Effect of Hormone-Induced Premature Parturition on Hemoglobin Switching in Sheep

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ABSTRACT. This study examines the effect of premature delivery on the switch from fetal ($\alpha_2 \tau_2$) to adult ($\alpha_2 \beta_2$) Hb, in lambs in which premature parturition was induced by the intrafetal infusion of ACTH or corticotrophin releasing hormone. Of 10 chronically cannulated ovine fetuses given ACTH at the rate of 79 ng/min for a 15 min period every 2 h starting at days 125 ($n = 9$) or 126 ($n = 1$) of gestation, three died *in utero* at days 131, 131, and 130, respectively. The remaining seven were born alive at 133 ± 1.6 days. Five control fetuses, treated with vehicle (0.9% NaCl, wt/vol) only were delivered at 149 ± 2.7 days of gestation, which is not significantly different from the duration of pregnancy in other chronically cannulated lambs in this flock. Hb switching, as measured by the globin synthesis ratio, β/α , was complete at term in the control lambs. The β/α globin synthesis ratio of the prematurely delivered lambs was not accelerated by birth, and was similar to that of control fetuses of the same gestational age still *in utero*. The four lambs surviving premature birth more than 70 days did not complete the switch to normal adult Hb until after 220 days postconception. (*Pediatr Res* 24: 688–692, 1988)

Abbreviations

CRH, corticotrophin-releasing hormone

For most of gestation sheep fetal red blood cells contain a fetal Hb, with two α and two τ globin chains. This fetal Hb has a higher oxygen affinity than does adult Hb with two α and two β globins (1, 2). There are two genetic variants of adult Hb in adult sheep (β^A and β^S) which differ in at least seven amino acids in the globin chain (3–5). Sheep may be homozygous for either or heterozygous. In those animals carrying the β^A gene a third type of Hb (β^C) is produced when sheep are made anemic (6, 7). This variant, linked to the β^A gene differs from β^A in having 141 rather than 145 amino acids in the globin chain, and a minimum of 18 substitutions in amino acid structure (3, 5).

The switch from fetal to adult Hb commences approximately 4 wk before term (142–152 days) and is complete by 170 days postconception at the latest (1, 2, 8, 9). When the capacity of reticulocytes to synthesize globin chains is assessed the switch is complete at term, but it is approximately 2 wk before all fetal

Hb disappears from neonatal blood (9). It was reported that in lambs carrying the β^A gene a small proportion of β^C (<15% of total β) appears transiently after birth and persists for several months (10). Many attempts have been made to influence the time of onset of the switch from fetal (τ) to adult (β) Hb but these have been largely unsuccessful (8, 9, 11, 12). Once commenced, the rate of the switching process has been shown to be able to be modulated. Hypophysectomy or bilateral adrenalectomy of the chronically cannulated fetus causes the completion of the switch to be delayed considerably (8, 9). In the adrenalectomized fetus replacement of cortisol in gradually increasing amounts returned the rate of adult Hb synthesis to normal (9).

An increase in fetal cortisol production precedes and indeed induces parturition at normal term in sheep (13). Premature parturition can be induced by the treatment of immature ovine fetuses with cortisol, ACTH, or CRF some 3 wk or more before normal term (14, 15). These fetuses show accelerated lung maturation. The purpose of this study was to investigate the effect of premature activation of the fetal adrenal on Hb switching in the ovine fetus. The hypothesis was that premature parturition, induced by the fetal infusion of ACTH or CRF, would accelerate the completion of the switch from fetal to adult Hb.

MATERIALS AND METHODS

A total of 54 blood samples (1 ml) from 20 fetuses chronically cannulated as described previously (16, 17) was used to establish α , β , and τ globin synthesis in normal intact animals from 103 days of gestation until term. Then 39 samples were collected from birth until 30 days postpartum, and three samples from one older lamb. Five of these fetuses received an infusion of sterile isotonic saline (0.9% NaCl) from day 125 to 135 of gestation, in a protocol identical to that used for ACTH infusion. In 11 other animals an attempt was made to induce premature parturition by the intrafetal infusion of ACTH or CRH. Starting on day 125 ($n = 9$) or 126 ($n = 1$) of gestation, synthetic ACTH_{1–24} was infused as a 15-min pulse (79 ng/min at the rate of 1.9 ml/h) every 2 h into the jugular veins of chronically cannulated fetuses until spontaneous parturition or fetal death *in utero* occurred.

Samples were also collected from one fetus or lamb delivered prematurely as the result of continuous intravenous infusion of ovine CRH, 2.4 μ g/h, as reported previously (15). A total of 19 samples was collected from these fetuses up until the time of birth. Then 46 samples were collected from the four surviving lambs over another 70–90 days. As a daily routine for all animals 1 ml of fetal arterial blood was withdrawn for the measurement of blood gases and hematocrit. A total of 8 ml blood was drawn daily during the hormone/saline infusion period for hormone assays (18). Fetal urine was drained from a bladder cannula into a sterile container for measurement of urine osmolality.

Globin synthesis/analysis. In all fetal and neonatal samples in

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which reticulocytes were present globin synthesis was measured; in those samples in which no reticulocytes were present the actual globin chains present in the sample were quantitated, after separation, by densitometry. All samples from one animal were analyzed by the same globin separation technique. Approximately equal numbers of both control and experimental samples were analyzed by each method. All samples were incubated with [^3H]leucine (0.05 or 5.0 mCi, Amersham International, Bucks, U.K.) for 2 h at 37° C, washed with 0.9% (wt/vol) NaCl and processed immediately or stored at -80° C. The higher concentration of [^3H]leucine was required in the samples processed by the gel method (see below) to obtain adequate counts in the smaller aliquots of globin run in this system. When globin chains were to be separated by ion-exchange chromatography, hemolysates were prepared by the addition of distilled water, and stroma was removed by centrifugation at 3000 rpm for 5 min at room temperature. Globin was prepared from stroma-free hemolysates by acid-acetone precipitation (19). In these samples the globin chains were separated by ion-exchange chromatography on CM-52, using a Na_2HPO_4 -urea buffer system at pH 6.7 (20). The majority of the globin chain separation was done by a modification of the method of Alter *et al.* (21) by electrophoresis on 0.75-mm thick mini slab-gels (Bio-Rad Laboratories, Cambridge, MA). The samples were lysed and loaded in PAPS buffer (0.5 mg Pyromin Y; 1.74 ml deionized 8 M urea, 200 μl 2-mercaptoethanol; 60 μl Nonidet P-40). The 10% resolving gel consisted of 1.67 ml acrylamide: DATD T60%: C14.5%; 0.5 ml acetic acid; 7.38 ml 8 M deionized urea; 0.2 ml Triton X-100; 0.265

ml deionized water. After degassing for 10 min 125 μl of TEMED and 15 μl 60% freshly prepared ammonium persulfate were added. The gels were poured, overlayed with electrophoresis buffer (5% acetic acid) and allowed to polymerize overnight. The next morning the overlay was discarded, the well former inserted, and a stacking gel of 4.9% was poured and allowed to polymerize for 1 h. The well former was removed, the wells carefully rinsed with 5% acetic acid, and preelectrophoresis carried out with the anode at the top for 60 min at 150 V. The anode buffer was removed, the wells rinsed as above and filled with 10^{-1} M 2-mercaptoethanol, fresh electrophoresis buffer was added, and the second preelectrophoresis carried out for 60 min at 100 V. The anode buffer was again discarded and the wells were thoroughly rinsed with 5% acetic acid before the samples (5 μl lysate) and standards were applied to the appropriate wells. On addition of fresh anode buffer electrophoresis was performed at 30 mA until the dye marker reached the resolving gel, and then at 60 mA to the bottom of the gel. Electrophoresis was continued for an extra 5 min. The gels were stained with 0.5% Serva Blue G for 30 min and destained in 7% acetic acid/30% methanol. The globin bands were cut out and dissolved in 500 μl 2% periodic acid overnight on a shaking platform. A total of 10 ml of Hionic-fluor scintillation fluid was added to each sample and counting was performed on a Packard beta counter for a minimum of 10 min or until 10,000 cpm accumulated. Hionic-fluor was obtained from United Technologies Packard, acetic acid and Nonidet P-40 from BDH, methanol from Mallinckrodt Inc., St. Louis, MO, and all other reagents from Bio-Rad.

Fig. 1. *Upper panel*, pattern of globin separation in the gel-electrophoresis method described in the text. 1, standard sample of sheep blood from a fetus heterozygous for β^A , β^B . 2, seven samples from one prematurely delivered lamb at (2) 154, (3) 164, (4) 168, (5) 178, (6) 185, and (7) 220 days postconceptional age. As τ globin disappears β^A globin is completely replaced by β^C which is still present in significant amounts at day 220. 8, sample from fetus homozygous for β^B at 149 days postconception. *Lower panel*, globin separation in a lamb, heterozygous for $\beta^A\beta^B$, born at normal term (150 days) at (1) 210, (2) 189, (3) 175, (4) 169, (5) 164, (6) 164 days postconception. In well 6 half as much sample was loaded as in well 5. In well 7 the standard sample is run. All τ had disappeared by this time and β^C appeared transiently between days 164 and 189.

Statistics. The values for experimental and control fetuses/lambs were analysed by the unpaired Student's *t* test.

RESULTS

Validation of method. The separation of the various sheep globins by electrophoresis are shown in Figure 1 for 2 sheep, heterozygous for β^A and β^B . The upper panel shows the emergence and persistence of the β^C globin in the prematurely delivered lamb, while the lower panel shows the much more restricted expression of β^C in the normal term lamb.

Validation of the modified electrophoretic gel method as a useful globin protein assay was achieved by comparing the ratios of $\tau/(\tau + \beta)$ and $(\tau + \beta)/\alpha$ in the standard Clegg assay and the new method. Table 1 shows the results for six samples between the two methods.

The reproducibility of the new assay was tested by assaying the one sample six times. The mean value of the $\tau/(\tau + \beta)$ ratio was 0.70 and the coefficient of variation was 4%. The mean value of the $(\tau + \beta)/\alpha$ ratio was 0.98 and the coefficient of variation was 5%. In all assays results were not used unless the non- α - to α -globin ratio was in the normal range, 0.8–1.2, as described previously (9).

Outcome of pregnancies. Of the 10 fetuses infused with ACTH, eight were born alive 178 ± 14 h after the commencement of the infusion, at 133 ± 1.6 days of gestation. The mean duration of pregnancy in this flock, for chronically cannulated fetuses is 149.6 ± 2.7 days (22). Thus parturition in this study occurred 2–3 wk prematurely. Three fetuses died *in utero* on days 130, 131, and 131, respectively, of cord constriction. Of the live-born lambs four died at days 133–145 postconception of unknown causes. Three survived for more than 4 months, and two are currently alive. One lamb was born due to CRF infusion, at 135 days of gestation and survived for more than 4 months (15).

Birth weight of the premature lambs was 2.2 ± 0.1 kg which was significantly less than that of normal full-term lambs, 3.1 ± 0.2 kg ($p < 0.01$).

During the period of ACTH/vehicle infusion the blood gases and urine osmolalities of all fetuses *in utero* were in the normal range for unstressed fetuses in this laboratory (23). For the fetuses receiving ACTH the values were — pH 7.0 ± 0.05 , $p\text{CO}_2$ 42.5 ± 2.7 mm Hg, $p\text{O}_2$ 24.4 ± 1.1 mm Hg, hematocrit $27 \pm 4\%$, and fetal urine osmolality 119 ± 14 mosmol/kg water. These values are the mean $\pm$ SD of the average daily values of 10 fetuses.

Arterial cannulae were not generally maintained in the lambs after birth, as the lambs remained with the ewes, and the risk of accidental hemorrhage was significant. In one prematurely delivered lamb and one control lamb blood gases were available for a restricted period. At postconception ages 154–176 days the $p\text{O}_2$ was 67.6 ± 6 mm Hg in the premature lamb, compared with 77 ± 7 mm Hg in the normal term lamb. In the age range 186–203 days the $p\text{O}_2$ values for the premature and control lambs were 80 ± 8 and 85 ± 9 mm Hg, respectively.

In Figure 2 there is shown the degree of adult Hb synthesis, as characterized by the β/α globin ratio for the control group and

Fig. 2. Normal increase in adult Hb as measured by the β/α globin synthesis ratio in control fetuses (hatched area) and the ratios of fetuses treated with ACTH/CRH in the (premature) perinatal period.

the prematurely delivered group about the time of delivery, or fetal death *in utero*. The shaded area depicting the normal range was drawn from the mean $\pm$ 2 SD. It can be seen that the values of the ACTH-treated fetuses lie predominantly within the normal range. The mean value of the β/α ratio for these animals at 130–140 days postconception was 0.45 ± 0.24 ($n = 9$) which was not significantly different from that of the control fetuses till in utero at that age, 0.34 ± 0.14 ($n = 10$). Both of these values were significantly lower than the β/α ratio at birth in the control animals, of 0.94 ± 0.18 (11).

Of the four fetuses which survived for a prolonged period after premature parturition, two were homozygous for β^A and two were homozygous for β^B . The extent of adult Hb synthesis after birth is shown by the β/α globin ratio in Figure 3. It can be seen that the switch to full adult Hb synthesis was not completed at the normal time. In the two β^B lambs the fetal τ globin persisted for an extended period. There was an unusual oscillation in the amount of adult Hb. In the two β^A lambs the τ globin synthesis ceased but abnormally high levels of β^C globin were found. The proportion of β^C , as a percentage of all β globin, is shown in Figure 4. It can be seen that β^C was present from 130–220 days postconception, in amounts from 2–50%. In contrast, in 28 samples from heterozygous lambs born at normal term, β^C appeared in only 10 samples, from 158–189 days, and never exceeded 3% of the total β globin.

DISCUSSION

The modified method for separation of globin chains described in this paper has the following advantages over the method of Alter *et al.* (21). Better separation of the various globin chains of sheep is possible; the amount of sample that is required is 1 μg , allowing multiple analyses of the one hemolysate; the gel is readily solubilized for counting of the ^3H -globin in a liquid scintillation spectrometer. Reproducibility is acceptable, and the accuracy is similar to that obtained using the ion exchange column (19, 20) which requires much larger amounts (mg) of globin for analysis.

In all fetuses the initiation of β -globin synthesis had occurred before the ACTH or CRH infusion began. This was both expected and desirable as it has been shown previously that the absence or presence of cortisol did not affect the onset of the

Table 1. Comparison of globin synthesis ratios by two methods*

Sample	$\tau/(\tau + \beta)$		$(\tau + \beta)/\alpha$	
	A	B	A	B
1	0.94	0.86		1.23
2	0.96	0.87	1.17	1.24
3	0.93	0.84	1.20	1.18
4	1.00	0.80	1.10	1.01
5	0.28	0.34	1.05	1.16
6	0.06	0.12	0.80	0.99

* Globin synthesis ratios in six individual blood samples from fetal sheep determined by A, the ion exchange method (19, 20) and B, by the electrophoresis method described in "Materials and methods."

switch from fetal to adult Hb (9). The onset of switching seems to be related to a developmental program inherent in the erythropoietic cell line (12). This may be conditioned by the number of cell divisions that stem cells undergo. The slightly accelerated onset of switching seen when fetal erythropoietic tissue was transplanted into irradiated adult sheep has been explained by the increased rate of red cell production which occurs in such conditions (11, 12).

Although the premature birth of some viable lambs could be achieved by the protocols used there was no acceleration of the completion of the switch from fetal to adult globin synthesis.

Fig. 3. Percent adult Hb as determined by the $(\beta^A + \beta^B)/(\beta + \tau)$ ratio (for lambs P and S) and by the $\beta^B/(\beta + \tau)$ ratio for lambs K₁ and K₂. These four lambs were born prematurely as the result of hormone treatment as described. The normal adult ratio of 100% was not achieved until more than 220 days postconceptional age.

The cortisol values rose to ones similar to those seen in normal term fetuses (MacIsaac RJ, personal communication), although over a shorter time period and at an earlier postconceptional age. There are a number of reasons why the switch might not be accelerated. As sheep erythroid progenitors require 3–6 days for maturation the period of 6–8 days of ACTH infusion may have been too short to see the effect. However, inasmuch as globin synthesis rather than content was measured, this is unlikely to be a major problem.

For a steroid such as cortisol to exert a biologic effect on gene expression there must be receptors in the target tissue, the inducible genes must be contained in DNAase-sensitive domains, and appropriate interactions between the chromosomal gene and the nuclear matrix appear essential (24, 25). Receptors for ³H-dexamethasone, but not for aldosterone, have been found in the bone marrow and liver of fetuses at 123–130 days of gestation (Wintour and Funder, unpublished results). In midgestation only the τ gene is in the conformation associated with active transcription, whereas both β and τ genes are in the DNAase-sensitive conformation in the bone marrow of newborn lambs (26). As β -synthesis had begun before the hormone infusions started, presumably the β -gene was in the appropriate conformation by this time. As the τ -gene remains in the active conformation the possibility exists that glucocorticoids can also affect translation of the globin mRNA, as demonstrated for α - and β -genes in differentiating Friend erythroleukemia cells (27, 28). Even if the previously demonstrated effect of cortisol is not on the globin genes directly but on some intermediate cell that produces a trans-acting factor, these arguments still apply.

It has been shown that chromatin structure in the region of the three goat β -globin genes, β^F , β^C , and β^A , changes in a manner which parallels their developmentally regulated expression (29). Further mapping of the fine structure of the chromatin of the goat β^F gene has delineated two further sites which differ in accessibility to specific restriction enzymes from corresponding sites on the goat β^C and β^A genes which were not expressed in fetal erythropoietic liver tissue (30). More studies are required in the fetal sheep erythroid tissues to help us to understand the possible time of action of trans-acting factors that may regulate the extent of expression of fetal and adult globin genes.

In the four premature lambs that survived for prolonged periods after birth normal adult Hb synthesis was not found until more than 220 days postconception. In the two lambs of the β^B genotype the τ -globin persisted for a prolonged period. The unusual oscillation of the amount of adult Hb may reflect the instability of the gene expression when it has lost the normal

Fig. 4. Percentage of β^B of the non- α -globin in two fetuses, heterozygous for $\beta^A \beta^B$, born prematurely as the result of hormone treatment as described. β^C was present at more than normal levels for an extended period after birth.

modulating effect of cortisol. In the two lambs of the β^A genotype τ -globin synthesis ceased but there was expression of the β^C gene at abnormally high levels for an abnormally long period of time. It is of interest that the order of decreasing oxygen affinity of sheep Hb is Hb-F, Hb-A_C, Hb-A_B, and Hb-A_A (31). Thus the premature lambs all expressed a Hb of higher than normal oxygen affinity in the perinatal period. Wardrop *et al.* (32) described the clinical features of tachycardia, tachypnea, dyspnea, and feeding difficulties in 53% of babies born at less than 32 wk of gestation. They ascribe these findings to the high oxygen affinity of the fetal Hb in these babies.

These experiments are being pursued because they may provide more evidence that exposure of the fetal lamb to an increasing concentration of cortisol at a critical time (135–145 days) is crucial for the normal completion of the Hb switch. The concept of critical time periods is one familiar to developmental biologists. So far there has been one report on a "time" gene. In mice a linkage has been found between a single gene (Prn-i) that affects scrapie incubation period and the prion which is the infectious agent (33).

Endocrine disturbances that affect the rate of Hb switching in man have also been described. Babies born to diabetic mothers had a mean β -globin synthesis of 14.8% at 36–38 wk of gestation compared with 44.6% in babies of healthy mothers, at the same gestational age (34). A significantly higher than normal proportion of fetal Hb has been reported in babies, more than 55 weeks postconception, who have died a sudden infant death or cot death (35). These clinical studies emphasize the importance of understanding the factors modulating the expression of globin genes.

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