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Effects of interleukin-1β, interleukin-13 and transforming growth factor-β on gene expression in human airway smooth muscle using gene microarrays

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Abstract

Inflammatory gene expression in airway smooth muscle may be influenced by its inflammatory milieu. We analysed the gene expression profile of airway smooth muscle cells cultured from human airways exposed to a pro-inflammatory cytokine, interleukin-1β, a T helper-2 cytokine, interleukin-13, and to a growth factor, transforming growth factor (TGF)β1 (10 ng/ml each) after 4 and 24 h using the Affymetrix GeneChip 95A array which detects ~12,500 genes and expression sequence tags (ESTs). Airway smooth muscle cells were responsive to each cytokine with distinctive patterns of gene expression for cytokines, chemokines, adhesion and signalling proteins, and transcription factors. Interleukin-1β induced the highest number of genes such as cytokines/chemokines including interleukin-8, growth-related oncogene (GRO)-α, -β and -γ, epithelial neutrophil activating protein (ENA)-78, monocyte chemotactic protein (MCP)-1, -2 and -3 and eotaxin. Using quantitative real-time reverse transcription-polymerase chain reaction, the expression of GRO-α, -β and -γ, interleukin-8 and eotaxin by interleukin-1β was confirmed, with good correlation with microarray data. Transforming growth factor (TGF)β1 induced other growth factors such as connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), insulin growth factor (IGF) and many structural and extracellular matrix proteins. Interleukin-13 was the weakest inducer, with stimulation of eotaxin and genes of unknown function. While many genes were co-regulated at 4 and 24 h, there were also differences in expression patterns. Interleukin-1β induces a predominantly pro-inflammatory profile while TGFβ1 can be linked to proliferative and matrix changes. The rich profile of mediators, growth factors and signalling molecules released from airway smooth muscle depends on the inflammatory milieu.

Keywords: Airway smooth muscle; Cytokine stimulation; Microarray; Growth factor; Airway remodelling

1. Introduction

The airway smooth muscle has been regarded for a long time as mainly passive, particularly responding to bronchoconstrictor mediators released from other cells by contracting, leading to narrowing of the airways and airflow obstruction. An increase in airway smooth muscle mass accounted for by hyperplasia and also by hypertrophy has been described (Dunnill, 1960; Ebina et al., 1993), which could contribute to exaggerated airway narrowing observed in asthma (Lambert et al., 1993; James et al., 1989). Airway smooth muscle cells can maintain a proliferative phenotype in response to inflammatory mediators and growth factors (Panettieri, 1998), and airway smooth muscle cultured from bronchial biopsies obtained from asthmatics are in an enhanced state of proliferation (Johnson et al., 2001).

Airway smooth muscle cells also exhibit a synthetic potential with the elaboration of inflammatory mediators,

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and can also respond to its inflammatory milieu by expressing and releasing proteins that renders the muscle cell a potentially active participant of the inflammatory response (Chung, 2000). Airway smooth muscle cells can also produce arachidonic acid metabolites such as prostaglandin E_2, chemokines such as regulated upon activation, normal T-cell expressed and secreted (RANTES), eotaxin, monocyte chemotactic protein (MCP)-1, -2 and -3, cytokines such as interleukin-5,-6 and -11, receptors for cytokines such as tumour necrosis factor-α, interleukins-4 and -13, adhesion molecules, and growth factors (Johnson and Knox, 1997). Interactions of airway smooth muscle cells with inflammatory and immune cells such as T-cells, eosinophils and mast cells have been proposed (Chung, 2000). For example, anti-CD3-stimulated peripheral blood T-cells adhere to airway smooth muscle cells and upregulate intercellular adhesion molecule (ICAM)-1 expression partly through interferon (IFN)γ and induce the expression of MHC Class II (Lazaar et al., 1997). The expression of CD40, a member of the tumour necrosis factor receptor family, is increased by tumour necrosis factor-α or IFNγ (Lazaar et al., 1998). Airway smooth muscle may also participate in airway wall remodelling through the secretion of structural and extracellular matrix proteins, and of growth factors (Amrani and Panettieri, 2003).

It is clear that the airway smooth muscle can be exposed to different inflammatory environments, as evidenced by potential interactions with different inflammatory cell types such as T cells and mast cells (Amrani and Panettieri, 2003). Little is known about other aspects of the inflammatory response required to transduce inflammatory signals in the airway smooth muscle and the integrated networks that operate to produce inflammatory and structural responses. During various inflammatory conditions in the airways, the response of the airway smooth muscle could be different, leading to distinct phenotypic changes (Chung, 2000). To examine this, we studied the effect of three different cytokines with distinct spectrum of activities such as interleukin-1β, a pro-inflammatory cytokine, interleukin-13, a T helper2-cytokine and transforming growth factor (TGF)β, a growth factor, on the expression of genes from human airway smooth muscle cells using gene microarray matrix. This comprehensive study shows that the transcriptional response of the airway smooth muscle is different and specific for these stimuli.

2. Methods

2.1. Airway smooth muscle cell culture and stimulation

Human bronchial tissue was obtained from two patients undergoing surgical resection for lung carcinoma. Airway smooth muscle cells were cultured as described previously (Hirst et al., 1992). Briefly, bands of smooth muscle from bronchi were cut out from surrounding connective tissue and were washed with Hank's balanced salt solution (HBSS), placed in 1 ml Dulbecco's modified Eagle's Medium (DMEM) containing 1 mg/ml collagenase and maintained in a humidified atmosphere at 37 °C in 5% CO_2/95% air (vol/vol) for 24 h. The resulting cell suspension was centrifuged ($200\times g$ for 5 min) and the pellet was resuspended in supplemented DMEM containing 10% foetal bovine serum. Once airway smooth muscle cells had grown to confluence, they were passaged and transferred into 175-mm^2 tissue culture flasks. Airway smooth muscle cells displayed typical 'hill and valley' growth patterns, and staining for smooth muscle α-actin, calponin and smooth muscle myosin heavy chain was positive for greater than 95% of cells in culture. Airway smooth muscle cells at passage 5 grown to confluence in 162 cm^2 tissue culture flasks were placed in supplemented serum-free DMEM. Following 24 h of serum deprivation, airway smooth muscle cells were incubated with interleukin-1β, interleukin-13 or TGF-β1, at 10 ng/ml each, for 4 or 24 h.

2.2. RNA extraction and microarray analysis

Total RNA was extracted from cells using TRIZOL (LifeTechnologies) as described by the manufacturer and further purified using the RNeasy mini-columns from Qiagen (Valencia, CA, USA). RNA samples were stored at −80 °C until being processed and hybridized to oligonucleotide microarrays as described (Lockhart et al., 1996). The HG-U95Av2 Array (Affymetrix, Santa Clara, CA, USA) containing probes for 12,500 annotated genes and expression sequence tags (ESTs) was used. Probe preparation and microarray hybridisation were performed in duplicates. A total of 32 microarrays were used in these experiments.

2.3. Microarray data analysis

Data analysis was performed by software developed by the Novartis Pharmacogenomics department and by using the Expressionist™ Version 3.1 software (GeneData, Basel, Switzerland). Initial analysis of data quality and reproducibility included the determination of unnormalised signal intensity distribution across all microarrays and the comparison of signals detected on duplicate chips. Data were then normalized for each microarray using the logarithmic mean of total signal intensity. The analysis of the normalised data included hierarchical clustering of all experiments using Expressionist with genes called present by GeneChip software (Affymetrix) and also by using all genes. The determination of genes differentially regulated between experiments (different cytokines, different time points) was performed by using both Expressionist and the NPGN software, both yielding essentially identical results. As the noise in the microarray data increases with decreasing signal intensity, generally a cut-off rate of >2-fold was used for signals

greater than the average normalised signal intensity and >3-fold for signals that were lower than the average normalised signal intensity. In our experience, with these cut-off values >90% of microarray hits can be verified by an independent method (e.g. TaqMan reverse transcription real-time polymerase chain reaction, RT-QPCR) at least qualitatively though not necessarily with identical differential expression values. For the annotation and functional grouping of hits several databases including the Ensembl Human Genome Browser at the Sanger Institue (http://www.ensembl.org/Homo_sapiens/) and the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/UniGene/) were used.

2.4. TaqMan analysis

Messenger RNA levels in total cellular RNA samples were measured by TaqMan RT-QPCR. First strand cDNA was synthesised using the TaqMan Reverse Transcription Reagents and random hexamer primers from Applied Biosystems as recommended by the manufacturer. Gene-specific primers and probes were designed using Primer Express™ software (Applied Biosystems). Primer-probe sets were designed across exon–intron boundaries to avoid amplification of genomic DNA whenever possible. The sequence of the primers and probes were as follows. Growth-related oncogene (GRO)-α: forward 5′-ATAGAGGCTGGCGGATCCA, reverse 5′-TACATTCCCCTGCCTTCACAA, probe 5′-CAAATGGCCAATGAGAT; GROβ: forward 5′-CCCATGGTTAAGAAAATCATCGA, reverse 5′-TTCCTTCTGGTCAGTTGGATTTG, probe 5′-AGATGCTGAAAAATG; GROγ: forward 5′-AGCGTCCGTGGTCACTGAA, reverse 5′-AGGTGAATTCCCTGCAGTGTCT, probe 5′-TGCGCTGCCAGTGC; interleukin-8: forward 5′-TTGGCAGCCTTCCTGATTTC, reverse 5′-TTTAGCACTCCTTGGCAAAACTG, probe 5′-CAGCTCTGTGTGAAGGT. All probes were synthesised using the minor groove binding (MGB) chemistry. Quantitative RT-QPCR reactions were performed in triplicate in 25 μl final volumes and contained final concentrations 1× Taqman Universal PCR master mix containing AmpliTaq Gold® DNA Polymerase (Applied Biosystems) with 10 ng of target cDNA. The primer concentrations were 900 nM for the forward and reverse primers and 250 nM for the labelled probe. All probes for the target genes were labelled with the reporter dye FAM (6-carboxy-fluorescein) and the internal control genes transferrin receptor (TrR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the reporter dye VIC. Experiments were performed using an ABI Prism® 7700 Sequence Detection System sequence detector (Applied Biosystems) and analysed using ABI PRISM 7700 Sequence Detection System software. Amplification conditions were as follows: 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Standard curves were generated for each target and internal control gene using

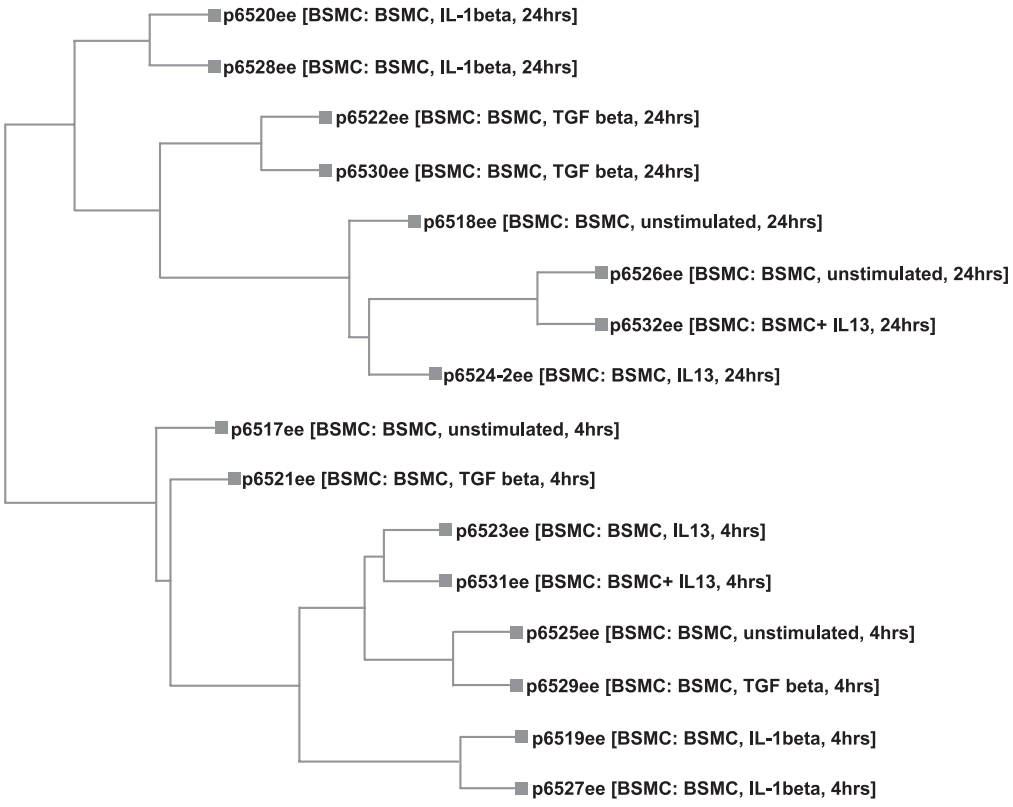

Fig. 1. Dendrogram of microarrays. Hierarchical clustering by positive correlation of all experiments was performed using normalised expression data for every gene from each stimuli and time point.

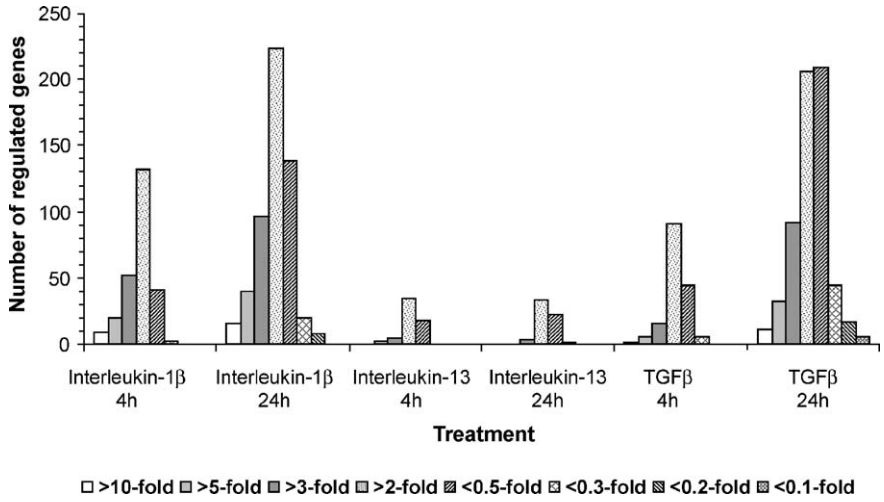

Fig. 2. Gene expression changes in response to cytokine stimulation. Genes that were differentially expressed between stimulated and control samples were identified by comparing normalised average signal intensities obtained from duplicate chips. The obtained lists were then manually curated and genes with unsatisfactory correlation between duplicates removed. Genes were then ranked by fold expression differences for each time point for each cytokine and the number of genes with greater then 2-, 5- and 10-fold induction or repression was plotted for each experiment.

serial dilutions of cDNA and data for the experimental samples determined using the standard curve, normalised to the internal control and expressed as fold increase/decrease of control samples.

3. Results

3.1. Global gene expression in airway smooth muscle cells upon interleukin-1β, interleukin-13 and TGFβ1 stimulation

Cluster analysis showed that interleukin-1β, interleukin-13 and TGFβ1 initiated different transcriptional programs. Duplicate samples, samples stimulated with the same cytokine, and samples stimulated for the same length of time clustered together (not shown). A few exceptions included samples stimulated with interleukin-13 for 24 h and with TGFβ1 for 4 h that clustered with unstimulated samples indicating a less significant global effect of interleukin-13 at 24 h and of TGFβ1 at 4 h on gene expression (Fig. 1). As a control, and to exclude the possibility that some of the observed effects were patient-specific, we repeated the experiment with samples obtained from a different individual. There was good correlation for the two patients, with the exception of a small subset of genes.

A summary of the data obtained when using cut-off rates of 2-, 3-, 5- and 10-fold is presented in Fig. 2. We focused our analysis on genes with the largest fold increases as these generally represent the most reproducible subset of the differentially expressed genes. Furthermore, the genes that are strongly induced are very likely to play important roles even though the functional significance of their differential expression cannot be established with the approaches we used.

Interleukin-1β at 4 and 24 h and TGFβ1 at 24 h have the strongest overall effect on gene expression in airway smooth muscle cells, whereas interleukin-13 at both time points and

Table 1
Number of genes upregulated (>2-fold increase) according to class at 4 and 24 h after interleukin-1β, TGFβ1 and interleukin-13

Molecules	Interleukin-1β (4 h)	Interleukin-1β (24 h)	TGFβ1 (4 h)	TGFβ1 (24 h)	Interleukin-13 (4 h)	Interleukin-13 (24 h)
Cell surface receptors	6	5	5	8	2	2
Cytokines/chemokines and cytokine/chemokine receptors	20	25	2	5	3	2
Growth factors	9	7	6	18	0	0
Adhesion molecules	3	2	1	8	0	3
Transcription factors and nuclear proteins	32	19	25	34	10	3
Signalling proteins	17	17	12	17	5	4
Proteases/protease inhibitors	9	8	2	8	2	1
Structural proteins and extracellular matrix proteins	3	3	2	28	1	0
Enzymes	12	15	3	35	4	9
Ion channels and membrane transport proteins	2	8	2	9	0	1

Table 2
Genes upregulated by interleukin-1β after 4 h (A) and 24 h (B) of stimulation

GeneBank accession	Description	Expression level in resting cells	Fold induction
(A)			
M36821	GRO3 oncogene	78.4	43.3
M36820	GRO2 oncogene	105.1	37.3
X03656	colony stimulating factor 3 (granulocyte)	22.5	25.1
M17017	interleukin-8	141.9	23.9
X04430	interleukin-6 (interferon, beta 2)	299.3	19.1
M13207	colony stimulating factor 2 (granulocyte-macrophage)	20.0	18.9
M24283	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	21.9	16.6
M59465	TNFalpha-induced protein 3 (A20, antipoptotic Zn-finger)	132.3	15.3
L24564	Ras-related associated with diabetes	23.2	10.2
M92357	tumor necrosis factor, alpha-induced protein 2 (B94 protein)	79.4	9.6
U64197	small inducible cytokine subfamily A (Cys–Cys), member 20 (MIP-3a)	20.0	8.0
X54489	GRO1 oncogene (melanoma growth stimulating activity, alpha)	468.7	7.6
AF059617	serum-inducible kinase	96.0	7.2
AB002344	EST; KIAA0346 protein	23.2	7.0
M60278	diphtheria toxin receptor (heparin-binding EGF-like growth factor)	33.9	6.0
M58603	nuclear factor of kappa light polypeptide gene enhancer in B-cells1 (p105)	223.4	5.8
U04636	prostaglandin-endoperoxide synthase 2	399.0	5.7
U12767	nuclear receptor subfamily 4, group A, member 3	23.5	5.7
M28225	small inducible cytokine A2 (monocyte chemotactic protein 1)	554.0	5.6
U45878	inhibitor of apoptosis protein 1 (HIAP1) (HIAP-1)	24.4	5.6
(B)			
M28130	interleukin-8	45.8	124.2
M36821	GRO3 oncogene	49.5	53.0
X03656	colony stimulating factor 3 (granulocyte)	27.7	50.7
U04636	prostaglandin-endoperoxide synthase 2	40.5	22.4
M36820	GRO2 oncogene	132.0	19.8
U19557	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 4	27.1	19.7
X72308	small inducible cytokine A7 (monocyte chemotactic protein 3)	77.9	17.9
U81234	small inducible cytokine subfamily B (Cys–X–Cys), member 6 (GCP-2)	385.5	17.1
X04430	interleukin-6 (interferon, beta 2)	350.5	17.0
X78686	small inducible cytokine subfamily B (Cys–X–Cys), member 5 (ENA-78)	298.0	16.4
S66896	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3	20.0	12.0
J04513	fibroblast growth factor 2 (basic)	32.3	11.7
AF010316	prostaglandin E synthase	158.0	11.6
X54489	GRO1 oncogene (melanoma growth stimulating activity, alpha)	498.1	11.3
U77735	pim-2 oncogene (Ser/Thr prot kinase)	34.9	10.3
M13207	colony stimulating factor 2 (granulocyte-macrophage)	20.0	9.8
X77956	inhibitor of DNA binding 1, dominant negative helix–loop–helix protein	109.1	9.7
AB008109	regulator of G-protein signalling 5	33.3	9.5
Y00630	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	68.8	9.5
U27768	regulator of G-protein signalling 4	47.0	8.9

TGFβ1 at 4 h induce the expression of fewer genes and, generally, to a lesser extent (Fig. 2).

We examined the number of induced molecules classified according to function (Table 1). Interleukin-1β was active in increasing chemokines, cytokines and their receptors, while TGFβ1 had the most pronounced inducing effect on the expression of growth factors, structural and extracellular matrix proteins, and enzymes. Both interleukin-1β and TGFβ1 stimulation resulted in a nearly equal activity at inducing transcription factors and nuclear proteins. By contrast, interleukin-13 was relatively less active than interleukin-1β and TGFβ1. The first 20 genes most strongly induced (>2-fold increase) at 4 and 24 h are shown in Tables 2–4.

3.2. Independent confirmation of microarray expression data for selected genes

Microarray experiments were performed in duplicate and the microarray analysis was repeated with a second independent set of duplicate samples to ensure reproducibility of our data. We have also performed quantitative RT-QPCR (TaqMan) analysis on some selected genes. We

Table 3
Genes upregulated by TGFβ1 after 4 h (A) and 24 h (B) of stimulation

GeneBank accession	Description	Expression level in resting cells	Fold induction
(A)			
M29039	jun B proto-oncogene	20.0	26.5
AF078077	growth arrest and DNA-damage-inducible beta, GADD45 (MYD118).	41.3	9.4
W29065	protein tyrosine phosphatase type IVA, member 3	20.0	5.6
AI524125	protocadherin 9	20.8	5.5
AF010193	EST; MAD, mothers against decapentaplegic homolog 7 (Drosophila)	131.6	5.5
AF035528	EST, MAD, mothers against decapentaplegic homolog 6 (Drosophila)	26.7	5.2
X15217	EST, SKI-like	24.2	4.6
J03764	serine (or cysteine) proteinase inhibitor, clade E (PA inhibitor type 1)	459.4	4.5
M60278	diphtheria toxin receptor (heparin-binding EGF-like growth factor)	33.9	3.9
Z19588	SKI-like (nuclear, unknown function)	39.0	3.7
X58377	interleukin-11	23.9	3.5
S81439	TGFB inducible early growth response (Zn-finger transcription factor)	119.9	3.4
U76702	follistatin-like 3 (secreted glycoprotein)	72.5	3.3
D79994	EST-KIAA0172 protein	97.9	3.1
AJ222801	sphingomyelin phosphodiesterase 2 (neutral sphingomyelinase)	24.3	3.1
X78947	connective tissue growth factor	1618.5	3.1
AB002344	EST-KIAA0346 protein	23.2	3.0
AB007902	autism-related protein 1	52.2	3.0
AL050125	EST; cDNA DKFZp586F071	26.2	2.9
M63896	TEA domain family member 1 (SV40 transcriptional enhancer factor)	25.8	2.9
(B)			
M35878	insulin-like growth factor binding protein 3	21.7	54.1
J03764	serine (or cysteine) proteinase inhibitor, clade E (PA inhibitor type 1)	36.8	40.3
U76702	follistatin-like 3 (secreted glycoprotein)	20.0	28.3
X77956	inhibitor of DNA binding 1, dominant negative helix–loop–helix protein	109.1	16.8
X52896	elastin (supravalvular aortic stenosis, Williams–Beuren syndrome)	20.0	15.7
M29039	jun B proto-oncogene	20.0	14.0
L32137	cartilage oligomeric matrix protein	21.7	13.4
X58377	interleukin-11	20.0	11.8
J00073	actin, alpha, cardiac muscle	23.1	10.9
X77956	inhibitor of DNA binding 1, dominant negative helix–loop–helix protein	358.7	10.5
M19267	tropomyosin 1 (alpha)	205.5	9.7
AF078077	growth arrest and DNA-damage-inducible beta, GADD45 (MYD118).	20.0	8.4
L07517	"Mucin 6, Gastric"	20.0	8.2
X68277	dual specificity phosphatase 1	47.9	8.1
AF010193	MAD, mothers against decapentaplegic homolog 7 (Drosophila)	28.8	7.7
X78947	connective tissue growth factor	743.9	7.2
AI924594	tetraspan 2	20.0	6.8
V01512	v-fos FBJ murine osteosarcoma viral oncogene homolog	20.0	6.7
AL021154	E2F transcription factor 2	349.7	6.4
AB018293	EST; KIAA0750 gene product	46.0	6.4

cultured and stimulated additional primary human bronchial smooth muscle cells for the Taqman experiments. There was a good correlation between the results of the Taqman experiments for GROα, GROβ, GROγ, interleukin-8 and eotaxin in response to interleukin-1β stimulation at 24 h with the microarray data (Fig. 3).

3.3. Interleukin-1β upregulates cytokines and chemokines

Interleukin-1β, as expected, induced many inflammation-related genes, including transcription factors such as members of the nuclear factor kappaB (NF-κB) family, adhesion molecules (ICAM-1), and a most striking number of secreted cytokines and particularly chemokines (Table 2). The chemokines upregulated by interleukin-1β at either time-point include interleukin-8, GROα, GROβ, GROγ, granulocyte chemotactic protein (GCP)-2 and epithelial neutrophil activating protein (ENA)-78, which are major neutrophil chemoattractants. In addition, chemokines mainly specific for monocytes and T cells, such as MCP-1, MCP-2, MCP-3 and macrophage inflammatory protein (MIP)-3α are also upregulated at both 4 and 24 h. Eotaxin is strongly induced at 24 h.

3.4. TGFβ1 induction of growth factors and structural protein genes

TGFβ1 upregulates many growth factors and related proteins, structural proteins, extracellular matrix and other secreted glycoproteins, which are potentially involved in

Table 4
Genes upregulated by interleukin-13 after 4 h (A) and 24 h (B) of stimulation

GeneBank accession	Description	Expression level in resting cells	Fold induction
(A)			
D25218	homolog of yeast ribosome biogenesis regulatory protein RRS1	36.7	5.6
M97676	msh homeo box homolog 1 (Drosophila)	37.0	5.6
AA195301	EST, hypothetical protein MGC2574	37.4	3.3
M29039	jun B proto-oncogene	20.0	3.2
D86979	EST; KIAA0226 gene product	52.7	2.9
AJ010046	neuroepithelial cell transforming gene1 (Rho G nucleotide-exchange factor)	20.9	2.9
M85085	cleavage stimulation factor, 3' pre-RNA, subunit 2, 64 kDa	35.9	2.6
Z34897	histamine receptor H1	30.5	2.6
U46573	small inducible cytokine subfamily A (Cys–Cys), member 11 (eotaxin)	231.2	2.5
U51224	U2 small nuclear ribonucleoprotein auxiliary factor, small subunit 1	63.3	2.5
X79389	glutathione *S*-transferase theta 1	38.0	2.5
M96686	Tenascin	61.0	2.5
M28225	small inducible cytokine A2 (monocyte chemotactic protein 1)	554.0	2.4
(B)			
U46573	small inducible cytokine subfamily A (Cys–Cys), member 11 (eotaxin)	363.1	4.8
AI677689	KIAA0685 gene product	30.7	3.5
D25218	homolog of yeast ribosome biogenesis regulatory protein RRS1	32.5	3.4
AJ224326	ribulose-5-phosphate-3-epimerase	58.0	2.9
AF089814	tumor suppressor deleted in oral cancer-related 1 (DOC1-related)	36.2	2.7
U88964	interferon stimulated gene (20 kDa)	20.0	2.7
X52008	glycine receptor, alpha 2	31.4	2.6
AL050154	EST; cDNA DKFZp586L0120	29.4	2.6
AF012023	integrin cytoplasmic domain-associated protein 1	30.3	2.6
M58597	fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)	23.8	2.5

remodelling processes (Table 3). The growth factors connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)2 and nerve growth factor (NGF) are induced at 4 h by 2- to 3-fold, with a further elevation at 24 h, in addition to the growth factors insulin growth factor (IGF1), endothelial cell growth factor (ECGF)1, bone morphogenic protein (BMP)1 and BMP2, with a 54-fold induction of IGF-binding protein (IGFBP)-3. TGFβ1 also upregulates structural and extracellular matrix proteins including tropomyosin, actin, cartilage oligometric matrix protein, collagens, elastin and dermatopontin. TGFβ1 has a less pronounced effect on cytokine and chemokine expression when compared to interleukin-1β but it selectively induces interleukin-6, -8 and -11.

3.5. Interleukin-13 induced gene expression

Interleukin-13 stimulation had only moderate effect on gene expression in airway smooth muscle cells when compared to interleukin-1β and TGFβ. We also identified genes whose upregulation by interleukin-13 has already been shown such as eotaxin, which is induced 2.5- and 4.8-fold at 4 and 24 h, respectively. Similarly, MCP-1 is strongly expressed in ASM cells and further induced by interleukin-13 stimulation, particularly at 4 h. Of the matrix proteins, tenascin was also induced by interleukin-13. Similar to TGFβ1 and interleukin-1β stimulated samples, we also found junB induction (3.2-fold above baseline at 4 h).

3.6. Co-regulation of genes by interleukin-1β, interleukin-13 and TGFβ1

A few genes were co-regulated by all three cytokines (Table 5) even though in most cases the level of induction varied significantly, with many of the genes of unknown function or only poorly characterised. However, they regulate the expression of some genes in a parallel fashion, such as Jun B, a component of the transcription factor activator protein (AP)-1. There is also increased production of interleukin-8 and -11, FGF-2 and tenascin in response to each of three cytokines.

4. Discussion

Using gene chip array technology, we show that different cytokines induce different transcriptional programs in airway smooth muscle. The differences in expression profiles with different cytokines was significantly more pronounced than the differences observed between the two individual primary cell lines studied; furthermore there was very little variability in the two replicate samples. While interleukin-1β caused the strongest induction of a very wide range of pro-inflammatory cytokines and chemokines,

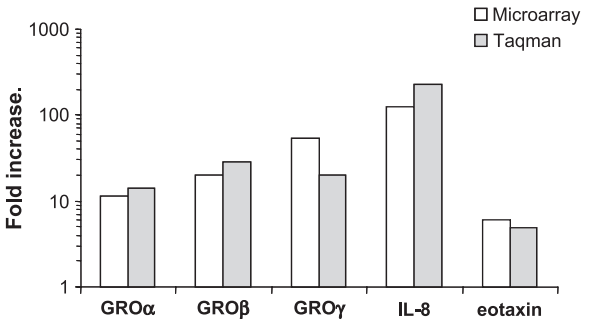

Fig. 3. Confirmation of microarray data using quantitative real time RT-PCR. Expression data obtained by microarray analysis were confirmed by Taqman RT-PCR. A comparison obtained by the two independent methods for chemokine expression after 24 h stimulation with interleukin-1β is shown as an example.

interleukin-13 generally caused a smaller and less intense spectrum of cytokine and chemokine expression but the expression of specific chemokines such as eotaxin and MCP-1 indicates a potential link between T helper 2 cell activation and chemokine generation. TGFβ, on the other hand, while stimulating less pronounced effects on cytokine and chemokine expression, provoked a wide range of growth factors and matrix protein components. Although there have been previous studies employing gene array technology on airway smooth muscle (Hakonarson et al., 2001), our study is the most comprehensive. We conclude that the airway smooth muscle responds differentially to different types of stimulation. Thus, interleukin-1β evokes a most potent and pluripotent pro-inflammatory response, interleukin-13 a weak chemokine response and TGFβ a growth and remodelling response, with some pro-inflammatory effects.

The effect of interleukin-1β on airway smooth muscle has been the most studied so far, and interleukin-1β is known to induce the secretion of many cytokines and chemokines including interleukin-8, RANTES, MCP-1, 2 and 3, eotaxin, interleukin-6, interleukin-11 and granulocyte-macrophage colony stimulating factor (GM-CSF), and also cyclo-oxygenase-2 and ICAM-1 (John et al., 1998; John et al., 1997; Pype et al., 1999; Chung et al., 1999; Elias et al., 1997; Belvisi et al., 1997). Using this microarray, we confirmed the upregulation of these genes and also identified chemokines such as GRO-α, β and γ, MIP-3α and ENA-78 as well as the cytokine granulocyte colony-stimulating factor (G-CSF) and interleukin-1 receptor antagonist. Various nuclear receptors and transcription factors such as JunB, NF-κB subunits and interferon regulatory factor (IRF)1, and also mitogen-activated protein kinase (MAPK) kinase kinase 8, endothelin and matrix metalloproteinase (MMP)-12 were over-expressed. Part of our data corresponds closely with the data of Hakonarson et al. (2001) who used lower concentrations of interleukin-1β (1 ng/ml) together with tumour necrosis factor-α (5 ng/ml) to stimulate airway smooth muscle cells. The pattern of cytokine and chemokine expression supports the notion that interleukin-1β induces a pro-inflammatory response in airway smooth muscle.

The major effect of interleukin-13 was to induce the expression of the chemokines eotaxin and MCP-1. Interleukin-13-induced eotaxin release has been previously reported (Moore et al., 2002). Lee et al. (2001) examined by gene array the effect of interleukin-13 on airway smooth muscle cells at 6 h and did not report eotaxin release, and the profile of the genes most highly induced was different from that found here. The differences are not easy to reconcile but their source of the cells was different (from Clonetics) and a 10-fold higher concentration of interleukin-13 (100 ng/ml) was used. Induction of eotaxin by interleukin-13 has been previously demonstrated in lung epithelial cells (Matsukura et al., 2001) and in airway fibroblasts (Wenzel et al., 2002) and its expression and induction in response to cytokine stimulation was also shown in airway smooth muscle cells (Ghaffar et al., 1999).

Tenascin, an extracellular matrix glycoprotein, is mainly expressed in epithelial cells in the basement membrane during lung development and transiently in regenerating tissue after epithelial injury (Ericson, 1993). Tenascin expression can be induced by various cytokines, including interleukin-4 in the epithelium (Harkonen et al., 1995). However, this is the first report on its upregulation in bronchial smooth muscle cells in response to interleukin-13. Tenascin-C may amplify airway smooth muscle response to growth factors and its deposition has also been associated with pulmonary hypertension (Cowan et al., 2000). This supports a role for interleukin-13 in airway wall remodelling.

The effect of TGFβ was stronger at 24 h than at 4 h and whether this is a direct effect by TGFβ or is due to the activation of downstream autocrine pathways remains to be elucidated. Typically, it upregulated many growth factors, structural proteins, extracellular matrix and other secreted glycoproteins including tropomyosin, actin, cartilage oligomeric matrix protein, collagens, elastin and dermatopontin. The very strong induction of IGF-binding protein 3 indicates that airway smooth muscle cells may have a strong and important growth-promoting role in the airways upon TGFβ stimulation since many lung cells are potential target cells of the secreted growth factors. In fact, the induction of some of these growth factors may indicate an autocrine regulation as for example TGFβ and BMP proteins use similar signalling pathways such as the Smad (Sma/Mothers against Decapentaplegic) pathway, Ras and MAPK pathway (Massague, 1998). IGFBP-3 has been shown to mediate the growth-promoting effects of TGFβ on airway smooth muscle cells (Cohen et al., 2000). The enhancing effects of TGFβ on its own production are also indicative of how an autocrine amplifying effect may be operating (Bouche et al., 2000). TGFβ-airway smooth muscle cell interactions may also contribute to airway remodelling by modulating the protease–anti-protease balance by regulating the levels of some proteases (MMP10)

Table 5
Genes co-regulated by interleukin-1β, TGFβ1 and interleukin-13 stimulation at 4 h (A) and at 24 h (B)[a]

GeneBank accession	Description	Fold induction or repression/Interleukin-1β	Fold induction/ TGFβ	Fold induction/ Interleukin-13
(A)				
M29039	jun B proto-oncogene	4.4	26.5	3.2
X58377	interleukin-11	4.9	3.5	1.6
S81439	TGFB inducible early growth response	1.8	3.4	1.8
AB002344	KIAA0346 protein	7.0	3.0	2.1
U27655	regulator of G-protein signalling 3	2.1	2.8	2.3
M85085	cleavage stimulation factor, 3' pre-RNA, subunit 2, 64 kDa	1.7	2.8	2.6
AF050110	TGFB inducible early growth response	1.6	2.7	1.6
AB023227	KIAA1010 protein	1.8	2.6	2.1
D14497	mitogen-activated protein kinase kinase kinase 8	4.4	2.4	1.6
V00568	v-myc myelocytomatosis viral oncogene homolog (avian)	2.5	2.4	1.9
J04513	fibroblast growth factor 2 (basic)	3.2	2.2	1.6
U50136	leukotriene C4 synthase	1.8	2.1	2.0
X02612	cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1	1.7	1.9	2.1
M17017	interleukin-8	23.9	1.8	2.6
M96686	Tenascin	2.2	1.7	2.5
U67319	caspase 7, apoptosis-related cysteine protease	2.0	1.5	2.0
(B)				
AF089814	tumor suppressor deleted in oral cancer-related 1	2.5	3.3	2.7
L40377	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 8	1.9	3.0	1.7
U80747	trinucleotide repeat containing 3	2.2	2.9	2.1
AF091092	weakly similar to glutathione peroxidase 2	2.6	2.6	1.6
AL022238	adenylosuccinate lyase	1.6	2.6	1.8
AI660225	coactivator-associated arginine methyltransferase-1	1.8	2.4	1.8
AI288757	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	1.8	2.2	2.1
AF070530	hypothetical protein, clone 24751	2.7	2.1	2.0
L13972	sialyltransferase 4A (beta-galactosidase alpha-2,3-sialytransferase)	3.6	2.0	1.7
AJ007041	KIAA0304 gene product	2.3	2.0	1.8
M17589	tyrosine hydroxylase	3.3	1.6	1.7
AF035444	tumor suppressing subtransferable candidate 3	3.3	1.5	1.8
AL049274	Homo sapiens mRNA; cDNA DKFZp564H203 (from clone DKFZp564H203)	2.3	1.5	1.5

[a] The genes are ranked by fold increase values in TGFβ stimulated cells. Those genes are shown for which an at least 2-fold difference has been detected for either cytokine.

and particularly via inducing several protease inhibitors such as nexin, tissue inhibitor of MMPs (TIMP)-3 and cystatin (Knox et al., 2001).

Although TGFβ had a less pronounced effect on cytokine and chemokine expression when compared to interleukin-1β, it selectively induced interleukin-8, and also strongly upregulated interleukin-6 and interleukin-11, confirming previous reports (Elias et al., 1997; Fong et al., 2000). Generally, interleukin-6 and interleukin-11 play different biological roles but interestingly they both also share some common function such as induction of acute phase proteins. Their receptors also comprise a shared β-chain, gp130 (Ozaki and Leonard, 2002). The transcription factor junB, a member of the leucine zipper family of proteins that constitute the AP-1 transcription factor is strongly upregulated. It may have antiproliferative functions (Shaulian and Karin, 2002), even though in some systems it can also induce proliferation through cyclin A activation (Andrecht et al., 2002). In lymphoid cells, interleukin-6 upregulates JunB expression (Sjin et al., 1999) and the induction of BMP-2 at 24 h can lead to a further elevation of junB mRNA levels (Lai and Cheng, 2002). This marked elevation of junB expression in airway smooth muscle cells may be due to an additive or even synergistic effect of the primary TGFβ and the secondary interleukin-6 and BMP-2 stimuli leading to an autocrine induction of junB.

Although the results of this study of gene microarray is limited to airway smooth muscle cells obtained from two patients because of the expense, we have shown that interleukin-1β, interleukin-13 and TGFβ induce different transcriptional programs in the airway smooth muscle, which may in turn lead to specific phenotypic changes in airway smooth muscle. This may be the basis for the airway smooth muscle contributing to airway inflammation in different ways depending on the temporally and spatially regulated cytokine milieu in distinct respiratory pathologies.

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