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Identification of a Unique Form of Protein C in the Ovine Fetus: Developmentally Linked Transition to the Adult Form

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ABSTRACT

To investigate fetal development of protein C, a pregnant ovine model was used. Protein C was isolated from ovine plasma, and a polyclonal antibody was raised. Citrated plasma was obtained from undisturbed chronically catheterized fetal lambs. On Western blot, nonreduced adult ovine protein C had a molecular mass of 70 kD. Fetal ovine protein C was determined to have a molecular mass of 4 to 6 kD larger than the adult molecule. Crossed immunoelectrophoresis demonstrated slightly increased anodal migration of the fetal form. Isoelectric focusing demonstrated a decreased pI of the fetal molecule (4.45 *versus* 4.6). The ovine protein C molecules were deglycosylated with *N*-glycanase. Deglycosylated fetal protein C migrated more similarly to the adult form, although a portion of the fetal form persisted. These experiments demonstrate the first example of a unique fetal form of a vitamin K-dependent protein and are compatible with increased glycosylation of fetal ovine protein C. It is speculated that altered posttranslational processing may exist as a general process by which certain coagulation proteins are modified during fetal development. mRNA was isolated from maternal and fetal hepatic tissue and analyzed by Northern hybridization. Fetal plasma concentration and hepatic mRNA for protein C were both 40% of normal maternal values from midgestation onward. At term, protein C mRNA increased to adult range ($p < 0.025$), although plasma protein C concentration decreased slightly ($p < 0.001$). A transition from fetal to adult protein C form was found beginning 6 d before term birth, with a doubling time of 24 h. These data are compatible with a gestationally determined maturation of ovine protein C. There was no evidence for very low plasma concentrations of protein C during normal fetal and neonatal development. Decreased plasma protein C concentration after birth associated with increased hepatic mRNA suggests increased turnover of protein C in the perinatal period. Further investigation of the transition from fetal to adult forms of protein C and altered hepatic mRNA expression will be necessary to understand, treat, and prevent complications of protein C deficiency in the neonate. (***Pediatr Res* 37: 365–372, 1995**)

Abbreviations

DFP, diisopropyl fluorophosphate

The hemostatic system of the fetus and neonate differs from that of the adult. Plasma concentrations of different coagulation factors achieve adult levels variably during maturation, such that by midgestation in the human, levels of factors V, VIII, fibrinogen, and the von Willebrand factor are within the normal adult range, whereas vitamin K-dependent factors II, VII, IX, and X do not achieve adult means until several weeks to months postnatally (1).

To date, the only fetal characteristic of the vitamin K-dependent proteins is the finding by Bovill *et al.* (2) of an increased production of des-carboxy prothrombin and protein C in term and preterm human neonates related to decreased activity of hepatic reductase. Protein C is a vitamin K-dependent coagulation zymogen that functions both to limit coagulation and to augment fibrinolysis (3). The ontogeny of protein C is of interest, because protein C seems to serve a critical role in the regulation of fetal hemostasis. Infants with homozygous protein C deficiency suffer spontaneous thromboses *in utero*, notably within the retina and the CNS (4, 5). A mean protein C concentration of 0.40 U/mL is found in healthy human infants at term, with an observed range of 0.25–0.76 U/mL (6). Levels of protein C rise slowly after birth and do not achieve adult levels until late childhood or adolescence (7, 8). Manco-Johnson *et al.* (6, 9) recently described protein C levels of less than 0.10 U/mL in 30% of preterm infants with respiratory distress syndrome as well as in some term infants born after diabetic or twin gestation; these infants exhibited an increased incidence of thromboses during the neonatal period. The neonates with very low levels of protein C did not demonstrate other evidence of disseminated intravascular coagulation, and the mechanism of protein C deficiency in these babies is not clear.

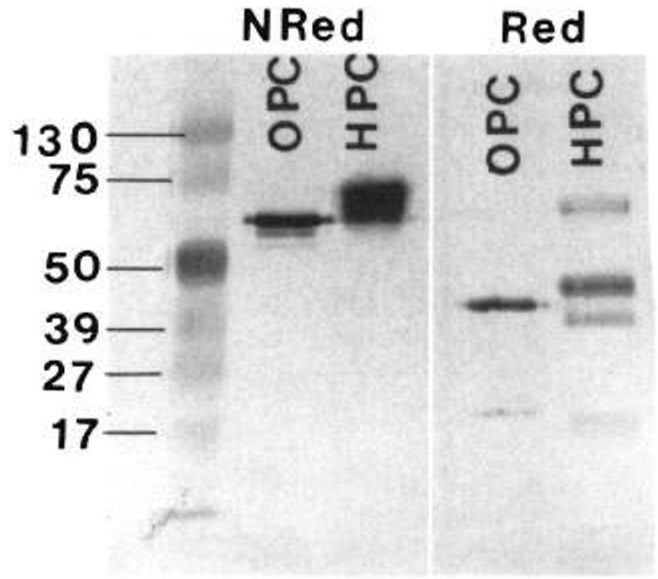

Figure 1. Western blot (SDS-PAGE, nonreduced and reduced) of ovine and human protein C. Nonreduced ovine protein C comprises a slightly smaller molecular mass polypeptide. Reduced ovine protein C demonstrates a minimal component of single-chain protein C compared with human protein C. In addition, note differences in migration of the heavy chain.

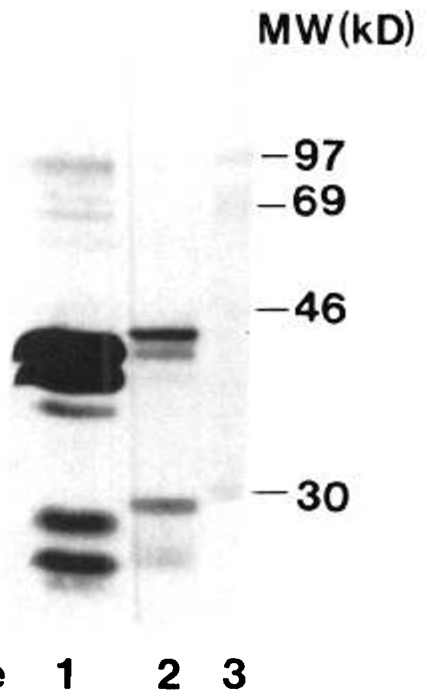

Figure 2. Adult and fetal ovine protein C. Western blot of reduced purified adult and fetal ovine protein C in *lanes 1* and *2*, respectively. See Table 1 for molecular masses.

Table 1. *Molecular masses of protein C components*

		Reduced		
	Nonreduced	SC	HC	LC
Human adult	73	70	45	20
			37	
Ovine adult	67	64	42	28
	59		38	26
	55		35	
Ovine fetal	73		45	29
	67		41	26
			38	

Molecular masses (in kilodaltons) were calculated from Western blotting of human and ovine protein C; SC, HC, and LC, α-, β-, and γ-components of the single, heavy, and light chains, respectively.

To study normal fetal development and regulation of protein C, we used a chronically catheterized fetal ovine model. We chose the ovine model because the pregnant ewe generally gives birth to a single fetus whose weight is 3 kg at term. This model allows serial sampling of the fetus from midgestation in an undisturbed state. Work by Kisker and colleagues (10), Massicotte *et al.* (11), and Moalic *et al.* (12) support the theory that maturation of levels of coagulation factors in the ovine fetus is similar to that of the human. Thus, the ovine model is appropriate for study of the ontogeny of coagulation factors in humans. One of the authors (J.W.S.) developed a technique of hepatic vein catheterization in the ovine fetus (13). This catheter remains intact during labor and delivery and allows uninterrupted collection of blood samples before, during, and after transition from fetal to extrauterine life.

Using the pregnant ovine model and the fetal hepatic vein catheters, we were able to determine the concentration and molecular forms of ovine protein C during fetal development and for the first 3 wk of postnatal life. This work describes the *in utero* maturation and regulation of levels and forms of ovine protein C. A developmentally regulated maturation of ovine protein C was found with transition from the fetal to adult form linked to term birth.

METHODS

Laboratory Methods

Purification of ovine protein C. Forty liters of ovine blood, obtained after humane killing at a local abattoir, were collected into 3.8% sodium citrate containing 100 U/mL heparin, 50 mM benzamidine, and 0.12 trypsin inhibitor units/mL aprotinin. The blood was centrifuged in 250-mL aliquots for 20 min at 12 000 rpm at 4°C. Twenty-four liters of ovine plasma were used in the purification. Vitamin K-dependent proteins were absorbed with 1.92 L of 1 M BaCl added drop-wise and mixed overnight at 4°C. The precipitate was washed three times with 0.15 M NaCl, 0.01 M BaCl, 0.02 M Tris, and 1 mM benzamidine, pH 7.0. The precipitate was eluted with 0.2 M EDTA, pH 7.4, containing 1 mM benzamidine and 5 mg/L aprotinin. The eluate was precipitated with 40% ammonium sulfate, and the precipitate was discarded. The supernatant was precipitated with 67% ammonium sulfate, and the supernatant was discarded. The precipitate was dissolved in 120 mL of distilled water and dialyzed in 0.1 M sodium phosphate, pH 6.0, containing 1 mM benzamidine. The material was applied to a 2.5 × 40-cm column containing diethylaminoethyl-Sephadex A50, washed with dialysis buffer, and eluted with a 800-mL NaCl gradient (0.15–0.55 M). The column eluents were collected in 10-mL fractions, which were monitored with coagulant assays for factors II, VII, IX, and X using specific human deficient plasma substrates. Protein C was monitored with a

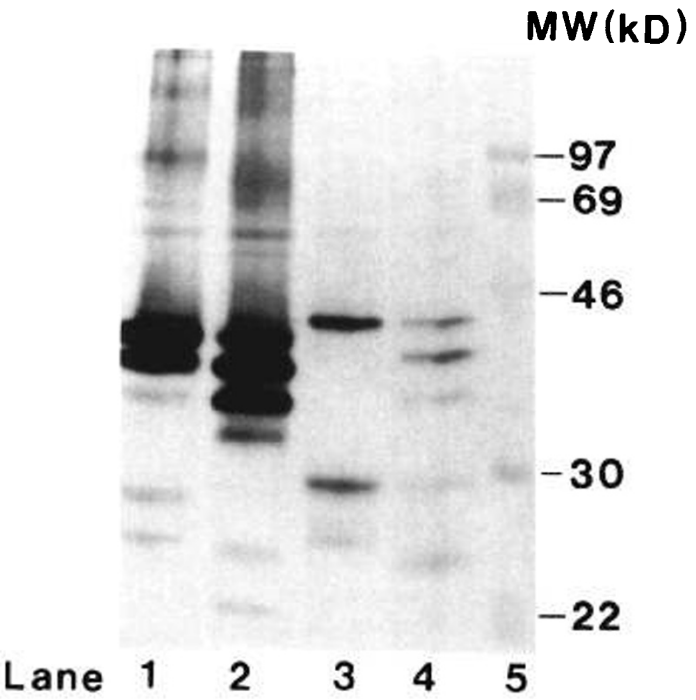

Figure 3. Glycosylation studies of ovine protein C. Western blot of purified adult ovine protein C incubated for 24 h with 0 and 30 U/mL of *N*-glycanase (*lanes 1* and *2*). Corresponding fetal samples are in *lanes 3* and *4*. Molecular mass markers are in *lane 5*.

Table 2. *Molecular masses of ovine protein C components after deglycosylation*

	No *N*-glycanase		*N*-glycanase	
	Adult	Fetal	Adult	Fetal
HC	42	45	42	45
	38	41	38	40
	35	38	35	35
			32	
LC	28	29	25	29
	26	26	22	24

Purified adult and fetal ovine protein C were incubated 24 h at 37°C with 0 or 30 U/ml *N*-glycanse. Molecular mass components (analyzed by reduced Western blot) are expressed in kilodaltons. HC and LC, α-, β-, and γ-components of the heavy and light chains, respectively.

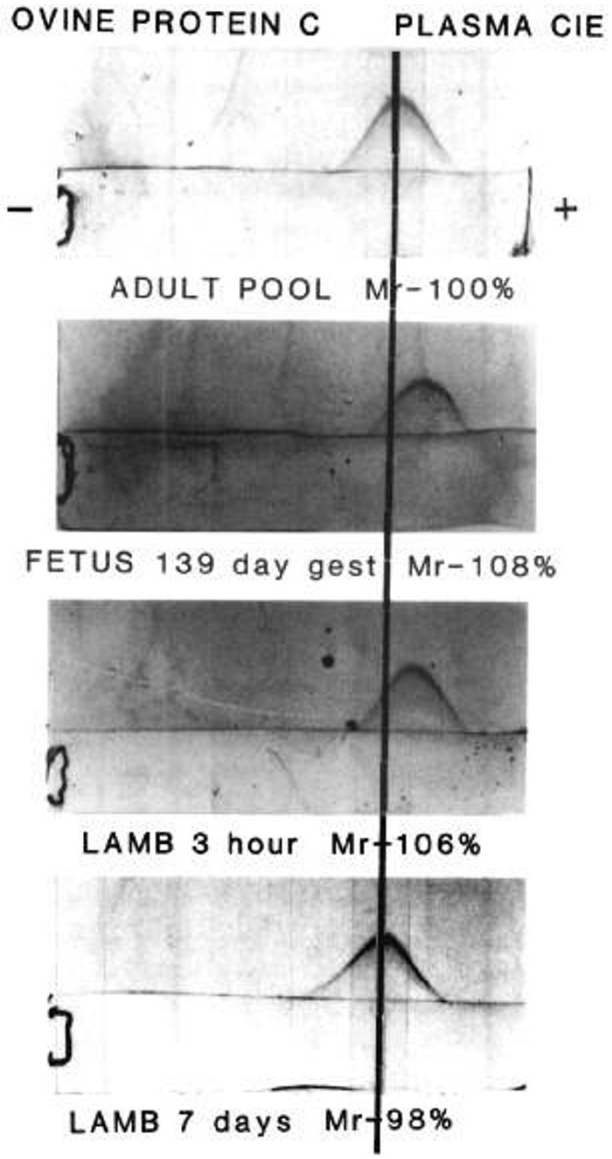

Figure 4. Crossed immunoelectrophoresis of ovine protein C. The *top* crossed immunoelectrophoresis (*CIE*) contains adult ovine plasma. The *second* CIE contains fetal ovine plasma (139 d gestation). The *third* CIE contains plasma from a 3-h neonatal lamb. Western blot of this sample showed distinct adult and fetal bands (data not shown). The CIE demonstrates total identity of the two molecular forms with the polyclonal antibody used. The *bottom* CIE contains plasma from a 7-d-old lamb; the relative migration of protein C on this crossed immunoelectrophoresis is equal to that of the adult sheep. Western blot of this sample shows migration equal to that of adult protein C.

chromogenic assay using activation with the snake venom Agkistrobon contortrix contortrix and detection with chromogenic substrate S-2366. Fractions 100–110 from the diethylaminoethyl-Sephadex A50 column containing protein C as well as factors II and IX were pooled, 1 mM DFP was added, and the material was dialyzed into 0.05 M imidazole, pH 6.0, containing 0.012% Na azide; 2.5 mM CaC1 was added and the material was applied onto a 1.5 × 30-cm heparin agarose column. The column was washed overnight with 0.05 M imidazole buffer, 2.5 mM $CaC1_2$, 1 mM benzamidine, 0.02% Na azide, pH 6.0, and eluted with a 360-mL NaCl gradient (0–1 M). Five-mL fractions were collected and tubes 100–115 were pooled. DFP was added to a final concentration of 1 mM, and the pool was dialyzed into 0.05 M imidazole, 0.02% Na azide, and 1 mM benzamidine, pH 6.3, and applied to a 1.5 × 36-cm blue Sepharose column. The column was washed with dialysis buffer overnight and eluted with a 500-mL NaCl gradient (0–2 M). Five-mL fractions (fractions 30–38) from the blue Sepharose column were pooled; 1 mM DFP was added, and the fractions were dialyzed into 0.02 M Tris and 0.145 M NaCl, pH 7.5. Protein C was isolated from 500 mL of fetal ovine plasma using similar techniques.

Determination of protein C activity. Plasma vitamin K-dependent proteins were absorbed with BaCl and eluted with EDTA as previously described (14, 15). Purified proteins and EDTA eluants were activated with Agkistrodon contortrix contortrix; activated protein C was determined using both a chromogenic assay, which used S-2366 (Kabi, Stockholm, Sweden), or by prolongation of an activated partial thromboplastin time coagulant assay.

Table 3. *Comparison of protein C concentrations in humans and sheep*

Group	*n*	Mean (mg/L)	SD (mg/L)	Observed range (mg/L)
Humans				
Normal adult controls	38	4.00	0.6	2.9–5.6
Males	17	4.08	0.6	2.9–5.0
Females	21	3.96	0.6	3.0–5.6
Normal term newborns	65	1.58	0.4	0.9–3.1
Normal preterm newborns	11	0.76	0.2	0.6–1.2
Sick preterm newborns	26	0.72	0.4	0.0–1.6
Sheep				
Normal adult controls	26	7.90	1.3	5.6–11.5
Males	7	8.00	1.9	5.6–11.5
Females	19	7.90	1.1	6.1–10.2
Pregnant ewes	111	7.80	1.4	4.0–12.6
Normal term newborns	89	3.30	1.0	1.3–5.4
Normal fetuses	141	3.70	0.8	2.0–5.8

Note that although ovine protein C levels are twice that of human, the relationship of fetus and newborn to adult is similar.

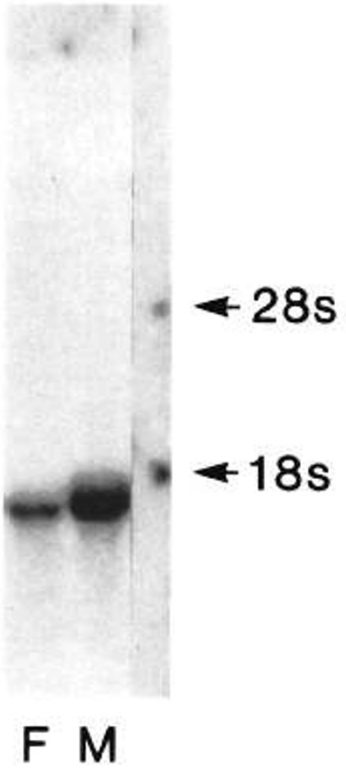

Figure 5. Northern blot of fetal (*lane 1*) and maternal (*lane 2*) RNA probed with a ^{32}P-labeled probe for protein C mRNA. Markers in the *right lane* are 18S and 28S RNA.

Production of rabbit antisheep protein C polyclonal antibody. Purified ovine protein C was used to immunize New Zealand White rabbits. The primary immunization was performed with 1 mL of 63 μg/mL protein C mixed with 1 mL of Freund's complete adjuvant and injected s.c. into eight sites per rabbit. Secondary immunizations using the same concentration of immunogen with incomplete adjuvant were performed monthly. Immunized rabbit serum was precipitated with 50% NH_4SO_4 and washed twice with the same. The precipitate was

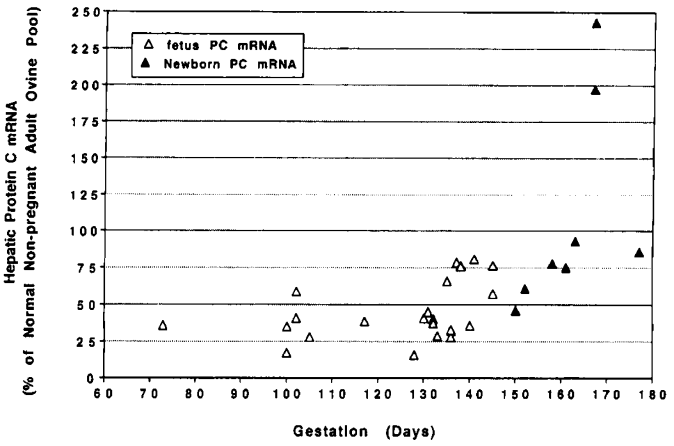

Figure 6. Abundance of hepatic protein C mRNA in normal ovine fetus and newborn expressed as a percent of normal adult.

dissolved in Tris-buffered saline to one third of the original volume and dialyzed extensively. Rabbit IgG was further isolated by protein A Sepharose conjugated with purified protein C for use in Western blot analysis. Purified human protein C and a monospecific rabbit antihuman protein C antibody were kindly provided by Richard Marlar (Veterans Administration Medical Center, Denver, CO).

Physical characterization. Adult and fetal ovine protein C were investigated using SDS-PAGE under reducing and nonreducing conditions, Western blot, and crossed immunoelectrophoresis using standard techniques (14–16). Isoelectric focusing was performed in a Bio-Rad (Richmond, CA) miniisoelectrofocusing chamber using Bio-Rad ampholytes. Glycosylation of ovine protein C was evaluated using purified proteins, which were incubated for 16 h at 37°C in incubation solution (Genzyme Corp., Cambridge, MA) containing 0–60 U/mL *N*-glycanase [peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase]. Protein concentration was estimated using a Lowry assay (Bio-Rad DC Protein Assay).

Determination of protein C concentration. The determination of protein C concentration by immunoelectrophoresis (Laurell technique) was as follows. Eighteen-mL blood samples were drawn from the external jugular vein by two-syringe technique into 2 mL of 3.8% sodium citrate anticoagulant. The samples were immediately placed on ice and centrifuged at 1800 × *g* for 20 min at 4°C. Twenty-six adult sheep, including 19 nonpregnant adult females, were sampled. After portioning an aliquot of the plasma, the remainder was pooled and used as ovine reference plasma. All individual and pooled samples were stored at −70°C until the time of assay.

A purified ovine protein C standard was quantified using a Lowry assay (Bio-Rad). This standard was then used to determine the protein C concentration of the pooled ovine plasma. Thereafter, three dilutions of pooled ovine plasma were used on each electrophoresis slide to construct a curve for the determination of protein C antigen, and results were determined as a percent of the normal ovine pool. Using the same procedures, protein C concentration was determined on a sample of purified human protein C kindly provided by Richard Marlar and used as a reference for results of normal human

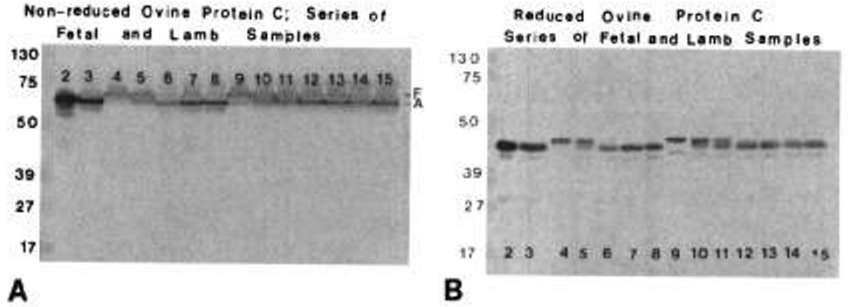

Figure 7. Western blot of transition of ovine protein C from fetal to adult form. Western blot of nonreduced (*A*) and reduced (*B*) ovine protein C samples. Plasma samples in *lanes 3–15* are BaCl precipitated and EDTA eluted. *Lane 1* contains molecular mass standards. *Lane 2* contains purified adult ovine protein C. *Lane 3* contains protein C derived from a normal adult ovine pool. *Lane 4* contains protein C derived from a normal fetal ovine pool. *Lane 5* contains protein C from fetus 1, 147 d gestation. *Lanes 6–8* contain protein C from lamb 1, 30 h, 5 d, and 9 d old. *Lane 9* contains protein C from fetus 2, 139 d gestation. *Lanes 10–15* contain protein C from lamb 2 at 2.8 h, 24 h, 3.5 d, 4.5 d, 5 d, and 7 d of age.

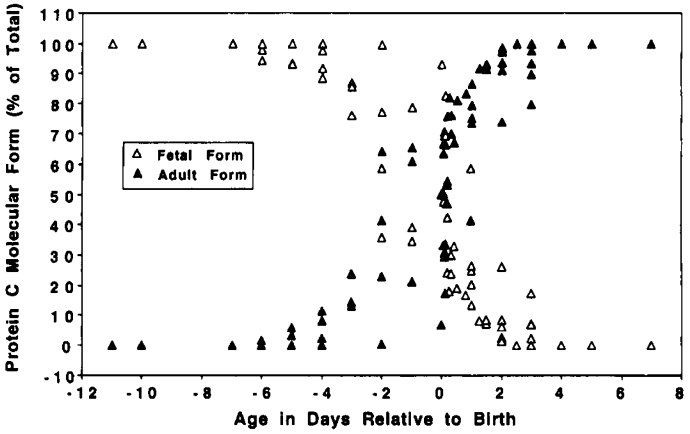

Figure 8. Transition of ovine protein C from fetal to adult form relative to birth.

adults. The normal range of human protein C was determined for a pool of 38 healthy adults who were receiving no medications.

Molecular forms of protein C. Molecular forms of protein C were determined by the Western blot method. Whole blood (1.8 mL) was drawn into 0.2 mL of 3.8% sodium citrate anticoagulant. The sample was immediately centrifuged at 1800 × *g* for 20 min at 4°C. Vitamin K-dependent clotting factors were precipitated from the plasma using BaCl. Protein C was eluted from the precipitate with EDTA as previously described by Aronson (17). The eluate was subjected to SDS-PAGE and transferred to nitrocellulose membranes using standard Western blot technique (16). Protein C was detected using the immunopurified rabbit antiovine protein C antibody as a probe. Protein C bands were quantified using scanning densitometry. Individual bands of protein C were measured as a proportion of the total signal. Results of protein C antigen concentration and Western blot analysis were examined as a function of postconceptual age and as a function of age in days from delivery.

Determination of mRNA for protein C. Liver tissue was obtained from the pregnant ewe and her fetus or neonatal lamb for the purpose of determining abundance of mRNA for protein C. Hepatic tissue, approximately 1 g, was removed within 5 min of killing, quick frozen in liquid nitrogen, and stored at −70°C. RNA was isolated by the rapid method of Chomczynski and Sacchi (18) and analyzed by Northern blot hybridization using a ^{32}P-labeled 900-bp bovine cDNA probe for protein C produced by cleavage of an internal *Pst* site of an 1150-bp sequence encoding bovine protein C nucleotides 247-1397 (kindly provided by Robert Wydro, Integrated Genetics, Boston, MA). The cDNA probe was prehybridized for 3 h in 0.2× standard saline citrate at 42°C. The blots were washed using high-stringency conditions (four washes using 0.2× standard saline citrate at 65°C). After autoradiography of the Northern blots, signal intensity of protein C mRNA was quantified by measuring the area under the curve using scanning densitometry. mRNA for β-actin and 18S RNA were determined using the same densitometric technique. To assess total RNA, Northern blots were stained with methylene blue, and the total area under the curve was measuring using scanning densitometry. A pool of RNA extracted from livers of 10 nonpregnant ewes was used in each Northern blot as a control. mRNA for protein C was determined in comparison with both β-actin and 18S mRNA as well as with total mRNA and was reported as a percentage of the protein C and reference signal intensity of the nonpregnant adult pool.

Animal Methods

Columbia-Rambouillet pregnant sheep with fetuses of known gestational age were operated on at or after 70 d gestation. Surgery was performed under i.v. pentobarbital sedation (5 mg/kg) and tetracaine spinal anesthesia (6 mg in 10% glucose). Using standard operating techniques previously described (13, 19–21), 20- or 22-gauge polyvinyl catheters were placed into the maternal and fetal arterial and venous circulations. Maternal sampling catheters were placed into the femoral vein and artery. Fetal catheters were placed into the abdominal aorta, a femoral vein, and the left hepatic vein, as previously described (13). All catheters were tunneled s.c. and kept within a plastic pouch attached to the mother sheep's skin. All protocols were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center. After surgery, the sheep were allowed to recover at least 4 d before study.

Healthy chronically catheterized pregnant ewes were allowed an *ad libitum* diet of alfalfa pellets, water, and mineral supplements. Blood samples were collected from the ewe and

her fetus on one or more occasion. The catheters were first cleared by removing 3 mL of blood, and then 0.9-mL blood samples were drawn into 0.1 mL of 3.8% sodium citrate anticoagulant. The samples were processed and stored as described above. In all, 141 fetal samples and 111 maternal samples were obtained from 63 ewes and their fetuses. These data included 17 mother-fetal pairs who were sampled three times a week for 2–8 wk. Fetal hepatic catheters remained intact during spontaneous vaginal delivery at term. Fifteen fetuses were allowed to deliver, and samples were collected every 1–3 d from 2 wk before term to 3 wk postnatally to determine the concentration and molecular forms of protein C during the transition to extrauterine life.

Data Analysis

Normal values for human and ovine plasma protein C concentrations were derived from healthy populations as described above using means, 2-SD ranges, and observed ranges. Results of maternal and fetal ovine plasma protein C concentrations were plotted as a linear function of time from conception. Abundance of ovine maternal and fetal hepatic mRNA was plotted as a linear function of time from conception. Mean values for concentrations of plasma protein C concentration and hepatic protein C mRNA were compared using a one-tailed *t* test.

The transition of protein C molecular forms was determined by plotting the fetal and adult percentages of total plasma protein C as determined by densitometry. Results were plotted both as a linear function of time from conception and as time from birth by spontaneous vaginal delivery.

RESULTS

Ovine protein C purification and molecular forms. The purification of adult ovine protein C produced material that yielded a single band on silver stain, showed no activity for factors II, VII, IX, or X, and cleaved S-2366 after activation with either Agkistrobon contortrix contortrix or a complex of thrombin and thrombomodulin. This protein was used to raise a polyclonal antibody. Figure 1 shows Western blots of nonreduced and reduced ovine protein C as well as purified human protein C blotted with an appropriate antibody for comparison. On reduction, almost all of the ovine protein C was found to circulate as a two-chain molecule. Of note, 10% of human protein C consisted of a single-chain molecule, in contrast to a minimal amount of single-chain ovine protein C.

Fetal form of ovine protein C. Migration of protein C bands on nonreduced and reduced Western blots was measured, and molecular mass was determined in comparison with standards. Calculated results are displayed in Table 1. Purification of fetal ovine protein C yielded a protein with a molecular mass of 6 kD greater than that found in the adult form on nonreduced SDS-PAGE (Fig. 2). Increased molecular mass was seen in both the heavy and light chains.

Further characterization of ovine protein C. Adult and fetal ovine protein C were deglycosylated with *N*-glycanase. After removal of N-linked carbohydrate residues, both heavy and light chains showed increased migration. However, a portion of the unique fetal form persisted and was resistant to cleavage by up to 60 U/mL glycanase. Figure 3 shows the Western blot of reduced adult and fetal ovine protein C before and after treatment with 30 U/mL *N*-glycanase; Table 2 displays the molecular masses of these protein C components calculated from Figure 3.

Crossed immunoelectrophoresis performed in the absence of calcium showed a slight increase in relative migration of the fetal form to 108% of adult migration (Fig. 4). Crossed immunoelectrophoresis of a mixture of adult and fetal protein C showed complete identity of the two forms with the polyclonal antibody. Isoelectric focusing of ovine protein C showed a pI of 4.6 for the adult molecule and 4.45 for the fetal molecule.

Plasma protein C concentration in the sheep. Table 3 compares the level of protein C in ovine plasma with that found in the human. Although concentrations of protein C in the sheep are twice those in humans, the two species exhibit proportionate ranges and no differences related to sex or pregnancy.

There is no change in plasma protein C antigen concentration in pregnant ewes during normal ovine gestation ($p > 0.5$). The range of protein C concentration determined in the ovine fetus, 25–75% of normal ovine adult, is the same as that reported for the human neonate at term. Of note, when first sampled at midgestation, the ovine fetus had a concentration of protein C as high as that found at term. In fact, there was a trend toward lower concentrations of fetal protein C during the third trimester. After birth, plasma concentrations of protein C in the neonatal lamb were lower than that found in the fetus ($p < 0.001$); this decrease persisted for the 3 postnatal wk sampled in the study.

Hepatic expression of protein C mRNA. Figure 5 shows a representative Northern blot of fetal and maternal hepatic mRNA for protein C. Only one transcript for fetal ovine protein C was demonstrated, which was identical to that found in adult hepatic tissue and slightly smaller than 18S RNA. Abundance of mRNA for β-actin increased with gestational age and was thus not appropriate as a reference for protein C. mRNA for protein C from a pool of normal nonpregnant sheep showed similar results when compared with 18S or total RNA (ratio of results using calculations based on 18S RNA *versus* total RNA, 1.16). mRNA for protein C from a pool of 10 pregnant ewes showed similar results (ratio, 1.22). Protein C mRNA from 14 individual fetal ovine liver samples quantified in comparison with 18S RNA was slightly higher than when total RNA was used for comparison (mean ratio, 1.35; SD, 0.36), but no variation with gestation was noted. The following results were based on calculations in comparison with 18S RNA.

mRNA for maternal hepatic protein C was 108% of values for healthy nonpregnant sheep ($p > 0.5$). Figure 6 shows a scatter gram of mRNA for protein C extracted from fetal and neonatal ovine hepatic tissues. Of note, fetal hepatic mRNA for protein C was 40% of the mean maternal abundance from midgestation until 6 d before term, when protein C mRNA abundance increased to a mean of 110% of maternal abundance and remained there for the first 14 postnatal d sampled in this study ($p < 0.025$). Correcting for the 10% decrease in fetal

protein C mRNA results obtained using 18S RNA rather than total RNA as a control, the results would be 44% fetal hepatic mRNA abundance compared with 121% mRNA abundance around the time of birth.

Transition of ovine protein C from fetal to adult form. A fetal form of protein C with a 3-kD increase in molecular mass relative to the adult form was identified on Western blot using the polyclonal antibody. This fetal form represented all of the plasma protein C detected by our antibody up to 6 d before birth. By 4 d after birth, all ovine protein C identified was of the adult form. Figure 7 displays a Western blot showing progressive transition from the fetal to the adult molecular form around the time of birth. Figure 8 displays the densitometry results as a percentage of total protein C measured as the adult and fetal forms. The transition from fetal to adult protein C began 6 d before spontaneous onset of labor at term, showed 50% of each form approximately 12 h before birth, and was complete by 4 d after birth. The sharp demarcation in time of transition was not found when results were plotted against postconceptual age.

DISCUSSION

Protein C is a pivotal coagulation regulatory protein, which functions both in the limitation of coagulation activation and in the promotion of fibrinolysis. Severe deficiencies of protein C, which are either genetic or acquired, predispose to disseminated intravascular coagulation, purpura fulminans, and large-vessel thrombosis. Plasma concentrations of protein C 3 SD or greater below the mean value for term gestation are found in 30% of sick, preterm infants and are associated with an increased risk of neonatal thrombosis in these infants. The etiology of nongenetic severe protein C deficiency in preterm infants is currently unknown, although developmental immaturity is frequently invoked (22).

In this study, the form(s) of protein C expressed in plasma from the ovine adult was examined and compared with that found in the ovine fetus. The ovine model was chosen because work by Kisker *et al.* (10), Massicote *et al.* (11), and Moalic *et al.* (12) have determined that the development of plasma levels of coagulation proteins in the ovine fetus and neonate are similar to that of the human, suggesting that ovine gestation may be a reasonable model to study the ontogeny of coagulation proteins. In addition, the ovine fetus, which is usually single and similar in size to the human fetus, can tolerate the placement and maintenance of indwelling vascular catheters by midgestation. This quality permits investigations of the development of coagulation in a steady state without disturbance to the fetus.

The current studies determined a unique form of protein C in fetal ovine plasma characterized by a 4- to 6-kD increased molecular mass, more anodal migration on crossed immunoelectrophoresis, and slightly lower pI. All of this is compatible with increased glycosylation of the fetal ovine protein C molecule. On removal of carbohydrate residues with *N*-glycanase, the migrations of a portion of the fetal protein C heavy- and light-chain polypeptides remained unchanged.

These fetal characteristics of the ovine protein C molecule resemble fetal properties described for human fibrinogen and plasminogen and may result from altered posttranslational processing in the fetus. This is the first report of unique fetal processing of a vitamin K-dependent protein.

The chronically catheterized pregnant ovine model was used to examine the maturation of protein C in the ovine fetus. This model demonstrates a relationship between adult and neonatal levels of protein C that is similar to that found in humans. The sheep was determined to express a single protein C mRNA transcript in the fetus, which was identical to that found in pregnant and nonpregnant adults. These data contrast with those of Hassan *et al.* (23), who reported two protein C mRNA transcripts in human fetal liver tissue.

In the fetal lamb, plasma concentrations of protein C were equal to or higher than that of the term neonate from midgestation onward. Liver mRNA for protein C was extracted and measured to evaluate the hepatic regulation of protein C in the ovine fetus. Hepatic expression of mRNA was proportionate to plasma protein level through the second half of gestation up to 6 d before spontaneous onset of labor. The plasma concentration of protein C decreased after birth and remained decreased for the 3 wk of study. Postnatal abundance of hepatic mRNA for protein C was elevated. However, this did not result in continuously increasing plasma concentrations of protein C.

Increased mRNA for protein C associated with decreased plasma protein C concentration after birth suggests increased protein C turnover. Alternatively, the translational efficiency of protein C mRNA may be decreased by some as yet undetermined mechanism(s) after birth. These hypotheses remain to be investigated.

Transition from the fetal to the adult form of protein C was distinctly linked to the spontaneous onset of labor at term and preceded labor by 6 d. During the time of transition, the relative abundance of the adult form doubled every 24 h; there was no net change in the total plasma protein C concentration (both forms). The signal initiating this transition is currently unknown; however, the time is not exactly coincident with the corticosteroid burst occurring in the ovine fetus 48 h before onset of labor. Nonetheless, Kisker *et al.* (24) reported that betamethasone administration was associated with increased concentrations of fetal ovine coagulation factors II, V, VII, IX, and X; in these experiments, protein C was not examined. Antenatal administration of corticosteroids has a variety of maturational effects on the fetus. For example, betamethasone has been used to accelerate maturation of fetal surfactant production, thus preparing the human fetus for a premature delivery, which cannot be otherwise avoided (25). Therefore, examination of a possible role for corticosteroids in the prenatal maturation of protein C may prove of therapeutic interest.

The precise timing demonstrated for the transition of protein C from fetal to adult forms suggests that the response is highly regulated. Knowledge of the signals stimulating the appearance of adult protein C 6 d before onset of spontaneous labor may be critical for understanding, treating, and ultimately preventing protein C-related coagulopathies in human premature neonates.

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