Ontogeny of Erythropoietin Gene Expression in the Sheep Fetus: Effect of Dexamethasone at 60 Days of Gestation

By Gaik Bee Lim, Kandiah Jeyaseelan, and E. Marelyn Wintour

We have used competitive reverse transcription and polymerase chain reaction (RT/PCR) to compare the levels of erythropoietin (Epo) mRNA in the liver and kidneys of the sheep fetus at 60, 80, 100, 130, and 140 days of gestation (term = 145 to 150 days). The effect of dexamethasone infusion in the ewe on Epo gene expression in the 60-day fetus was also investigated. Epo mRNA levels were highest at 60 days of gestation, the earliest age studied, in both liver and kidney. In the liver, Epo mRNA expression declined as gestation proceeded. Kidney Epo mRNA was maintained at a high level until 100 days of gestation, declining significantly in the 130-day fetus (P < .01). Treatment of ewes carrying 60-day fetuses with 0.76 mg/h dexamethasone for 48 hours resulted in a significant decrease in fetal plasma Epo values and Epo mRNA levels in both the liver and kidney. In the dexamethasone-treated fetuses, Epo mRNA in the liver was 52% of control values (P < .05), and in the kidney, 33% of control (P < .001). The results suggest that the kidney may play a more important role as a site of Epo synthesis in the early gestation sheep fetus than previously thought. Glucocorticoids may have a role in the regulation of Epo gene expression.

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MATERIALS AND METHODS

Experimental Animals

All experimental and surgical procedures had been given prior approval by the Institute’s Animal Experimentation and Ethics Committee.

To determine whether Epo mRNA was distributed evenly throughout the liver, preliminary studies were performed on several fetuses of 100 to 140 days of gestation, as well as two fetuses (120 and 140 days of gestation) that had been chronically cannulated for 20 to 40 days and had become hypoxic for several days before the fetus was killed.

For ontogeny studies, ovine fetuses of various gestational ages were used. These were grouped into 60-, 80-, 100-, 130-, and 140-day age groups, with four animals in each group. In the dexamethasone studies, five animals from the 60-day group were used as controls, and another five for the dexamethasone infusion. Twin fetuses were treated as independent animals.

Ewes were killed by an overdose of sodium pentobarbital (Lethabar, Arnolds of Reading, Boronia, Victoria, Australia). Fetal livers and kidneys were removed and snap frozen in liquid nitrogen. Blood samples were taken for plasma Epo assay. Epo levels were determined by radioimmunoassay.

Dexamethasone Treatment

Five ewes were infused with dexamethasone (Decadron, Phosphate Shock Pak; Merck, Sharpe, and Dohme, Pty Ltd, Australia) at 0.76 mg/h for 48 hours. Controls were infused with saline at 0.38 mL/h for 48 hours.
EPO GENE EXPRESSION IN OVINE FETUS

Competitive Reverse Transcription / Polymerase Chain Reaction (RT/PCR) assay

<table>
<thead>
<tr>
<th>total RNA + cRNA standard</th>
<th>co-reverse transcription</th>
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<tr>
<td>cDNA</td>
<td>polymerase chain reaction</td>
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<tr>
<td></td>
<td>30 cycles</td>
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<tr>
<td></td>
<td>[5'-32P]dCTP</td>
</tr>
<tr>
<td>amplified product</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>quantitation of radioactive bands</td>
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BAS 2000 Bio-imaging analyzer

Fig 1. RT/PCR protocol for the determination of Epo mRNA levels.

RNA Preparation

Total RNA was prepared by the guanidinium isothiocyanate/cesium chloride centrifugation procedure. Frozen tissue was weighed and homogenized in 5 x volumes of 4 mol/L guanidinium isothiocyanate with 0.1 mol/L β-mercaptoethanol, 5 mMol/L TRIS Cl and 1.2% N-lauryl-sarcosine using an Ultrasomix homogenizer (Jenke and Kunkel, Staufen, Germany). Total RNA was isolated by centrifugation through 5.7 mol/L CsCl, resuspended in TE buffer (10 mMol/L TRIS HCl pH 7.5, 1 mMol/L EDTA) with 0.1% sodium dodecyl sulfate and precipitated in ethanol. The RNA was recovered in TE buffer and concentrations were determined by spectrophotometry. Total RNA was stored in ethanol at -70°C. Aliquots of RNA were electrophoresed in a 1% agarose/formaldehyde gel to check their integrity.

Competitive Reverse Transcription and Polymerase Chain Reaction (RT/PCR)

A schematic for the protocol is shown in Fig 1. RT. Total RNA (10 or 20 μg) was reverse transcribed together with 10 or 20 ng of a synthetic cRNA internal standard using an Epo-specific antisense primer (5' CGTGACAGCCTTGCACACCAGCAGCGCCACG 3') (50 ng/μL) in 1 x PCR buffer (10 mMol/L TRIS HCl pH 8.3, 50 mMol/L KCl and 1.5 mMol/L MgCl2), 1 mMol/L dithiothreitol, 0.2 mMol/L deoxynucleotide triphosphate (dNTP), 8 U of RNasin (Promega, Madison, WI) and 4 to 5 U avian myeloblastosis virus reverse transcriptase (Promega) in a total volume of 20 μL. Reverse transcription was performed at 42°C for 1 hour and terminated by heating at 94°C for 10 minutes.

PCR. A PCR cocktail mix consisting of 1 x PCR buffer, 100 μMol/L of each dNTP, 100 ng each of antisense and sense (5' ATCTGTGACAGCCAGTCTGGAGAGGTAC 3') primers, and 1.5 U Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT) was added to the RT product to a total volume of 50 μL. PCR was performed on a Perkin Elmer-Cetus thermal cycler for 30 cycles after an initial denaturation step at 94°C for 5 minutes. The cycle conditions were: 1 minute denaturation at 94°C, 1 minute annealing at 55°C, and 1 minute extension at 72°C. There was an extension segment of 10 minutes at 72°C after the final cycle.

Separation of bands and quantitation. Samples were electrophoresed on 6% non-denaturing polyacrylamide gels in TBE buffer (0.09 mol/L TRIS-borate, 0.002 mol/L EDTA, pH 8.0). The gels were then dried and exposed on a phosphoimaging plate. Radioactivity was quantitated on a Fuji BAS2000 Bio-Imaging Analyzer (Berthold Australia, Bundooora, Victoria, Australia), and the amount of Epo mRNA present in the sample was determined by calculating the ratio of the sample to the standard in the same lane. To validate the data obtained from the bio-imaging analyzer in initial experiments, bands that had been quantitated on the analyzer were subsequently excised and counted in a Packard 1900CA Liquid Scintillation Analyzer (Canberra Packard, Mount Waverly, Victoria, Australia).

Statistical analysis. All samples for the ontogeny studies were quantitated five times. Mean values of the sample to standard ratio were obtained for each animal. The values of the 4 animals in each age group were analyzed by a one-way analysis of variance to establish if a difference occurred over age. Tukey-Kramer post hoc test was used for pairwise comparison between different ages. The effect of dexamethasone treatment, for which experiments were performed four times, was assessed by unpaired Student's t-test. The SYSTAT statistical analysis program (SYSTAT Inc., Evanston, IL) was used.

One sample was analyzed five times in one assay to calculate intra-assay coefficient of variation. Interassay coefficients were obtained for each sample in five assays.

RESULTS

Epo mRNA Expression in the Liver

Portions of liver representing the central and peripheral portions of the right and left lobes of the liver were taken from a number of normal and hypoxic fetuses to determine whether there was any difference in Epo synthesis in these regions. Epo mRNA was expressed in all sections of the liver (data not shown) despite the unequal oxygenation states of the different lobes. There were no differences between the amounts of Epo mRNA in the various sections. Subsequently, liver sections were taken from the central portion of the liver.
Ontogeny of Epo mRNA Expression

Table 1 shows body weights of fetuses and weights of livers and kidneys at different gestational ages. Epo mRNA in the liver was highest at the 60-day period (Fig 3). By 80 days, Epo mRNA levels had decreased significantly ($P < .05$). From 80 days onward, there was a gradual decrease in the amount of Epo mRNA present per microgram of total RNA. In the kidney (Fig 4), Epo mRNA was expressed at a high level from 60 to 100 days of gestation. There was a significant drop in expression only in late gestation, in the 130- and 140-day-old fetal kidneys when compared with the 60-day kidneys ($P < .01, P < .001$, respectively).

Relative contributions of the kidney and liver. The Epo:standard ratio appears to be much higher for the kidney compared with the liver, by about one order of magnitude. However, it has to be noted that the liver is on average four to five times the mass of the kidney. The average weights of the two organs at different ages is shown in Table 1. In addition, the amount of total RNA

![Image of a graph showing correlation between BAS 2000 Phosphorimager and liquid scintillation counting.](image-url)

**Fig 2.** Correlation between results from the BAS 2000 Bio-imaging analyzer and liquid scintillation counting.

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recovered per gram of liver tissue is at least twice that of the kidney. Thus, the liver also makes a large contribution to the total Epo synthesis.

The amount of total RNA synthesized by a given cell may change as a function of gestational age. In addition, throughout most of gestation, the liver functions primarily as a hematopoietic organ, and much of the liver mass consists of hematopoietic tissue. The population of parenchymal cells, the site of hepatic Epo synthesis, makes up a smaller proportion of liver tissue in relation to its weight compared with later in gestation, so estimating the output of individual cells in the liver based on its weight may underestimate the actual output of the cells in the younger fetus.

**Dexamethasone Infusion Experiments**

Dexamethasone infusion of the ewe led to the complete suppression of endogenous plasma adrenocorticotrophic hormone values in the fetus. Plasma Epo levels were significantly lower ($P < .01$) in the dexamethasone-treated fetuses, being $9 \pm 0.4$ mU/mL ($n = 5$) in control and below the limit of detection of the assay (3 mU/mL) in dexamethasone-treated fetuses. There was a marked effect on the expression of the Epo gene (Fig 5). Epo gene expression in dexamethasone-treated animals was decreased to 33% and 52% of control values in the kidneys and livers, respectively. Both values were highly significant ($P < .001$ and $P < .05$, respectively).

**DISCUSSION**

*Expression of Epo mRNA in the Sheep Fetus*

It has long been thought that, in the sheep fetus, the liver is the main source of Epo until late in gestation. Zanjani et al. reported that in the sheep, the kidney began synthesizing Epo at about 120 days of gestation (term = 150 days) and was the main organ of synthesis by 40 days after birth. However, these studies involved organ ablation as well as hemorrhage to stimulate Epo production to levels detectable by bioassay. In this study, the RT/PCR method enabled study of Epo gene expression in uninduced, intact fetuses. We found that both the kidney and liver expressed Epo mRNA at a relatively high level at earlier ages, thereafter dropping to the low levels prevalent in the adult. At 60 days, Epo mRNA levels were relatively high in both organs. In the liver, Epo mRNA levels began dropping by 80 days, whereas in the kidney, Epo mRNA concentrations were relatively constant until the 300-day period before decreasing markedly by 130 days.

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Kidney Weight (g)</th>
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<tr>
<td>58-66</td>
<td>70 ± 25</td>
<td>5.2 ± 0.7</td>
<td>1.0 ± 0.1</td>
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<tr>
<td>78-81</td>
<td>252 ± 1</td>
<td>15.5 ± 0.5</td>
<td>2.8 ± 0.4</td>
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<tr>
<td>100-104</td>
<td>960 ± 30</td>
<td>45.6 ± 3.6</td>
<td>11.5 ± 1.5</td>
</tr>
<tr>
<td>126-130</td>
<td>2,720 ± 73</td>
<td>74.1 ± 3.6</td>
<td>19.0 ± 0.7</td>
</tr>
<tr>
<td>139-141</td>
<td>3,770 ± 330</td>
<td>90.6 ± 10.0</td>
<td>23.3 ± 1.8</td>
</tr>
</tbody>
</table>

Number of animals used for each group was $n = 4$, and figures represent mean ± SEM values.
In the postnatal rat, Eckardt et al. have shown that both liver and kidney contain Epo mRNA two days after birth. Epo mRNA levels, determined by ribonuclease protection, thereafter declined with increasing age to reach adult levels by 28 days after birth.

Correlation with plasma Epo levels. In this study, we found that plasma-immunoreactive Epo concentrations, at 60 to 65 days of gestation were 9.0 ± 0.4 mU/mL. These are not significantly different from those that we previously reported in chronically cannulated fetuses at 110 to 125 days of 11.4 ± 3 mU/mL. They are higher than values found in chronically cannulated fetuses close to term, 5.2 ± 0.4 and 6.1 ± 2.3 mU/mL, but lower than values in adult sheep, 15 ± 4 mU/mL. Epo mRNA levels are higher in the 60-day fetus than in fetuses close to term and adult sheep. Analogous studies in the rat showed that the ratio of serum immunoreactive Epo to total Epo mRNA increased with the age of the animal. This may reflect a difference in the efficiency of translation of the mRNA, or a difference in the half-life of the hormone. Studies on Epo pharmacokinetics in fetal and neonatal sheep showed increased distribution and clearance rates of the hormone in the fetus and neonate compared with adults.

Another possible reason for the low plasma Epo levels in relation to Epo mRNA levels is that Epo produced in the liver may be acting in a paracrine fashion. Because the liver is the site of erythropoiesis during the time that Epo is synthesized by the liver, Epo would be cleared by the target cells as well as by the liver itself, and would not appear in the general circulation.

Organ ablation studies. Earlier studies in which kidneys were removed and plasma Epo measured after hemorrhage suggested that the liver was the sole source of Epo until 120 days of gestation. However, this study was conducted with a bioassay that may have detected factors other than Epo because the values in control animals were at least 80-fold that of Epo values measured by a sensitive and specific radioimmunoassay in our previous studies. Our results clearly show that the kidney contains Epo mRNA from much earlier in gestation. However, it is not known with certainty if the Epo message is translated with similar efficiency in both kidney and liver. It is likely that studies based on organ ablation may not reflect the situation in the intact animal. The liver may be able to compensate for the absence of kidneys when stimulated by hemorrhage. When chronically cannulated fetuses at 110 to 125 days of gestation were bled to produce a decrease in hematocrit from 29% to 20%, Epo mRNA increased in both liver and kidney (manuscript in preparation). Lack of response by the liver to hemorrhage after 120 days of gestation may be caused by a change in
the sensitivity of the liver to hemorrhage at this age — this proposal is under investigation currently. Previous studies have shown that a hemorrhage of 20% of the blood volume is more effective in increasing plasma Epo concentrations in early- rather than late-gestation ovine fetuses. 18,19

Other species. In the rat,17 livers of animals exposed to normobaric hypoxia had increased levels of Epo mRNA in the first 2 weeks of life, whereas the kidneys did not respond to the same stimulus until 2 weeks after birth. Thereafter the kidney was the main source of Epo mRNA in response to normobaric hypoxia. Carbon monoxide administration led to high levels of Epo mRNA in both liver and kidney at all ages studied. In earlier studies in the rat that involved organ ablation and stimulation of Epo synthesis, the apparent switch from the liver to the kidney as the major organ of Epo production was at about 4 weeks. A similar compensatory mechanism may have been operating in the nephrectomized and hemorrhaged sheep fetus.5 Clemons et al.,22 measuring serum-immunoreactive Epo in fetal and neonatal rats, found intact rats that the switch from liver to kidney production was accelerated by hypoxia. Thus, the switch may merely reflect the change in relative sensitivity of the two organs to stimulation of the Epo gene.

Dexamethasone Infusion Studies

Dexamethasone clearly had a marked effect on Epo mRNA levels in both the liver and kidney of the intact 60-day fetus. Glucocorticoids have been shown to affect erythropoiesis in vitro, both potentiating23 and suppressing24 it, and dexamethasone directly interacts with the erythroid transcription factor GATA-1 via the glucocorticoid receptor.25 Negative regulation of the Epo gene itself may represent another mechanism by which dexamethasone inhibits erythropoiesis in vivo.

The decrease in Epo gene expression with dexamethasone was greater in the kidney compared with the liver. This may be, at least in part, caused by the diuretic effect of dexamethasone in the fetal kidney,26 giving rise to a large increase in urine output. Previous studies with cortisol infusion showed that the diuresis caused by glucocorticoids is mediated by a decrease in proximal tubular reabsorption,27 which would decrease oxygen consumption, and therefore, raise local oxygen tension in the cells synthesizing Epo. Studies using diuretic drugs that preferentially inhibit sodium absorption at different regions of the nephron, indicate that proximal tubular function affects Epo production.28

Steroid receptor half-sites have been identified on oxygen-responsive elements of the human Epo gene, a 53-bp promoter region, and a 3' enhancer region of 43 bp.29 These half-sites were found to be necessary for the function of the enhancer, but did not appear to be affected by steroid hormones. Artificial constructs of the 3' enhancer coupled to the luciferase gene transiently transfected in Hep3B cells failed to show any response, either in the uninduced or induced state, with various steroid hormones, including dexamethasone, at physiologic concentrations. More recently, glucocorticoid response elements have been reported several kilobases upstream of the human Epo gene.30 However, there is as yet no information as to whether these sites exist in the sheep. Dexamethasone may be acting directly on the Epo gene or by an indirect mechanism.

ACKNOWLEDGMENT

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