Asporin Gene Expression in Mouse Alveogenesis

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ABSTRACT

Objective: To identify the temporal gene expression of asporin, one of the extracellular matrix (ECM) molecules, during mouse alveogenesis or lung alveolar formation.

Methods: C57BL/6 mouse lungs at postnatal days (P) 1, 7, 14, 21, 28 and 60 were harvested. RNA purification and quantitative real time - PCR were used for indentifying the temporal pattern of asporin gene expression. Mouse lungs were inflated with 10% buffered formalin at 25 cmH_2O and further processed for histological study of airspace development.

Results: The peak of asporin gene expression in mouse lungs was observed on P14, which related to the alveolar stage of mouse lung development. The level of asporin gene expression on P14 was significantly elevated, twentyfold and sixtyfold higher, in comparison with P1 (saccular stage) and P60 (adult age), respectively. Alveogenesis was also observed from mouse lung histology on P7 and P14.

Conclusion: Highly regulated asporin expression in the lung during alveogenesis suggested that asporin expression affects alveogenesis

Keywords: Asporin, alveogenesis, airspace enlargement, lung development

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INTRODUCTION

The development of functional alveoli or alveogenesis is related with the septation from pulmonary saccule which is a big primitive air sac.[1] Alveogenesis in mice takes place after birth between postnatal days (P) 4 to P21, whereas in humans alveogenesis takes place prenatally in the third trimester of gestation (28^{th} - 40^{th} gestational weeks) and continues up to 8 years of age.[2] Disruption of alveogenesis results in interrupted and arrested lung maturation. Recently, the production of new alveoli in adult mice was reported. Previous studies supported the concept that alveolar regeneration shared some aspects of alveolar development.[3] Thus, factors involved in alveolar septation are of high interest.

Several extracellular matrix (ECM) proteins have been reported to regulate alveogenesis. ECM proteins transmit essential information to pulmonary cells and regulate their differentiation, organization, and function.[4] Importantly, alteration of ECM gene expression, including the small leucine-rich proteoglycan (SLRP) family, may affect the pattern of alveogenesis. Asporin, decorin and biglycan are ECM proteins and belong to the SLRP family.[5] A previous study reported the expression of asporin mRNA in human lungs of fetuses and adults, using human RNA hybridized blots.[6] In both fetal and adult human lungs, there are moderate expression levels of asporin gene. However, the expression levels were higher in fetal than adult lungs, suggesting that asporin is involved in fetal lung development. We therefore determined the temporal expression of asporin gene in mouse lungs during the saccular stage and alveolar stage of lung development including adult age.

MATERIALS AND METHODS

The animals used in this study were C57BL/6 mice at postnatal day (P) 1, 7, 14, 21, 28 and 60. C57BL/6 mice were obtained from the National Laboratory Animal Center, Mahidol University. All animals were supplied with food and water ad libitum. All experimental procedures using laboratory animals were approved by Siriraj Animal Care and Use Committee (SI-ACUC) (005/2553) and were conducted following national guidelines for the care and protection of animals.

RNA isolation and quantitative real time RT-PCR

Total RNA was prepared from whole lung tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA). RNase-free DNase I (deoxyribonuclease) (New England

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Biolabs, Ipswich, MA) digestion was used to treat 10 µg of total RNA to eliminate contaminating genomic DNA. Reverse transcription was performed using iScript reverse transcriptase, oligo dT primers and random hexamer primers from commercial cDNA synthesis kit (iScriptTM cDNA synthesis kits, Bio-Rad, Hercules, CA). Quantitative real time PCR (qPCR) was performed using oligonucleotide primers for mouse asporin and peptidylprolylisomerase A (PPIA), SYBR green chemistry (VeriQuestTM SYBRR Green qPCR Master Mix, Affymetrix, Santa Clara, CA) on an Mx3005P real-time PCR system (Stratagene, La Jolla, CA). The expression level of asporin gene in each sample was normalized to the level for the endogenous control PPIA gene and calculated using the mean of difference in cycle threshold between asporin and PPIA genes. The mean expression of asporin was calculated and represented as a fraction of that normalized by PPIA expression.

Lung histology

After mice were euthanized, an intravenous catheter (JelcoR, Smiths Medical International Ltd., Rosendale, NY) was inserted into their trachea and secured with a 4/0 nonabsorbable surgical suture (Pearsalls Ltd., Taunton, Somerset, U.K.). Lungs were exposed and perfused with 5 mL NSS using a 25-gauge needle (Terumo, Tokyo, Japan) which was inserted into the outflow tract of their right ventricle. The right lung was then tied off at the hilar region with a suture and cut off distal to that suture. The left lung was inflated via the tracheal cannula to a fixed pressure of 25 cm H_2O with 10% buffered formalin for at least 15 minutes. At the end of the fixation period, the catheter was removed and the trachea was ligated by suture. The heart and inflated lung were removed *en bloc* and immersed in 10% buffered formalin for 48 hours fixation. After fixation, lungs were sectioned mid-sagitally and embedded in paraffin for histological and morphometrical analysis. Sections were cut into 5-µm thick with a microtome and stained with hematoxylin and eosin. The lung slice was imaged at 200 times (200X) magnification [objective lenses (10X) and eyepiece lenses (20X) in the microscope] using the Zeiss AxioCam MRc digital camera for microscopy (Carl Zeiss Inc., Göttingen, Germany).

Statistical analyses

The data were analyzed by SPSS software version 11.5 (SPSS Inc.). ANOVA followed by Bonferroni correction was used for the statistical analysis among age groups. All data were presented as mean ± standard error of measurement (S.E.M). The *P*-values less than 0.05 were considered statistically significant.

RESULTS

Mouse lung histology from birth to adult

We detected the histology of mouse lungs at P1, 7, 14, 21, 28, 60 by hematoxylin and eosin staining and found that airspace size was different in various ages of mouse. Mouse lungs at P1 had thick alveolar walls with large size of airspace or primitive saccule (Fig 1A). Mouse lungs at P7 had thinner alveolar wall and smaller alveolar size than P1 (Fig 1B). At P7, we noted the protrusion of secondary crests initiated from the walls of terminal sacs to form alveoli. When mouse lung developed from P1 to P14, the alveolar size was smaller and the alveolar wall was thinner (Fig 1A-C). We did not observe any apparent difference in alveolar wall thickness and alveolar size of mouse lungs between P21, P28 and P60 (Fig 1D-F).

Highly regulated asporin gene expression pattern during alveogenesis

To investigate the temporal expression of asporin gene during alveogenesis, we detected the asporin expression in mouse lungs at P1, 7, 14, 21, 28, 60 by real time PCR. The age range between P7 to P28 was selected in order to include the stage of mouse lung alveogenesis. The results showed that lung asporin gene expression was significantly increased in mice at P7 compared with P1 (143.81 ± 15.08 vs. 48.74 ± 5.91 [10^{-3} arbitrary unit]; *P*-value < 0.0001) (Fig. 2). The peak of asporin expression was observed at P14 compared with all other groups (947.21 ± 69.07 [10^{-3} unit]; P-value < 0.0001). Asporin expression level was then found to be decreased at P21 (39.19 ± 2.42 [10^{-3} unit]) and P28 (27.96 ± 6.36 [10^{-3} unit]). There was no significant difference among P1, P21 and P28. Lung asporin expression was decreased in mice at P60 compared with all other groups (14.41 ± 0.52 [10^{-3} unit]; *P*-value < 0.005), except P28.

DISCUSSION

Asporin is one of the ECM proteins. It is known that several ECM proteins are essential for alveolar

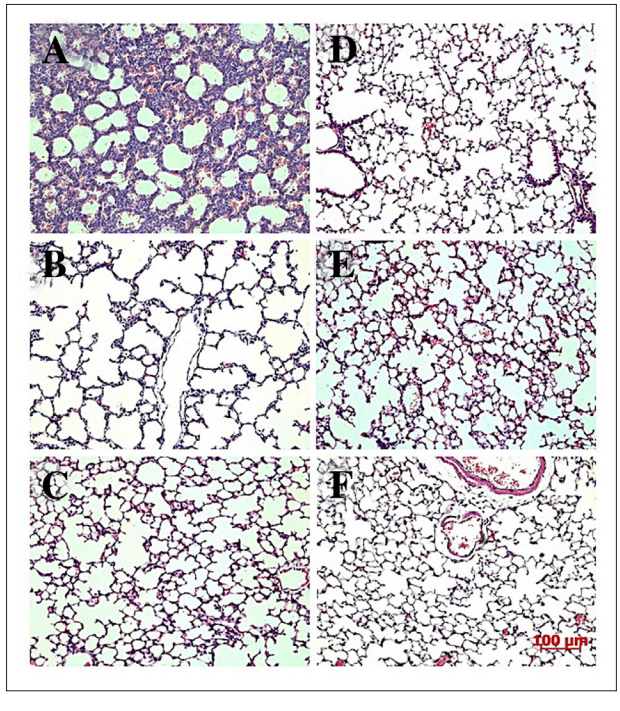

Fig 1. Histology of mouse lung alveogenesis. Representative hematoxylin and eosin stained images of 5 µm paraffin section of C57BL/6 mouse lungs at P1 (A), P7 (B), P14 (C), P21 (D), P28 (E), and P60 (F) taken with similar magnification (200X). Scale bar of 100 µm is shown.

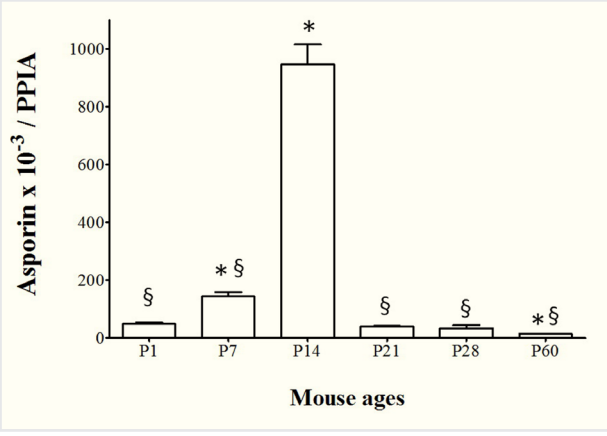

Fig 2. Asporin gene expression in mouse lungs by quantitative real time – PCR. Y axis represents relative asporin gene expression normalized by PPIA. Data are expressed as mean + SEM and determined by one-way ANOVA and Bonferroni post hoc test. (n= 6/group)

* indicates *P*-value < 0.0001 compared with P1

§ indicates *P*-value < 0.0001 compared with P14

development, maintenance and repair.[4] However, the study of asporin and lung development was not evident. To determine the asporin gene expression during lung development, we used quantitative real time PCR and found that mouse lung asporin expression was significantly increased at P7-P14, corresponding with the stage of alveogenesis in mice. Alveogenesis was apparently observed from mouse lung histology on P7 and P14.

Alveogenesis belongs to the final stage of mammalian lung development. In this stage, the alveolar septum is initiated by protrusion from the pulmonary saccule which is a big primitive air sac. The development of alveoli accompanying the capillary change leads to the effacement of primitive alveolar capillary membrane, contributing to effective gas exchange.[1] Disruption of alveogenesis results in interrupted and arrested lung maturation, with respiratory insufficiency as evidenced in premature birth. Furthermore, impairment of alveolar maintenance and repair following lung injury may lead to the loss of functional alveoli, associated with respiratory disorders known as bronchopulmonary dysplasia (BPD)[7] and pulmonary emphysema.[8] Recently, the production of new alveoli in adult mice was reported, suggesting that mechanisms of lung repair exist.[9,10] Moreover, signals that regulate programs of alveolar turnover are conserved from rodents to humans.[11] Previous studies supported the concept that alveolar regeneration shared some aspects of alveolar development.[3] Many chronic lung diseases would benefit from therapeutic approaches that recapitulate the process of alveogenesis to increase the surface area of gas exchange. However, our current lack of understanding of the mechanisms controlling lung development, maintenance, and repair limits the therapeutic approaches to combat lung disease.

Mariani et al. studied the pattern of gene expression in mouse lung development by microarray experiment and reported that clusters of ECM and fibroblast growth factor receptor types 3 and 4 (FGFR3/4) have been shown to share the gene expression pattern, implicating their roles on alveogenesis in mice.[12] These genes were highly expressed at P4-P20 which is related to mouse alveogenesis. The gene cluster analysis also revealed a relationship between FGFR3/4 and all other genes during alveogenesis suggesting that FGF signalings are involved in the regulation of ECM formation in the lungs. Mice harboring mutated FGFR3/4 compound knockout exhibit the enlarged and primitive lung saccule consistent with cessation of alveolar septation, similarly observed in BPD.[13,14] The data illustrated the role of FGFR3/4 on controlling ECM expression that, in turn, affects alveogenesis. Interestingly, asporin gene expression in FGFR3/4 compound knockout mice is significantly elevated when compared with age-matched wild type and compound heterozygote control groups suggesting that FGFR3 and 4 signalings regulate asporin expression.[14]

Previous study reported the expression of asporin mRNA in several human tissues both in fetuses and adults, using human RNA hybridized blots.[6] The high level expression was found in human aorta and uterus. These data suggested that asporin has been expressed in smooth muscle - rich tissue. In both fetal and adult human lungs, there are moderate expression levels of asporin gene. Interestingly, asporin gene expression levels were higher in fetal than adult human lungs, whereas the expression levels in other tissues of fetus and adults were not different. These data suggested that asporin might be involved in fetal lung development. However, the age of harvested human tissues both of fetal and adult specimens was not defined in the study. Moreover, if the fetal tissues belonged to a preterm, one cannot warrant whether the development of lungs were normal or any diseases in fetuses perhaps affected the lung development. Therefore, we cannot verify that an increase in asporin gene expression in fetal lung is related to a normal development process or the effect of other factors. In this study, mouse lungs at P1 which is related to the saccular stage of lung development showed minimal level of asporin expression. Highly expressed mouse asporin mRNA was observed at P7 and P14 which is related to alveolar stage or alveogenesis. Asporin gene expression was then significantly decreased at P21 and P28. The lowest level of asporin expression was shown in adult mouse lungs at P60. Therefore, these observations of asporin peak expression during P7-P14 suggested that asporin may involve alveogenesis. However, the role of asporin on alveogenesis needs further investigation.

Previous studies showed failure of alveogenesis in FGFR3/4 compound knockout mice, resulting in abnormal airspace enlargement.[13,14] These data suggested that FGFR3 and 4 are important for alveogenesis. However, neither FGFR3 or FGFR4 knockout mice, exhibited impaired alveolar development. FGFR3 mutant mice developed skeletal dysplasia, without failure of alveolar septation.[15] Mice with FGFR4 mutant have no apparent abnormalities.[13] These data suggested that FGFR3 and FGFR4 collaborate with each other for controlling alveolar development. Over-expression of asporin gene was detected in the lungs of FGFR3/4 compound knockout mouse.[14] Moreover, an aberrant increase in α-smooth

muscle actin (α-SMA) – positive cells was detected in FGFR3/4 compound knockout mouse lungs. During alveogenesis, myofibroblast is spatially present at the tips of secondary crests detected by positive staining for α-SMA, a marker of smooth muscle cells. The findings in FGFR3/4 compound knockout mouse lungs suggested abnormal cellular differentiation leading to abnormal postnatal lung development. The findings of highly increased asporin gene expression and loss of spatial expression of α-SMA – positive cells at the tips of secondary septae in FGFR3/4 compound knockout mouse lungs suggested that the expression of asporin and myofibroblast may be regulated by FGFR signaling. Disruption in asporin expression would perhaps impair alveogenesis. Thus, the association of asporin and myofibroblast should be further determined to explain the cooperation in the pathology observed in FGFR3/4 compound knockout mouse lungs. In conclusion, asporin has been reported to have a crucial role on the development of several tissues. The data gathered from this study suggested asporin as an ECM molecule related to lung development. Further investigations are needed to demonstrate asporin protein/spatial expression during alveogenesis as well as the involvement of asporin expression and alveolar enlargement.

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REFERENCES

1. Joshi S, Kotecha S. Lung growth and development. Early Hum Dev. 2007 Dec;83(12):789-94.
2. Warburton D, Gauldie J, Bellusci S, Shi W. Lung development and susceptibility to chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2006 Nov;3(8):668-72.
3. Perl AK, Gale E. FGF signaling is required for myofibroblast differentiation during alveolar regeneration. Am J Physiol Lung Cell Mol Physiol. 2009 Aug;297(2):L299-308.
4. McGowan SE. Extracellular matrix and the regulation of lung development and repair. FASEB J. 1992 Aug;6(11):2895-904.
5. Henry SP, Takanosu M, Boyd TC, Mayne PM, Eberspaecher H, Zhou W, et al. Expression pattern and gene characterization of asporin. a newly discovered member of the leucine-rich repeat protein family. J Biol Chem. 2001 Apr;276(15):12212-21.
6. Lorenzo P, Aspberg A, Onnerfjord P, Bayliss MT, Neame PJ, Heinegard D. Identification and characterization of asporin. a novel member of the leucine-rich repeat protein family closely related to decorin and biglycan. J Biol Chem. 2001 Apr;276(15):12201-11.
7. Hislop AA. Bronchopulmonary dysplasia: prenatal and postnatal influences. Pediatr Pulmonol. 2001;Suppl 23:107-9.
8. Tuder RM, McGrath S, Neptune E. The pathobiological mechanisms of emphysema models: what do they have in common? Pulm Pharmacol Ther. 2003 Apr;16(2):67-78.
9. Narayanan M, Owers-Bradley J, Beardsmore CS, Mada M, Ball I, Garipov R, et al. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. Am J Respir Crit Care Med. 2012 Jan;185(2):186-91.
10. Chen L, Acciani T, Le Cras T, Lutzko C, Perl AK. Dynamic regulation of platelet-derived growth factor receptor alpha expression in alveolar fibroblasts during realveolarization. Am J Respir Cell Mol Biol. 2012 Oct;47(4):517-27.
11. Massaro D, Massaro GD. Toward therapeutic pulmonary alveolar regeneration in humans. Proc Am Thorac Soc. 2006 Nov;3(8):709-12.
12. Mariani TJ, Reed JJ, Shapiro SD. Expression Profiling of the Developing Mouse Lung: Insights into the Establishment of the Extracellular Matrix. Am J Respir Cell Mol Biol. 2002 May;26:541–8.
13. Weinstein M, Xu X, Ohyama K, Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development. 1998 Sep;125(18):3615-23.
14. Srisuma S, Bhattacharya S, Simon DM, Solleti SK, Tyagi S, Starcher B, et al. Fibroblast growth factor receptors control epithelial-mesenchymal interactions necessary for alveolar elastogenesis. Am J Respir Crit Care Med. 2010 Apr;181(8):838-50.
15. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet. 1996 Apr;12(4):390-7.