Developmental and glucocorticoid regulation of surfactant protein mRNAs in preterm lambs

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ANTENATAL GLUCOCORTICOID therapy is routinely used clinically to reduce the incidence of respiratory distress syndrome and other morbidities of premature infants. Efficacy of this treatment requires at least a 24-h exposure to glucocorticoids before delivery. However, there is little information regarding the duration of benefit from this treatment, and it is not known whether retreatment is needed when delivery of an infant is delayed more than a week (11, 13, 23). Current clinical practice varies from a single course of antenatal corticosteroid treatment to multiple treatments at weekly intervals even in the absence of premature labor and impending delivery. Although a single course of antenatal steroids is considered safe, concerns about adrenal suppression, fetal growth retardation, and increased susceptibility to infection with exposure to repetitive courses have been raised (4, 23).

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Pulmonary surfactant is a complex mixture of lipids and proteins that reduces the surface tension at the alveolar air-liquid interface, thereby stabilizing the terminal air sacs and preventing alveolar collapse during expiration. Adequate synthesis and secretion of pulmonary surfactant accompanying maturation of the lung during late gestation are essential in preventing respiratory distress in newborn infants. Surfactant-associated proteins (SP) account for approximately 10% of pulmonary surfactant mass, and four surfactant proteins (SP-A, SP-B, SP-C, and SP-D) have been isolated and described (12). SP-A and SP-D are hydrophilic oligomeric glycoproteins whose primary roles in vivo appear to be nonimmune host defense in the lung (20). SP-B and SP-C are low-molecular-weight lipophilic proteins that are believed to play a major role in the surface tension-reducing properties of surfactant phospholipids, and SP-B is essential for normal surfactant function in vivo. Congenital deficiencies of SP-B, with associated deficiency of mature SP-C, result in severe respiratory failure at birth in term infants and transgenic mice (9, 24, 36).

Glucocorticoids regulate both lipid and protein components of surfactant (25). In the model system of cultured fetal lung, glucocorticoid effects occur pretranslationally, and these responses are reversible with removal of hormones (1, 15, 25, 34, 36). The fetal sheep model has been used to examine the timing and duration of glucocorticoid effects in vivo because of the relatively long gestation. Exposure of fetal lambs to glucocorticoids results in anatomic and biochemical maturation of the fetal lung, characterized by thinning of the alveolar walls, improved lung function, and increased content of surfactant (3, 16, 17, 27–30). Polk et al. (28) have shown that short-term glucocorticoid treatment of fetal lambs (24 h before delivery) increased mRNA for SP-A, SP-B, and SP-C, but in other studies, this treatment regimen did not alter the lavage fluid content of SP-A and SP-B (3). On the other hand, repetitive weekly doses of betamethasone prior to preterm delivery increased surfactant lipid and proteins in both lung tissue (2- to 3-fold over preterm control) and lavage fluid (10- to 15-fold over preterm control) (3). The effect of repetitive in vivo betamethasone treatment on induction of SP mRNAs is not known.

The goals of this study were to investigate the ontogeny of SP mRNAs in the fetal lamb and the effect of different in utero glucocorticoid treatment regimens.
The results provide new information related to the timing and reversibility of glucocorticoid induction of SP gene expression in vivo.

**METHODS**

Animals. Protocols for these experiments were approved by the animal use and care committees at both Harbor University of California, Los Angeles Medical Center and The Western Australia Department of Agriculture. A description of the animals and treatment procedures has been reported elsewhere (16). Three separate glucocorticoid treatment protocols, each with their own control animals, were utilized in these experiments (Fig. 1). Two short-term treatments (protocols A and B) and one long-term treatment (protocol C). Date-mated ewes with singleton pregnancies were identified by ultrasound at 60 days gestation and were treated with 150 mg of medroxyprogesterone (Depo-Provera, Upjohn, Kalamazoo, MI) at 101 days gestation to reduce the occurrence of preterm labor and abortion after glucocorticoid treatment. Ewes were randomized to receive maternal intramuscular injections or ultrasound-guided intra-amniotic or fetal intramuscular injections at the assigned times as designated by the treatment protocol. Glucocorticoid-treated animals received 0.5 mg/kg maternal weight of betamethasone (Celestone, Schering Pharmaceuticals, Kenilworth, NJ), which was previously found to be the most effective dose (17, 30).

Treatment protocol A: 15-h exposure. Pregnant sheep were treated with betamethasone given to either the ewe (maternal intramuscular, n = 7) or the fetus (fetal intramuscular, n = 7) or the equivalent volume of diluent (normal saline solution, control, n = 6) 15 h prior to delivery. All fetuses in this protocol were delivered by cesarean section at 123 days gestation and received bevacizumab (Suvanta, Ross Laboratories, Columbus, OH; 4 ml/kg intratracheal administration).

Treatment protocol B: 48-h exposure. Date-mated ewes were randomized into one of four treatment groups as per Fig. 1: 1) intra-amniotic betamethasone at 123 days gestation (48 h intra-amniotic, n = 9); 2) maternal intramuscular betamethasone at 123 days gestation (48 h maternal intramuscular, n = 10); 3) maternal intramuscular betamethasone at 123 and 124 days gestation (48/24 h maternal intramuscular, n = 10); and 4) saline injection either intra-amniotic (n = 7) or intra-amniotic (n = 5). Results for the two groups of control animals (intramuscular and intra-amniotic) were comparable, and the data were pooled. The animals remained undisturbed except for the injections, and all fetuses were delivered by cesarean section at 125 days gestation.

Treatment protocol C: repetitive dosing. Date-mated ewes were randomized into one of five treatment groups of 11 animals each to receive either intramuscular betamethasone for 1–4 doses or the equivalent intramuscular volume of normal saline solution given weekly starting at 104 days gestation as per Fig. 1. Control animals received four weekly intramuscular saline injections at 104, 111, 118, and 124 days gestation. The animals were not handled except for the injections, and all fetuses were delivered by cesarean section at 125 days gestation.

Control animals. For comparison with the premature animals of the study groups, lung tissue was collected at delivery from seven term, untreated, and unmanipulated newborn lambs of 145–150 days gestation born to date-mated ewes. For the ontogeny study, lung tissue was collected at delivery from 12 untreated fetuses of varying gestational ages and postnatally from term lambs. These animals were all untreated controls available from other protocols.

Preterm delivery and postnatal evaluation. The deliveries and postnatal care were by methods reported previously for lambs delivered at 125 days gestation (16). The lambs were killed with an overdose of pentobarbital sodium followed by exsanguination after 4 h of ventilation (protocol A) or 40 min of ventilation (protocols B and C). Pieces of the unlavaged right lower lobe were removed from each lamb and then snap-frozen for assay of surfactant protein mRNA levels. It was observed previously that short-term ventilation of prematurely delivered fetal lambs did not affect levels of SP mRNAs (38).

Isolation of RNA and DNA hybridization. RNA was extracted from lung tissue (generally 50–100 mg wet wt) using the acid guanidium-phenol-chloroform method as previously described (8). To assess mRNA size and integrity, total RNA was size-fractionated by electrophoresis in 1% agarose-formaldehyde gels and then transferred to nitrocellulose membranes by blotting. Specific mRNA content was determined by hybridization using a dot blott apparatus as previously described (18, 34). The membranes were hybridized as described below to homologous ovine SP-A, SP-B, and SP-C cDNA fragments after random oligonucleotide labeling using [32P]dCTP and Ready To Go DNA labeling kit (Pharmacia Biotech, Piscataway, NJ). The SP-A probe was a 322-bp fragment of cloned ovine SP-A corresponding to the neck and globular carbohydrate recognition domains of SP-A. The SP-B probe was a 280-bp fragment of a cloned ovine SP-B containing the full-length coding region of the mature SP-B. The SP-C probe was subcloned (202 bp) from a 802-bp fragment that represents the complete ovine SP-C clone. The sequence and characterization of these cDNAs are described in a separate study (G. A. Braems, L.-J. Yao, K. Inchley, A. Brickenden, V. K. M. Han, A. Grolla, J. R. G. Challis, and F. Possmayer, unpublished data).

The hybridization reaction occurred at 42°C overnight under stringent conditions. Nitrocellulose membranes were washed twice under high-stringency conditions at 50°C with 0.2× saline-sodium citrate buffer and 0.1% SDS. Blots were exposed to storage phosphor screens and then scanned and analyzed.

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### Table 1: Glucocorticoid Treatment Protocols

<table>
<thead>
<tr>
<th>Protocol A</th>
<th>Control</th>
<th>15 h maternal IM</th>
<th>15 h fetal IM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 6</td>
<td>n = 7</td>
<td>n = 7</td>
</tr>
<tr>
<td>Protocol B</td>
<td>Control</td>
<td>48 h intraamniotic</td>
<td>48 h maternal IM</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
<td>n = 9</td>
<td>n = 10</td>
</tr>
<tr>
<td>Protocol C</td>
<td>Control</td>
<td>1 dose maternal IM</td>
<td>2 doses maternal IM</td>
</tr>
<tr>
<td></td>
<td>n = 11</td>
<td>n = 11</td>
<td>n = 11</td>
</tr>
<tr>
<td>Term Control</td>
<td>Gestational Age (days)</td>
<td>n = 8</td>
<td>148 delivery</td>
</tr>
</tbody>
</table>

Fig. 1. Three glucocorticoid treatment protocols used to evaluate short-term (protocols A and B) and long-term (protocol C) glucocorticoid administration are outlined. Normal saline injections are represented by open rectangles and betamethasone injections (0.5 mg/kg) are represented by the filled rectangles given at specified gestational ages. Animals in protocol A were delivered at 123 days gestation, whereas animals in protocols B and C were delivered at 125 days gestation. IM, intramuscular; n, no. of animals.
quantitated on the STORM phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). All blots were boiled in water for 10 min and reprobed with 32P-labeled cDNA for human β-actin (15) for comparison within and between various groups.

Data analysis. Data for SP mRNA levels in preterm animals are expressed as percent of the mean value obtained for RNAs present on all blots of individual term newborn animals. All values are given as means ± SE for each treatment group. SP mRNA content data were also normalized to β-actin, which gave similar results to unnormalized data. Comparisons between groups were done by ANOVA using Fisher’s exact test, and significance was accepted at P < 0.05.

RESULTS

Three different glucocorticoid treatment protocols were used in these studies. The effect of each treatment protocol on maternal and newborn weights, cord blood gas values, lung weight, protein and DNA content, and pulmonary function have been reported previously (16, 29). Only fetuses receiving two to four weekly doses of maternally administered betamethasone in the repetitive treatment protocol (protocol C) experienced significant decreases in body and lung weight.

Northern analysis with sheep cDNA probes were used to assess SP mRNAs in preterm (125 days gestation control and betamethasone treated) and term (148 days gestation) sheep lung (Fig. 2). A single major band was found for SP-A (~2.0 kb) and SP-C (~1.0 kb). For SP-B, a major band was found at 3 kb, with a second minor band at 1.8 kb. These sizes are similar to those observed in other species except for the higher SP-B band, which has been reported previously in sheep (10, 19, 26, 31, 32, 37).

Ontogeny. To examine the developmental profile of SP mRNAs, lung tissue was obtained from control, untreated animals throughout the third trimester of gestation. These results are shown in Fig. 3. The mRNA content for all three SPs was low or undetectable at 99–100 days gestation and increased progressively after ~120 days gestation to term (145–150 days gestation), with an apparent postnatal increase for SP-A. SP-C mRNA content appeared to increase somewhat faster than that for SP-A or SP-B. Human β-actin mRNA did not vary with development, and normalization of the data with β-actin yielded similar results (data not shown).
Effect of betamethasone treatment. The content of SP mRNAs for animals of protocol A (15-h exposure) are shown in Fig. 4A. mRNA levels in control preterm animals were 15–30% of term values. All surfactant protein mRNAs were significantly increased (~2- to 2.5-fold) in response to 48-h and 48/24-h maternal glucocorticoid administration, whereas only SP-A and SP-B mRNAs were significantly increased (~2-fold) with 48 h of intra-amniotic betamethasone administration. Previously, we found no effect of 48-h betamethasone exposure on SP-A content in lung tissue; however, SP-B was significantly increased (~2.5-fold) (3).

Results for repetitive maternal glucocorticoid dosing from protocol C are shown in Fig. 4C. mRNA levels in control preterm animals for this protocol were 15–40% of term. Only four weekly doses of betamethasone, with the last dose given 24 h before delivery, significantly increased SP-A, SP-B, and SP-C mRNA content compared with preterm control levels (3-, 2.5-, and 2-fold, respectively). These findings are in contrast to SP content for both lung tissue and lavage fluid in these same animals: a 2- to 3-fold increase in SP-A and SP-B content in tissue and a 10- to 15-fold increase in SP-A and SP-B concentration in lavage fluid were maintained after 2–4 weekly doses of betamethasone (3).

Summary data showing the mean SP mRNA content as a function of the time of the last exposure to glucocorticoid prior to delivery are shown in Fig. 5. The kinetics of the inductive response were similar for each SP. Stimulation was maximal 24–48 h after treatment, and mRNA content returned to near control levels 7 days after betamethasone exposure.

**DISCUSSION**

In these studies, we investigated the ontogeny of SP mRNAs in fetal sheep and the effect of different in utero glucocorticoid treatment regimens. Our results demonstrate a coordinated developmental profile for all three SP-A, SP-B, or saturated phosphatidylcholine; however, lung function was significantly improved over that in control preterm animals (3).
SP mRNAs, with a detectable increase in levels around 120 days gestation and a progressive increase until term. The major increase in SP mRNAs occurs after 130 days gestation and parallels the developmental increase in surface-active material in lung lavage fluid (22). At 125 days gestation, the content of SP-A and SP-B mRNAs was ~20 and 30%, respectively, of the term level compared with levels of 5–10 and 1–2% for the respective proteins (3). This may indicate that translation and/or processing of SPs, in particular SP-B and SP-C, is developmentally delayed compared with gene expression, similar to the findings in human fetal lung (5, 33).

The developmental profiles of SPs in sheep are generally similar to those previously found for rats (31) and rabbits (10, 26, 37) in that SP-C gene expression precedes that for SP-A or SP-B. SP-C mRNA is detected by gestational day 17 in rats (term 21 days) and gestational day 19 in rabbits (term 31 days), with the appearance of SP-A and SP-B following on day 18 in the rat and days 24 and 26, respectively, in the rabbit. We observed a continued postnatal increase in content of SP-A mRNA, but not of SP-B or SP-C, in the lamb, not unlike that seen in the newborn rat (31). SP-A mRNA has also been examined in the baboon, with levels just detectable at midgestation and markedly increasing after 125 days (term 184 days) (32). In human lung, mRNAs for SP-B and SP-C are detected as early as 13 wk gestation (term 40 wk), with increases noted from 16 to 24 wk gestation (SP-B greater than SP-C), whereas mRNA for SP-A remains low or undetectable even at 24 wk gestation (2, 18, 35).

Developmental changes in mRNAs for SPs have been previously examined in fetal sheep using human cDNA probes (38). In nonventilated fetal sheep, a 10-fold increase in SP-A mRNA and a 2-fold increase in SP-C mRNA levels were demonstrated between 120 and 139 days gestation, with no change found for SP-B mRNA content over the same period. Using ovine cDNA probes, we also found a greater developmental increase for SP-A than for SP-C (5-fold vs. ~2.5-fold between 125 days and term, respectively). In contrast to the previous report (38), we found a similar developmental change in SP-B mRNA (~3-fold) that is consistent with the pattern for SP-B protein in both lung tissue and lavage fluid of fetal sheep. It is possible that the failure of Woods et al. (38) to observe a change in SP-B mRNA relates to the use of a nonhomologous SP-B cDNA.

SP genes have been shown previously to be steroid responsive in cultured lung and in a number of animal models; however, the persistence of this effect in vivo is not known (2, 5, 10, 14, 15, 18, 21, 28, 32). In human fetal lung explants, there is rapid reversibility of glucocorticoid effects. Transcription rates for SP-B and SP-C decrease by 4 h after removal of cortisol, and mRNA levels reach control values by 48 h (1, 18). In the current study, we investigated the effect of three different and clinically relevant glucocorticoid treatment regimens on mRNA levels in preterm sheep. There was a rapid and maximal induction of all SP mRNAs (2-to 3-fold) by 24–48 h after glucocorticoid exposure, and these responses were fully reversible after an interval of 7–21 days from the last treatment prior to delivery. By comparison, maximal induction of SP-A and SP-B was achieved only after repetitive doses of betamethasone, and levels were elevated in lung tissue for at least 2 wk after treatment (3). The rapid reversibility of SP mRNA induction is consistent with the time course for betamethasone, which is cleared from the fetal circulation within 24 h after maternal administration (6). The persistence of elevated protein vs. mRNA levels for SP-A and SP-B likely reflects longer half-lives of the proteins.

Previously, Polk et al. (28) examined SP mRNA levels in 128-day-gestation lambs exposed to fetally administered intramuscular betamethasone 7 days and/or 24 h prior to delivery. Similar to our findings, SP-A, SP-B, and SP-C mRNAs all increased two- to threefold after 24 h of glucocorticoid exposure. However, these investigators also found a two- to threefold increase in SP-A and SP-C mRNAs after a single dose of betamethasone given 7 days before delivery and reported that these mRNA levels were not different from control if both treatments were given. SP-B mRNA content, on the other hand, increased approximately twofold with 24-h betamethasone exposure but was not elevated with treatment 7 days before delivery. The reasons for the discrepancies in results from this and the Polk et al. study are not known but could relate in part to the mode of administration of betamethasone (maternal vs. fetal, respectively), which influences the kinetics and levels of circulating betamethasone (6).

The rapid induction of SP mRNAs, plus the finding that the magnitude of response for SP mRNA and protein in tissue is similar, provides evidence that glucocorticoid regulation is primarily at the pretranslational level. In other species, glucocorticoids influence regulation of SP mRNAs by effects on both transcription rate and mRNA stability (25). It is of interest to note that repetitive glucocorticoid treatment did not suppress SP-A mRNA content, which is the dominant response to glucocorticoids in cultured human and baboon lungs (15, 32).

The need for retreatment is an important current topic in the clinical use of antenatal corticosteroids. Our data along with previous reports indicate complete reversibility of SP mRNA induction by glucocorticoid in fetal sheep, although levels of SP-A and SP-B (and saturated phosphatidylcholine) remain elevated for at least 2 wk after treatment (3). If the process is similar in the human fetus, these findings support a need for retreatment when delivery is delayed >2 wk. However, multiple courses of antenatal corticosteroids appear to be associated with adverse effects. A recent post hoc analysis of infants born between 25 and 32 wk gestation found no evidence for increased benefit with additional courses of antenatal corticosteroids compared with a single course with regard to incidence of respiratory distress syndrome, intraventricular hemorrhage, or chronic lung disease (4). Moreover, neonates who received three or more courses of antenatal corticosteroids had slightly lower birth weights, greater as well
as more prolonged adrenal suppression, and increased risk of death. Thus risk as well as possible benefit must be considered in contemplating retreatment with antenatal glucocorticoid.

In summary, expression of the SP genes in fetal sheep is developmentally and hormonally regulated. In vivo natal glucocorticoid treatment rapidly induces a maximal developmentally and hormonally regulated. In vivo natal glucocorticoid.

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