β together with serum may synergistically induce proliferation, possibly explaining the previously reported proliferative response. Stewart *et al.* found an increase in thymidine incorporation using TNF-α at very low concentrations (5.0 pg/ml to 0.5 ng/ml) in human ASM cells, but at higher concentrations (5.0 ng/ml) this effect was abolished.[12] De *et al.* also found that IL-1 in the absence of indomethacin inhibited proliferation of ASM cells.[13] We did not observe proliferative effects in either the absence or in the presence of indomethacin, again suggesting that considerably low concentrations of serum-derived factors may synergistically contribute to ASM cell mitogenesis.

The upregulation of c-fos expression, in human ASM cells, by the pro-inflammatory cytokines investigated in our study, was thus insufficient for the induction of cell proliferation. Radiolabelled thymidine incorporation demonstrated that serum-induced DNA biosynthesis in cultured human ASM cells, which is a well-established phenomenon in ASM cell culture. IL-5 stimulation of human ASM cell proliferation has not previously been reported, although ASM cells have been reported to express and secrete IL-5 in response to the serum obtained from allergic asthma patients.[36] We found that IL-5 (2 ng/ml) is not mitogenic for human ASM cells, even in the presence of indomethacin.

The c-fos protein, which is able to translocate to the nucleus and bind to c-jun, forms a heterodimeric complex known as activator protein-1 (AP-1). This transcription factor can activate gene transcription by

binding to the AP-1 site in the promoter region of target genes. Several recent studies have shown that ASM cell stimulation by cytokines leads to the expression and subsequent release of a number of inflammatory cytokines and chemokines.[28,37–42] The majority of these pro-inflammatory cytokines contain AP-1 binding sites in the promoter region of their genes. We have recently shown that c-fos induction precedes IL-6 protein secretion by human ASM cells.[28] The transient upregulation of c-fos that we measured in this study may contribute to the induction and release of cytokines, such as IL-6, thereby playing a role in the regulation of inflammatory responses in asthmatic airways. IL-6 is upregulated in the bronchoalveolar lavage fluid from symptomatic asthma patients and has pro-inflammatory effects that may be relevant to airway wall inflammation. These include mucus hypersecretion, the terminal differentiation of B cells into antibody producing cells, upregulation of IL-4-dependent IgE production, and differentiation of immature mast cells.[15–18]

The present study suggests that ASM cells may play a role in the regulation of airway inflammation through the production of cytokines, like IL-6. This may occur via upregulation of different genes, including cytokines and chemokines such as TGF-β_1, IL-8, granulocyte macrophage-colony stimulating factor or RANTES.[27,28,37–39] Although the individual cytokines investigated in our study did not induce human ASM cell proliferation, we cannot exclude that their concerted actions in asthmatic airways may contribute to ASM remodelling. Evidence for the importance of combinations of mediators has recently been published.[43] Whether the endogenous generation of cytokines by ASM cells, and especially the release of IL-6, contributes to the pathogenesis of airway inflammation and remodelling is unclear, but the capacity of ASM cells to produce IL-6 suggests that these cells could participate in local inflammatory events in the airways.

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