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Change of connexin 37 in allergen-induced airway inflammation

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Abbreviations: BAL, bronchoalveolar lavage; Cx, connexin; ICAM-1, intercellular adhesion molecule-1; OVA, ovalbumin; R_L, airway resistance; VCAM-1, vascular cell adhesion molecule-1

Abstract

Gap junction channels formed with connexins directly link to the cytoplasm of adjacent cells and have been implicated in intercellular signaling. Connexin 37 (Cx37) is expressed in the gas-exchange region of the lung. Recently, Cx37 has been reported to be involved in the pathogenesis of inflammatory disease. However, no data are available on the role of Cx37 in allergic airway inflammatory disease. In the present study, we used a murine model of ovalbumin (OVA)-induced allergic airway disease and primary murine epithelial cells to examine the change of Cx37 in allergic airway disease. These mice develop the following typical pathophysiological features of asthma: airway hyperresponsiveness, airway inflammation, and increased IL-4, IL-5, IL-13, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, eotaxin, and RANTES levels in lungs. Cx37 protein and mRNA expression were decreased in OVA-induced allergic airway disease. Immunoreactive Cx37 localized in epithelial layers around the bronchioles in control mice, which dramatically disappeared in allergen-induced asthmatic lungs. Moreover, the levels of Cx37 protein in lung tissues showed significantly negative correlations with airway inflammation, airway responsiveness, and levels of Th2 cytokines in lungs. These findings indicate that change of Cx37 may be associated with the asthma phenotype.

Keywords: asthma; connexin 37; epithelial cells; gap junctions; inflammation mediators; models, animal; respiratory system

Introduction

Connexins (Cxs), connexons, and gap junctions are involved in numerous processes contributing to the maintenance of normal cell growth and differentiation (Saez *et al.,* 2003; Jiang and Gu, 2005). The gap junction channels formed with Cxs directly link to the cytoplasm of adjacent cells and have been implicated in intercellular signaling that may regulate the functions of cells. The gap junction channels allow intercellular passage of ions, second messengers (such as Ca^{2+}, IP_3, cAMP, and cGMP), and small metabolites. Cxs are members of a family of proteins encoded by at least 20 different mammalian genes that are expressed in a wide variety of tissues (Saez *et al.,* 2003; Sohl and Willecke, 2004). Previous studies have shown that at least eight Cxs are expressed in the gas-exchange region of the lung; these include Cx26, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45, and Cx46 (Paul, 1995; Lee *et al.,* 1997; Abraham *et al.,* 1999). Recently, Cx37 has been reported to play a role in the pathogenesis of various inflammatory diseases (Wagenaar *et al.,* 2004; Chanson and Kwak, 2007). Moreover, proinflammatory cytokines are shown to reduce expression of Cx37, resulting in a decrease in gap junction-mediated communication (van Rijen *et al.,* 1998). Asthma is a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils, T lymphocytes, and airway epithelial cells (National Institutes of Health, 1995; Lilly *et al.,* 2005). In susceptible individuals, the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli. However, no data are available on the role of Cx37 in pathogenesis of allergic airway inflammatory disease and the precise localization of Cx37 has not been fully determined in lungs.

In the present study, we used ovalbumin (OVA)-inhaled mice to examine the change of Cx37 in allergic airway disease, and we also evaluated whether Cx37 is expressed in airway epithelial cells.

Materials and Methods

Animals and experimental protocol

Female C57BL/6 mice, 8 to 10 wk of age and free of murine specific pathogens, were obtained from the Orientbio Inc. (Seoungnam, Korea), were housed throughout the experiments in a laminar flow cabinet, and were maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Standard guidelines for laboratory animal care were followed (Institute of Laboratory Animal Research, 1996). Mice (8 mice per group) were sensitized on days 1 and 14 by i.p. injection of 20 μg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200 μl, as previously described with some modifications (Kwak *et al.,* 2003; Lee *et al.,* 2006a, b). On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (wt/vol) OVA in saline (or with saline) using an ultrasonic nebulizer (NE-U12, Omron, Japan). Bronchoalveolar lavage (BAL) was performed at indicated time after the last challenge. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc. Aguada, Puerto Rico) in order to examine the cell differentials.

Isolation and primary culture of murine tracheal epithelial cells

Murine tracheal epithelial cells were isolated from 5 independent mice under sterile conditions, as previously described (Kwak *et al.,* 2003). The trachea proximal to the bronchial bifurcation was excised and adherent adipose tissue was removed. The trachea was opened longitudinally and cut into three pieces. The isolated tracheas were incubated in DMEM containing 0.1% protease overnight at 4°C. Following tissue digestion, FBS (10% final concentration) was added to the medium to deactivate enzymes, undigested fragments of tissue were removed, and tracheal epithelial cells were harvested by centrifugation at 100 × *g* for 5 min. The epithelial cells were seeded onto 35-mm collagen-coated dishes for submerged culture. The growth medium DMEM/F-12 (Sigma-Aldrich) containing 10% FBS, penicillin, streptomycin, and amphotericin B was supplemented with insulin, transferrin, hydrocortisone, phosphoethanolamine, cholera toxin, ethanolamine, bovine pituitary extract, and BSA. The cells were maintained in a humidified 5% CO_2 incubator at 37°C until they adhered.

Western blot analysis

Lung tissues or cultured airway epithelial cells were homogenized in the presence of protease inhibitors to obtain extracts of proteins. Protein concentrations were determined using the Bradford reagent (Bio-Rad Laboratories Inc., Hercules, CA). Samples (30 μg of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to PVDF membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% non-fat dry milk in Tris-buffered saline Tween 20 (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h, and the blots were then incubated with an anti-Cx37 Ab (Alpha Diagnostic International Inc., San Antonio, TX), anti-IL-4 Ab (Serotec Ltd, Oxford, UK), anti-IL-5 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-13 Ab (R&D Systems, Inc., Minneapolis, MN), anti-intercellular adhesion molecule-1 (ICAM-1) Ab (Santa Cruz Biotechnology), and anti-vascular cell adhesion molecule-1 (VCAM-1) Ab (Santa Cruz Biotechnology), anti-eotaxin Ab (Abcam Ltd., Cambridge, UK), or anti-RANTES Ab (Abcam Ltd.) overnight at 4°C. Anti-rabbit or anti-mouse HRP conjugated IgG was used to detect binding of antibodies. The membranes were stripped and reblotted with anti-actin Ab (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibodies was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

RNA isolation and RT-PCR

Total RNA from lung tissues was isolated using a rapid extraction method (TRI-Reagent) as previously described (Chomczynski and Sacchi, 1987). RNA was quantified by measuring absorption at 260 nm and stored at − 80°C until use. Total RNA

(4 μg) was reverse-transcribed to cDNA in a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT; 0.5 μg random hexanucleotide primers, 2.5 mM dNTP, 40 U RNase inhibitor, and 50 U/μl SuperScript II RT (Invitrogen, Carlsbad, CA), in a final volume of 20 μl. This mixture was incubated for 50 min at 42°C and then digested with 2 U/μl *E. coli* RNase H for 20 min at 37°C. The first-strand cDNAs were used for PCR amplification of Cx37 or the housekeeping gene, GAPDH. PCR amplification was performed by mixing 3 μl of the reverse transcription reaction mixture with 47 μl of buffer containing 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) and 30 pmol of specific primer pairs for mouse cDNA of Cx37 or GAPDH, designed from published mouse gene sequences. The primers used were as follows: Cx37 (predicted length 136 bp) sense: 5'-AGCTCTGCATCCAAGAAGCAGTA-3', antisense: 5'-AGTTGTCTCTGAGGTGCCTTTGA-3' and GAPDH (predicted length 609 bp) sense: 5'-GCCATCAACGACCCCTTCATTGAC-3', antisense: 5'-ACGGAAGGCCATGCCAGTGAGCTT-3'. PCR reactions were performed in a thermocycler (GeneAmp® PCR System 2400, Applied Biosystems, Foster City, CA) using the following reaction conditions; after an initial incubation for 2 min at 95°C, samples were subjected to 35 cycles of 1 min at 94°C, 2 min at 54°C (GAPDH) or 68°C (Cx37) and 1 min at 72°C. A final extension step at 72°C for 10 min was performed. The RT-PCR products were electrophoretically fractioned on 2% agarose gels stained with ethidium bromide. DNA bands were visualized under UV light.

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The sequences of primers used were as follows: Cx37 sense: 5'-AGCTCTGCATCCAAGAAGCAGTA-3', antisense: 5'-AGTTGTCTCTGAGGTGCCTTTGA-3' and β-actin sense: 5'-CAGATCATGTTTGAGACCTTC-3', antisense: 5'-ACTTCATGATGGAATTGAATG-3'. Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol. The data have been normalized to the expression of β-actin.

Histology and immunohistochemistry

At 72 h after the last challenge, lungs were removed from the mice after sacrifice. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. Lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4-μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI) stain. Inflammation score was graded by three independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (Tournoy *et al.,* 2000). For immunohistochemistry of Cx37, the deparaffinized 4-μm sections were incubated sequentially in accordance with the R. T. U. Vectastain Universal Quick kit instruction from Vector Laboratories (Burlingame, CA). Briefly, the slides were incubated in Endo/Blocker (Biomeda, Foster City, CA) for 5 min and then in pepsin solution for 4 min at 40°C. The slides were incubated in normal horse serum for 15 min at room temperature. The slides were then probed with an anti-Cx37 Ab (Alpha diagnostic International Inc.), overnight at 4°C, and were incubated with prediluted biotinylated pan-specific IgG for 10 min. The slides were incubated in streptavidin/peroxidase complex reagent for 5 min, and then in 3-amino-9-ethylcarbazole substrate kit for 12 min. For the control, sections of lung tissue from mice were treated without the primary Ab under the same conditions. After immunostaining, the slides were counterstained with Gill's hematoxylin in 20% ethylene glycol for 1 min and then mounted with Aqueous Mounting Medium (InnoGenex, San Ramon, CA) and photomicrographed (Vanox T; Olympus, Tokyo, Japan).

Determination of airway responsiveness

Airway responsiveness was also assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere (Takeda *et al.,* 1997). Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected intraperitoneally. The trachea was then exposed through midcervical incision, tracheostomized, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H_2O to achieve a mean lung volume close to that during

spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5 to 50 mg/ml in saline). After each methacholine challenge, the data of calculated airway resistance (R_L) were continuously collected. Maximum values of R_L were selected to express changes in airway function which was represented as a percentage change from baseline after saline aerosol.

Measurement of Th2 cytokines

Levels of IL-4, IL-5, and IL-13 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (IL-4; BioSource International, Inc. Camarillo, CA, IL-5; Endogen, Inc., Woburn, MA, IL-13; R&D Systems, Inc.). Sensitivities for IL-4, IL-5, and IL-13 assays were 5, 5, and 1.5 pg/ml, respectively.

Densitometric analysis and statistics

All immunoreactive signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad Laboratories Inc.). Data were expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffe's test. Pearson's correlation was calculated to assess the correlation between data. Significant differences between two groups were determined using the unpaired Student's *t* test. Statistical significance was set at $P < 0.05$.

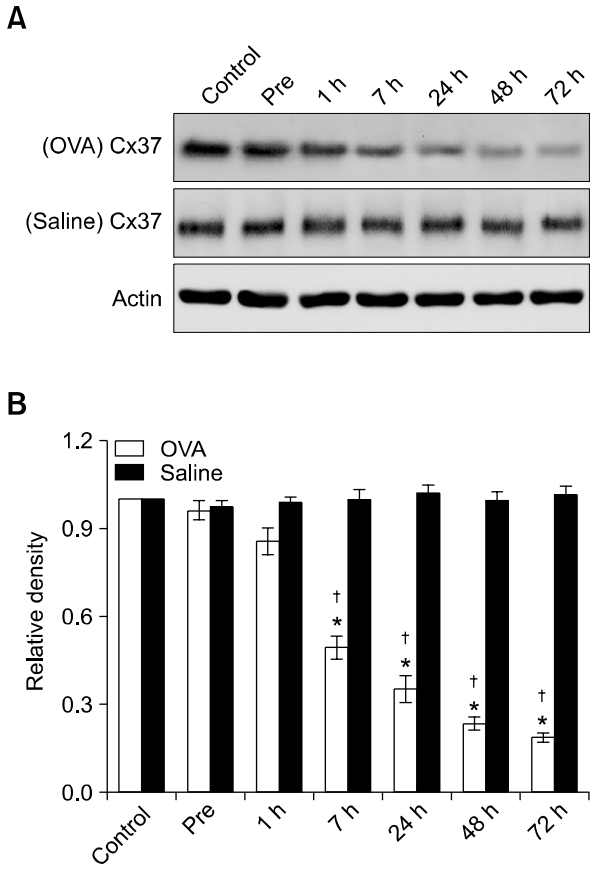

Figure 1. Levels of Cx37 protein in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed in lung tissues from sensitized mice challenged with OVA or saline. (A) Western blot analyses of Cx37 protein. (B) Densitometric analyses are presented as the relative ratio of Cx37 to actin. The relative ratio of Cx37 in the lung tissues of control group is arbitrarily presented as 1. Data represent mean ± SEM from 8 mice per group. 1, 7, 24, 48, and 72 h are time periods after the last challenge. Control, no treatment; Pre, 1 h before the first challenge; †$P < 0.05$ versus Pre; *$P < 0.05$ versus saline inhalation.

Results

Cx37 protein levels in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that Cx37 protein levels in lung tissues were decreased approximately 0.89-, 0.51-, 0.36-, 0.24-, and 0.19-fold at 1, 7, 24, 48, and 72 h after OVA inhalation, respectively, compared with the prechallenge period (Figure 1). In contrast, no significant changes in the Cx37 protein level were observed after saline inhalation.

Cx37 mRNA expression in lung tissues of OVA-sensitized and -challenged mice

Real-time RT-PCR analysis revealed that Cx37 mRNA expression was decreased approximately 0.58-, 0.44-, 0.32-, 0.26-, and 0.26-fold at 1, 7, 24, 48, and 72 h after OVA inhalation, respectively, compared with the prechallenge period (Figure 2). In contrast, no significant changes in the Cx37 mRNA expression were observed after saline inhalation.

Localization of immunoreactive Cx37 in lung tissues of OVA-induced allergic airway disease

Immunoreactive Cx37 localized in epithelial layers around the bronchioles of control mice (Figure 3A), which dramatically disappeared in allergen-induced airway inflammatory lungs (Figure 3B).

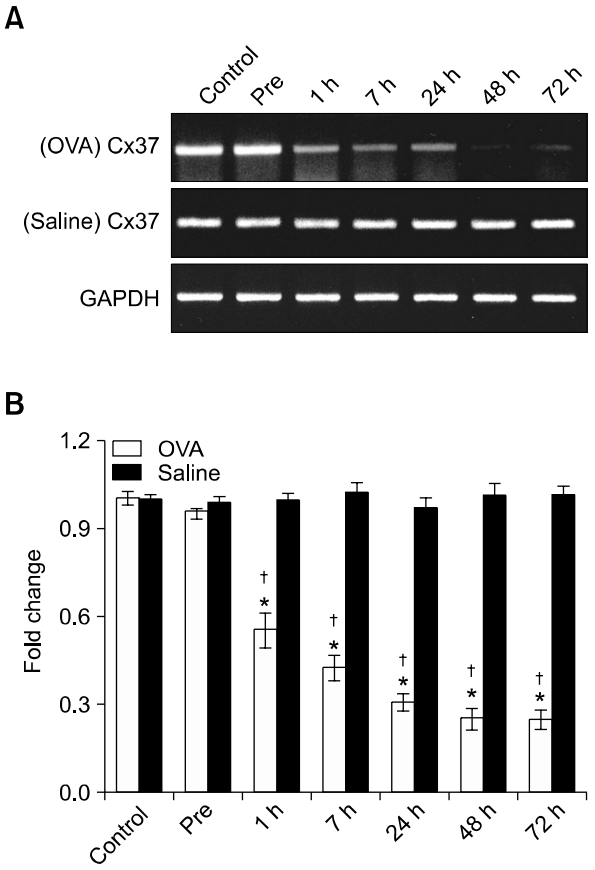

Figure 2. Levels of Cx37 mRNA in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed in lung tissues from sensitized mice challenged with OVA or saline. (A) Representative RT-PCR analysis of Cx37 mRNA expression. (B) Quantitative analysis of Cx37 mRNA expression by means of real-time RT-PCR. The relative ratio of Cx37 in the lung tissues of control group is arbitrarily presented as 1. Data represent mean ± SEM from 8 mice per group. 1, 7, 24, 48, and 72 h are time periods after the last challenge. Control, no treatment; Pre, 1 h before the first challenge; $^{\dagger}P < 0.05$ versus Pre; $^{*}P < 0.05$ versus saline inhalation.

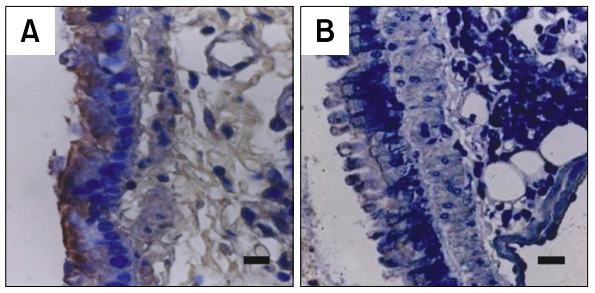

Figure 3. Localization of immunoreactive Cx37 in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in lung tissues from sensitized mice challenged with saline (A) and from sensitized mice challenged with OVA (B). Representative light microscopy shows Cx37-positive cells in the bronchioles. The dark brown color indicates Cx37-positive cells. Bars indicate 10 μm.

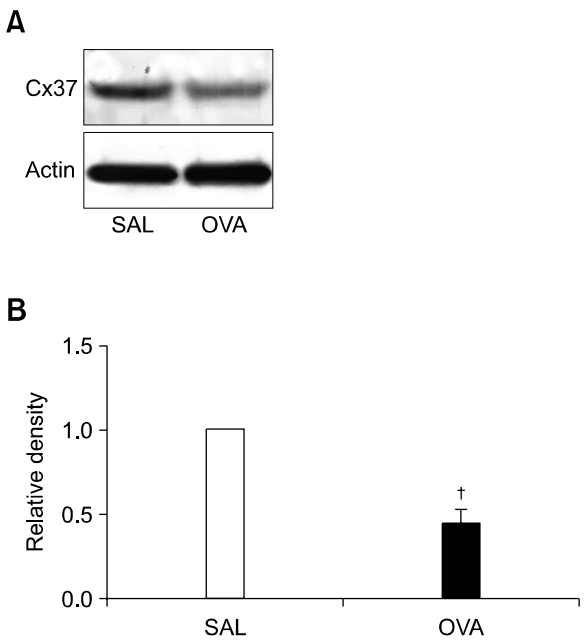

Figure 4. Levels of Cx37 protein in tracheal epithelial cells. (A) Western blot analyses of Cx37 protein in tracheal epithelial cells of saline-sensitized and -challenged mice (SAL) and OVA-sensitized and -challenged mice (OVA). (B) Densitometric analyses are presented as the relative ratio of Cx37 to actin. The relative ratio of Cx37 in the epithelial cells of SAL is arbitrarily presented as 1. Data represent mean ± SEM from 5 independent experiments. $^{\dagger}P < 0.05$ versus SAL.

Cx37 protein levels in primary murine tracheal epithelial cells

Western blot analysis revealed that Cx37 protein levels in tracheal epithelial cells were significantly decreased after OVA inhalation compared with the levels in control mice (Figure 4).

Cellular changes in BAL fluids after OVA inhalation

Numbers of total cells, eosinophils, and neutrophils were increased in a time-dependent manner, reaching maximum levels in BAL fluids at 48 h after OVA inhalation, and the numbers of total cells, eosinophils, lymphocytes, and neutrophils were significantly greater from 24 h to 72 h after OVA inhalation compared with cell numbers before the inhalation or those in the control group (Figure 5A). The levels of Cx37 protein in lung tissues showed significantly negative correlations with the numbers of total cells ($r = -0.825$, $P < 0.05$), eosinophils ($r = -0.736$, $P < 0.05$), lymphocytes ($r = -0.819$, $P < 0.05$), and neutrophils ($r = -0.761$, $P < 0.05$) in BAL fluids (Figure 5B, C, D, and E).

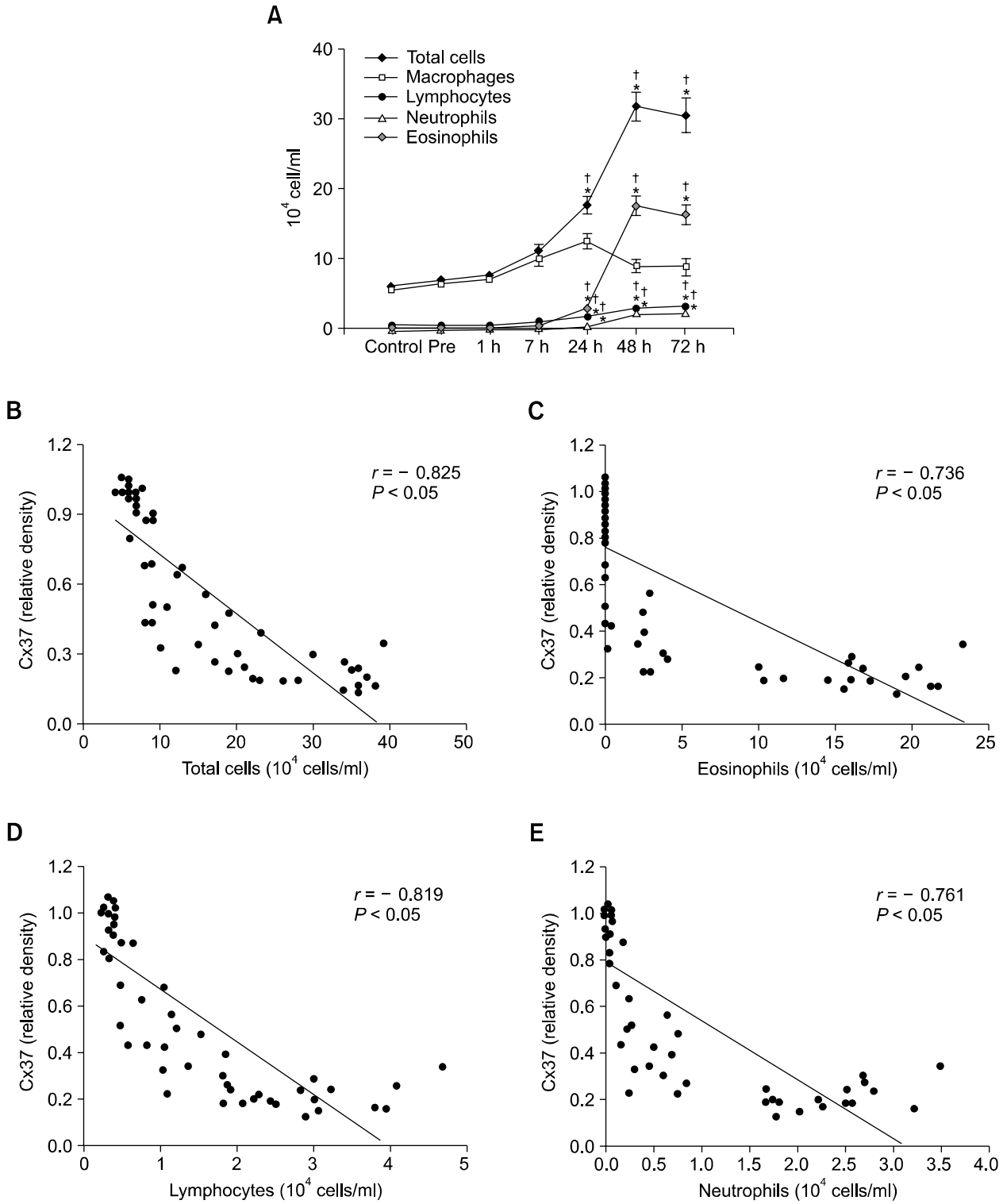

Figure 5. Cellular changes in BAL fluids after OVA inhalation. (A) Effect of OVA challenge on total cells and differential cellular components in BAL fluids. The numbers of each cellular component of BAL fluids from mice were counted at indicated time after the last challenge with OVA or saline. 1, 7, 24, 48, and 72 h are time periods after the last challenge. Data represent mean ± SEM from 8 mice per group. Control, no treatment; Pre, 1 h before the first challenge; $^{\dagger}P < 0.05$ versus Pre; $^{*}P < 0.05$ versus Control. (B-E) Correlations of Cx37 protein levels in lung tissues with numbers of total cells (B), eosinophils (C), lymphocytes (D), and neutrophils (E) in BAL fluids.

Pathological changes of OVA-sensitized and -challenged mice

Histologic analyses revealed typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Figure 6B). In contrast, no significant changes were observed in the control mice (Figure 6A). The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 h after OVA inhalation compared with scores after saline inhalation (Figure 6C).

Airway responsiveness after OVA inhalation

Airway responsiveness was assessed as a percent increase of R_L in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent R_L shifted to the left compared with that of control mice (Figure 7A). In addition, the percent R_L produced by methacholine administration at dose of 50 mg/ml increased significantly in the OVA-sensitized and -challenged mice compared with the controls. These results indicate that OVA induce airway hyperresponsiveness. In addition, the levels of Cx37 protein in lung tissues showed significantly negative correlation with the values of R_L ($r = -0.883$, $P < 0.05$) (Figure 7B).

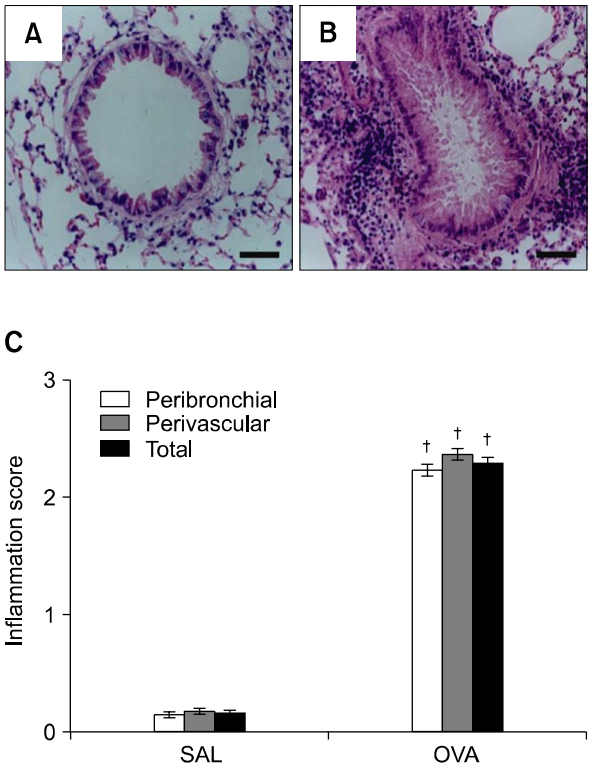

Figure 6. Pathologic changes after OVA inhalation. (A and B) Representative hematoxylin and eosin-stained sections of the lungs. Sampling was performed at 72 h after the last challenge in saline-sensitized and -challenged mice (A) and OVA-sensitized and -challenged mice (B). Bars indicate 50 μm. (C) Peribronchial, perivascular, and total lung inflammation. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores in saline-sensitized and -challenged mice (SAL) or OVA-sensitized and -challenged mice (OVA). Data represent mean ± SEM from 8 mice per group. $^{\dagger}P < 0.05$ versus SAL.

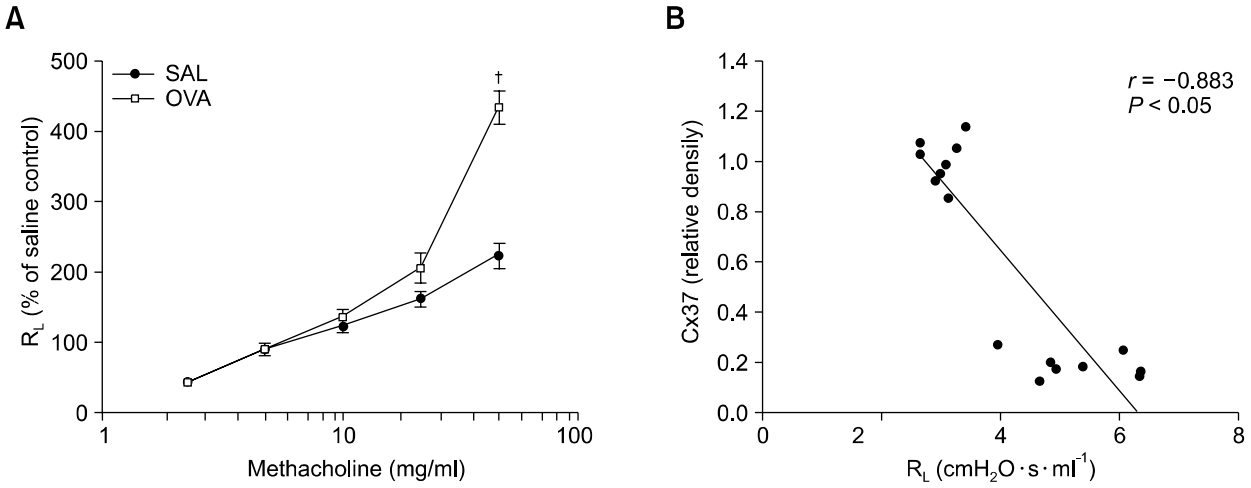

Figure 7. Airway responsiveness after OVA inhalation. (A) Effect of OVA challenge on airway responsiveness. Airway responsiveness was measured at 72 h after the last challenge in saline-sensitized and -challenged mice (SAL) or OVA-sensitized and -challenged mice (OVA). Data represent mean ± SEM from 8 mice per group. $^{\dagger}P < 0.05$ versus SAL. (B) Correlation between the levels of Cx37 protein in lung tissues and airway responsiveness. Data of airway responsiveness were the values of R_L measured by methacholine challenge at dose of 50 mg/ml.

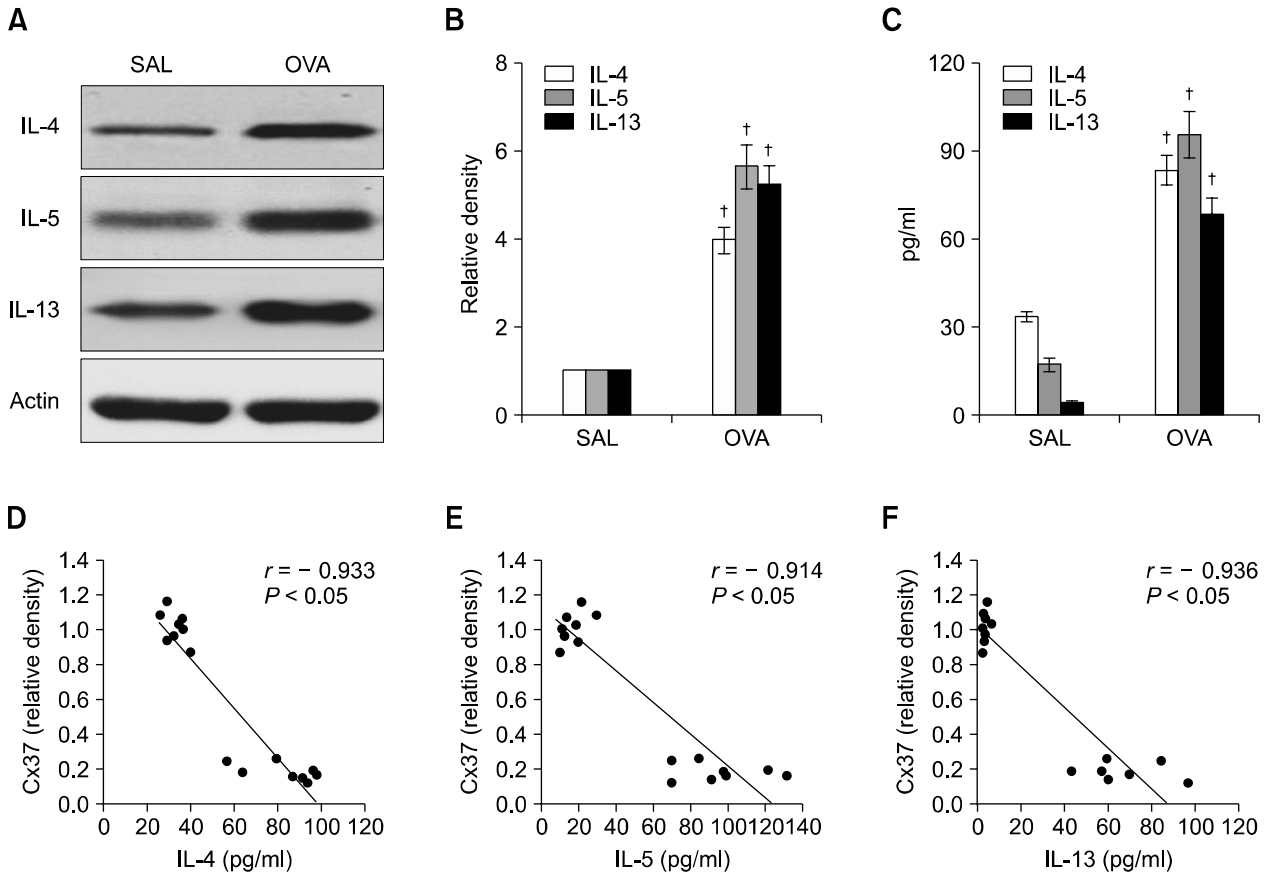

Figure 8. Levels of Th2 cytokines in lung tissues and BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-sensitized and -challenged mice (SAL) and OVA-sensitized and -challenged mice (OVA). (A) Western blotting of IL-4, IL-5, and IL-13 in lung tissues. (B) Densitometric analyses are presented as the relative ratio of IL-4, IL-5, and IL-13 to actin. The relative ratio of each molecule in the lung tissues of SAL is arbitrarily presented as 1. (C) Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids. Data represent mean ± SEM from 8 mice per group. $^{\dagger}P < 0.05$ versus SAL. (D-F) Correlations of Cx37 protein levels in lung tissues with levels of IL-4 (D), IL-5 (E), and IL-13 (F) in BAL fluids.

Levels of Th2 cytokines (IL-4, IL-5, and IL-13) in lungs of OVA-sensitized and -challenged mice

Western blot analysis revealed that the protein levels of IL-4, IL-5, and IL-13 in lung tissues were significantly increased at 72 h after OVA inhalation compared with the levels in the control mice (Figure 8A and B). Consistent with the results obtained from the Western blot analysis, enzyme immunoassays also showed significant increases in the levels of IL-4, IL-5, and IL-13 in BAL fluids at 72 h after OVA inhalation compared with the levels in the control mice (Figure 8C). The levels of Cx37 protein in lung tissues showed significantly negative correlations with the levels of IL-4 ($r = -0.933$, $P < 0.05$), IL-5 ($r = -0.914$, $P < 0.05$), and IL-13 ($r = -0.936$, $P < 0.05$) in BAL fluids (Figure 8D, E, and F).

Levels of adhesion molecules (ICAM-1 and VCAM-1) and chemokines (eotaxin and RANTES) in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that the protein levels of ICAM-1, VCAM-1, eotaxin, and RANTES in lung tissues were significantly increased at 72 h after OVA inhalation compared with the levels in the control mice (Figure 9).

Discussion

To our knowledge, this report is the first to describe the change of Cx37 in a murine model of allergen-induced airway inflammation. Gap junctions contain channels that allow the intercellular passage of ions and small molecules (Giepmans, 2004). At least twenty different Cxs have been reported, and they show different patterns of tissue distribution and developmental expression, as well as forming

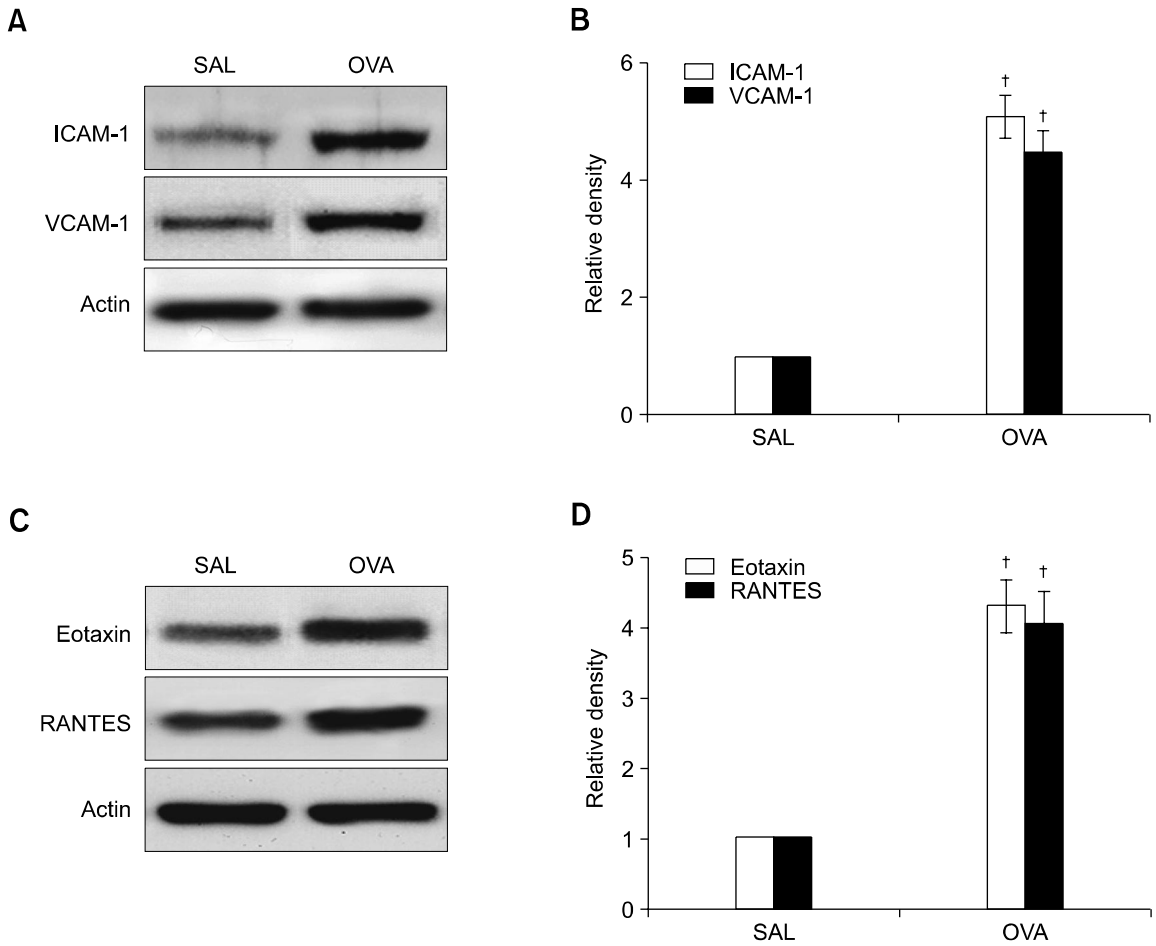

Figure 9. Levels of adhesion molecules and chemokines in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 72 h after the last challenge in saline-sensitized and -challenged mice (SAL) and OVA-sensitized and -challenged mice (OVA). (A) Western blotting of ICAM-1 and VCAM-1 in lung tissues. (B) Densitometric analyses are presented as the relative ratio of ICAM-1 and VCAM-1 to actin. (C) Western blotting of eotaxin and RANTES in lung tissues. (D) Densitometric analyses are presented as the relative ratio of eotaxin and RANTES to actin. The relative ratio of each molecule in the lung tissues of SAL is arbitrarily presented as 1. Data represent mean $\pm$ SEM from 8 mice per group. $^{\dagger}P < 0.05$ versus SAL.

channels with different biophysical characteristics. Unlike some other gap junction proteins, Cx37 is a subunit gap junction protein which exhibits limited expression in a few organs of the body including heart, lung, uterus, and ovary. Endothelial cells are a predominant cell type shown to express Cx37 (Reed *et al.,* 1993; Carter *et al.,* 1996; Yeh *et al.,* 1997). Cx37 may also play important roles in other cell types in organs such as the ovary where it is found between cumulus cells and the oocyte; Cx37 knock out mice exhibit female infertility due to impaired maturation of ovarian follicles (Reed *et al.,* 1993). Cx37 forms some of the most voltage-sensitive channels among known gap junctions and shows conductance, permeability, and gating properties that are distinct from other Cxs (Reed *et al.,* 1993; Veenstra *et al.,* 1994). Cx37 has been detected abundantly in lung including endothelial cells, fibroblastic cells, and airway smooth muscle cells (Willecke *et al.,* 1991; Nakamura *et al.,* 1999).

Many studies on Cx expression in alveolar tissue and alveolar epithelial cell cultures have uncovered complex patterns of protein expression with at least six different Cxs: Cx26, Cx32, Cx37, Cx40, Cx43, and Cx46 (Haefliger *et al.,* 1992; Lee *et al.,* 1997; Carson *et al.,* 1998; Abraham *et al.,* 1999, 2001; Guo *et al.,* 2001; Isakson *et al.,* 2001a, b). Changes in Cx expression in alveolar epithelial cells have been associated with developing or post-injury airways (Carson *et al.,* 1995, 1998; Abraham *et al.,* 2001), phenotypic changes inherent in alveolar cell culture (Lee *et al.,* 1997; Abraham *et al.,* 1999; Isakson *et al.,* 2001b), or changes in extracellular signaling molecules, including growth factors and matrix proteins (Abraham *et al.,* 1999; Alford and Rannels, 2001;

Guo *et al.,* 2001; Isakson *et al.,* 2001b). Although alveolar epithelial cells display a complex regulation of Cx expression, it is unclear how or whether gap junctional communication contributes to physiologic changes in the lung.

In this study, Cx37 protein and mRNA expression were decreased in lung tissues of a murine model of OVA-induced allergic airway disease. Immunoreactive Cx37 localized in epithelial layers around the bronchioles of control mice, which dramatically disappeared in allergen-induced asthmatic lungs. In addition, Cx37 protein levels in tracheal epithelial cells were significantly decreased after OVA inhalation compared with the levels in control mice. These findings suggest that the epithelial layer is one of the sites of Cx37 expression in lungs. Our present study with OVA-induced murine model of allergic airway disease has revealed the following typical pathophysiological features: airway hyperresponsiveness, airway inflammation, and increased levels of Th2 cytokines (IL-4, IL-5, and IL-13), adhesion molecules (ICAM-1 and VCAM-1), and chemokines (eotaxin and RANTES) in lungs. These features of asthma induced in a mouse model are similar to the human asthma (National Institutes of Health *et al.,* 1995; Lilly *et al.,* 2005) and make the mouse model suitable for investigations of the pathophysiology and immunology of asthma. In this study, the levels of Cx37 protein in lung tissues showed significantly negative correlations with airway inflammation, airway responsiveness, and levels of Th2 cytokines in lungs. These findings suggest that alterations in Cx37 levels are associated with the asthma phenotype.

Previous studies have reported that inhibitors of Cx37 channels increase leukocyte adhesion (Zahler *et al.,* 2003; Wong *et al.,* 2006; Chanson and Kwak, 2007). In addition, the recruitment of Cx37-deficient inflammatory cells is enhanced compared with that of Cx37-expressing cells, and restoration of Cx37 expression in Cx37-deficient cell line by transfection of Cx37 cDNA decreases leukocyte adhesion (Wong *et al.,* 2006). These studies suggest that Cx37 is involved in regulating recruitment and adhesion of inflammatory cells. Adhesion molecules (ICAM-1 and VCAM-1) and chemokines (eotaxin and RANTES) play central roles in asthma through regulating adhesion and migration of inflammatory cells into airway (Romagnani, 2002; Yusuf-Makagiansar *et al.,* 2002). Our present study has shown that the protein levels of adhesion molecules (ICAM-1 and VCAM-1) and chemokines (eotaxin, and RANTES) were increased. In addition, the levels of Cx37 protein correlated inversely with the levels of these molecules in lung tissues (data not shown). Adhesion molecules and chemokines are expressed by various airway cells including eosinophils, bronchial epithelium cells, endothelial cells, T cells, mast cells, and alveolar macrophages (Wardlaw *et al.,* 1994; Bisset and Schmid-Grendelmeier, 2005). Expression of ICAM-1 or VCAM-1 is modulated by Th2 cytokines in airway inflammation (Bochner *et al.,* 1995; Striz *et al.,* 1999; Henderson *et al.,* 2000). Th2 cytokines are also able to stimulate the production of chemokines in airway cells (Mochizuki *et al.,* 1998; Li *et al.,* 1999; Teran *et al.,* 1999; Bisset and Schmid-Grendelmeier, 2005). In this study, the levels of Cx37 protein in lung tissues showed significantly negative correlations with the numbers of airway inflammatory cells, which are the source of adhesion molecules or chemokines, and the levels of Th2 cytokines, which induce the expression of these molecules. Therefore, the increase in the expression of adhesion molecules and chemokines associated with decreased Cx37 levels may be due to the increased airway inflammation and Th2 cytokines levels in lungs of allergic airway disease.

In summary, we have shown that Cx37 expression was decreased in lung tissues of a murine model of allergic airway disease, which is mimicking human bronchial asthma, and that Cx37 was localized in airway epithelial cells. Moreover, levels of Cx37 protein in lung tissues showed significantly negative correlations with airway inflammation, airway responsiveness, and levels of Th2 cytokines in lungs. Our results suggest that alterations in Cx37 levels are associated with airway inflammation and bronchial hyperresponsiveness.

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