The Erythropoietin Gene Is Expressed Strongly in the Mammalian Mesonephric Kidney

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In the ovine fetus at 41 days of gestation (term is 150 days), there are two sets of kidneys, mesonephroi and metanephroi. We have examined the expression of the erythropoietin (Epo) gene in both types of kidneys by competitive reverse transcriptase-polymerase chain reaction and hybridization histochemistry and compared the expression to that of the 60-day fetal metanephros. At 41 days, the Epo gene was expressed in both mesonephros and metanephros, as well as in the fetal liver. The cells expressing the Epo gene in the mesonephros were interstitial cells in the vicinity of the proximal tubules.

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N THE OVINE fetus, the first functional kidney is the mesonephros, which begins excretory activity by day 18, reaches its maximum development by days 27 to 30, and regresses over days 40 to 57, where the term is ≈150 days.1 The metanephric kidney starts to develop with the budding of the metanephric duct or ureteric bud by days 27 to 30 and becomes the permanent kidney, with nephrogenesis being complete by ≈130 days of gestation.4

It has recently been found that the metanephros expresses the gene for erythropoietin (Epo) from as early as day 60 of gestation in the ovine fetus and that renal Epo gene expression can be increased by hemorrhage and decreased by glucocorticoid treatment.1,2 The fetal liver also expresses the Epo gene to a greater extent than does the adult liver in normoxic sheep.2 The expression of the Epo gene is higher earlier than later in gestation in both liver and kidney. The aims of these studies were (1) to determine whether the Epo gene was expressed in kidneys in very early gestation (0.27) in both meso- and meta-nephros; (2) to compare the relative expression levels in renal and liver tissue; and (3) to determine the site of expression of the Epo gene in immature kidneys.

MATERIALS AND METHODS

Experimental Animals

All experiments were approved by the Animal Ethics Committee of the Howard Florey Institute in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Six pregnant ewes, of known mating date (41 days of gestation), were killed with an overdose of sodium pentobarbitone (Lethabarb, Ceva-siq Melbourne, Padcviile, Victoria 3052, Australia). With the use of a dissecting microscope, the paired mesonephroi and metanephroi were frozen immediately in liquid nitrogen. Fetal mesonephroi and metanephroi from a further two fetuses were taken into 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), fixed overnight, and paraffin embedded.

Fetal livers were frozen immediately in liquid nitrogen. Fetal mesonephroi and metanephroi from as early as day 60 of gestation were fixed overnight, embedded in paraffin, and sectioned at 4-μm. By light microscopic examination, the mesonephroi were identified and sectioned into 10-μm sections. The sections were stained with hematoxylin and eosin. The sections were examined at ×200 magnification and the site of Epo gene expression was determined.

RNA Preparation

Because of the small size of the fetal kidneys (≈10 mg), paired mesonephroi or metanephroi were extracted immediately by the method of Chomezynski and Sach.8 The mesonephroi and metanephroi were taken into 1 mL of solution D, containing 4 mol/L guanidinium thiocyanate, 25 mol/L sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 mol/L 2-mercaptoethanol, and homogenized at room temperature, with a glass Teflon homogenizer (Wheaton, NJ). The mixture was transferred to a 4-mL polypropylene tube to which was added 0.1 mL of 2 mol/L sodium acetate (pH 4), 1 mL of phenol (water saturated), and 0.2 mL of chloroform:isoamyl alcohol mixture (49:1) and mixed thoroughly. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes, then centrifuged at 10,000g for 20 minutes at 4°C. After centrifugation, the aqueous phase, containing the RNA, was transferred to a fresh tube, mixed with 1 mL of isopropanol, and left at −20°C for at least 1 hour to precipitate the RNA. The mixture was then centrifuged at 10,000g for 20 minutes, and the resulting pellet dissolved in 0.3 mL of solution D, transferred to a 1.5-mL Eppendorf tube and precipitated with 1 vol of isopropanol at −20°C for 1 hour. After centrifugation for 10 minutes at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (15 minutes), and dissolved in 50 μL of 0.5% sodium dodecyl sulfate (SDS) for 10 minutes. RNA from the larger livers was prepared by the guanidinium isothiocyanate/cesium chloride centrifugation procedure,2 as detailed before.9

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

RT. Total RNA (5 μg) was reverse transcribed together with 5 fg of a synthetic cRNA internal standard6 using an Epo-specific antisense primer (5'-CGTGACAGCTTGTCACATGTCCGCGCAG3') in 1× 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 (dNTP), 8 U of RNAasin (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 mix of 1× PCR buffer, 100 mmol/L of each dNTP, 100 ng each of antisense and sense (5'-ATCTGGACAGCCGAGCTCGTCCTGGAGAGGTAC3') 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, and after the last cycle, a final extension at 72°C for 10 minutes.
Samples were electrophoresed on 6% nondenaturing 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 BAS 2000 Bioimaging Analyser (Berthold Australia, Bundoora, 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. All samples were processed in the same assay, and samples of RNA from the metanephros and liver of a 60-day fetus were also included. The intraassay coefficient of variation of this assay has been determined previously as 15%.

Hybridization Histochernistry

Sections (4 μm) of paraffin-embedded tissues were treated with pronase E (Sigma, St Louis, MO) at a concentration of 125 μg/mL for 10 minutes at 37°C, postfixed 10 minutes in 4% paraformaldehyde, and dehydrated before addition of the cRNA probe. These slides were hybridized overnight at 50°C in a humidified chamber, washed in formamide buffer for 3 hours, and treated with RNase (Sigma) at a concentration of 1.9 mg/mL for 1 hour at 37°C. Slides were then washed in 2X SSC at 65°C for 90 minutes and dehydrated before dipping in Ilford K5 emulsion (Ilford, Cheshire, UK). Slides were stored at room temperature and developed after 13 and/or 21 days in D19 developer (Kodak, Rochester, NY) and fixed in Ilford rapid fixer (Ilford, Cheshire, UK); the slides were then counterstained with hematoxylin-eosin.

The cRNA probes (sense and antisense) were generated from a 520-bp of the ovine cDNA (exons II to IV), which had been cloned into Bluescript in the polylinker region.6 The antisense probe was generated using T7 RNA polymerase and labeled with 35S-UTP (37 x 104 Gbq/mmol; NEN Research Products, Boston, MA), and the sense probe from the erythropoietin sequence was synthesized using T3 RNA polymerase as detailed previously.11 As positive control, sections of metanephros (day 60) and kidney from an adult hemorrhaged sheep2 were included with tissues from the 41-day fetuses.

Image Acquisition

Light micrographs were acquired on a Sony 3CCD (charge coupled device) colour video camera DXC-930P (Sony Australia Ltd, Melbourne) coupled to a Nikon Microphot microscope (FSE Pty Ltd, Melbourne, Australia). The images were analyzed using the MCID (microcomputer imaging device) M2 image analyzer (Imaging Research Inc, St Catharines, Canada), color enhanced where necessary in Adobe Photoshop (Adobe Systems Inc, California) and printed on a Fujix Bas HG-printer (Berthold Australia Pty Ltd, Bundoora, Victoria, Australia).

Fig 1. Epo gene expression in mesonephros, metanephros, and liver of 4 ovine fetuses at 41 days of gestation compared with a control metanephros and liver from a 60-day gestation fetus and a reagent blank. Upper panel, Bioimaging Analyser-generated image; lower panel, ratio of Epo mRNA to standard (5 μg total RNA, 5 fg standard-RT-PCR) expressed as mean ± SEM.
Fig 2. Transverse section of fetal sheep at 41 days of gestation, showing mesonephros (MS), metanephros (MT), gonad (G), pancreas (Pa), stomach (St), and spinal cord (SpC). Original magnification ×40.

RESULTS

RT-PCR

In Fig 1, there is the Bioimaging Analyser-generated image of the Epo mRNA and standard for livers, mesonephroi, and metanephroi from four fetuses at 41 days of gestation and from one 60-day fetus. The mean ± SEM for the ratio of Epo mRNA to standard is shown for each group, as well as the individual values for 60-day fetal liver and kidney. The ratio of Epo mRNA to standard ranged between 1.5 and 2.3 in the metanephros, with a mean ± SEM of 1.9 ± 0.21. For the mesonephros the values were more variable, ranging from 3 to 18.1, with a mean ± SEM of 10.5 ± 4. Since every value for the mesonephros was greater than any value for the metanephros, the difference was statistically significant (P < .05) despite the large variation. In the liver, the mean ± SEM for this ratio was 16.9 ± 2.2.

Morphology orid Hybridization Histochemistry

In Fig 2, the location of the mesonephroi and metanephroi are indicated in a cross-section of a whole fetus at 41 days of gestation. The mesonephroi have partially regressed at this stage, and the metanephroi are at a very early stage of development. In Fig 3B, the expression of the Epo gene can be seen to occur in a group of interstitial cells in the vicinity of the proximal tubules, identified by cytoplasmic blebbing and vacuoles therein. No labeling is seen when the sense probe was used (Fig 3A). As a positive control, the antisense probe labeled the interstitial cells of the metanephros at 60 days (Fig 3C and D) and sections of a kidney from an adult hemorrhaged sheep (data not shown).

DISCUSSION

This is the first demonstration of expression of the Epo gene in a mammalian mesonephros. The mesonephros is the permanent kidney of fish and also the site of erythropoiesis in the fish. There is also evidence suggesting that an erythropoietin-like activity and gene exist in fish mesonephroi. Thus, there is a strong suggestion that “ontogeny recapitulates phylogeny” and the kidney is a “natural” organ of expression for the Epo gene. So rather than seeking to find why Epo gene expression switches from liver to kidney after birth, the question should be—why is liver expression so high before birth?

The exact cell type expressing the Epo gene in the adult kidney has been controversial. While some authors have suggested that the glomerulus or proximal tubules might be the site of synthesis of erythropoietin, the consensus view now is that peritubular interstitial cells are the normal site of Epo gene expression in the mesonephros of mice, rats, and sheep. The current study shows that this is also true of the mesonephros of the fetal sheep. These cells have been identified as fibroblasts in mice, as they have the appropriate ultrastructure and they also express 5′-ectonucleotidase. The liver was not subjected to hybridization histochemistry, as we have found previously that the ovine fetal liver labels nonspecifically with all probes used, including the sense Epo probe.

The ability to detect Epo gene expression in the mesonephros at 41 days and the metanephros at 60 days by hybridization histochemistry, whereas the Epo gene expression cannot be so detected in normal adult kidneys, is due to the fact that Epo gene expression occurs at a much higher level.
Fig 3. (A and B) Light microscope image of mesonephros at 41 days of gestation ovine fetus hybridized with sense (A) and antisense (B) 35S-labeled riboprobes for ovine Epo; arrow indicates a group of interstitial cells in the vicinity of proximal (P), not distal (D) tubules, showing Epo gene expression. (C and D) Light (C) and dark (D) field micrographs of a section of ovine metanephros, from a 60-day gestation fetus, probed with antisense 35S-labeled riboprobe for ovine Epo. The sites of Epo gene expression are groups of interstitial cells labeled with black dots, arrows (C), or white dots in dark field (D). All sections were counterstained with hematoxylin-eosin. Original magnification ×400.

 (>10-fold) in the fetal than the adult kidneys.\(^5\) The interesting facet of the expression, however, is that it is restricted to the same cell type as in the adult kidney. This is in contrast to the expression of other genes in fetal kidneys, eg, renin and angiotensinogen. Both of these genes are expressed in fetal kidneys at levels much higher than in adult animals,\(^22\),\(^23\) but the sites of expression in the fetal metanephros are much more widespread than in the adult kidney. Angiotensinogen is expressed throughout the proximal tubule of the ovine mesonephros and metanephros at 41 days of gestation.\(^24\) Renin synthesis occurs in the mesonephros of humans, pigs, and sheep,\(^24\),\(^26\) indicating that in these species the mesonephros may have important endocrine functions.

In conclusion, the data reported here show that not only is the Epo gene expressed in the ovine metanephric kidney extremely early in gestation (41 days), but it is also expressed in the transient renal structure—the mesonephros. Although the Epo gene is expressed, at quite high levels, in the liver at this early stage of gestation (≈0.27), there is no time so far determined when the liver is the sole source of Epo in the developing sheep.

REFERENCES
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