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Characterization of pulmonary surfactant apoproteins in the diabetic mouse

Kenneth Dean McCarty

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CHARACTERIZATION OF PULMONARY SURFACTANT APOPROTEINS

IN THE DIABETIC MOUSE

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master in Science

in

Biology

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by

Kenneth Dean McCarty
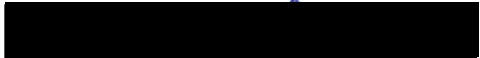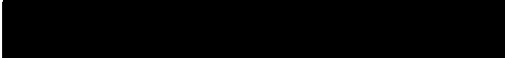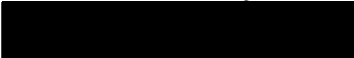
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Approved by:

Dr. Richard Fehn	11/15/89
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Abstract

Pulmonary surfactant proteins SP-A, B, and C were studied in mice exhibiting diabetes mellitus. Type I diabetes mellitus was produced via injection of streptozotocin (STZ) in C57 BL/KsJ db/m mice. Genetically diabetic C57 BL/KsJ db/db mice were used as a model for type II diabetes mellitus. The mice were also grouped based on age as immature and mature. Lung lavage was collected and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and immunostaining were performed to characterize the proteins. Endoglycosidase F and neuraminidase type X were used to assess the degree of protein glycosylation. The results confirmed the presence of SP-A in 36 and 43 kDa sizes, and identified SP-B/C as a 14 and 72 kDa protein in the mouse. Deglycosylation with endoglycosidase F and neuraminidase confirmed that SP-A had been glycated with mannose and sialic acid. No phenotypic difference in molecular weights for SP-A was found between type I diabetic and vehicle-treated mice, and type II diabetic and normal mice. However, the relative amount of SP-A was less in type II diabetic animals than in normal animals (0.70 ± 0.49 ug versus 1.74 ± 1.01 ug, $P \leq 0.05$). Age-related increases for the molecular weights of SP-A within the phenotypes and treatment groups were present and appeared to be caused by protein glycosylation.

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Chapter 1: Introduction

Much of the prior investigation regarding pulmonary surfactant has centered around factors which affect the biosynthesis of the lipid components, primarily dipalmitoyl phosphatidylcholine (DPPC), phosphatidylglycerol (PG), phosphatidylinositol (PI), and sphingomyelin (S) (1-11). However, more recent investigation has involved the study of apoproteins present as a small percentage of the total surface-active mixture, surfactant proteins A, B, and C (SP-A, SP-B, SP-C). These apoproteins are thought to aid extracellular type II pneumocyte lipid transport and increase the adsorption of the lipid portion of the pulmonary surfactant to the alveolar interface, thus forming a primary link between the alveolus and the surface-tension-reducing lipids.

SP-A, the quantitatively predominant protein, is present in a diversity of mammalian species (12), has a basic pH (12-16), and has a monomeric molecular weight (Mr) of between 26 and 36 kDa (12-14,17-20). Generally, this range of molecular weights is thought to be due to variable degrees of post-translational glycosylation which influences protein mobility during polyacrylamide gel electrophoresis (PAGE). Studies of glycosylation show neuraminidase- and endoglycosidase-sensitive forms which indicate the presence of sialic acid and mannose moieties on the rat, dog, and human protein core (13-18,21-25). Rat SP-A also contains disulfide bonds which appear to be required for biological activity (18,26,27). Synthesis of SP-A is dexamethasone- (28-30) and cAMP-inducible (22) in humans and rabbits, respectively, and has been identified in alveolar and bronchiolar cells (15,16,21,24,31-34). While it was originally thought that SP-A might function with the surfactant lipids to increase alveolar adsorption, recent findings reveal that this is not the case (35,36). Rather, SP-A acts with the divalent

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cation calcium to increase lipid aggregation as evidenced by increased liposome clustering (37,38). These results have led to the present hypothesis that the primary functions of SP-A are to act as a transport ligand to direct surfactant lipids to type II pneumocytes and to increase lipid uptake by type II pneumocytes (39). Regulation of surfactant turnover appears to be the main physiological role of SP-A.

While the lower molecular weight surfactant proteins, SP-B and SP-C, have been studied less extensively, more information has recently become available. SP-B in reduced forms appears as an 8 kDa monomer in the pig and a 16 kDa form in the rat (40). It also has multiple hydrophobic sequences and intrachain cysteine-containing disulfide bonds (41). SP-C is encoded on chromosome 8 in humans (42) and has a monomeric molecular weight of 5-6 kDa in the cow, rat, and human (32,43-45). It contains valine-, phenylalanine-(45), leucine-, and isoleucine-rich (46) hydrophobic amino acid sequences. Intracellularly, SP-C is contained in lamellar bodies within the alveolar cells (46). These proteins are believed to be responsible for enhancing lipoalveolar adsorption and reducing alveolar surface tension as evidenced by surface balance measurements (36,47-49). These effects have been reported to increase arterial oxygenation (P_aO_2), decrease pulmonary inflation pressures, and increase lung compliance (48). Since SP-B and SP-C appear to play an important role in alveolar patency, and thus in pulmonary physiologic function, greater research is now being undertaken to determine not only their functional characteristics but whether they play an etiologic role in various pathophysiologic conditions.

Researchers have begun to investigate the effect of metabolic disturbances such as diabetes mellitus on the surfactant proteins. When amniocentesis was

performed to deduce fetal pulmonary status in controlled maternal diabetes mellitus compared with nondiabetic patients, no significant difference was found (50). However, when the study was repeated for uncontrolled maternal diabetes, SP-A quantity was less in diabetic patients than in nondiabetic patients (51). Total amniotic SP concentration was also assayed for use as an indicator of pulmonary maturity, and reportedly correlates better than previously used indices such as lecithin/sphingomyelin (L/S) ratios (52). Clinically, diabetic patients have reduced arterial oxygenation (P_aO_2) and pulmonary diffusion capacities (D_LCO) when compared with nondiabetic patients. These results are consistent with alveolar collapse, and hence have been attributed to a possible functional alteration in surfactant (53), such as a deficiency and/or functional change in apoproteins. In addition, Alloxan-induced diabetic rats showed decreased surfactant proteins, which has been attributed to possible changes in lung protein or carbohydrate metabolism (40).

Uncontrolled type I and type II diabetes produce an absolute or relative lack of insulin, respectively, which leads to hyperglycemia. In addition, type I-associated hypoinsulinemia decreases amino acid transport into cells, reduces transcription and translation, and increases protein catabolism (54). Such alterations in protein and carbohydrate metabolism, when combined with the fact that surfactant proteins are glycosylated post-translationally, make it likely that diabetes may alter surfactant proteins via changes in biosynthesis and/or glycosylation.

While SP-A has been identified in mice (12,31,55) and has been found to have multiple isoforms with isoelectric points from 4.4-5.6 (12), studies to determine the basis for the heterogeneity of the protein in this species have not

been performed. Surfactant proteins B and C have not been identified nor characterized in the mouse.

The objectives of this study were to examine the surfactant proteins in terms of molecular weight and quantity in the presence of type I (insulin-dependent) and type II (noninsulin-dependent) diabetes mellitus. The C57 BL/KsJ db/db diabetic mouse, *Mus musculus*, serves as a model of type II (noninsulin-dependent diabetes mellitus) because it exhibits hyperglycemia, hyperinsulinemia, hyperphagia, and obesity (56). The model was used to assess the possibility of nonenzymatic glycation of surfactant proteins in the presence of insulin. Heterozygous C57 BL/KsJ db/m littermates which do not exhibit type II diabetes mellitus, were used as nondiabetic control animals. To assess the possibility of nonenzymatic glycation of surfactant proteins in the hypoinsulinemic and hyperglycemic mouse, heterozygous C57 BL/KsJ db/m mice were injected with streptozotocin (STZ) to selectively destroy pancreatic beta cells and reduce serum insulin levels. Therefore, the effect of hyperglycemia in the presence and absence of insulin on surfactant protein glycosylation and quantity was investigated using these two models. Different ages of mice were examined to permit varying periods of exposure to high levels of serum glucose to ascertain whether length of exposure to hyperglycemia influenced the degree of protein glycosylation.

Therefore, the purpose of this investigation was to confirm the presence of SP-A in the C57 BL/KsJ mouse, to ascertain whether SP-B and SP-C were present, to discern to what extent post-translational glycosylation of these proteins occurred, and to evaluate the effect of type I and type II diabetes on these surfactant proteins.

Chapter 2: Materials and Methods

Experimental Animals

Homozygous C57 BL/KsJ db/db mice were used as a model for type II diabetes mellitus while heterozygous (db/m) littermates served as nondiabetic control animals. Streptozotocin (STZ) was used to induce type I diabetes via intraperitoneal injection (0.008 ml/g, STZ/Citrate-NaCl 70 mg/ml, 0.05 M Citrate, 0.15 M NaCl, pH 4.5) following 24-hour fasting in eight-week-old non-diabetic C57 BL/KsJ db/m mice. Heterozygous C57 BL/KsJ db/m mice injected with vehicle (0.05 M Citrate, 0.15 M NaCl, pH 4.5) were used as control animals. Mice were further grouped as either immature (8-12 weeks of age), or mature (over 12 weeks of age) to represent early or stable long-term diabetes, respectively. Five immature and five mature animals were included for study in each of the following groups: untreated nondiabetic, vehicle-treated nondiabetic, untreated type II diabetic, and STZ-induced type I diabetic. Each group was housed at 23-27°C, 30-60% relative humidity, in 6 X 9 X 12-inch shoebox cages on hardwood chip bedding. Automatic lighting furnished 14 hours of light and 10 hours of dark per day. Food and water were provided *ad libitum*.

Lavage Procedure

Mice were anesthetized with Nembutal (50mg/ml, 60mg/Kg body weight, intraperitoneally) and their lungs lavaged with 1.0 ml of 0.9% NaCl. After confirming the anesthetic plane via extinction of the corneal reflex, a cervical dislocation was performed and an incision made in the abdominal wall just below the anterior rib cage and extended laterally until the diaphragm was visible. A diaphragmatic puncture was then performed to allow visualization of the lungs. An incision was made in the neck approximately overlying the thyroid cartilage,

and the musculature dissected away until the trachea was visible, after which a 1.0 mm tracheotomy was made and a 22-gauge X 1 inch Teflon angiocatheter (Becton Dickinson) inserted approximately 0.5 inch into the tracheal lumen. After ligating the angiocatheter in place, a 1.0 ml syringe containing 0.5 ml of 0.9% NaCl was attached to the angiocatheter and used to lavage the lungs. Each sample was injected into and withdrawn from the lungs two times, quantified for recovered volume, and stored in an ice bath. The lavage samples were then centrifuged at 1500 RPM (500 X g) for 15 minutes at 4°C in a Beckman Accuspin FR centrifuge to remove cells and debris. The resultant supernatants were transferred to 1.5 ml polypropylene microcentrifuge tubes for storage at -80°C.

Quantitation of Blood Glucose

Just prior to the lavage procedure, blood was obtained via cardiac puncture for the determination of serum glucose. The blood was obtained with an unheparinized 1 ml syringe and transferred to microcentrifuge tubes which were placed at 4°C for 1 hour to allow clotting. After coagulation, the blood was placed in a Beckman centrifuge and spun at 1500 RPM (500 x g) for 15 minutes to separate the serum and nonserum components. Serum was transferred to fresh microcentrifuge tubes and stored at -20°C. Serum glucose (in mg/dL) was determined by hexokinase colorimetric method (Sigma diagnostics).

Apoprotein Preparation and Analysis

Protein separation was performed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels (Laemmli) with Hoefer Scientific Instruments (HSI) Mighty Small SE-250 vertical slab gel units. A 100 ul sample of each lavage was evaporated to dryness using a Savant Speed-Vac (Model SVC100H) and resuspended in 15 ul of treatment buffer

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(0.125 M Tris, 4% SDS, 20% Glycerol, 10% 2-Mercaptoethanol, pH 6.8).

Molecular weight standards (Sigma diagnostics, #MW-SDS-70L) were added in a

gel lane during each run to allow interpolative calculation of the molecular weights

of the unknown samples, as follows: bovine serum albumin (BSA), 66 kDa;

ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic

anhydrase, 29 kDa; trypsinogen, 24.1 kDa; trypsin inhibitor, 20.1 kDa; alpha-

lactalbumin, 14.2 kDa; and a range of myoglobin fragments (Sigma diagnostics,

#MW-SDS-17) from 6.21 to 16.95 kDa (Myoglobin backbone, 16.95 kDa;

fragments I and II, 14.4 kDa; fragment I, 8.16 kDa; fragment II, 6.21 kDa).

Resuspended lavage samples were heated to 100°C for 3 minutes prior to

loading the gel. Ten microliter (10 ul) volumes were loaded into the wells using a

Pipetman micropipet, and a small volume of bromphenol blue (BPB) tracking dye

was added to each sample to aid visualization. Constant current gel

electrophoresis was performed (20 mA/gel) at 4°C on each gel in tank buffer

(0.025 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3) until the BPB tracking dye

front reached the bottom of the gel (approximately 1 hour/gel).

Protein identification was confirmed via staining with Coomassie Brilliant

Blue R-250 (0.125% in Methanol/Acetic Acid) for 30 minutes, followed by

destaining in 50% Methanol/10% Acetic Acid (Destain I) for one hour, and 5%

Methanol/7% Acetic Acid (Destain II) to term.

Specific identification of surfactant proteins was by Western blotting

following electrophoretic transfer of gel-separated proteins using an HSI TE-42

transfer apparatus. Samples which were electrophoresed into the Laemmli gel

matrix but not stained were electrophoretically transferred in 0.025 M Tris,

O.192 M Glycine, 20% Methanol, pH 8.3 to nitrocellulose (Micron Separations

Inc., Nitroplus 2000). Biotinylated molecular weight standards (phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa)(Biorad) were added to a gel lane during each run to allow calculation of molecular weights after Western blotting. The unstained gels were layered in the transfer apparatus after uniform soaking of blotter paper, nitrocellulose, gels, and sponges in transfer buffer. This apparatus was then placed in the transfer chamber with the nitrocellulose closest to the cathode, the transfer chamber filled with buffer, and electrophoresed at a constant 250 V at $4^{\circ}C$ for 2 hours. After the transfer, the nitrocellulose was removed from the apparatus and transferred to self-sealing plastic containers for the immunostain procedure. The proteins were then labeled with either rabbit-anti-human SP-A or rabbit-anti-bovine SP-B/C polyclonal antiserum (provided by Dr. William Taeusch, Dr. Alan Waring, and Dr. B. R. Fan, King/Drew Medical Center, Los Angeles, Ca.).

Immunostaining of the proteins transferred to the nitrocellulose took place in the following manner. In the case of the SP-A blots, the nitrocellulose was submersed in BLOTTO (5% w:v Carnation nonfat dry milk in tris buffered saline {TBS}) at $37^{\circ}C$ with shaking for 2 hours to block nonspecific protein binding. After discarding the old BLOTTO, polyclonal rabbit antibody to SP-A was added to the nitrocellulose (1:1000 in BLOTTO; 50 ul:50 ml) and incubated at room temperature with shaking overnight (12-18 hours). After discarding the BLOTTO/surfactant-antibody mix, the nitrocellulose was rinsed with fresh BLOTTO three times at $37^{\circ}C$ with constant shaking, 10 minutes per rinse. Biotinylated goat-anti-rabbit IgG (Sigma)(1:10,000 in BLOTTO; 5 ul:50 ml) was added and incubated for 2 hours at $37^{\circ}C$ with shaking, after which the

nitrocellulose was rinsed three times with TBS for ten minutes each at 37°C with shaking. A 1:1000 (50 ul:50 ml) mix of Avidin horseradish peroxidase (AvHRP; Biorad) in TBS was next added and incubated for 2 hours at 37°C with shaking, after which the blot was rinsed three times with TBS over 10 minutes at room temperature. After discarding the TBS rinse, 60 mg of 4-Chloro-1-Napthol in 20 ml Methanol and 60 ul of 30% H2O2 in 100 ml of TBS were mixed and added to the blot at room temperature and allowed to colorize. The immunostain procedure for the SP-B/C blots was essentially identical to the SP-A blots with two modifications. The nitrocellulose was initially blocked with a 1% preparation of gelatin/TBS, and the polyclonal rabbit anti-bovine SP-B/C antibody was incubated at 37°C with shaking in 1:1000 (50 ul:50 ml) 1% gelatin/TBS. Positive identification and location of the surfactant apoproteins on nitrocellulose resulted in blue-colored bands indicative of antibody/protein binding. These bands were compared to simultaneously run CBB-stained gels for identification, analytical determinations, and positional referencing within the total banding pattern.

To ascertain the extent of surfactant protein glycosylation, additional gel electrophoretic runs were performed on lavage samples following the addition of neuraminidase type X (Sigma diagnostics, #N-2133) and endoglycosidase F (Sigma diagnostics, #E-1262) to remove the sialic acid and mannose groups from the protein core.

Neuraminidase (0.3 units/ml) in 0.2M Tris-Cl, 0.1% Nonidet P-40, pH 7.4 was added to the untreated lavage sample (10- 50 ug total protein) and incubated at 37°C with constant shaking for 18 hours prior to SDS-PAGE and Western blotting. Before treatment with Endoglycosidase F (0.2 units/assay), the untreated lavage sample was added to 50 ul solubilization buffer (100 mM NaH2PO4, 50

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mM EDTA, 1.0% SDS, pH 6.1), and heated at 95°C for 3 minutes. Three microliters of Endoglycosidase F in 20 mM Potassium Phosphate, 50 mM EDTA, Sodium Azide, pH 7.2, and 10 ul of 10% Triton X-100 were then added to the sample before incubating 4 hours at 37°C with shaking. SDS-PAGE and Western blotting of the samples were then performed as previously described. Deglycosylated samples were then compared to original samples for determinations of molecular weights.

Stained gels and immunoblots were assessed for optical density and position of each band using a BioRad 620 Video densitometer. Protein banding in sample lanes and molecular weight standard lanes was evaluated positionally via the calculation of relative mobility (Rm). A plot of Rm values on the abscissa versus natural log (ln) molecular weight on the ordinate was performed with a line of least squares drawn. Subsequent Rm values were then calculated for surfactant protein bands and compared against the line of least squares of ln molecular weight standards for interpolation of molecular weight. Densitometric measurement of total optical density per lane was used, in combination with the Coomassie Brilliant Blue method total protein assay (Spectrum, #132920), to quantitate total protein concentration in lavage and relative protein concentrations of surfactant proteins.

Data Analysis

Statistical evaluation of data was by analysis of variance and the Student-Newman-Keuls range test (57). Regression analysis was performed using Lotus 1-2-3 (Lotus Corporation).

Chapter 3: Results

Serum Glucose

Values of serum glucose for untreated normal and diabetic mice are listed in table I. Immature and mature normal mice had significantly lower levels of glucose than their type II diabetic counterparts (191.5 ± 88.7 mg/dl versus 486.0 ± 184.1 mg/dl; $P \leq 0.05$). Within each phenotype, there were no significant differences in blood glucose between age groups. Blood glucose comparisons for the type I diabetic (STZ-treated) and vehicle-treated groups showed no significant differences (Table II).

Characterization of Pulmonary Surfactant Apoproteins

SDS-PAGE (Figure 1) followed by Western blot and immunostaining (Figure 2) of mouse lung lavage with polyclonal antibody to SP-A characteristically revealed four bands (B_1-B_4) having molecular weights of approximately 91, 77, 45, and 35 kDa, respectively. When mouse serum was subjected to the same procedures, two bands comigrated and were visualized, corresponding to bands B_1 and B_2 of mouse lung lavage (Figure 3).

Blotting and immunostaining of normal mouse lung lavage with polyclonal antibody to SP-B/C revealed two major bands with molecular weights of approximately 14 and 72 kDa (Figure 4) with no evidence of cross reactivity to mouse serum. A more complete characterization of SP-B/C was inconclusive due to inconsistent immunostaining results.

Phenotypic comparison of SP-A bands B_1 through B_4 showed no significant molecular weight differences between normal and type II diabetic subjects (Table III). However, age-related comparisons within each phenotype revealed a significant increase in the molecular weights for B_3 and B_4 when comparing

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Table I. Serum Glucose in Type II Diabetic Mice versus Normal Mice

	Serum Glucose (mg/dl)	
Normal (Immature)	159.2 $\pm$ (5)	33.2 [a]
Normal (Mature)	223.8 $\pm$ (5)	118.2 [a]
Diabetic (Immature)	585.6 $\pm$ (5)	197.0 [b]
Diabetic (Mature)	386.4 $\pm$ (5)	112.4 [b]
Normal (Immature and Mature)	191.5 $\pm$ (10)	88.7 [a]
Diabetic (Immature and Mature)	486.0 $\pm$ (10)	184.1 [b]

Values are Mean $\pm$ S.D.

Treatment groups having different superscripts are statistically distinguishable at $P \leq 0.05$

Number in parentheses denotes number of samples in group

Table II. Serum Glucose in Streptozotocin (STZ)-Induced Diabetic Mice versus Vehicle-Injected Mice

	Serum Glucose (mg/dl)	n
STZ	135.6 $\pm$ 58.4 [a]	10
Vehicle	219.6 $\pm$ 140.9 [a]	9

Values are Mean $\pm$ S.D.
n denotes number of samples in group
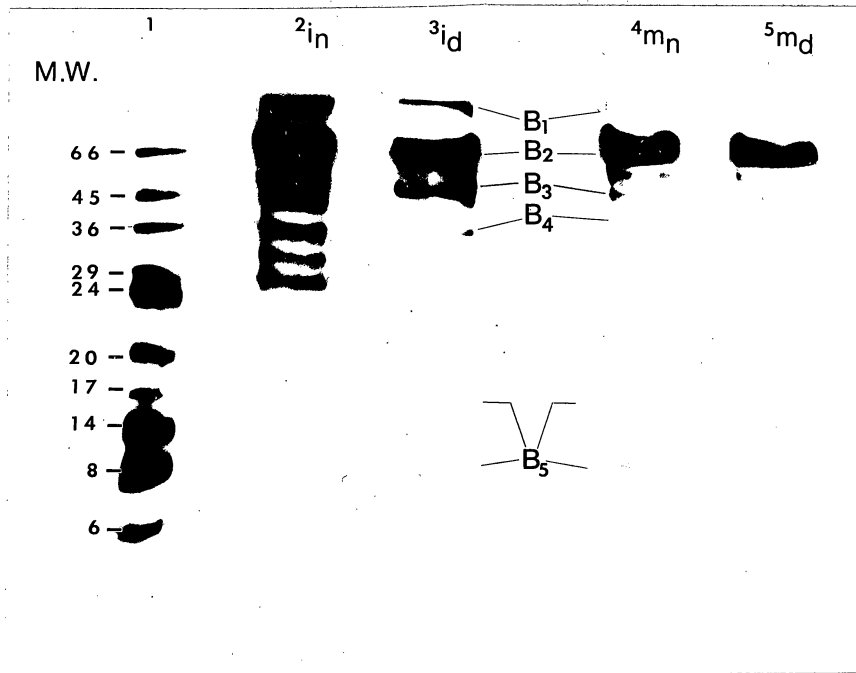
Figure 1. Coomasssie Brilliant Blue Gels - Bands 1 through 4 in Type II Diabetic versus Normal Mice. Coomassie Brilliant Blue-stained gels of lung lavage representing immature and mature type II diabetic mice and normal mice. Bands B_1 through B_4 are labeled corresponding to SP-A, with B_5 corresponding to SP-B/C. Molecular weights standards: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24.1 kDa; trypsin inhibitor, 20.1 kDa; myglobin backbone, 16.95 kDa; myoglobin fragments I and II, 14.4 kDa; alphalactalbumin, 14.2 kDa; myglobin fragment I, 8.2 kDa; and myoglobin fragment II, 6.2 kDa (lane 1). Immature normal mouse (i_n)(lane 2), immature diabetic mouse (i_d)(lane 3), mature normal mouse (m_n)(lane 4), and mature diabetic mouse (m_d)(lane 5) are shown.

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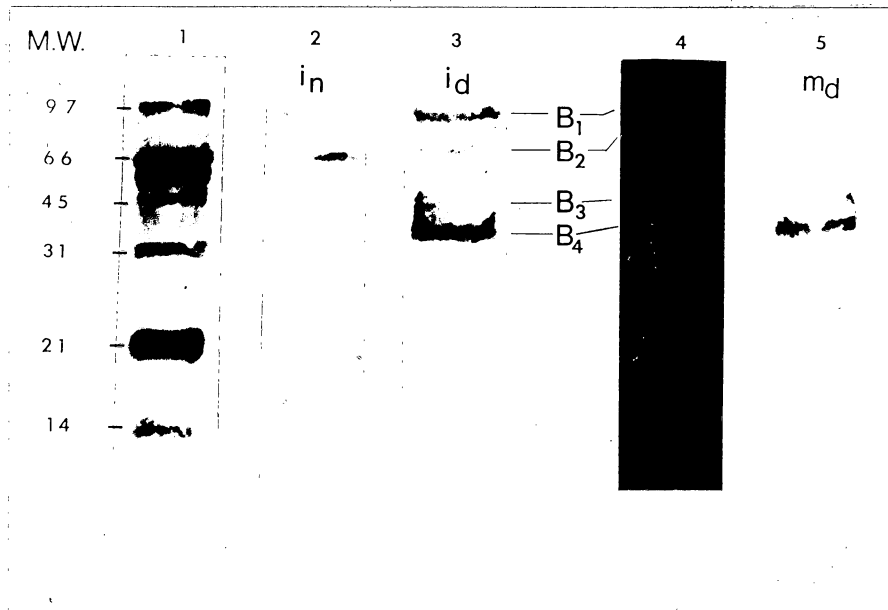
Figure 2. Immunostain of SP-A - Type II Diabetic versus Normal Mice.
Immunostain of bands B_1 through B_4 representing SP-A in immature and mature
type II diabetic mice and normal mice. Biotinylated molecular weight standards:
(phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa;
carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme,
14.4 kDa)(lane 1). Immature normal mouse (i_n)(lane 2), immature diabetic
mouse (i_d)(lane 3), mature normal mouse (m_n)(lane 4), and mature diabetic
mouse (m_d)(lane 5) are shown.

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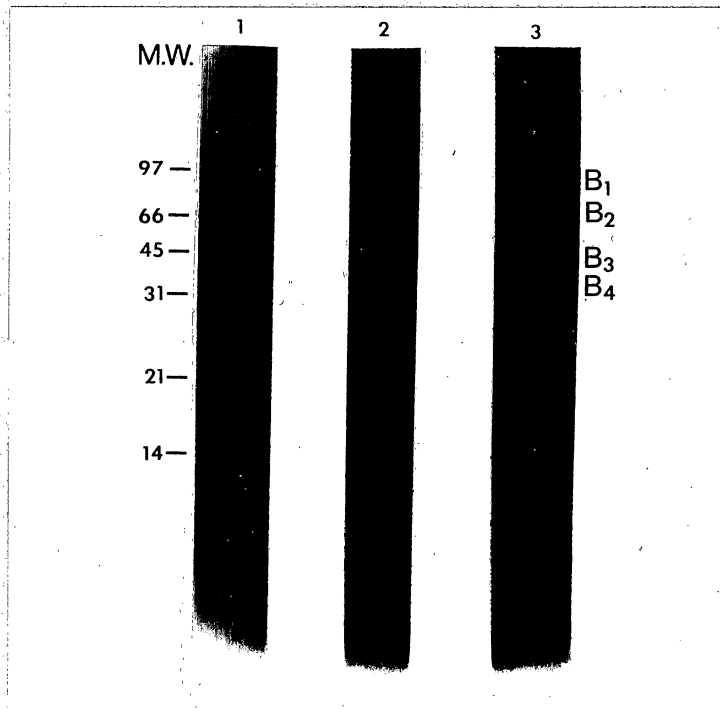
Figure 3. Immunostain of SP-A - Mouse Serum versus Lung Lavage. SP-A immunostain comparison of cross-reactivity between mouse serum and lung lavage. Biotinylated molecular weight standards (lane 1) (phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa). Bands B_1 and B_2 representing anti-SP-A immunostained mouse serum appear in lane 2. Bands B_1 (91 kDa), B_2 (77 kDa), B_3 (45 kDa), and B_4 (35 kDa) from mouse lung lavage immunostained with anti-SP-A antibody are shown in lane 3.
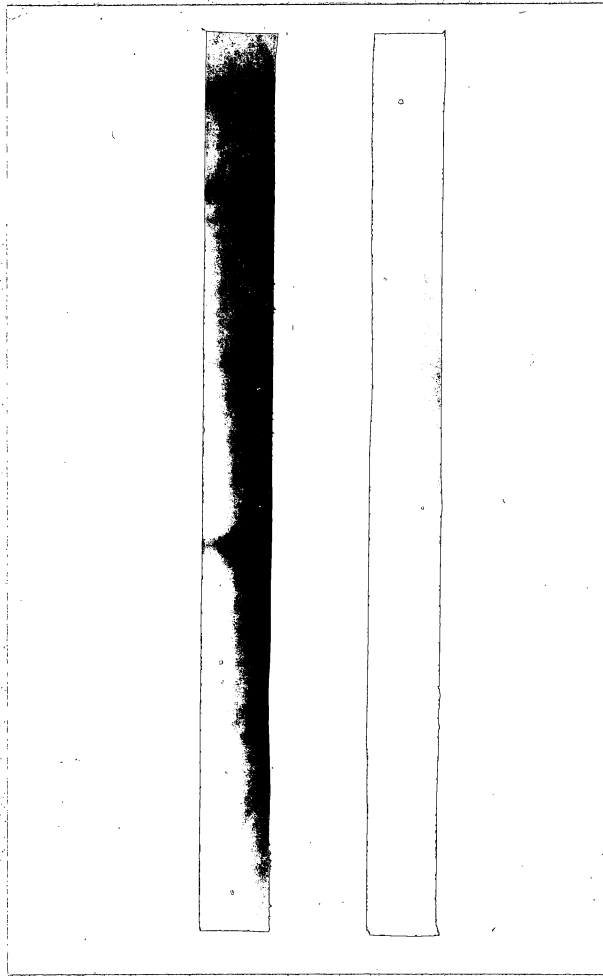
Figure 4. Immunostain of Mouse SP-B/C. Immunostain of bands representing SP-B/C in the C57 BL/KsJ db/m mouse. Biotinylated molecular weight standards (lane 1)(phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa). SP-B/C is represented by 14 kDa and 72 kDa bands in lane 2 whose position is highlighted by the asterisks.

Table III. SP-A Molecular Weight Comparison (kDa) in Type II Diabetic Mice versus Normal Mice

<div align="center"><u>Band</u></div>

Immature Group	B_1	B_2	B_3	B_4
Normal	85.6 [a] $\pm$ 3.32 (5)	70.0 [b] $\pm$ 4.29 (5)	42.6 d $\pm$ 2.21 (5)	34.6 [f] $\pm$ 1.9 (5)
Diabetic	92.3 [a] $\pm$ 6.42 (5)	74.5 [b] $\pm$ 7.21 (5)	43.0 d $\pm$ 2.05 (5)	36.0 [f] $\pm$ 1.31 (5)
Mature Group				
Normal	92.3 [a] $\pm$ 6.93 (4)	79.3 c $\pm$ 1.55 (5)	54.9 e $\pm$ 0.86 (5)	46.2 g $\pm$ 1.42 (5)
Diabetic	95.3 [a] $\pm$ 5.59 (5)	83.0 c $\pm$ 2.62 (5)	55.4 e $\pm$ 1.41 (5)	46.7 g $\pm$ 0.55 (5)

Values reported are Mean $\pm$ S.D.

Number of samples in group in parentheses

Values having different superscripts are statistically distinguishable at $P \leq 0.05$

immature with mature mice (Table III). Values for B_3 in immature mice were 42.6 $\pm$ 2.21 kDa in normal animals, and 43.0 $\pm$ 2.05 kDa in diabetic mice as compared to 54.9 $\pm$ 0.86 kDa and 55.4 $\pm$ 1.41 kDa, respectively, for their mature counterparts ($P \leq 0.05$). B_4 values in immature mice were 34.6 $\pm$ 1.9 kDa in nondiabetic, and 36.0 $\pm$ 1.31 kDa for diabetic mice, versus 46.2 $\pm$ 1.42 kDa and 46.7 $\pm$ 0.55 kDa, respectively, for mature mice ($P \leq 0.05$).

Glycosidase cleavage resulted in the reduction of the molecular weights of SP-A bands which were stained positively by the anti-SP-A antibody (Figure 5). The addition of neuraminidase type X reduced the molecular weight of B_3 and B_4 by approximately 2 kDa. Comparison of deglycosylation, using endoglycosidase F, between normal and type II diabetic mice showed a reduction in molecular weight from 46.1 to 44.2 kDa in normal subjects as compared to 48.5 to 44.2 kDa for diabetic mice.

Protein assays for total lavage, absolute SP-A quantity, and relative SP-A quantity for type II diabetic versus normal mice are listed in table IV. Total lavage protein was less in diabetic than in normal animals (10.17 $\pm$ 6.27 ug versus 16.17 $\pm$ 8.42 ug, $P \leq 0.05$), as was the absolute amount of SP-A (0.70 $\pm$ 0.49 ug versus 1.74 $\pm$ 1.01 ug, $P \leq 0.05$) and the relative amount of SP-A (10.38 $\pm$ 3.49 % compared to 19.38 $\pm$ 10.64 %, $P \leq 0.05$). While differences in percent SP-A were not suppported statistically between type I diabetic and vehicle-treated groups (immature: type I 18.1 % versus vehicle 21.1 %, and mature: type I 11.8 % versus vehicle 11.0 %), the mature subjects of each group appeared to have lower relative amounts of SP-A than their younger counterparts.

Within-group serum glucose levels were inconsistent in the streptozotocin- and vehicle-treated subjects for determination of type I diabetes (Table II). While

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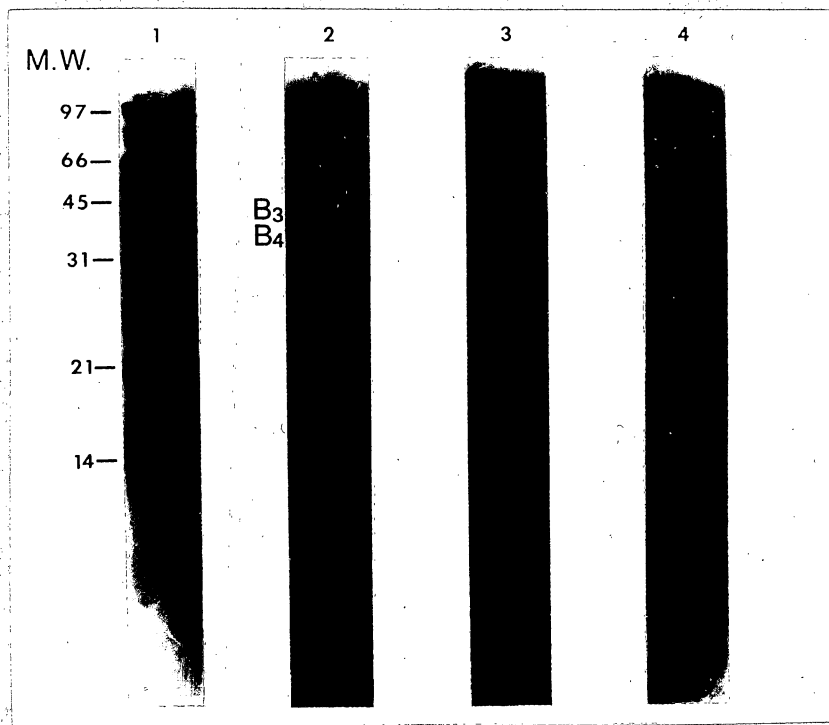
Figure 5. Post-glycosidase Immunostain of Mouse SP-A. Biotinylated molecular weight standards (lane 1)(phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa). Untreated mouse lung lavage (B_3 45 kDa and B_4 37 kDa)(lane 2). Mouse lung lavage treated with neuraminidse type X (lane 3)(B_3 43 kDa and B_4 35 kDa). Mouse lung lavage treated with endoglycosidase F (lane 4)(B_3 42 kDa and B_4 35 kDa).

Table IV. Quantitation of Pulmonary Lavage Protein (SP-A) in Type II Diabetic and Normal Mice

	Group	
Total Protein	Normal	16.17 ± 8.42 ug* (6)
	Diabetic	10.17 ± 6.27 ug* (6)
SP-A Absolute	Normal	1.74 ± 1.01 ug* (6)
	Diabetic	0.70 ± 0.49 ug* (6)
Percent SP-A	Normal	19.38 ± 10.64 %* (6)
	Diabetic	10.38 ± 3.49 %* (6)

* Indicates significant difference ($P \leq 0.05$) between normal and diabetic group

Values are Mean ± S.D.

Number of samples in parentheses

no difference was found between streptozotocin- and vehicle-treated mice in terms of the molecular weights of bands 1 through 4, an age-related increase was exhibited between bands 3 and 4 within each group (Table V). Values for B_3 in immature vehicle-treated mice were 40.1 ± 2.66 kDa as compared to 47.2 ± 2.39 kDa ($P \leq 0.05$) for their mature counterparts, while values for B_3 in immature streptozotocin-treated mice were 43.6 ± 5.63 kDa versus 50.8 ± 3.10 kDa ($P \leq 0.05$) for the mature mice. B_4 molecular weights for immature vehicle- and streptozotocin-treated groups were 35.2 ± 1.87 kDa and 35.4 ± 2.83 kDa, respectively, versus 39.6 ± 2.62 kDa and 41.7 ± 3.34 kDa for the mature subjects ($P \leq 0.05$). Correlation coefficients for molecular weight versus serum glucose showed no correlation between these parameters ($r = 0.07$ B_1; $r = 0.16$ B_2; $r = 0.44$ B_3; $r = 0.50$ B_4).

Table V. SP-A Molecular Weight Comparison (kDa) in Type I Diabetic Mice versus Vehicle-Treated Mice

	Band			
Immature Group	B_1	B_2	B_3	B_4
Vehicle	74.6 [a] $\pm$ 4.92 (5)	61.1 [b] $\pm$ 3.63 (5)	40.1 [c] $\pm$ 2.66 (5)	35.2 [e] $\pm$ 1.87 (5)
Type I Diabetic	75.6 [a] $\pm$ 8.12 (5)	63.3 [b] $\pm$ 4.80 (5)	43.6 [c] $\pm$ 5.63 (5)	35.4 [e] $\pm$ 2.83 (5)
Mature Group				
Vehicle	75.4 [a] $\pm$ 11.92 (4)	63.1 [b] $\pm$ 7.09 (4)	47.2 [d] $\pm$ 2.39 (4)	39.6 [f] $\pm$ 2.62 (4)
Type I Diabetic	77.3 [a] $\pm$ 5.82 (5)	65.4 [b] $\pm$ 4.14 (5)	50.8 [d] $\pm$ 3.10 (5)	41.7 [f] $\pm$ 3.34 (5)

Values reported are Mean $\pm$ S.D.

Number of samples in parentheses

Values with different superscripts are statistically distinguishable at $P \leq 0.05$

Chapter 4: Discussion

Other investigators have reported a group of proteins from 60 to 65 kDa in mouse lung lavage which are labeled by immunoblotting with anti-SP-A antibody (12). The results of this study comparing the immunostain of mouse serum and lung lavage with polyclonal anti-SP-A antibody show cross-reactivity of mouse serum with this antibody at similar molecular weights corresponding to bands 1 and 2. In addition, post-glycosidase molecular weights of B_1 and B_2 reported in this study correlate with molecular weights reported for serum albumin in other investigations (58). This indicates possible serum contamination of the original SP-A used as an antigen when the SP-A anitbody was prepared, or comigration of serum and lavage proteins of similar molecular weights which are not resolved by one-dimensional PAGE. Thus, the resulting bands appearing as B_1 and B_2 on anti-SP-A blots likely represent serum contaminants of the lung lavage. If so, these are most likely present as a result of the surgical procedure used to obtain the lung lavage, or due to transudation of fluid across the alveolar capillary membrane from pulmonary capillaries during the lavage. If these higher molecular weight bands are SP-A, then they may represent a dimeric form of the protein or a form in various stages of post-translational modification.

While B_1 and B_2 may represent serum proteins, the absence of cross-reactivity between mouse serum and bands 3 and 4 indicate that these are not serum contaminants. Indeed, the molecular weights of 35 kDa and 43 kDa for immature normal and diabetic mice reported here for these bands substantiate findings by other investigators regarding the molecular weight of SP-A (12).

The results of this investigation, showing a reduction in molecular weight of SP-A using neuraminidase type X and endoglycosidase F, support studies of other species involving SP-A glycosylation in which sialic acid and high mannose forms

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were cleaved from the protein core via treatment with these glycosidases (13-18,21-25). Nonenyzmatic glycation of proteins has been reported in diabetes (59,60) and is reported to be possible in any protein with a free-reacting lysine or valine in the presence of glucose (59). This effect has been found to alter protein function (61) and possibly play a role in various complications of diabetes through deactivation of enzymes, decreased proteolysis, and alteration of regulatory molecules (60,62). While no difference between phenotypes was observed in this study regarding the molecular weights of SP-A, a significant increase in molecular weight of SP-A was evident between immature and mature subjects within each phenotype. Since treatment with endoglycosidase F reduced these heterogeneous weights to a common value of approximately 44.2 kDa, glycosylation of the protein core might be the cause for this age-related alteration. However, since there was no phenotypic difference between normoglycemic and hyperglycemic animals, it seems likely that the glycosylation is independent of intravascular serum glucose levels and involves intracellular processing and glycosylation consistent with Golgi body function. Although studies to date have not been performed to deduce the amino acid sequence for mouse SP-A to determine whether the free-reacting amino acids valine or lysine are present, bovine, canine, and human surfactant proteins have been found to contain valine (45). Considering the importance of these proteins, phylogenetic conservation might suggest that mouse surfactant proteins also be expected to contain valine.

It has been reported that nonenzymatic glycosylation of serum albumin occurs at a higher level in diabetics than in nondiabetic individuals (58), and that its measurement is an index for short-term diabetic control. Since bands 1 and 2 of the immunoblots may in part represent serum albumin, a comparison of these

bands between diabetic and normal mice may represent the level of the diabetic state. Although the differences listed in table 3 for bands 1 and 2 are not supported statistically, the diabetic subjects appear to have a higher molecular weight for these bands than normal mice. Further investigation using a larger sample size might validate this finding and, if so, may serve as an additional index of diabetic state. Results of deglycosylation with endoglycosidase F confirm that both groups have the same relative mobility, indicating that they are modified by mannose post-translationally and likely contain a homologous protein core.

Published reports have established a decrease in surfactant protein in type I diabetic rats compared with control subjects (40). While that study did not specify whether the reduction was in SP-A, B, or C, due to the fact that it was published prior to standardization of the nomenclature, their reference was to a protein of molecular weight 16 kDa which most likely refers to either a dimeric form of SP-B or a trimeric form of SP-C. The decreased amounts of absolute SP-A and SP-A relative to total lavage protein in diabetic versus normal mice in this study justify these investigators' hypothesis that diabetes may alter pulmonary protein metabolism, and provides new information regarding SP-A in this model.

The function of SP-A appears to be the regulation of lipid transport to and from type II pneumocytes (39). Since the function of the lipid components of surfactant is known to be responsible in part for reducing surface tension and preventing alveolar collapse, a decrease in this protein might help explain reported clinical abnormalities observed in diabetic patients such as lower P_aO_2 and reduced diffusion capacity (53) caused by altered lipid transport and turnover.

The serum glucose assay for mice treated with streptozotocin and vehicle was inconsistent as an indicator of type I diabetes. However, using serum albumin

as an indicator of diabetic state, the increased molecular weights of bands 1 and 2 of STZ-treated versus vehicle-treated mice may indicate that type I diabetes was present and that the method used to determine serum glucose was not able to confirm the presence of the induced disease. The similar results obtained for the molecular weight characterizations of B_1 through B_4 in type I and type II diabetes may indicate that the increase in molecular weight of SP-A within each phenotype was a result of protein glycation. Alternatively, the analysis of serum insulin would have allowed positive confirmation of type I diabetes; however, since this was not done it was not possible to confirm or deny the presence of insulin-dependent diabetes mellitus.

The results of SDS-PAGE and immunoblotting for surfactant proteins B/C showed that this antibody does recognize a protein or proteins in mouse lung lavage presumed to be these surfactant proteins. However, since this antibody was not specific for one protein versus the other, an absolute differentiation between the bands identified and the proteins was not possible. The molecular weight of 14 kDa for the lower protein lies close to values for SP-B and dimeric SP-C reported in other species (32,40,43-45). Therefore, this band may represent an altered form of either protein B or C. The larger (72 kDa) molecular weight band identifed may also represent an oligomeric form of either SP-B or SP-C.

The difficulty in this study in isolating and characterizing mouse SP-B and SP-C versus SP-A may be accounted for by a variety of causes. Other investigators have reported that SP-B and SP-C occur in very small quantites in lung lavage, and this study indirectly confirms these reports in that identification of these proteins required the concentration of entire lavage volumes for immunostain resolution. For this reason, larger volumes of lung lavage may be necessary to further identify

these proteins in this species. Additionally, the use of BLOTTO as a primary blocking agent for the nitrocellulose and as a carrier for the anti-SP-B/C antibody appeared to interfere with the immunoblot procedure, as its use consistently resulted in a lack of banding. This may have been due to cross-reactivity between the rabbit-anti-bovine SP-B/C antibody and the Bovine Lacto Transfer Technique Optimizer (BLOTTO), in which the antibody was bound in the milk preparation and prevented from reacting with the nitrocellulose-complexed surfactant proteins. The use of a 1% gelatin/TBS mixture as a replacement for BLOTTO resulted in banding of SP-B/C during immunostaining, although variability in the success of the process was still evident. This inconsistency may be due to a low antibody specificity towards mouse surfactant proteins. While the antibody was developed in rabbit against bovine SP-B/C, no prior investigation had been done to determine its effectivness in the mouse.

Results of SDS gels stained with CBB and immunostained by the available heterologous antiserum on which mouse lung lavage was run showed the presence of protein bands at molecular weights consistent with the expected location of SP-B and SP-C reported by other investigators. However, without specific antibody confirmation, differentiating between mouse SP-B or SP-C was not possible. Further characterization of these proteins will likely require the development of homologous antiserum to mouse pulmonary lavage proteins.

Chapter 5: Summary

The results of this study confirmed the presence of SP-A, and identified SP-B/C in the C57 BL/KsJ db/db and db/m mouse. SP-A was present as a glycated form, containing mannose and sialic acid moeities. Phenotypically, no difference in molecular weights for SP-A was found between type I diabetic and vehicle-treated mice, or between type II diabetic and normal mice. The relative amount of SP-A was less in type II diabetic animals than in normal animals, suggesting a possible cause for the observed alteration in lung function in diabetes mellitus. Age-related increases for the molecular weights of SP-A within the phenotypes and treatment groups were present and appeared to be caused by protein glycosylation. Since glycosylation may adversely affect protein function, its role in producing a decline in pulmonary function in aging warrants further investigation in diabetic as well as normal individuals.

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