ONLINE DATA SUPPLEMENT –

Intratracheal Recombinant Surfactant Protein D Prevents Endotoxin Shock in the Newborn Preterm Lamb

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rhSP-D and Its Biological Funciton

rhSP-D was synthesized by transfection of CHO DHFR cells with a cDNA encoding full-human SP-D. Transfected cells were selected with increasing concentrations of methotrexate. Transfected pools were cloned by limiting dilution and high expressing clones were identified using an ELISA designed specifically for this purpose. A high expressing SP-D clone was grown in roller bottles in medium containing serum and then switched to serum free medium for bioproduction. To avoid high shear rates associated with large-scale buffer exchange methods, the protein was captured from conditioned medium using anion ion exchange chromatography to concentrate the sample and remove glucose. Following extensive washing to remove impurities, the rhSP-D was eluted from the arion exchange column, diluted and calcium was added to a final concentration of 5 mM. The rhSP-D was then affinity purified on maltose agarose using previously described methods (E1). To minimize endotoxin levels in the final preparation, the anion exchange resin and all chromatography equipment was sanitized by exposure to 0.2 N NaOH and the maltose agarose was treated with an acid-ethanol mixture. Purified rhSP-D migrated as a multimer of greater than 1×10^6 daltons on size exclusion chromatography. On SDS-PAGE gels, the protein migrated as a trimer under nonreducing conditions and fully converted to an ~48 kDa monomeric form when reduced (Fig. E1). Recombinant hSP-D bound and aggregated *E.coli* 055:B5 LPS *in vitro* in a calcium-dependent manner (data not shown). The rhSP-D used in these experiments was at a concentration of 0.5 mg/ml in 20mM Tris, 200mM NaCl, 1 mM EDTA pH 7.4. The endotoxin level in the rhSP-D preparations ranged from 0.1- 0.5 EU/ml (Limulus Lysate Assay, Charles River Laboratories, Wilmington, MA). In a preliminary study, instilling a treatment dose of rhSP-D into normal adult mice and

premature lambs did not induce lung inflammation (data not shown). Thus, the endotoxin level in rhSP-D either was below levels that induce inflammation or the endotoxin present was bound to rhSP-D and unable to elicit a response.

Processing of Lungs. The thorax was opened, the lungs were inflated with air to 40 cmH_20 pressure for 1 min, and the maximal lung volume recorded. The lungs were deflated and lung gas volume was measured at 20, 15, 10, 5 and 0 cmH_20. Lung tissue of the right lower lobe was frozen in liquid nitrogen for RNA isolation. Bronchoalveolar lavage (BAL) was performed on the left lung by filling it with 0.9% NaCl at 4^oC until visually distended, and the lavage was repeated five times. BAL fluid (BALF) was pooled and aliquots saved for determination of total protein (E2).

Alveolar Cells. BALF was centrifuged at 500 xg for 10 min and the cells in the pellets were counted using trypan blue. Differential cell counts were performed on stained cytospin preparations (Diff-Quick; Scientific Products, McGraw Park, IN). Activation of the cells recruited to the airways was assessed by measuring hydrogen peroxide using an assay based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by hydrogen peroxide under acidic conditions (Bioxytech H_2O_2 – 560 assay; OXIS International, Portland, OR).

Apoptotic cells are detected by annexin V and proprium iodide staining (Pharmigen, Mountain View, CA) and analyzed by flow cytometry as described previously (E3).

rhSP-D in BALF, Lung Tissue and Serum. Levels of rhSP-D in BALF, the supernatant of lung homogenate after centrifugation and in serum collected at 5h of age were analyzed by ELISA. For immunoblotting, 10 μl of BALF was loaded on a SDS/PAGE gel, transferred to nitrocellulose and the blots probed with rabbit anti-rhSP-D serum that does not crossreact with ovine SP-D, allowing an estimate of the level of exogenous rhSP-D in the samples.

Lung Histology. The right upper lobe was inflation fixed with 10% formalin at 30 cmH_20 pressure. Paraffin embedded tissues were sectioned (9 μm) and stained with hematoxylin and eosin. Immunohistochemical detection of IL-6, IL-8 and IL-1β on lung tissues was performed as previously described (E4) using rabbit polyclonal antibody for ovine IL-6 (Chemicon, Temecula, CA), mouse polyclonal antibody for ovine IL-8 (Chemicon) and rabbit polyclonal antibody for ovine IL-1β.

Endotoxin and Cytokines in Plasma. LPS was quantified in plasma at 0 (cord blood), 30 min, 1h, 2h and 5h with the Limulus amebocyte lysate assay (Bio Whittaker, Walkersville, MD). ELISA was used to determine IL-8 and IL-1β in plasma using antibodies from Chemicon.

RNA Analysis in Lung, Spleen and Liver. Total RNA was isolated from the right lower lung lobe, spleen and the liver by guanidinium thiocyanate-phenol-chloroform extraction. Spleen and liver tissue were used to evaluate whether the intratracheally-administered

LPS induced a systemic inflammatory response. RNase protection assays were performed using RNA transcripts of ovine IL-6, IL-1β, IL-8, IL-10 and TNFα as described previously (E5). Ovine ribosomal protein L32 was the reference RNA. Densities of the protected bands were qualified on a phosphorimager using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

REFERENCES

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Column Procedure Yields Highly Purified rhSP-D

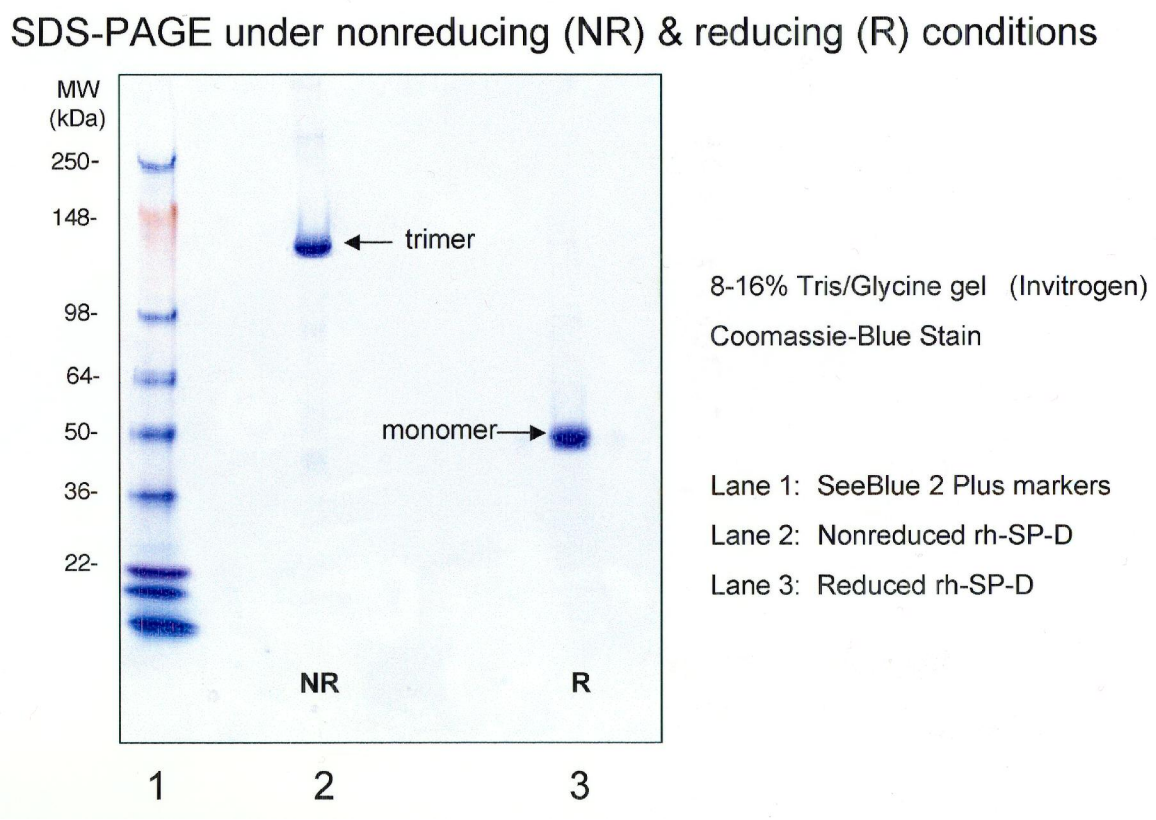

FIGURE E1