INTRODUCTION

Emphysema is one of the main pathological conditions of chronic obstructive pulmonary disease (COPD), and is characterized by enlargement of alveoli and reduction of lung elasticity. In general, once alveoli are damaged they cannot recover (Deslee et al., 2009). At the present time, the therapy for emphysema is only symptomatic, and it is highly desirable to develop an effective cure.

In studies to find effective targets for curative emphysema therapy and to evaluate new candidate compounds, many kinds of model animals for emphysema have been used, for example a model in which animals are exposed to cigarette smoke for a few months (Mahadeva and Shapiro, 2002). Another model in which mice are given postnatal treatment of dexamethasone (Dex) has been widely used in the last two decades because the procedure is simple and easy (Stinchcombe and Maden, 2008). In the Dex model, it is thought that Dex disturbs lung development and induces thinning of the alveoli walls (Massaro and Massaro, 1986). However, the detailed mechanism remains unclear.

In general, alveolar elastic fibers are essential for respiratory function in the lungs. Elastic fibers actively develop in the lungs around the time of birth as described below (Adrian and Robert, 2006). At the beginning of the process, fibrillin-1 (Fbn1) forms microfibrils which are the scaffold for assembly of elastin fibers. Then, monomeric tropoelastin (Tropo) is deposited on the microfibrils, and these complexes are reinforced with elastin microfibril interfacer 1 (Emilin-1: Emn1) and fibulin-5 (Fbln5) to form high molecular weight complexes (Hirai et al., 2007; Yanagisawa and Elaine, 2010). Finally, the complexes become mature with the formation of cross-linkages within and/or between the complexes, which is catalyzed by lysyl oxidase (Lox). However, the actions of postnatal treatment of Dex on assembly of alveolar elastic fibers in this emphysema model in mice are not clear.

Letter

Effects of postnatal dexamethasone treatment on mRNA expression profiles of genes related to alveolar development in an emphysema model in mice

Midori Kamei, Atsushi Miyajima, Mari Fujisawa, Yuka Matsuoka and Takashi Hirota

ABSTRACT — Emphysema can be induced in animals by postnatal treatment with dexamethasone (Dex) and such models have been widely used for various research. However, it is not clear what are the effects of Dex on assembly of alveolar elastic fibers in the emphysema model in mice. This study compared the expression profile of genes related to alveolar development between Dex treated and control mice during the treatment from postnatal day 3 (P3) to P14 with a 2-day break. From morphological observation of lung sections on P42, we confirmed the induction of emphysema in the treated mice. The mRNA expression level of fibrillin-1, which consists of microfibrils as a scaffold to form elastic fibers, and fibulin-5, which is a key protein reinforcing the fibers, reached maximum on P7 in control mice. However, in the Dex group, expression levels both types of mRNA were much lower with no clear expression peak. On the other hand, mRNA expression of tropoelastin, the main component in elastic fibers, reached maximum on P5 in the Dex group, which was 9 days earlier than in the control group. At this time, the amount of microfibrils might not be enough for tropoelastin to be deposited completely in Dex treated mice. This imbalance in the expression of tropoelastin and microfibril might interfere with the efficient formation of elastic fibers.

Key words: Emphysema-model, Mouse, Dexamethasone, Elastin

INTRODUCTION

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In this study, mRNA expression profiles of genes related to alveolar development were compared between Dex treated and control mice.

MATERIALS AND METHODS

Chemicals and reagents
Dex was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents in this study were commercially available and were either extra pure, molecular biology or biochemical grade.

Animals
Pregnant ICR mice (day 14 of gestation) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Their offspring were used regardless of sex. Postnatal day 1 (P1) was defined as the day after birth. All mice were housed in a temperature- (23 ± 1°C) and humidity- (55 ± 5%) controlled room with 12-hr light/dark cycle. Water and food were available ad libitum throughout the study. All experimental animals were handled in accordance with the institutional and national guidelines for the care and use of laboratory animals.

Study design and dosing
Six litters of newborn mice were divided equally on P3 to two groups, Dex-treated and control groups, keeping the integrity of the litters. The new born mice received the following treatment. In the Dex-treated group, mice were administered Dex solution subcutaneously from P3 to P14 daily with a 2-day break on P8 and P9 at a dose of 0.4 μg (20-μl injection) per animal. Control mice were treated with vehicle only. Dex solution was prepared as follows; Dex was dissolved in dimethylsulfoxide at a concentration of 4 mg/ml, and diluted with phosphate buffered saline (pH 7.4) to 20 μg/ml. To evaluate mRNA expression, one mouse from each of the three dams in each group was killed and the lungs were isolated on P3, P5, P7, P14, P21 and P42, and the body and lung wet weights were measured. The lungs isolated on P5, P7, P14 and P21 were used for the western blot to evaluate the protein expression level of tropoelastin. Only on P0 to obtain the baseline value before treatment, 3 dams were selected at random, regardless of group allocation, and one newborn mouse from each dam was used. For the preparation of histopathology sections of the lung, in the separate experiments, four litters of new born mice and dams were equally allocated to control and Dex groups and received the treatment described above. From two new born mice from each dam, the lungs were isolated on P42.

Histological and morphometrical analyses
The lung sections were prepared on P42 as follows. The trachea of isolated lungs was intubated and inflated to standard pressure of 20 cm H₂O with 1/2-Karnovsky’s solution (2% paraformaldehyde and 2.5% glutaraldehyde) for 10 min at room temperature. Then, the lungs were placed in the same solution for more than 48 hr for complete fixation. After that, the samples were embedded in paraffin and sliced at 5 μm thickness. The sections were stained with the Elastica-van Gieson method, and were observed with a light microscope (200 ×).

The alveolar mean chord length (Lm) was calculated from images of the sections using image analyses software, Image J (National Institutes of Health, Bethesda, MD, USA) as an index of the distance between alveolar walls (Dunnill, 1962). Briefly, a section was divided into six areas and average of the chord lengths of five non-overlapping viewing fields per each area was calculated. Then the average of the values in the six areas was

Table 1. Primers used for realtime RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession No.</th>
<th>Primer sequences</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropoelastin</td>
<td>U08210</td>
<td>CTTTGACTTTTCTCCCAATTATCC</td>
<td>Starcher, et al. (2008)</td>
</tr>
<tr>
<td>(Trop)</td>
<td></td>
<td>GGTCCCGAGAAGATCATTTTCTC</td>
<td></td>
</tr>
<tr>
<td>Fibulin-5</td>
<td>NM011812</td>
<td>TGTGACCCAGAGATGAAACTTGGAG</td>
<td>Nguyen et al. (2004)</td>
</tr>
<tr>
<td>(Fbn1)</td>
<td></td>
<td>AGCCCCCTTGTAGATTGTAGCA</td>
<td></td>
</tr>
<tr>
<td>Emelin-1</td>
<td>NM133918</td>
<td>TGTGCTAGGGTAGCATTTTTC</td>
<td>Huang et al. (2008)</td>
</tr>
<tr>
<td>(Emn1)</td>
<td></td>
<td>GAGGCTGAAGAAGCCCGAG</td>
<td></td>
</tr>
<tr>
<td>Fibrillin-1</td>
<td>NM007993</td>
<td>GGGGCGCTCAGCAGCGATG</td>
<td>Hartner et al. (2004)</td>
</tr>
<tr>
<td>(Fbn1)</td>
<td></td>
<td>GCCCAAGGTCGGCAAGCA</td>
<td></td>
</tr>
<tr>
<td>Lyxyl Oxidase</td>
<td>M65142</td>
<td>GCACCTGCACACACACAGGAG</td>
<td>Kumarasamy et al. (2009)</td>
</tr>
<tr>
<td>(Lox)</td>
<td></td>
<td>TTAGTCTGGCTCTCAGG</td>
<td></td>
</tr>
<tr>
<td>β-2 microglobulin</td>
<td>NM009735</td>
<td>GACTGTCCTTCTCTATACCTCGG</td>
<td>Trempus et al. (1997)</td>
</tr>
<tr>
<td>(β2M)</td>
<td></td>
<td>CTTCTGCGTGCATAAAATTG</td>
<td></td>
</tr>
</tbody>
</table>
defined as the Lm for each mouse.

**Real time RT-PCR**

Total RNA was purified from the lungs with RNeasy Plus Mini (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer’s protocol. For each sample, cDNA was produced from 1 μg of purified RNA with Prime-Script II 1st stand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s protocol. Real time PCR was performed with THUNDERBIRD™ SYBR® qPCR Mix (TOYOBO, Osaka, Japan) with Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA) according to their instructions. Briefly, 40 cycles of real time PCR were carried out under the following conditions: denaturing at 95°C for 30 sec, annealing at 60°C for 15 sec, and extension at 72°C for 20 sec for Fbn1, Fbln5 and Emn1. For Lox, Tropo and β-2-microglobulin (β2M, a housekeeping gene) the annealing and extension conditions were modified to 55°C for 15 sec and 72°C for 30 sec respectively. The primers used for each PCR are listed in Table 1. The data were analyzed by the delta delta Ct method to calculate the fold changes in gene expression. It was confirmed that the Ct values of β2M were not affected by sex, age or treatment of Dex in this study.

**Western blot**

About 30 mg of lung tissue was homogenized with extraction buffer containing Halt Protease inhibitor Single-Use Cocktail (Thermo Fisher Scientific, Waltham, MA, USA) on ice. The homogenate was shaken at 4°C for 1 hr and centrifuged at 14,000 g for 15 min at 4°C. The total protein concentration in each supernatant was determined by Protein Assay Standard II (Bio-Rad, Hercules, CA, USA). An amount of 30 μg protein of each sample was separated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene fluoride membrane (Merck Millipore, Bellerica, MA, USA). The membrane was blocked with 3% skim milk and incubated overnight at 4°C with a primary antibody, Polyclonal Antibody to Front Half of Mouse Tropoelastin (Elastin Products Company, Owensville, MO, USA, Cat. PR385, Lot.719, 1:5,000). After the washing with tris-buffered saline plus Tween (TBST) at room temperature, the membrane was incubated with ECL Rabbit IgG, HRP-Linked Whole Ab (GE Healthcare, Buckinghamshire, UK, Cat No.NA934, 1:50,000) as a secondary antibody at room temperature for 1 hr. Then after washing with TBST, the membrane was incubated in 1 ml of Luminata™ Forte Western HRP Substrate (Merck Millipore, MA, USA) at room temperature for 5 min. Chemiluminescence was detected with LAS4000 Mini (GE Healthcare). For stripping antibodies, the membrane was incubated with stripping buffer (pH 6.8) containing SDS and 2-mercaptoethanol at 50°C for 30 min. Then all the steps to detect the beta actin were performed in the same manner as described above using Anti-beta Actin antibody (Abcam, Cambridge, UK, Cat. ab8227, Lot.1017839, 1:5,000) as a primary antibody.

**Statistical analyses**

Statistical analyses were performed using Welch’s t-test. Differences were considered to be significant when \( p < 0.05 \).

**RESULTS AND DISCUSSION**

To confirm the effect of postnatal treatment with Dex
on the lung morphology, the lung sections of mice in the control and Dex groups were observed microscopically on P42. In the Dex group, fewer and larger alveoli were clearly observed compared with the control group (Fig. 1). Moreover, the average Lm, as a quantitative measure of emphysema, was 83.7 μm in the Dex group, which is approximately 3.3 times longer than in the control group (25.3 μm, p < 0.01, Fig. 2). These results were consistent with the previous reports using an emphysema model based on Dex treatment (Hind and Maden, 2004; Stinchcombe and Maden, 2008). Also, it was confirmed that postnatal Dex treatment induced emphysema in mice. The weight of the lung in Dex treated mice was significantly lighter than that in control mice from P3 to P42, while the body weights in control and Dex treated mice were not significantly different except for P7 and P21 (Fig. 3). From these results, Dex-treatment mainly affected the lung.

In this study, the expression levels of the following five components, Tropo, Fbn1, Fbln5, Lox and Emn1, were measured over time up to P42. In control group, the mRNA expression levels for Fbn1 and Tropo reached a maximum on P7 and P14, respectively (Fig. 4A and B). In addition, mRNA for Lox had no clear peaks during this study period (Fig. 4D). These results are consistent with the report by Mariani et al. (2002), demonstrating the various profiles of Tropo, Fbn1, and Lox during lung development.

Although Fbn1 and Fbln5 mRNA expression levels in the control group reached a peak on P7, the levels in the Dex group were much lower and did not show clear peaks during the study period (Fig. 4B and C). On the other hand, the mRNA expression level of Tropo reached a peak on P5 in the Dex group, 9 days earlier than in the control group (Fig. 4A). In addition, the change in the expression profile of tropoelastin protein was similar to that of mRNA (Fig. 5). These results were different from the results of in vivo studies demonstrating the induction by Dex of Tropo in fibroblasts of rat lung and Fbn1 in human skin fibroblasts (Nakamura et al., 2000; Barnett et al., 2011). Considering these facts, expression of Tropo, Fbn1 and Fbln5 was thought to be regulated by com-

![Fig. 2](image-url)  
**Fig. 2.** Effect of postnatal Dex treatment on the alveolar mean chord length. Data are shown as mean ± S.D., n = 4, *p < 0.05 vs control group analyzed by t-test.

![Fig. 3](image-url)  
**Fig. 3.** Weight of body (A) and lung (B) of the control and Dex-treated mice. The sex of mice on P1 to P7 was not determined. On P14, P21 and P42, the male-to-female ratios in control mice were 3:0, 2:1 and 2:1, respectively. The ratios in Dex treated mice were 2:1, 1:2 and 2:1 respectively. Data are shown as mean ± S.D., n = 3, *p < 0.05 vs control group analyzed by t-test.
**Fig. 4.** Effect of postnatal Dex treatment on mRNA expression profiles of Tropoelastin (A), Fibrillin-1 (B), Fibulin-5 (C), Lysyl oxidase (D), Emilin-1 (E). Data are shown as mean (ratio to β2M) ± S.D., n = 3. *p < 0.05 vs control group analyzed by t-test.

**Fig. 5.** Effect of postnatal Dex treatment on protein expression profile of tropoelastin in lungs. Western blot analysis was performed on P5, 7, 14 and 21 (n = 3). Representative data from both groups on each sampling day are shown. C, control; D, Dex-treated.
complicated mechanisms in vivo. Meanwhile, there were no differences in Lox and Emn1 mRNA expression levels between control and Dex groups (Fig. 4D and E). This result suggests that these genes are not critical genes for emphysema in the Dex model.

This study demonstrated that Dex accelerated the timing of peak mRNA expression levels of Tropo and reduced Fbn1 and Fbln5 expression levels (Fig. 4A and C). These results suggest that there are not enough microfibrils as a scaffold for the amount of Tropo produced on P5 in the Dex group. This imbalance in the expressions of Tropo and microfibril might interfere with the efficient formation of elastic fibers, resulting in thinning of alveolar walls.

ACKNOWLEDGMENT

We would like to thank Dr. Donald Hinman for scientific advice and editing the manuscript.

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