

The Fast Skeletal Muscle Myosin Light Chain Is Differentially Expressed in Smooth Muscle Cells of OVA-challenged Mouse Trachea

Ho-Young Kim^{1,†}, TaiYoun Rhim^{1,†}, Mi-Hyun Ahn¹, Pyoung-Oh Yoon¹, Soo-Ho Kim¹, Sang-Han Lee^{2,*}, and Choon-Sik Park^{1,*}

¹ Genome Research Center for Allergy and Respiratory Diseases, Soonchunhyang University Hospital, Bucheon 420-767, Korea;

² Department of Biochemistry, College of Medicine, Soonchunhyang University, Cheon-An 330-721, Korea.

(Received June 29, 2007; Accepted July 26, 2007)

In a search for new molecular pathways associated with asthma, we performed an mRNA differential display analysis using total RNA extracted from the tracheal tissues of ovalbumin (OVA)-challenged mice and sham controls. cDNAs corresponding to mRNAs for which expression levels were altered by OVA-challenge were isolated and sequenced. Twenty-eight genes differentially expressed in sham and OVA challenged mice were identified. A GenBank BLAST homology search revealed that they were related to cytoskeleton remodeling, transcription, protein synthesis and modification, energy production, and cell growth and differentiation. Two were selected for further characterization. Up-regulation of both the perinatal skeletal myosin heavy chain (skMHC) and fast skeletal muscle myosin light chain (skMLC) genes was confirmed by RT-PCR of trachea tissue from OVA challenged mice. Overexpression of skMLC protein was observed in the smooth muscle layers of OVA-challenged mice by immunohistochemistry, and the surface areas stained with skMLC antibody increased in the OVA-challenged mice. The overexpression of skMLC in murine asthma may be associated with the changes of bronchial smooth muscle.

Keywords: DD-PCR; OVA Induced Asthma; skMLC; Smooth Muscle.

Introduction

Asthma is a complex disease influenced by multiple genetic and environmental factors (Holgate, 1999; Sanctis et al., 1999) with significant morbidity and mortality. It is characterized by narrowing of the conducting airways of the lung with airway hyper-responsiveness (AHR). Although several abnormalities can cause AHR, the latest developments have led to the view that chronic inflammation and the remodeling of the allergic airway contribute to its pathophysiology (Oosterhout and Nijkamp, 1990; Snapper, 1990). Airway inflammation, a prominent feature of asthma, leads to bronchoconstriction, airway hyper-responsiveness (AHR), and ultimately airway remodeling, which includes goblet cell metaplasia/hyperplasia (Beasley et al., 1989), subepithelial fibrosis (Beasley et al., 1989; Roche et al., 1989), increased vascularity and smooth muscle hypertrophy (Ebina et al., 1993). The complexity of asthma originates from the interaction of a number of genes with environmental factors (Cookson, 1999). The majority of pathophysiological changes in asthma are probably due to altered expression of biologically plausible genes associated with transcriptional pathways, inflammatory mediators, chemokines, cytokines, apoptosis and cell proliferation (Busse and Rosenwasser, 2003; Shore, 2004). Therefore, identifying asthma-specific genes is important, since such genes will ultimately help in early detection, prognosis and treatment of the disease.

Airway is responsible for inducing sensitization to inhaled antigen, leading to airway inflammation. The mechanisms of airway inflammation in asthma have been

[†] These authors contributed equally to this work.

* To whom correspondences should be addressed.

Tel: 82-32-621-5105; Fax: 82-32-621-5023

E-mail: L1037624@sch.ac.kr (SHL)/

mdcspark@unitel.co.kr (CSP)

Abbreviations: DD-PCR, differential display-polymerase chain reaction; OVA, chicken ovalbumin; skMHC, skeletal muscle myosin heavy chain; skMLC, skeletal muscle myosin light chain.

studied in animal models (Hessel et al., 1995). Antigen sensitization and subsequent airway challenge with the antigen produces the typical characteristics of asthmatics including AHR and airway inflammation. The ovalbumin (OVA)-sensitized / challenged murine model (MacLean et al., 1999) displays Th2 allergic inflammation, the characteristic pathophysiological feature seen in asthmatics (Zhang et al., 1997).

This murine model also develops airway remodeling in response to repeated and long-term challenge with OVA (Collins et al., 2003). Hence it may be useful for searching for novel molecular pathways in asthma development. Differential display-polymerase chain reaction (DD-PCR) has been widely used for identifying genes differentially expressed between cell or tissue types (Ali et al., 2001; Kilty and Vickers, 1999; Liang and Pardee, 1992; 1995). We have applied this approach to the tracheal and main bronchial tissues of OVA-sensitized and -challenged mice, and have identified 28 gene changes that may contribute to the asthmatic condition of airways. Two of the genes were confirmed by RT-PCR and immunohistochemistry.

Materials and Methods

Experimental animals and ovalbumin (OVA) challenge Six-week-old male BALB/c (Charles River Technology Inc., Korea) mice were maintained in pathogen-free conditions. They were kept at 22°C and 20–50% humidity with a 12 h light period. Food and water were presented *ad libitum*. For the OVA-sensitized / challenged group, mice were sensitized on days 1 and 14 by intraperitoneal injection of 100 µg OVA (Sigma, USA) emulsified in 20 mg of aluminum hydroxide (Sigma) in a total volume of 200 µl. On days 15, 16, and 17 after the initial sensitization, the mice were challenged by intranasal instillation of 1.5 mg OVA dissolved in 1 ml of endotoxin free saline (Fig. 1A). The control group mice received an equivalent volume of endotoxin-free water. Airway resistance was measured by one-chamber plethysmography (ALL Medicus, Korea) and expressed as fold increase of enhanced pause (Penh_{MCh}) in response to an increasing concentration of methacholine (MCh) over Penh value in response to PBS challenge (Penh_{PBS}). Two days after the last challenge, the mice were killed by intraperitoneal injection of pentobarbital sodium (100 mg/kg). Blood samples were obtained via the inferior vena cava. Anti-OVA specific IgE antibodies were measured by ELISA as described (Zhang et al., 1997). Briefly, 96-well flat-bottom ELISA plates (ICN Costa Mesa, USA) were coated overnight with 20 µg/ml of OVA in a 0.1 M NaHCO₃ buffer (pH 8.3) at 4°C. Nonspecific binding was blocked with 1% bovine serum albumin. After incubation of the test sera for 2 h, the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgE (Pharmingen, USA) for 1 h at room temperature. The reaction was developed with a tetramethylbenzidine substrate. The optical density was measured at 450 nm. At the time of lavage, 48 h after

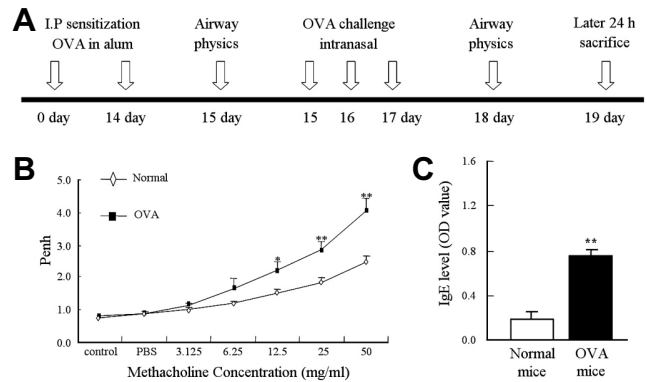


Fig. 1. Profiles of the OVA- sensitized / challenged murine asthma model. **A.** Outline of the protocol. **B.** Penh values as a function of increasing concentrations of methacholine in sham (n = 8, open square) and OVA-challenged mice (n = 8, closed square) (* P < 0.05 and ** P < 0.01). **C.** Comparison of serum OVA-specific IgE concentrations in sham (open bar) and OVA-challenged mice (closed bar) (** P < 0.01).

last challenge, the mice were killed by intraperitoneal injection of pentobarbital sodium (100 mg/kg body weight). The trachea and the right main bronchi were excised and immediately frozen in liquid nitrogen for RNA extraction. The left lung was fixed with 4% paraformaldehyde in phosphate buffered saline and paraffin-embedded for immunohistochemistry and morphometric analyses. The tissue sections were subjected to Luna's staining to identify infiltrating eosinophils.

Differential display-polymerase chain reaction (DD PCR)

The trachea was dissected free of other tissues, immediately frozen in liquid nitrogen, and the frozen trachea was homogenized using a homogenizer. Total RNA was isolated with a Total RNA extraction kit (Ambion, USA), and treated the RNA with RNase-free DNase I (Boehringer Mannheim, USA) for 30 min at 37°C. After phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation, the RNA was suspended in DEPC-treated H₂O. Differential display analysis was carried out as described (Ali et al., 2001; Liang and Pardee, 1995). Briefly, 400 ng of DNA-free total RNA was reverse-transcribed using T₁₁VV primer where V represents a degenerate mixture of dA, dG and dC (Table 1). Amplification reactions were performed in 20 µl volumes containing 10 mM Tris-HCl, pH 8.3, 10 mM KCl, 5 mM MgCl₂, 200 µM of each deoxyribonucleoside triphosphate (dNTP), 0.5 µM of primer and 1 unit of Stoffel fragment (Perkin-Elmer, USA). PCR amplification was carried out on a thermal cycler (Perkin-Elmer DNA Thermal Cycler 480) for a total of 40 cycles at 94°C for 30 s, at 40°C for 60 s, and at 72°C for 60 s, followed by 7 min post-extension at 72°C. PCR amplification products were analyzed by electrophoresis on nondenaturing 10% polyacrylamide mini-gels. After silver staining, the bands of interest ranging from 150 to 600-bp were recovered from the gels and reamplified in 50 µl reaction mixtures with the same pair of primers used in the differential display reaction.

Table 1. Oligonucleotide primers used in the mRNA differential display.

Arbitrary primer	Sequence	Arbitrary primer	Sequence	Anchor primer	Sequence
1	5'-GATCATAGCC-3'	13	5'-TGGATTGGTC-3'	1	5'-TTTTTTTTTTTAA-3'
2	5'-CTGCTTGATG-3'	14	5'-GGAACCAATC-3'	2	5'-TTTTTTTTTTTAC-3'
3	5'-GATCCAGTAC-3'	15	5'-GATCAATCGC-3'	3	5'-TTTTTTTTTTTATAG-3'
4	5'-GATCGCATTG-3'	16	5'-TCGGTCATAG-3'	4	5'-TTTTTTTTTTTCA-3'
5	5'-AAACTCCGTC-3'	17	5'-GATCTGACTG-3'	5	5'-TTTTTTTTTTTCC-3'
6	5'-TGGTAAAGGG-3'	18	5'-TCGATACAGG-3'	6	5'-TTTTTTTTTTTCG-3'
7	5'-GATCATGGTC-3'	19	5'-TACAACGAGG-3'	7	5'-TTTTTTTTTTTGA-3'
8	5'-TTTTGGCTCC-3'	20	5'-GATCAAGTCC-3'	8	5'-TTTTTTTTTTTGC-3'
9	5'-GTTTTTCGAG-3'	21	5'-GATCTCAGAC-3'	9	5'-TTTTTTTTTTTGG-3'
10	5'-TACCTAAGCG-3'	22	5'-GGTACTAAGG-3'		
11	5'-GATCTGACAC-3'	23	5'-GATCACGTAC-3'		
12	5'-GATCTAACCG-3'	24	5'-CTTCTACCC-3'		

Cloning and cDNA sequence analysis Amplified cDNA fragments were resolved by gel electrophoresis using 6% polyacrylamide gel. The gel-purified cDNAs were directly cloned into the TA cloning vector of pGEM-T system (Promega, USA). DNA inserts from positive colonies were amplified with T7 and SP6 universal primers and sequenced using an ABI Prism Big-Dye Terminator Cycle Sequencing kit and an ABI 310 Genetic Analyzer (Perkin Elmer, USA). DNA sequences were compared to the GenBank databases utilizing the Blast program available at <http://www.ncbi.nlm.nih.gov>.

Reverse transcription-polymerase chain reaction (RT-PCR)

Two genes of interest showing altered expression in the DD-PCR were further examined by RT-PCR. The cDNA was synthesized from 1 µg of total RNA using a Superscript II kit (Invitrogen) and amplified by PCR. Reactions were performed at 94°C for 3 min and then at 94°C for 30 s, 52°C for 60 s, and 72°C for 60 s for a total of 40 cycles. The primers for perinatal skeletal myosin heavy chain (skMHC), fast skeletal muscle myosin light chain (skMLC), and mouse GAPDH (glyceraldehydes 3-phosphate dehydrogenase), respectively, were as the followings: perinatal skMHC, forward 5'-AACAATCCAACGCCAACCTG-3' and reverse 5'-AGTAAACCCAGAGAGGCAAG-3'; fast skMLC, forward 5'-TTTCTGCCCATGATGCAAGC-3' and reverse 5'-TGAGATTATCAACTGTAGGC-3'; mGAPDH, forward 5'-TGCTGAGTATGTCGTGGAGTCTA-3' and reverse 5'-AGTGGGAGTTGCTGTTGAAGTCG-3'. The mouse GAPDH mRNA served as an internal and amplification control. PCR products were electrophoresed on a 1% agarose gel in Tris-borate EDTA buffer at 100 V for 25 min and visualized under UV light.

Immunohistochemical identification of skMHC, skMLC and α -smooth muscle actin (α SMA), and morphometric analysis

To detect skMHC, skMLC and α -smooth muscle actin (α -SMA) proteins, we performed immunohistochemistry on lung tissue

using anti-skMHC antibody, anti-skMLC antibody (Santa Cruz, USA) and anti- α -smooth muscle actin antibody (Spring Bioscience, USA), respectively. 4 µm-thick transverse sections were incubated with 0.3% hydrogen peroxide in methanol at RT for 20 min to block endogenous peroxidase, followed by 1.5% normal serum in TBS at RT for 30 min. Tissue sections were then treated with goat anti-human skMHC IgG (1:100), rabbit anti-human skMLC IgG (1:200), or rabbit anti-human α SMA IgG (1:100), overnight at 4°C. After washing with TBS, the slides were incubated for 30 min with biotinylated goat anti-IgG or rabbit anti-IgG (1:200, Vector Laboratories, USA), and exposed to avidin-biotin peroxidase complex (ABC kit, Vector Laboratories). Color was developed by staining with 3,3'-diaminobenzidine tetrachloride (DAB; Zymed Laboratories Inc., USA). Negative controls were incubated with isotype-matched antibodies (Vector Laboratories). The areas of expression of skMHC, skMLC and alpha smooth muscle actin were measured from the immunohistochemically stained areas of the intrapulmonary bronchi in one longitudinally-sectioned lung (average: 6 bronchi per lung). Images were acquired with a Nikon DXM1200 digital camera attached to a Nikon Eclipse E600 microscope. They were transferred in JPEG format and analyzed with Image Pro Plus 4.0 software (Media Cybernetics, USA). The stained areas (As) and length of each bronchus were measured. The length of the basement membrane was defined by the basement membrane perimeter (Pbm) of the bronchus. The thickness of the stained area was calculated as As over Pbm as described previously (Snapper, 1990). As and pbm were obtained from 126 bronchi of eight sham mice and 135 bronchi of eight OVA-sensitized/ challenged mice.

Data analysis Data are presented as means $\pm$ SEM. The Mann-Whitney U test was applied to evaluate the difference between two groups. Differences were considered to be significant when the probability was less than 0.05.

Table 2. cDNA fragments corresponding to the differentially expressed mRNAs in OVA-challenged mice tracheas.

Category	Gene name	Genbank accession No.	cDNA level
Cell adhesion & cytoskeleton remodeling	Periplakin gene	AF116523.1	Up
	Perinatal skeletal myosin heavy chain mRNA	M12289.1	Up
	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit	NM_005398.2	Up
	Myosin light chain, alkali, fast skeletal muscle (Myf)	XM_129801.1	Up
Transcription factor	Nascent-polypeptide-associated complex alpha polypeptide	BC029830.1	Up
	Homo sapiens SMARCA4 isoform (SMARCA4) gene	AF254822.1	Up
	Nuclear receptor subfamily 2	XM_133568.1	Up
Protein modification	Cytidine monophospho-N-acetylneuraminic acid synthetase	XM_132934.1	Up
	Ubiquitin-conjugating enzyme E2E 3	BC011477.1	Up
Protein synthesis	Homo sapiens, mitochondrial ribosomal protein S15	BC031336.1	Up
	Homo sapiens muscle specific gene (M9)	NM_013234.1	Up
Energy production	Cytochrome c oxidase	BC034302.1	Up
	Adult male hippocampus cDNA	AK013631.1	Up
Cell growth	C lectin-related protein E pseudogene	AF320597.1	Up
Differentiation	CRP-ductin; muclin; vomeroglandin	XM_124983.1	Up
Signal Transduction	Rattus norvegicus synaptojanin 2 binding protein	NM_022599.1	Up
Unknown protein	mRNA for integral plasma membrane protein	MMU310638	Up
	Homo sapiens CGI-145 protein	XM_113258.1	Up
	Homo sapiens Nebulette (Actin-binding Z-disk protein)	XM_165566.1	Down
Cell adhesion & cytoskeleton	Traube (Trb)	XM_109804.1	Down
Transcription factor	Zinc fingers and homeoboxes protein 1 (Zhx1)	NM_009572.1	Down
Cell growth	Mitochondrial SDS-stable vimentin-bound DNA 1	AJ297014.1	Down
Differentiation	Sel-1 suppressor of lin-12-like	NM_005065.3	Down
Protease inhibitor	Ovomacroglobulin	XM_132895.1	Down
Unknown protein	MUM (muddled meiosis) 2 protein	XM_126252.1	Down
	EMSY protein	XM_133691.1	Down
	Solute carrier family 25	XM_122089.1	Down
	KIAA0144 gene product	BC007179.1	Down

Results

Mechanical measurements (Penh) and airway inflammation in bronchial trees of the OVA-induced murine asthma model and sham mice Airway resistance to increasing concentrations of methacholine was expressed in terms of fold increase of enhanced pause (Penh) over baseline. In the OVA - challenged group, Penh increased in response to 12.5 mg or higher concentrations of methacholine when compared with those in sham control mice ($P < 0.05$ – 0.01) (Fig. 1B). Microscopic analysis of the OVA challenged mice revealed a significant number of inflammatory cells including eosinophils and mononuclear cells infiltrated into the peribronchial tissues and submucosal layer (Fig. 3A-b), while infiltrating cells were only occasionally found in the sham mice (Fig. 3A-a). The serum OVA-specific IgE level was also elevated in the OVA-challenged mice ($P < 0.01$) (Fig. 1C).

Isolation of differentially expressed mRNA and identification of gene The tracheas and right main bronchi of

eight OVA challenged mice and of eight sham mice were pooled for the RNA extraction and analysis of DD-PCR. To identify differentially expressed gene, 24 arbitrary 10-mer primers were used in combination with all 9 downstream T₁₁VV primers. By using all the primer sets (Table 1), we identified 69 differentially expressed cDNA fragments with ≥ 2 fold changes, and excised them from the gels. cDNAs of 42 of these were cloned and sequenced and 28 genes were identified by BLAST (NCBI) analysis. Of these, 18 were up-regulated, and 10 down-regulated. Twenty-two genes could be functionally classified using the protein knowledge base in the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB) (Table 2). They encoded: 1) transcription factors; SMARCA4 isoform protein, nascent-polypeptide-associated complex alpha polypeptide (Yotov *et al.*, 1998) and nuclear receptor subfamily 2 (Sacchetti *et al.*, 2001), increased by OVA-challenge, and zinc fingers and homeoboxes protein 1 (ZHX1) decreased (Shou *et al.*, 2003) by OVA-challenge; 2) protein synthesis factors; mitochondrial ribosomal protein S15 (Galloni, 2003)

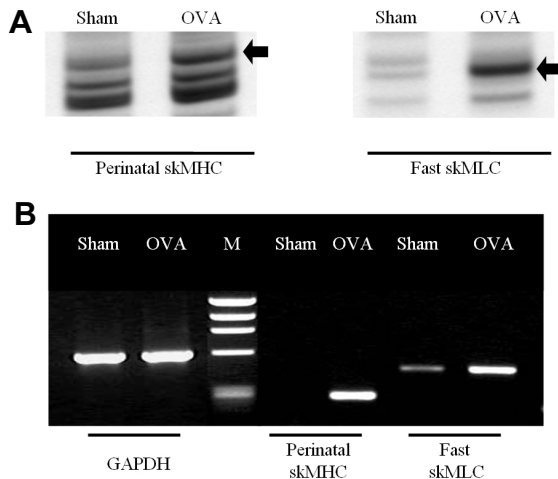


Fig. 2. Messenger RNA differential display and RT-PCR analysis of perinatal skMHC and fast skMLC genes in sham and OVA-challenged mice. Total RNA was isolated from the tracheas of each group ($n = 8$). **A.** Differential display analysis showing up-regulation of perinatal skMHC and fast skMLC genes. The arrows indicate the differentially expressed cDNA for each gene. **B.** mRNA expression of perinatal skMHC and fast skMLC genes in trachea confirmed by RT-PCR using sequence-specific primers. The experiments were repeated more than three times. GAPDH mRNA served as control.

and a muscle specific protein (M9) (Martinuzzi et al., 1999) both up-regulated; 3) ovomacroglobulin (Molla et al., 1987), a protease inhibitor decreased; 4) synaptotagmin 2 binding protein (Nemoto and De Camilli, 1999) involved in signal transduction, cytochromic oxidase (Kadenbach et al., 2000) and adult male hippocampus protein (Campbell and Macqueen, 2004) involved in energy production all up-regulated; 5) cytidine monophospho-N-acetylneuraminic acid synthetase (Puente-Polledo et al., 1998) and ubiquitin conjugating enzyme E2E3 (Burger and Seth, 2004; Hershko et al., 1983) involved in protein modification both increased; 5) Cell adhesion and/or cytoskeleton remodeling proteins such as periplakin (PPL) (Leung et al., 2002), perinatal skeletal myosin heavy chain (skMHC) (da Costa et al., 2002), and fast skeletal muscle myosin light chain (skMLC) (Nieznanka et al., 2002) all increased. The remaining six genes with altered expression encoded products of uncharacterized or unknown functions.

Validation of the change of genes expression in the OVA - induced asthma model Among the genes with altered expression, perinatal skMHC, and fast skMLC gene were chosen to validate the results of DD-PCR by RT-PCR and immunohistochemistry (Fig. 2A). Marked up-regulation of the perinatal skMHC and fast skMLC genes by OVA-challenge was confirmed by RT-PCR (Fig. 2B).

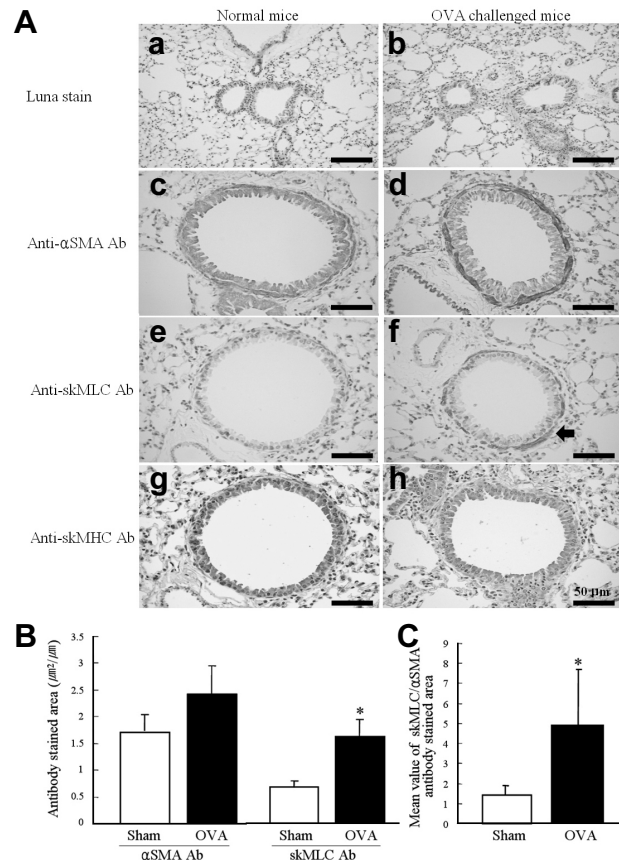


Fig. 3. Expression of skMHC, skMLC, and α SMA in the intrapulmonary bronchi of sham and OVA-challenged mice. **A.** Histology showing the marked infiltration of mononuclear cells and eosinophils visualized by Luna's stain in the peribronchial layer in OVA-challenged mice (b) compared with sham mice (a) (Magnification $\times 200$). Smooth muscle layers stained with HRP-labeled rabbit anti-human α SMA antibody (c, d), rabbit anti-human skMLC antibody (e, f), and goat anti-human skMHC (g, h). The expression of skMLC protein was markedly higher in the bronchial smooth muscle layer (arrow) of OVA-challenged mouse (f) than of sham controls (e) (Original magnification $\times 200$). **B.** The mean area of the smooth muscle layer staining for skMLC per peribronchial diameter is higher in OVA-challenged mice ($n = 8$) than in sham mice ($n = 8$) (* $P < 0.05$). **C.** The mean ratio of skMLC stained area to α SMA stained area is also significantly increased in OVA-challenged mice (* $P < 0.05$).

In the immunohistochemical analysis, skMLC was clearly stained in the smooth muscle layer of the bronchus (Fig. 3A-f), and the intensity of staining was greater in the OVA-challenged mice (Fig. 3A-e). The area stained for skMLC per length of basement membrane increased from $0.65 \pm 0.11 \mu\text{m}^2/\mu\text{m}$ for the control group, to $1.79 \pm 0.33 \mu\text{m}^2/\mu\text{m}$ for the OVA-challenged group ($P < 0.05$). There was no significant difference in the area stained for α -SMA in the two groups ($P > 0.05$) (Fig. 3B). The ratio of skMLC and α -SMA stained areas was also elevated in the

OVA-challenged mice ($P < 0.05$) (Fig. 3C). skMHC was diffusely stained in the epithelium, smooth muscle layer, and inflammatory cells, and its intensity also increased in the OVA-challenged mice (Fig. 3A-gh).

Discussion

In the present study we detected changes in the mRNA levels of 28 genes in the airways of OVA-challenged mice. Two genes affecting airway smooth muscles were chosen to validate these results because their products change in a characteristic way in asthmatics. Up-regulation of perinatal skMHC and fast skMLC mRNAs were confirmed using RT-PCR and protein expression analysis. The increase in both fast skMLC mRNA and protein indicates that the protein level reflects the induction of transcription in response to OVA-challenge. To our knowledge, enhanced expression of these two genes has not been reported previously in OVA-challenge mice.

The expression of skeletal muscle proteins in smooth muscle and epithelium has not previously been described. In this study, we demonstrated that airway smooth muscle cells express perinatal skMLC and that airway epithelial cells express fast skMHC. The simultaneous expression of skMHC and skMLC in different cells suggests that the products of skeletal muscle myosin genes are present in non-skeletal muscle, including epithelium and smooth muscle. The expression of skeletal muscle myosin genes increases during lung inflammation.

Myosins are the molecular motors responsible for movement in all eukaryotes. Conventional myosin expressed in non-smooth muscle cells has been shown to be involved in various cellular functions, from cytokinesis to morphogenesis (Fukui et al., 1990). Skeletal myosin (skM), the major myofibrillar protein, consists of two heavy (skMHC) and four light (skMLC) chains. skMHCs comprise eight isoforms (2a, 2x, 2b, embryonic, perinatal, slow/I β , extraocular, and α), each encoded by a separate gene, and five major skMLC isoforms (two slow, MLC_{1s} and MLC_{2s}, and three fast, MLC_{1f}, MLC_{2f}, and MLC_{3f}), respectively (Weiss and Leinwand, 1996). They are expressed in a tissue- and stage-specific manner during development. This differential expression is likely to reflect differences in the physiological state of individual muscles, and indicates that the regulation of muscle gene is altered in asthmatics.

Gordon et al. reported that periods of increased or decreased muscle activity alter the expression of the skMHC and/or skMLC isoforms (Lutz et al., 2002; Reggiani et al., 1997; Sweeney et al., 1988). Although the expression of skeletal muscle genes in smooth muscle cell has not been reported, they are known to be expressed in other cell types and tissues. Skeletal myosin heavy chains are expressed in myofibroblast of pulmonary and kidney me-

sangial cells, and skeletal muscle myosin enzymatic activity is required for pulmonary myofibroblast contractility (Mayer and Leinwand, 1997; Rice and Leinwand, 2003). The fetal cardiac/skeletal slow-twitch muscle-specific beta-MHC ranscript was detected in mesenteric lymphatics. Moreover skeletal alpha-actin exists in both mesenteric lymphatics and arterioles (Muthuchamy et al., 2003). Blockade of mitogen-activated protein kinase increases perinatal skMHC mRNA in rat skeletal muscle cultures (Higginson et al., 2002), and mitogen - activated protein kinase is enhanced in asthmatic airways (Pelais et al., 2005). A shared myosin light chain is expressed in skeletal and smooth muscles, and in brain, at an early stage (Kawashima et al., 1987), and the smooth muscle myosin light chain gene is expressed in smooth, skeletal, and nonmuscle tissues (Taubman et al., 1987). In addition, a smooth muscle myosin light chain kinase isoform is present in skeletal muscle (Dalla Libera et al., 1997; Herring et al., 2000). Thus it is possible that skeletal myosins exist in non-skeletal muscle. As mentioned above, the skeletal myosin heavy chain has contractile functions in non-skeletal muscle cells. Arterial endothelium injury leads to proliferation of vascular smooth muscle cells induced by the accumulation of connective tissue. The changes involved include altered expression of the myosin heavy chain isoform (Gallagher et al., 2000). These data suggest that endothelium injury is related to the contraction of smooth muscle and expression of myosin heavy chain. Consequently, we supposed that the skMLC gene can be expressed in lung epithelial cells as a result of injury, or by contraction in smooth muscle.

Airway smooth muscle (ASM), which is involved in the regulation of airway movement, is found from the trachea to the terminal bronchioles. ASM undergoes marked phenotypic modulation in asthmatic conditions, as well as during lung development. The contraction of ASM regulates airway diameter and modulates airway resistance (Wiggs et al., 1990). In addition, ASM mass increases in asthma and may represent an injury-repair response due to inflammation (Lambert et al., 1993). Hence contractile tissue may become an active force in the airway remodeling in asthma (Opazo et al., 2000). Airway remodeling, defined as hypertrophy and hyperplasia of the airway smooth muscle, thickening of the basement membrane, and collagen deposition, is thought to play a prominent role in asthma (Lambert et al., 1993). In particular, the increase in airway smooth muscle content is believed to account for most of the airway luminal narrowing and the permanent reduction of airway caliber in asthma and bronchial hyper-reactivity. The proportions and distributions of cytoskeletal and contractile proteins in the smooth muscle change during airway remodeling. The majority of these effects may ultimately depend on the interactions of unknown genes (Halayko and Amrani, 2003).

Acknowledgment This study was supported by a grant from the Korea Health 21 R&D Project Ministry of Health & Welfare Republic of Korea (A010249).

References

- Ali, M., Markham, A.F., and Isaacs, J.D. (2001). Application of differential display to immunological research. *J. Immunol. Methods* 250, 29–43.
- Beasley, R., Roche, W.R., Roberts, J.A., and Holgate, S.T. (1989). Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.* 139, 806–817.
- Burger, A.M. and Seth, A.K. (2004). The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications. *Eur. J. Cancer* 40, 2217–2229.
- Busse, W.W. and Rosenwasser, L.J. (2003). Mechanisms of asthma. *J. Allergy Clin. Immunol.* 111, S799–804.
- Campbell, S. and Macqueen, G. (2004). The role of the hippocampus in the pathophysiology of major depression. *J. Psychiatry Neurosci.* 29, 417–426.
- Collins, R.A., Sly, P.D., Turner, D.J., Herbert, C., and Kumar, R.K. (2003). Site of inflammation influences site of hyperresponsiveness in experimental asthma. *Respir. Physiol. Neurobiol.* 139, 51–61.
- Cookson, W. (1999). The alliance of genes and environment in asthma and allergy. *Nature* 402, B5–11.
- da Costa, N., Blackley, R., Alzuherri, H., and Chang, K.C. (2002). Quantifying the temporospatial expression of postnatal porcine skeletal myosin heavy chain genes. *J. Histochem. Cytochem.* 50, 353–364.
- Dalla Libera, L., Podhorska-Okolow, M., Martin, B., Massimino, M.L., Brugnolo, R., and Cantini, M. (1997). Smooth muscle myosin light chain kinase is transiently expressed in skeletal muscle during embryogenesis and muscle regeneration both *in vivo* and *in vitro*. *J. Muscle Res. Cell Motil.* 18, 295–303.
- De Sanctis, G.T., Merchant, M., Beier, D.R., Dredge, R.D., Grobholz, J.K., Martin, T.R., Lander, E.S., and Drazen, J.M. (1995). Quantitative locus analysis of airway hyperresponsiveness in A/J and C57BL/6J mice. *Nat Genet.* 11, 150–154.
- Ebina, M., Takahashi, T., Chiba, T., and Motomiya, M. (1993). Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am. Rev. Respir. Dis.* 148, 720–726.
- Fukui, Y., De Lozanne, A., and Spudich, J.A. (1990). Structure and function of the cytoskeleton of a Dictyostelium myosin-defective mutant. *J. Cell Biol.* 110, 367–378.
- Gallagher, P.J., Jin, Y., Killough, G., Blue, E.K., and Lindner, V. (2000). Alterations in expression of myosin and myosin light chain kinases in response to vascular injury. *Am. J. Physiol. Cell Physiol.* 279, C1078–1087.
- Galloni, M. (2003). Bonsai, a ribosomal protein S15 homolog, involved in gut mitochondrial activity and systemic growth. *Dev. Biol.* 264, 482–494.
- Halayko, A.J. and Amrani, Y. (2003). Mechanisms of inflammation-mediated airway smooth muscle plasticity and airway remodeling in asthma. *Respir. Physiol. Neurobiol.* 137, 209–222.
- Herring, B.P., Dixon, S., and Gallagher, P.J. (2000). Smooth muscle myosin light chain kinase expression in cardiac and skeletal muscle. *Am. J. Physiol. Cell Physiol.* 279, C1656–1664.
- Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983). Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J. Biol. Chem.* 258, 8206–8214.
- Hessel, E.M., Zwart, A., Oostveen, E., Van Oosterhout, A.J., Blyth, D.I., and Nijkamp, F.P. (1995). Repeated measurement of respiratory function and bronchoconstriction in unanesthetized mice. *J. Appl. Physiol.* 79, 1711–1716.
- Higginson, J., Wachterhage, H., Woods, N., Schjerling, P., Ratkevicius, A., Grunnet, N., and Quistorff, B. (2002). Blockades of mitogen-activated protein kinase and calcineurin both change fiber-type markers in skeletal muscle culture. *Pflügers Arch.* 445, 437–443.
- Holgate, S.T. (1999). Genetic and environmental interaction in allergy and asthma. *J. Allergy Clin. Immunol.* 104, 1139–1146.
- Kadenbach, B., Huttemann, M., Arnold, S., Lee, I., and Bender, E. (2000). Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome c oxidase. *Free Radic. Biol. Med.* 29, 211–221.
- Kawashima, M., Nabeshima, Y., Obinata, T., and Fujii-Kuriyama, Y. (1987). A common myosin light chain is expressed in chicken embryonic skeletal, cardiac, and smooth muscles and in brain continuously from embryo to adult. *J. Biol. Chem.* 262, 14408–14414.
- Kilty, I.C. and Vickers, P.J. (1999). Studies of differential gene expression in clinically derived eosinophil populations. *Clin. Exp. Allergy* 29, 1671–1680.
- Lambert, R.K., Wiggs, B.R., Kuwano, K., Hogg, J.C., and Pare, P.D. (1993). Functional significance of increased airway smooth muscle in asthma and COPD. *J. Appl. Physiol.* 74, 2771–2781.
- Leung, C.L., Green, K.J., and Liem, R.K. (2002). Plakins: a family of versatile cytolinker proteins. *Trends Cell Biol.* 12, 37–45.
- Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257, 967–971.
- Liang, P. and Pardee, A.B. (1995). Recent advances in differential display. *Curr. Opin. Immunol.* 7, 274–280.
- Lutz, G.J., Sirsi, S.R., Shapard-Palmer, S.A., Bremner, S.N., and Lieber, R.L. (2002). Influence of myosin isoforms on contractile properties of intact muscle fibers from Rana pipiens. *Am. J. Physiol. Cell Physiol.* 282, C835–844.
- MacLean, J.A., Sauty, A., Luster, A.D., Drazen, J.M., and De Sanctis, G.T. (1999). Antigen-induced airway hyperresponsiveness, pulmonary eosinophilia, and chemokine expression in B cell-deficient mice. *Am. J. Respir. Cell Mol. Biol.* 20, 379–387.
- Martinuzzi, A., Schievano, G., Nascimbeni, A., and Fanin, M. (1999). McArdle's disease. The unsolved mystery of the reappearing enzyme. *Am. J. Pathol.* 154, 1893–1897.
- Mayer, D.C. and Leinwand, L.A. (1997). Sarcomeric gene expression and contractility in myofibroblasts. *J. Cell Biol.* 139, 1477–1484.
- Molla, A., Matsumura, Y., Yamamoto, T., Okamura, R., and

- Maeda, H. (1987). Pathogenic capacity of proteases from *Serratia marcescens* and *Pseudomonas aeruginosa* and their suppression by chicken egg white ovomacroglobulin. *Infect Immun.* 55, 2509–2517.
- Muthuchamy, M., Gashev, A., Boswell, N., Dawson, N., and Zawieja, D. (2003). Molecular and functional analyses of the contractile apparatus in lymphatic muscle. *FASEB J.* 17, 920–922.
- Nemoto, Y. and De Camilli P. (1999). Recruitment of an alternatively spliced form of synaptotagmin 2 to mitochondria by the interaction with the PDZ domain of a mitochondrial outer membrane protein. *EMBO J.* 18, 2991–3006.
- Nieznanska, H., Nieznanski, K., and Stepkowski, D. (2002). The effects of the interaction of myosin essential light chain isoforms with actin in skeletal muscles. *Acta Biochim. Pol.* 49, 709–719.
- Opazo Saez, A.M., Seow, C.Y., and Pare, P.D. (2000). Peripheral airway smooth muscle mechanics in obstructive airway disease. *Am. J. Respir. Crit. Care Med.* 161, 910–917.
- Pelaia, G., Cuda, G., Vatrella, A., Gallelli, L., Caraglia, M., Marra, M., Abbruzzese, A., Caputi, M., Maselli, R., Costanzo, F.S., et al. (2005). Mitogen-activated protein kinases and asthma. *J. Cell Physiol.* 202, 642–653.
- Puente-Polledo, L., Reglero, A., Gonzalez-Clemente, C., Rodriguez-Aparicio, L.B., and Ferrero, M.A. (1998). Biochemical conditions for the production of polysialic acid by *Pasteurella haemolytica* A2. *Glycoconj J.* 15, 855–861.
- Reggiani, C., Potma, E.J., Bottinelli, R., Canepari, M., Pellegrino, M.A., and Stienen, G.J. (1997). Chemo-mechanical energy transduction in relation to myosin isoform composition in skeletal muscle fibres of the rat. *J. Physiol.* 502, 449–460.
- Rice, N.A. and Leinwand, L.A. (2003). Skeletal myosin heavy chain function in cultured lung myofibroblasts. *J. Cell Biol.* 163, 119–129.
- Roche, W.R., Beasley, R., Williams, J.H., and Holgate, S.T. (1989). Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1, 520–524.
- Sacchetti, P., Mitchell, T.R., Granneman, J.G., and Bannon, M.J. (2001). *Nurr1* enhances transcription of the human dopamine transporter gene through a novel mechanism. *J. Neurochem.* 76, 565–572.
- Shore, S.A. (2004). Direct effects of Th2 cytokines on airway smooth muscle. *Curr. Opin. Pharmacol.* 4, 235–240.
- Shou, Z., Yamada, K., Inazu, T., Kawata, H., Hirano, S., Mizutani, T., Yazawa, T., Sekiguchi, T., Yoshino, M., Kajitani, T., et al. (2003). Genomic structure and analysis of transcriptional regulation of the mouse zinc-fingers and homeoboxes 1 (*ZHX1*) gene. *Gene* 302, 83–94.
- Snapper, J.R. (1990). Inflammation and airway function: the asthma syndrome. *Am. Rev. Respir. Dis.* 141, 531–533.
- Sweeney, H.L., Kushmerick, M.J., Mabuchi, K., Sreter, F.A., and Gergely, J. (1988). Myosin alkali light chain and heavy chain variations correlate with altered shortening velocity of isolated skeletal muscle fibers. *J. Biol. Chem.* 263, 9034–9039.
- Taubman, M.B., Grant, J.W., and Nadal-Ginard, B. (1987). Cloning and characterization of mammalian myosin regulatory light chain (RLC) cDNA: the RLC gene is expressed in smooth, sarcomeric, and nonmuscle tissues. *J. Cell Biol.* 104, 1505–1513.
- Van Oosterhout, A.J. and Nijkamp, F.P. (1990). Lymphocytes and bronchial hyperresponsiveness. *Life Sci.* 46, 1255–1264.
- Weiss, A. and Leinwand, L.A. (1996). The mammalian myosin heavy chain gene family. *Ann. Rev. Cell Dev. Biol.* 12, 417–439.
- Wiggs, B.R., Moreno, R., Hogg, J.C., Hilliam, C., and Pare, P.D. (1990). A model of the mechanics of airway narrowing. *J. Appl. Physiol.* 69, 849–860.
- Yotov, W.V., Moreau, A., and St-Arnaud, R. (1998). The alpha chain of the nascent polypeptide-associated complex functions as a transcriptional coactivator. *Mol. Cell Biol.* 18, 1303–1311.
- Zhang, Y., Lamm, W.J., Albert, R.K., Chi, E.Y., Henderson, W.R.Jr., and Lewis, D.B. (1997). Influence of the route of allergen administration and genetic background on the murine allergic pulmonary response. *Am. J. Respir. Crit. Care Med.* 155, 661–669.