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Circadian Rhythm of Surfactant Protein A, B and C mRNA in Rats

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Background : All organisms have developed an internal timing system capable of reacting to and anticipating environmental stimuli with a program of appropriately timed metabolic, physiologic and behavioral events. The alveolar epithelial type II cell of the mammalian lung synthesizes, stores, and secretes a lipoprotein pulmonary surfactant, which functions to stabilize alveoli at low lung volumes.

Methods : The authors investigated the diurnal variation of surfactant protein A, B and C mRNA accumulation. The diurnal variation on gene expression of surfactant protein A, B and C was analysed using filter hybridization at 9 a.m., 4 p.m. and 11 p.m. Lung SP-A protein content was determined by double sandwich ELISA assay using a polyclonal antiserum raised in rabbits against purified rat SP-A.

Results : 1. The accumulation of SP-A mRNA at 4 p.m. was significantly decreased by 23.5% compared to the value at 9 a.m. *($p<0.05$)*.

2. The accumulation of SP-B mRNA at 4 p.m. and 11 p.m. was decreased by 15.1% and 5.7%, respectively, compared to the value at 9 a.m. *($p=0.07$, $p=0.69$)*.

3. The accumulation of SP-C mRNA at 4 p.m. and 11 p.m. was decreased by 6.8% and 7.7%, respectively, compared to the value at 9 a.m. *($p=0.38$, $p=0.57$)*.

4. Total lung SP-A content at 4 p.m. and 11 p.m. was increased by 5.3% and 15.9%, respectively, compared to the value at 9 a.m. *($p=0.64$, $p=0.47$)*.

Conclusion : These findings represent the diurnal variation of surfactant proteins mRNA expression in vivo. These results indicated that the diurnal variation of significant gene expression is observed in hydrophilic surfactant protein rather than in hydrophobic surfactant proteins.

Key Words : Gene expression, Surface-active agents, Proteins, Circadian rhythm

INTRODUCTION

Surfactant consists of 80~90% phospholipid and 5~10% protein and carbohydrate. Phosphatidylcholine occupies 70~80% of phospholipid, of which 60~70% is dipalmitoly phosphatidylcholine (DPPC) that functions to lower surface tension at low lung volumes[1]. Phosphatidylglycerol occupies 5~10% of phospholipid and plays an important role in surfactant metabolism and lipid-protein interaction[2]. 10~15% of lipid-protein is phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin. Neutral lipid includes cholesterol, fatty acid and triacylglycerol. Surfactant consists of 5~10% protein, which is hydrophilic protein of the surfactant protein A (SP-A) and surfactant protein D (SP-D) and hydrophobic protein of surfactant protein B (SP-B) and surfactant protein C (SP-C). SP-A is mixed with SP-B and SP-C complex to stimulate phospholipid adsorption to air liquid interface[3]. SP-A, SP-B and SP-C have crucial roles in surfactant activity.

First, surfactant especially maintains the function of macro-

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phage properly through interaction between SP-A and macrophage[4]; second, it controls lots of mediators being involved in inflammation[5, 6]; third, it has an antioxidant function essential to surfactant protein itself[7]; fourth, it removes particles from pulmonary alveoli during expiration[8], which influences the pulmonary defense mechanism. Impairment in any or all of these functions may be important in the pathogenesis of acute respiratory distress syndrome (ARDS).

Circa means about and dies means a day, by which "circadian rhythm" indicates a diurnal rhythm changing daily and is adopted for hormone secretion, body temperature, sleep-wake, cognitive functions[9]. These circadian rhythms are endogenous so that they act even without environmental visual signal factors. Endogenous circadian rhythm of surfactant protein has not been reported yet.

The alveolar epithelial type II cell consists of cytosol, nucleus, lamellar body, multivesicular body, mitochondria, Golgi complex and hybrid composition. The volume of alveolar epithelial type II cell is minimal at 8~9 a.m. and maximal at 4 p.m[10, 11]. Surfactant is synthesized and secreted from the alveolar epithelial type II cell and the endogenous circadian rhythm of surfactant protein mRNA is closely related with the volume of type II cell.

We investigated the volume of the alveolar epithelial type II cell at 9 a.m. when it is minimal, at 11 a.m. when medium and at 4 p.m when maximal using rats, respectively, and examined the gene expression of SP-A, SP-B and SP-C by filter hybridization and lung SP-A protein content by double sandwich enzyme-linked immunosorbent assay (ELISA) assay, which was to observe the endogenous circadian rhythm to the gene expression of SP-A, SP-B and SP-C in rats in vivo.

MATERIALS AND METHODS

1. Laboratory animals

This study used 300~400 gm Sprague-Dawley rats (Grand Island, Nebraska) purchased from Sasco Co. which were acclimatized to a new environment for 2 weeks before the study. Each 7 rats under intraperitoneal injection of pentobarbital 0.2 cc/rat were sacrificed at 9 a.m, 11 a.m. and 4 p.m. To get their pulmonary tissues, they were anesthetized with pentobarbital and lung tissue was harvested by removing the lungs en bloc and was all collected. To examine mRNA, 500~750 mg pulmonary tissues were put in 10 mL solution D (4M guanidium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and homogenized by Tissumizer (Tekma, Cincinnati, Ohio) at a high speed for 30~60 seconds before storage in a freezer at -70℃. Tissue samples were frozen on dry ice and stored at -70℃ for ELISA.

2. Preparation of RNA

Whole RNA was purified by the acid phenol method of Chomczynski and Sacchi[12]. 50 μL 2M sodium acetate of pH 4.0 was added to 500 μL solution D in an Eppendorf tube and acidified before it was buffered with 0.1M Tris of pH 7.5 and 10 mM ethylenediaminotetraacetic acid (EDTA) of pH 7.5 and mixed with phenol 500 μL. It was mixed well with vortex and added again with 100 μL solution of chloroform and isoamyl alcohol in the ratio of 49:1 and mixed again by vortexing. This composition in the Eppendorf tube was put in ice for 15 minutes and centrifuged by 10,000 G microfuge for 5 minutes at room temperature. Aqueous phase was removed into the Eppendorf tube with a micropipette and the same amount of isopropanol was added to precipitate RNA. After being latent at -20℃ for at least 2 hours, the precipitate was collected by centrifugation at 10,000 G for 5 minutes. Supernatant was decanted and its precipitate was redissolved in 150 μL solution D with the same amount of isopropanol at -20℃ overnight. After centrifugation by 10,000G for 5 minutes, precipitates were collected and washed once with 70% ethanol. They were dried and resuspended in 0.1% diethyl-pyrocarbonate (DEPC) treated 1 mM EDTA (pH 8). Isolated RNA was quantitatively measured by spectrophotometry 260 nanometers. The quality of RNA was assessed with formaldehyde/agarose denaturing gel stained with ethidium bromide, so that degradated specimens were excepted from analysis.

3. RNA Hybridization Assays

The abundance of each mRNA was quantitatively estimated as a fraction of total RNA fractions and in relation to β-cytoskeletal actin mRNA by a filter hybridization assay. The complete coding regions of each rat SP-A, SP-B and SP-C to the complementary DNA (cDNA) were subcloned individually into pGem 3Z or 4Z. Anti-sense or sense transcripts were obtained by using SP6 RNA polymerase. Yields from transcription reaction averaged 20 to 30 μg of full-length transcript per microgram (μg) of linealized vector.

0, 0.1, 0.5, 1.0, 2.5 and 5.0 ng sense transcripts and 1 μg whole lung RNA were applied in triplicate to 13 mm nitrocellulose filters (0.45 μm in pore size) (Schleicher and Schuell, Keene, NH) in 10x standard saline citrate (SSC)/ 50% formamide after denaturing at 65℃ for 10~15 minutes. Filters were baked at 80℃ for 2 hours and prehybridized by shaking prehybridization fluid in a quantity of 0.2~0.5 mL per filter, which included 1 M sodium chloride, 10% dextran sulfate, 50% formamide, 1% sodium dodecyl sulfate (SDS) in 50 cc Falcon centrifuge tubes at 56℃ for 12~14 hours. After prehybridization, 4xSSC, 1xDenhardt's solution, 45% formamide, 10% dextran sulfate, 0.5% SDS and 0.1 mg/mL

sonicated denatured salmon sperm DNA fluid were added in a quantity of 0.2~0.5 mL per filter. It was shaken with the specific cDNA probe of SP-A, SP-B, SP-C and the untranslated 3' region of human beta actin, which was labeled with ^{32}P of $5x10^6$ cpm/mL by random oligonucleotide primed second-strand DNA synthesis using a kit from BRL at 56℃ during 17~20 hours for hybridization. All filters were washed together three times with 2xSSC and 0.2% SDS fluid at a room temperature, and three times with 0.1xSSC and 0.2% SDS fluid at 65℃. Filters were each counted by scintillation vial after being dried at room temperature. The specific mRNA was calculated from the standard curve using the regression equation.

4. Determination of SP-A Protein content

Protein content was determined from 0.025~0.050 mL of the lung homogenate by the bicinchoninic acid method of Smith et al.[13] at 490 nm using microtiter autoreader EL 307 (Biotech Instruments). Lung SP-A protein content was determined by double sandwich ELISA assay using a polyclonal antiserum raised in New Zealand white (Hazelton) rabbits against purified rat SP-A. Weighed lung fragments were homogenized in 4 mL 1% Triton X-100/phosphate-buffered saline (PBS) using a polytron and then sonicated briefly. Untreated microtiter plates (Dynatech Laboratories, Alexandria, VA)were incubated with a 0.1 mL/well rabbit anti-rat SP-A Ig G fraction (0.1 ㎍ /mL and 0.1 M $NaHCO_3$, pH 9.3) overnight at 22℃. The antibody solution was removed and the wells were incubated with PBS without calcium or magnesium of pH 7.4 containing 1% Triton X-100 and 3% bovine serum albumin for 30 minutes, then washed twice with the same buffer. Then 0.1 mL of various dilutions of sample lung homogenate (1:250 to 1:1,000) were added to the wells and incubated at 37℃ for 90 minutes. The wells were then washed 3 times with 3% albumin/PBS/1% Triton X-100, and 0.1 mL of rabbit anti-rat SP-A IgG fraction conjugated with horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) was added to each well and incubated for 90 minutes at 37℃. Wells were washes 4 times with 1% Triton X-100/PBS, and 0.1 mL of substrate solution (0.1% (wt/vol) o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) and 0.03% (vol/vol) hydrogen peroxide in 0.1 M citrate buffer at pH 4.6 were added to each well. Plates were incubated in the dark at 22℃ for 10 minutes. The reaction was stopped by the addition of 0.1 mL of 2 M H_2SO_4 per well. Adsorption at 460 nm was determined with a microtiter autoreader EL307 (Biotech Instruments).

5. Result Analysis

Statistical evaluation was performed with unpaired Student's *t*-test between mRNA values of normal control group and each group. Regression equations were derived using Epistat statistical package. Probability value <0.05 was significant.

RESULTS

1. Standard curve of sense transcript to SP-A, SP-B and SP-C

Standard curve for SP-A, SP-B, SP-C sense transcript 0, 0.1, 0.5, 1.0, 2.5, 5 ng to CPM and correlation coefficient (r) are as follows: standard curve for SP-A sense transcript was Y=0.001X-0.027 (X=CPM, Y=SP-A mRNA transcript) and r was 0.99 (Figure 1). Standard curve for SP-B sense transcript was Y=0.0008X-0.026 (X=CPM, Y=SP-B mRNA transcript) and r was 0.99 (Figure 2). Standard curve for SP-C sense transcript was Y=0.0019X-0.103 (X=CPM, Y=SP-C mRNA transcript) and r was 0.99 (Figure 3).

2. SP-A, B, C mRNA Diurnal Rhythm

The accumulation of SP-A mRNA at 4 p.m. significantly decreased by 23.5% compared to the value at 9 a.m. ($p<0.05$) (Figure 4). The accumulation of SP-B mRNA decreased by 15.1% at 4 p.m and 5.7% at 11 p.m, respectively, compared to the value at 9 a.m, which was not statistically significant ($p=0.07$, $p=0.69$) (Figure 5). The accumulation of SP-C mRNA decreased by 6.8% at 4 p.m and 7.7% at 11 p.m compared to the value at 9 a.m, which was not statistically significant ($p=0.38$, $p=0.57$) (Figure 6).

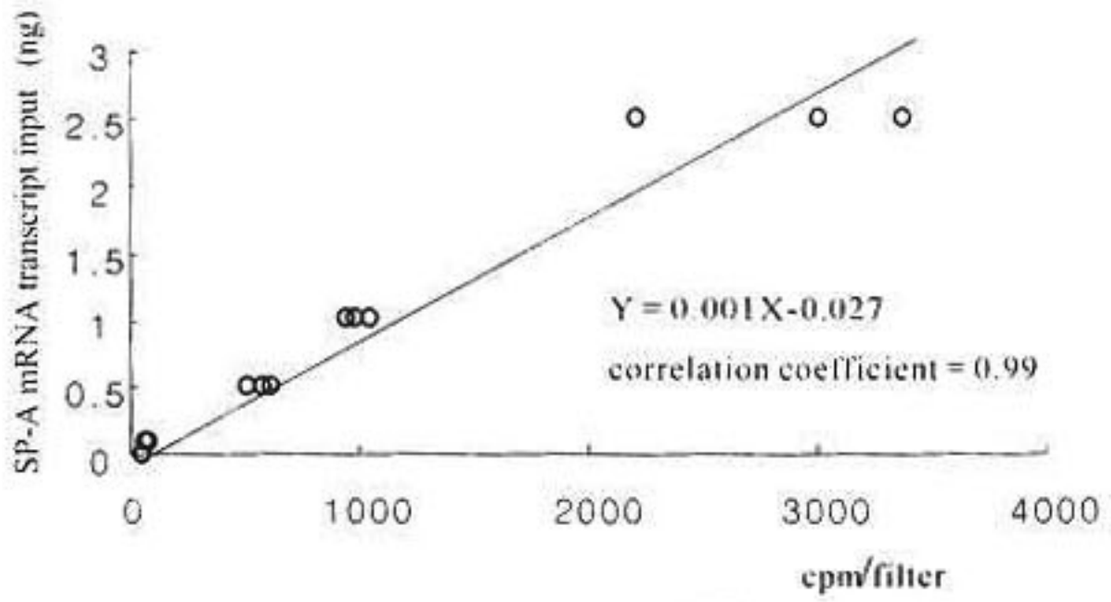

Figure 1. Standard curve for SP-A mRNA transcript input

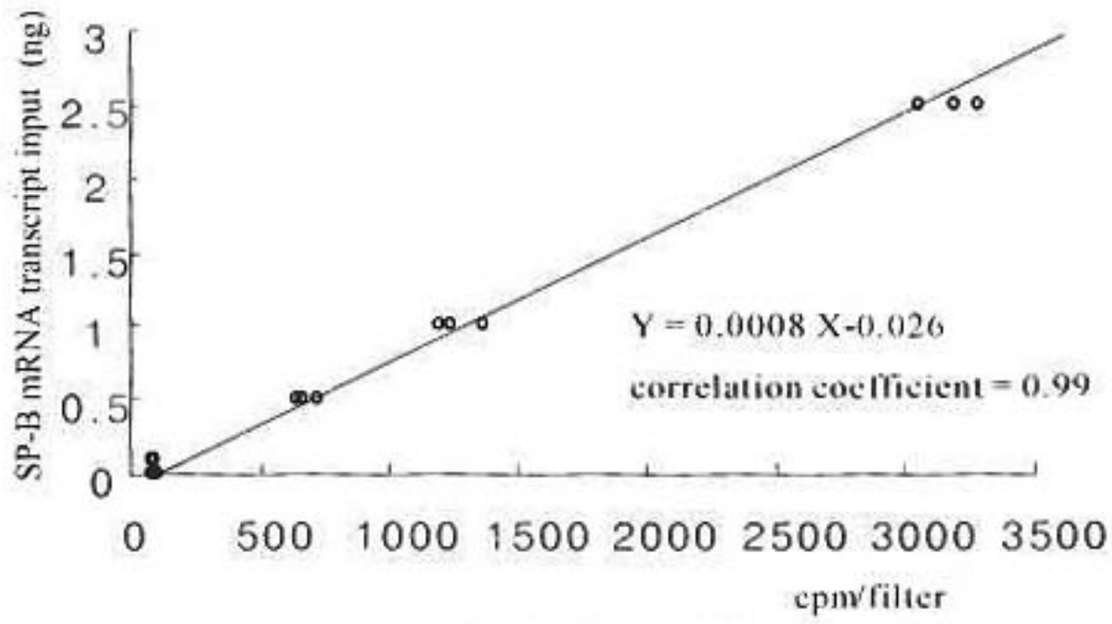

Figure 2. Standard curve for SP-B mRNA transcript input

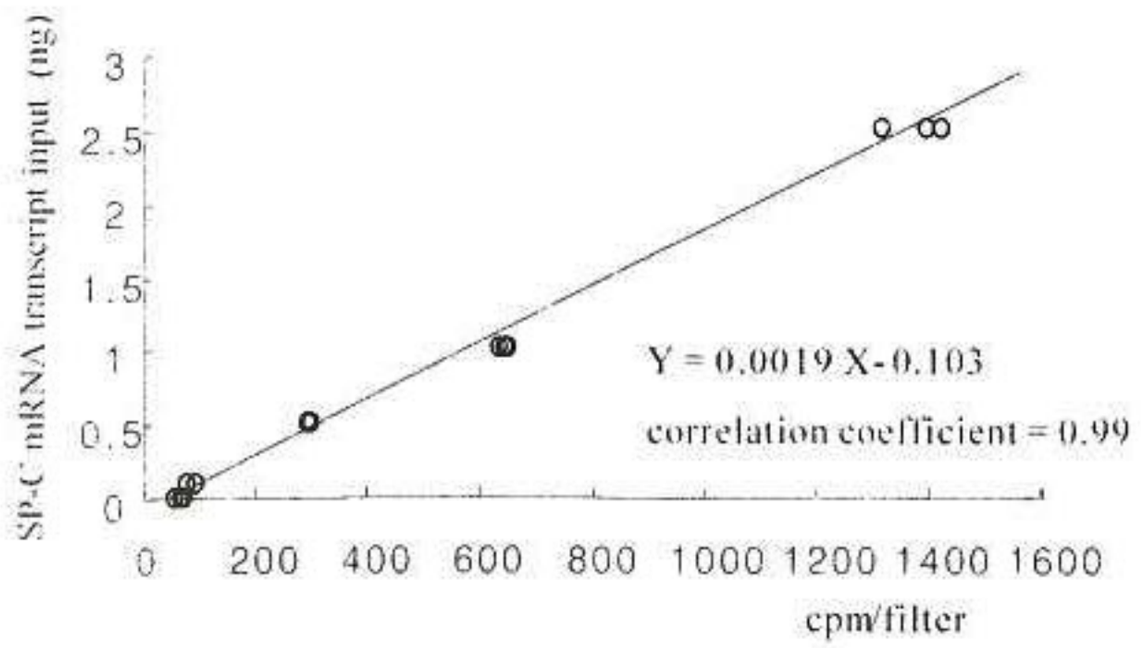

Figure 3. Standard curve for SP-C mRNA transcript input

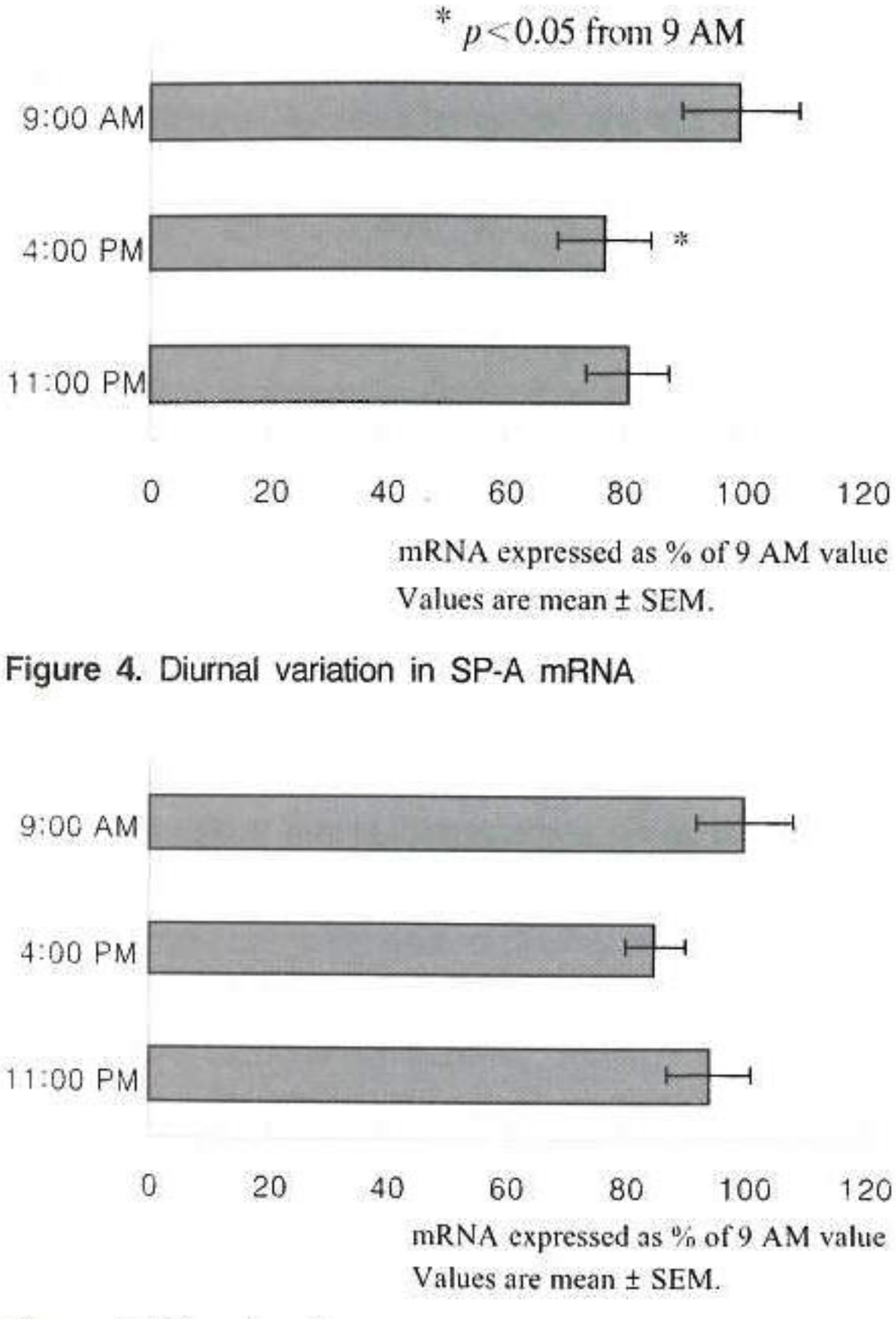

Figure 4. Diurnal variation in SP-A mRNA

Figure 5. Diurnal variation in SP-B mRNA

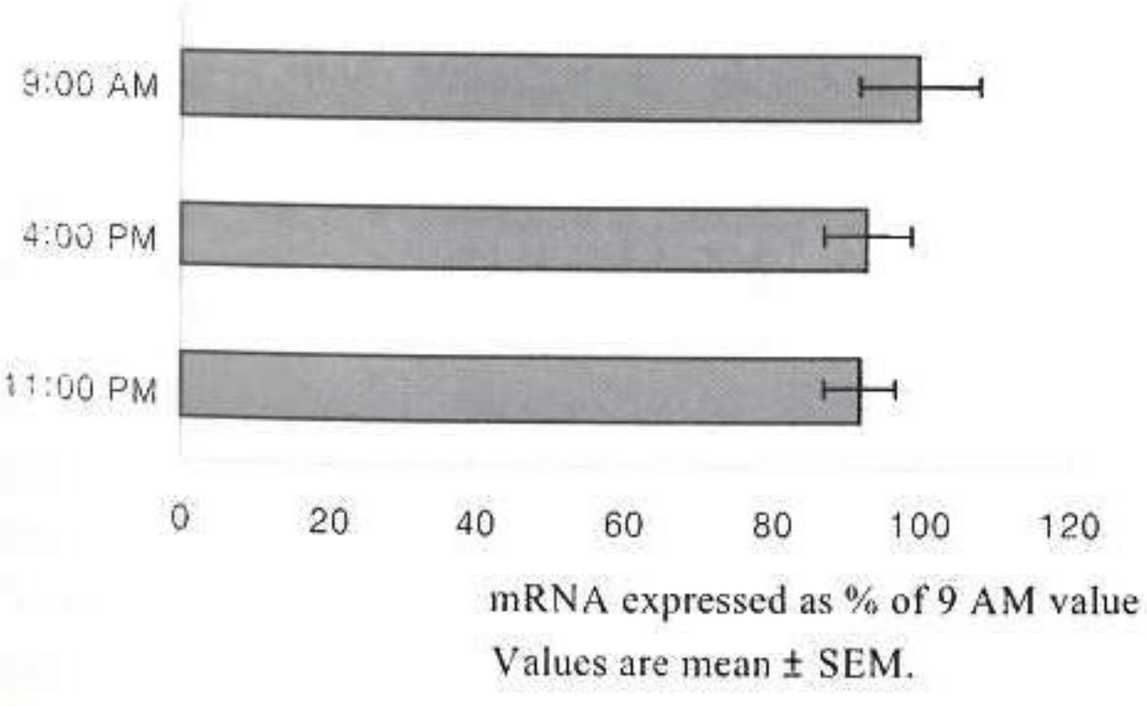

Figure 6. Diurnal variation in SP-C mRNA

The accumulation of β-actin mRNA was not significantly changed between normal control group and each group and it was uniform.

3. Measurement of SP-A content

SP-A protein content increased by 5.3% at 4 p.m. and 15.9% at 11 p.m. compared to the value at 9 a.m., which was not statistically significant (p=0.64, p=0.47) (Figure 7).

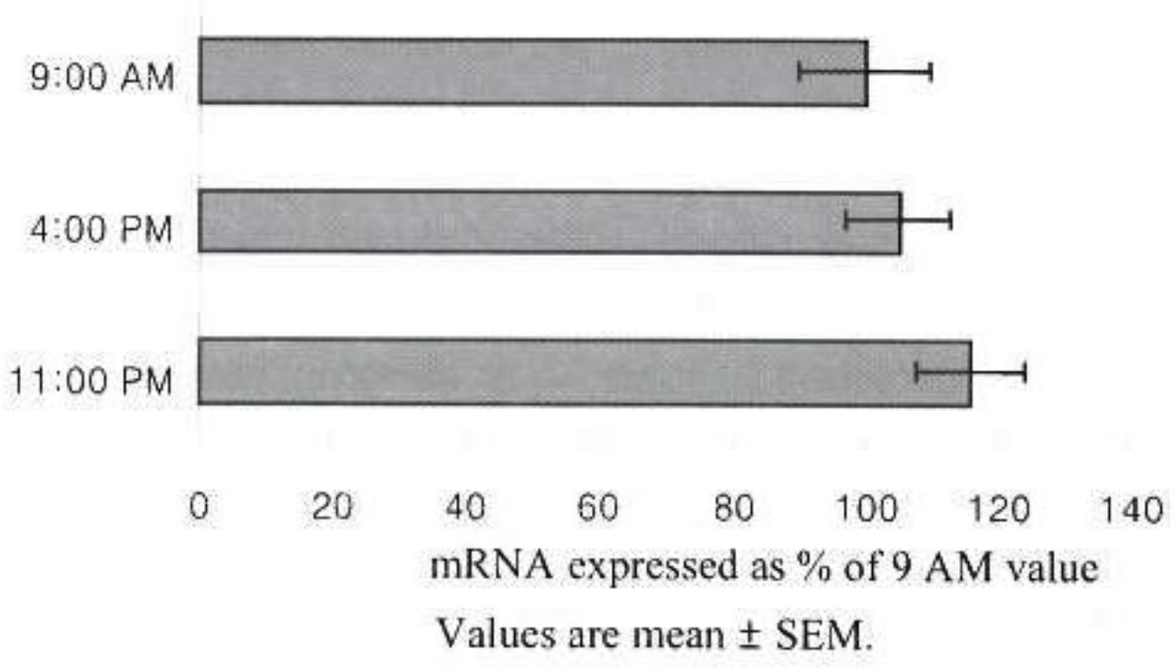

Figure 7. Diurnal variation in SP-A protein

DISCUSSION

The accumulation of normal mRNA for whole RNA was obtained by standard curve using sense transcript. Unlike this, the accumulation of β-actin mRNA was uniform in each group all the time. When cpm value forms a linear relationship with RNA loaded on each filter and hybridization is specific, each cpm per filter at 9 a.m., 4 p.m. and 11 p.m. may be compared to evaluate the relative quantity. The filter hybridization used in this study is more sensitive to small changes than Northern blot or slot blot with high reproducibility and handy usage. Each nitrocellulose filter used in this study can combine with 80 $\mu g/cm^2$ RNA and perform with lots of specimens at a time. But this nitrocellulose filter is so easy to break and fall that it requires careful handling[14].

The diurnal rhythm has an endogenous one made under the circumstances without environmental time signal factor. The organ in vivo that makes endogenous diurnal rhythm is called diurnal vibrator or mediator and a representative one is suprachiasmatic nucleus (SCN). It is proved with the facts that a diurnal rhythm is not observed if SCN is removed from rodents. Electrophysiological diurnal rhythm is expressed if SCN separated from the organism is cultured in a lab tube. If SCN of a mutant hamster living in the cycle of 22 hours is damaged and transplanted with SCN of a hamster living in the cycle of 24 hours under the abscence of diurnal rhythm, by which the mutant hamster comes to live in the cycle of 24 hours, not 22 hours[15-17].

When a diurnal vibrator is influenced by an external impulse and varied in cycle and phase of endogenous diurnal rhythm, it is called a synchrony. External factors which cause a synchrony are time signal factors, which are day and night cycles, social factors, food, surrounding temperature and rough activities. The diurnal indicator expressed later than the standard point is phase delay and the one expressed earlier is phase advance, of which phase delay and phase advance are called phase shift together[9, 15].

Surfactant lipid synthesis is produced on the pathway of cytidine diphosphate choline of the alveolar epithelial type II cell. Surfactant is saved in phospholipid bilayer within the lamellar body and packed to be released from the cell. Lamellar body is an intracellular organ storing surfactant within a cell and functions to secrete. After secreting from the cell, lamellar body forms tubular myelin storing phospholipid for alveolar monolayer. An unique lattice-shaped tubular myelin includes SP-A and SP-B[18]. Tubular myelin is considered as a precursor of phospholipid monolayer full of DPPC[1]. It exists as tubular myelin outside the cell and is discharged from air liquid interphase to monolayer. 90~95% surfactant is recycled, reproduced, refined and reassembled for resecretion. Alveolar lipid pool is secreted by 11~40% per hour[1]. Alveolar material is cleared by 7~15% per hour, which may be influenced by ventilation, phospholipid composition and ventilation rate[19]. If the ventilation rate is higher, secretion and clearance are stimulated to change surfactant pool. Surfactant lipid is absorbed by alveolar epithelial type II cell, of which absorption ratio is different by age. Phosphatidylcholine is reutilized by 90% in 3-day-old rabbits, but only 23% is reutilized in grown-up rabbits[20].

Surfactant protein indicates alveolar epithelial type II cell functions and occupies about 10% of cells in the lung[21]. SP-A of surfactant protein is glycoprotein and the multiple isoforms of monomeric SP-A have a molecular weight of 28 ~36 kD_a. It occupies 30~40% of whole surfactant protein[22] and SP-A mRNA is found in the bronchiolar cell, clara cells and pre-type II cells of the fetus during 19~20 weeks. SP-A is glycosylated in the endoplasmic reticulum. It concerns maintenance of pulmonary monolayer and associates with SP-B and SP-C to increase adsorption of DPPC to pulmonary monolayer and prevents its contamination[23]. SP-A is required for formation and stabilization of tubular myelin in the presence of calcium, while it contributes to formation and structure of another surfactant groups. SP-A facilitates adsorption of phospholipid from alveolar epithelial type II cell and regulates lipid turnover by controlling surfactant secretion from alveolar epithelial type II cell. SP-A plays an important role in secretion, synthetic and recirculation of surfactant. SP-B is a hydrophobic protein and the molecular weight under non-reducing conditions is 18 kD_a. It essentially contributes to the whole manufacturing process of SP-C and packaging of phospholipid. SP-B mRNA is found only in alveolar epithelial type II cells and clara cells[24]. If the biological stain test is performed at 26 weeks of pregnancy, it is found in alveolar epithelial type II cells and in bronchiolar epithelial cell (clara cells)[25]. SP-B is essentially involved in facilitating surfactant film formation by adsorbing and spreading surfactant phospholipid in air liquid interface during cycling[3]. SP-B associates with SP-A to help formation of tubular myelin and is essential for lamellar body formation and surfactant function. There is a report that congenital SP-B deficiency causes death[26, 27]. The progress of newly-formed SP-B and SP-C within cells goes through a similar route. Inactivation of SP-B gene disrupts the secretion route of surfactant lipids, thereby causing an abnormal manufacturing progress of SP-C[27]. Various types of contamination in the lung disrupt SP-B gene expression or SP-B homeostasis[28-30]. SP-C is a hydrophobic protein and the molecular weight under non-reducing conditions is 5~8 kD_a. Compared to SP-A and SP-B, it is very lipotropic. SP-C mRNA exisits only in alveolar epithelial type II cells and is found in the lung of a 13-week-old fetus. SP-C is found in the lamellar body and pulmonary alveoli. It is involved in adsorption of phospholipid at air liquid interface and pulmonary monolayer. SP-C stimulates absorption of phospholipid on the surface and changes the structure of phospholipid within film to calculate a small compact space[31], and recirculates in a similar route with DPPC as a lamellar body in the pulmonary alveoli. SP-B and SP-C cooperate with SP-A in facilitating film formation of surfactant to reduce surface tension of monolayer[32].

SP-A, SP-B and SP-C function to facilitate release and adsorption of DPPC to air liquid interface of pulmonary alveoli and stimulate release of phospholipid to monolayer in order to reduce surface tension and to control the surfactant homeostasis. SP-A, SP-B and SP-C help DPPC out of tubular myelin and another surfactant aggregates to be released fast from the pulmonary monolayer. Like this, surfactant protein plays a very important role in determining the physical properties of surfactant and in regulating metabolism. Surfactant phospholipid is similar in composition ratio, but different in protein composition. It is not certain how a different protein composition is related with surfactant metabolism.

In this study, SP-A mRNA significantly decreased by 23.5% at 4 p.m. compared to the value at 9 a.m. SP-B mRNA decreased by 15.1% at 4 p.m. and 5.7% at 11 p.m., respectively, compared to the value of 9 p.m. SP-C mRNA decreased by 6.8% at 4 p.m. and 7.7% at 11 p.m., respectively, compared to the value of 9 p.m. SP-A protein content increased by 5.3% at 4 p.m.

and 15.9% at 11 p.m. compared to the value at 9 p.m. These results indicated that the diurnal variation of significant gene expression is observed in hydrophilic surfactant protein rather than in hydrophobic surfactant proteins.

Surfactant lipids and proteins are synthesized in endoplasmic reticulum of type II cells to be transported to Golgi apparatus by an unknown mechanism. After transportation, carbohydrate substances are added to SP-A and lipids appear in the lamellar body. Protein is transported through a multivesicular body. Small lamellar body gets bigger with phospholipid. The volume change in alveolar epithelial type II cells is related with formation, accumulation and secretion of lamellar body. The accumulation of lamellar body is related with a resting phase of a rat, but its secretion with very active hours. Such a volume change of alveolar epithelial type II cells is related with diurnal variation of surfactant protein. Alveolar epithelial type II cell volume is maximal at 4 p.m. and minimal at 8 a.m[10, 11]. Multivesicular body volume increases from 4 a.m. to 8 a.m., while lamellar body volume increases from 4 p.m to 8 p.m. In this study, SP-A mRNA showed a significant decrease by 23.5% at 4 p.m. compared to the control value at 9 a.m. when the alveolar epithelial type II cell volume is minimal. Meanwhile, the alveolar epithelial type II cell volume is maximal at 4 p.m. and the lamellar body volume increases from 4 p.m to 8 p.m[11]. On the basis of these reports, it is thought that SP-A mRNA more secretes than being accumulated in the alveolar epithelial type II cell or lamellar body, thereby decreasing within cells. Also, the accumulation of SP-A mRNA significantly decreased by 23.5% at 4 p.m. compared to the value at 9 a.m. but SP-A protein content increased, which was influenced by several factors in post-translation. No reports are made about surfactant protein mRNA diurnal rhythm and it should be further studied how surfactant protein mRNA diurnal rhythm is changed by different factors like diseases and hormones.

From the above study, it is clear that diurnal rhythms of SP-A, SP-B and SP-C gene expression are each different and a significant diurnal rhythm change is noticed in hydrophilic protein SP-A rather than in hydrophobic protein SP-B and SP-C.

SUMMARY

In this study, we investigated SP-A, SP-B and SP-C gene expression by filter hybridization using rats sacrificed at 9 a.m, 4 p.m and 11 p.m. and also examined lung SP-A protein content by double sandwich ELISA assay. Thereby, we observed diurnal variation on gene expression of SP-A, SP-B and SP-C and determined surfactant A protein content as follows.

1. The accumulation of SP-A mRNA significantly decreased by 23.5% at 4 p.m. compared to the value at 9 a.m. ($p<0.05$).
2. The accumulation of SP-B mRNA decreased by 15.1% and 5.7% at 4 p.m. and 11 p.m, respectively, compared to the value at 9 a.m. ($p=0.07$, $p=0.69$).
3. The accumulation of SP-C mRNA decreased by 6.8% and 7.7% at 4 p.m. and 11 p.m, respectively, compared to the value at 9 a.m. ($p=0.38$, $p=0.57$).
4. Total SP-A protein content increased by 5.3% and 15.9% at 4 p.m. and 11 p.m., respectively, compared to the value at 9 a.m. ($p=0.64$, $p=0.47$).

The above result suggested a specific diurnal variation on gene expression of SP-A, SP-B and SP-C according to the internal timing system of rats and a significant change in diurnal rhythm of the gene expression is observed in hydrophilic protein SP-A rather than in hydrophobic proteins SP-B and SP-C in vivo.

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