Impermeability of the Ovine Placenta to ^{35}S-Recombinant Erythropoietin

JOHN A. WIDNESS, THOMAS A. MALONE, AND R. ALLAN MUFSON

Department of Pediatrics, Women & Infants' Hospital of Rhode Island, Brown University Program in Medicine, Providence, Rhode Island 02905 [J. A. W., T. A. M.] and Genetics Institute, Cambridge, Massachusetts 02138 [R. A. M.]

ABSTRACT. Controversy exists regarding the placental permeability of erythropoietin (Ep), a glycoprotein hormone that regulates red blood cell production in the fetus, newborn, and adult after tissue hypoxia. The purpose of the current study was to determine the placental permeability of biologically active ^{35}S-labeled human recombinant erythropoietin given by bolus injection into the circulation of the fetal lamb. Specific radioactivity in fetal plasma trichloroacetic acid protein precipitate fractions increased 13-fold from preinfusion levels at 6 h (47 ± 1.3 to 679 ± 237 cpm/mL) and thereafter fell progressively until the study was terminated at 45 h. In contrast, maternal trichloroacetic acid protein precipitate fractions demonstrated no detectable increase in radioactivity at any time. Based on the counting precision, a rise in maternal plasma radioactivity of more than 3 cpm/mL would have been detected (i.e. 0.5% of the 582 cpm/mL rise in the fetal protein precipitate counts at six h). Similar data were obtained with simultaneously administered unlabeled human urinary Ep. We conclude that physiologically significant amounts of Ep do not cross from fetus to mother; hence, maternal Ep level functions as a separate indicator of the adequacy of tissue oxygenation in the maternal compartment. (Pediatr Res 25: 649–651, 1989)

Abbreviations

Ep, erythropoietin
rhEp, recombinant human erythropoietin
TCA, trichloroacetic acid
SaO₂, arterial whole blood oxygen saturation

Ep is a glycoprotein hormone (mol wt = 34 000 D) that regulates red blood cell production in the fetus, newborn, and adult (1). In all developmental periods, the primary physiologic stimulus for the release of this hormone is tissue hypoxia, the origin of which can be anemia, hypoxemia, and/or ischemia. The recent availability of recombinant DNA-derived human Ep from engineered mammalian cells has allowed μg amounts of pure, isotopically labeled Ep to be used for biologic studies (2, 3).

During human pregnancy, fetal and maternal plasma levels of Ep are increased relative to nonpregnant adults suggesting the presence of relative tissue hypoxia (4–7). Although fetal Ep levels near term before the onset of labor are only slightly increased above those of normal nonpregnant adults (6), in a variety of

pathologic states before and during labor fetal Ep levels may be extraordinarily elevated (8). The nature of the relationship of Ep levels in the fetus and mother has been the subject of several studies the majority of which indicate that Ep does not cross the placenta (9–13). However, a recent report in which ^{125}I-labeled rhEp in the late gestation pregnant mouse was infused in the mothers indicated that intact Ep does cross from mother to fetus (14). The purpose of the current study was to determine the placental permeability from fetus to mother of radiolabeled ^{35}S-rhEp injected into the fetal lamb.

MATERIALS AND METHODS

Pregnant ewes (n = 5) were all operated on at 123 d of gestation under general anesthesia using intravenous ketamine hydrochloride (400–500 mg/h). At surgery, catheters were inserted into a fetal femoral artery and vein as well as a maternal femoral artery. In the two twin preparations, only one twin was catheterized. Before study, catheter patency was maintained with a concentrated heparin solution (1 000 U/mL). Ampicillin (200 mg/kg) and gentamicin (5 mg/kg) were administered daily to the mothers for the 3 to 4 d after surgery before study. At the end of the study, the ewes and fetuses were killed using ketamine sedation followed by intraarterial thiamylal sodium.

To ascertain the baseline metabolic and oxygenation status, fetal and maternal arterial whole blood samples were drawn for pH, PaO₂, PaCO₂, bicarbonate, hematocrit, Hb, and oxygen saturation (SaO₂) before the bolus infusion of ^{35}S-rhEp. To assess stability of the preparations, identical determinations were repeated just before termination of the study. Arterial pH, blood gases, and bicarbonate were measured at 37° C using a Corning 168 blood gas analyzer (Corning Glass Works, Medfield, MA). Hematocrit was measured on whole blood centrifuged for 5 min at 13 400 × g. Hb and SaO₂ were measured using a Radiometer OSM-2 hemoximeter (Radiometer America, Inc., Westlake, OH).

Fetal and maternal Ep levels were determined using a double antibody RIA technique in which human and sheep Ep cross-react (4). Because exogenous human Ep was administered, Ep values were read from a human standard curve in a single assay. In the human assay, linear values for Ep concentrations are obtained between 10 and 150 mU/mL. Intraassay coefficients of variation for three samples of pools of human plasma were between 6 and 10%.

rhEp used for bolus injection was intrinsically labeled with ^{35}S-cysteine produced in a line of Chinese hamster ovary cells containing highly amplified copies of the human Ep cDNA and purified as previously described (3). This ^{35}S-rhEp was 95% radiochemically pure as determined by PAGE, had a sp biologic act of 250 000 IU/mg protein as determined by in vitro bioassay (15), and had a radio sp act of 150–200 cpm/fmol protein. On the day of study, ^{35}S-rhEp in a dose of 0.8–1.4 × 10^6 cpm was injected into the fetal femoral vein over a 1- to 2-min period.

Maternal and fetal arterial blood samples were obtained at selected intervals after the ^{35}S-rhEp bolus: 6, 12, 21, 30, and 45 h. (Only three of the five samples were available for study at the 45-h interval.) The duration of study was selected to allow ample time for the passage of Ep from fetus to mother; the $t_{1/2}$ disappearance rate of Ep in adult sheep has been reported as 9.1 ± 3.9 h (mean $\pm$ SD) (16). To simulate conditions under which high endogenous fetal plasma Ep levels could contribute to maternal plasma Ep levels, 125 IU of unlabeled Ep (step I human urinary Ep in PBS, Terry Fox Labs, Vancouver, BC, Canada) was injected immediately after the bolus of ^{35}S-rhEp.

Fetal and maternal whole blood samples were centrifuged to remove red cells and 1.0-mL plasma samples were precipitated in 7-mL screw-top tubes with an equal vol of 20% TCA. After 20 min at 4° C, plasma samples were centrifuged at 4° C at 1 000 rpm for 15 min. Supernatant fractions were decanted into 20-mL scintillation vials with 16 mL of Aquasol-2 added (New England Nuclear, Boston, MA). The remaining TCA protein precipitates were vortexed for 30 s in 3.0 of mL Protosol (New England Nuclear). To dissolve the resulting protein suspension fully, the 7-mL screw-top tubes were capped and placed in a 55° C dry bath overnight. The resulting clear solution was transferred to glass scintillation vials with 16 mL of Aquasol-2 added. The TCA supernatant and precipitate fractions were counted in a liquid scintillation spectrometer (Packard Tri-Carb, model 3385, Hewlett-Packard Co., Palo Alto, CA), using window settings for [14]C. All samples were counted for 50–100 min, which produced a mean counting error (2 SD) of 0.5 to 4.1% of the total cpm/mL. In absolute terms, this error was 1.3 to 2.0 cpm/mL for all maternal and for the fetal prebolus baseline samples.

Statistical analyses were performed using microcomputer software (Statview II, Abacus Concepts Inc., Berkeley, CA). Within and between group comparisons were done using Student's paired and unpaired t tests. For multiple within-group comparisons with the baseline preinfusion values, repeated measures ANOVA (1-tailed) was used with Dunnett's *post hoc* testing.

RESULTS

There were no differences between the initial baseline and final fetal or maternal arterial measurements of pH, PaO_2, $PaCO_2$, bicarbonate, hematocrit, Hb, or SaO_2 (Table 1). All were within normal limits for pregnant ewes and fetal lambs. The fetal and maternal baseline Ep levels were not significantly different (10.0

$\pm$ 2.7 and 11.1 ± 4.8 mU/mL, respectively). At the termination of study, the wt of the fetuses ranged from 3.1 to 3.8 kg.

Plasma sp act of the maternal TCA protein precipitate and supernatant fractions demonstrated no measurable increase in radioactivity at any of the five sampling times after the bolus injection of ^{35}S-rhEp (Table 2). This was in marked contrast to the cpm/mL measured in the fetal plasma protein precipitate fractions. These manifest a 13-fold increase from preinfusion levels at 6 h: 47 ± 1.3 to 679 ± 237 cpm/mL ($p < 0.01$). All subsequent fetal TCA protein precipitable counts were significantly increased relative to baseline.

Similar findings were observed for maternal and fetal levels of unlabeled plasma Ep measured by RIA (Table 3). Maternal unlabeled Ep levels remained unchanged throughout the study; fetal levels increased significantly 6 h after the bolus infusion of 125 IU human urinary Ep ($p < 0.01$). In contrast to the fetal ^{35}S TCA precipitate findings, fetal plasma RIA Ep values measured after this time were not significantly different from baseline values.

DISCUSSION

The present study provides direct evidence that the placenta is impermeable to the passage of erythropoietin from fetus to mother. Our data are consistent with the majority of previous work which was performed before the availability of pure, biologically active, labeled Ep (9–13). An exception is the report of Finne (17), in which he suggested that endogenously produced Ep could pass from the fetus to the mother in severely affected Rh sensitized pregnancies and from mother to fetus in pregnant mice administered large doses of exogenous Ep. These previous studies have all relied on a variety of less sensitive, less specific bioassays for measuring endogenous and exogenous Ep.

In contrast in the present study, ^{35}S-rhEp was administered to late gestation fetal sheep. Our precision in TCA precipitate counting combined with an estimated pipetting error of $\approx 2\%$ allowed us to detect a rise in maternal plasma radioactivity of as little as 3 cpm/mL. This value represents only 0.5% of the 582 cpm/mL rise observed in the fetal TCA protein precipitate at 6 h. Although there was a 20-fold wt difference between the fetus and ewe, the conceptus (fetus and placenta) has a 50% more blood vol/wt than the ewe (18). Moreover, because TCA treatment results in the precipitation of low mol wt proteins, including potential Ep breakdown products, TCA precipitate fractions

Table 1. *Fetal and maternal (n = 5) metabolic and oxygenation parameters at start and end of study (mean ± SD)*

	pH$_a$	PaO$_2$ (mm Hg)	PaCO$_2$ (mm Hg)	HCO$_3$ (mEq/liter)	Hematocrit (%)	SaO$_2$ (%)	Hb (g/dL)
Mothers*							
Initial	7.488 ± 0.018	104.3 ± 18.8	31.7 ± 2.4	25.7 ± 2.4	34.4 ± 2.0	96.3 ± 2.4	8.8 ± 1.0
Final	7.458 ± 0.022	100.5 ± 12.42	32.9 ± 2.7	24.7 ± 2.3	32.0 ± 3.3	96.5 ± 0.8	8.9 ± 1.1
Fetuses*							
Initial	7.364 ± 0.041	19.2 ± 1.7	44.8 ± 4.6	27.1 ± 1.2	38.5 ± 3.9	54.0 ± 7.2	9.9 ± 1.0
Final	7.354 ± 0.042	18.9 ± 2.5	48.8 ± 4.0	28.8 ± 1.9	35.3 ± 0.9	54.9 ± 7.0	9.2 ± 0.9

* p = NS for all within group comparisons of initial and final values.

Table 2. *Fetal and maternal (n = 5) plasma specific radioactivity (mean ± SD) of TCA fractions (cpm/mL)*

	0 h	6 h	12 h	21 h	30 h	45 h
Maternal						
Precipitate	47.5 ± 1.4	47.7 ± 1.6	47.5 ± 2.2	49.6 ± 4.7	47.2 ± 2.1	47.8 ± 0.8
Supernatant	36.0 ± 1.4	36.7 ± 1.2	36.2 ± 0.8	36.7 ± 1.1	36.5 ± 0.9	36.5 ± 1.2
Fetuses						
Precipitate	47.7 ± 1.3	630* ± 237	365* ± 98	274* ± 47	253* ± 54	214 ± 50
Supernatant	36.8 ± 1.5	53 ± 5.8	49 ± 3.6	47 ± 3.8	47 ± 5.3	44 ± 3.9

* $p < 0.01$ relative to 0 h (repeated measures ANOVA).

Table 3. *Fetal and maternal (n = 5) plasma Ep levels (mU/mL) at study intervals (mean ± SD)*

	0 h	6 h	12 h	21 h	30 h	45 h
Maternal	11.1 ± 4.8	10.6 ± 3.1	17.3 ± 9.7	11.4 ± 3.2	11.6 ± 3.9	9.2 ± 2.2
Fetuses	10.0 ± 2.8	47.7* ± 20.3	16.1 ± 8.9	11.3 ± 6.9	9.3 ± 2.9	14.3 ± 11.2

* $p < 0.01$ relative to 0 h (repeated measures ANOVA followed by Dunnett's *post hoc* testing).

overestimate the amount of intact, biologically active Ep in fetal and maternal plasma. In parallel studies of 6- to 8-d-old newborn lambs, we have observed qualitative differences in the gel filtration profiles obtained from the ^{35}S-rhEp infusate compared to postinfusion plasma samples.

Our results differ from those of Koury *et al.* (14), who also administered labeled Ep during pregnancy. These investigators administered biologically active ^{125}I-rhEp to pregnant mice and observed a rise in fetal levels of less than 10% of the rise in simultaneously measured maternal levels. The authors confirmed with HPLC and PAGE that fetal plasma radioactivity was Ep and not metabolic breakdown products of Ep. If confirmed, these small amounts of Ep transferred to the fetus may be of potential physiologic significance, as some investigators have reported that fetal erythroid precursors are more sensitive than those of the adult to Ep (19).

Several possible reasons exist for the discrepancy between the findings of Koury *et al.* (14) and those of the present study. In the study of Koury *et al.*, Ep infusate was administered into the maternal circulation; in the present study, it was infused into the fetal circulation. Binding to Ep receptors located on the maternal side of the placenta could be the first step in an endocytotic process similar to that of IgG in many mammalian species (20). Of interest in this respect was the finding of Koury *et al.* that Ep receptors were present in washed placental extracts. This finding, however, differed from that reported of Pekonen *et al.* (21), who were unable to detect evidence of Ep receptors in human placental tissue.

Because, with the exception of IgG, molecular size is inversely correlated with placental permeability, it is not unexpected that we did not find Ep crossing from the fetal to the maternal circulation. In an *in vitro* study in guinea pigs, the syncytiotrophoblast acted as an absolute barrier to proteins larger than 17 000 mol wt infused into the fetal side of the circulation (22). Gitlin *et al.* (23) found in the human that of five proteins, albumin, transferrin fibrinogen, and IgM, only IgG crossed from mother to fetus in appreciable amounts. Based on our results and those of others, it appears that in the absence of a specific placental transport receptor for Ep, Ep does not cross the placenta in physiologic amounts.

Morphologic differences in placentas of different species may also account for the discrepancy of our findings with those of Koury *et al.* (14). The ovine placenta is four-layered and epitheliochorial as compared with that of the mouse, which is hemotrichorial (24). Although the choice of the best species for comparison of placental function with the human remains controversial, the sheep may be as appropriate a model as is the guinea pig (25).

Using a highly sensitive assay system in the near term pregnant sheep, we were unable to demonstrate any passage of ^{35}S-rhEp across the placenta from fetus to mother. Qualitatively similar findings were observed for simultaneously administered unlabeled human urinary Ep. Thus, although the potential for passage of Ep in the opposite direction, *i.e.* from mother to fetus remains open, physiologically significant amounts of Ep do not cross from fetus to mother. We conclude that the maternal level of Ep is an indicator of the adequacy of tissue oxygenation in the maternal compartment independent of fetal oxygenation. We speculate that elevated plasma Ep levels in human pregnancies reflect true increases in maternal tissue oxygen requirements.

REFERENCES

1. Jelkmann W 1986 Renal erythropoietin: properties and production. Rev Physiol Biochem Pharmacol 104:139–215
2. Jacobs K, Shoemaker C, Rurensdorf R, Neill SD, Kaufman RJ, Mufson H, Seehra J, Jones SS, Hewick R, Fritsch EF, Kawakita M, Shimizu T, Miyake T 1985 Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature 313:806–810
3. Mufson RA, Gesner TG 1987 Binding and internalization of recombinant human erythropoietin in murine erythroid precursor cells. Blood 69:1485–1490
4. Garcia JF, Ebbe SN, Hollander L, Cutting HO, Miller ME, Cronkite EP 1982 Radioimmunoassay of erythropoietin: circulating levels in normal and polycythemic human beings. J Lab Clin Med 99:624–635
5. Cotes PM, Canning CE, Lind T 1983 Changes in serum immunoreactive erythropoietin during the menstrual cycle and normal pregnancy. Brit J Obstet Gynaecol 90:304–311
6. Widness JA, Clemons GK, Garcia FJ, Oh W, Schwartz R 1984 Increased immunoreactive erythropoietin in cord serum after labor. Am J Obstet Gynecol 148:194–197
7. Widness JA, Clemons GK, Garcia JF, Schwartz R 1984 Plasma immunoreactive erythropoietin in normal women studied sequentially during and after pregnancy. Am J Obstet Gynecol 149:646–650
8. Teramo KA, Widness JA, Clemons GK, Voutilainen P, McKinlay S, Schwartz R 1987 Amniotic fluid erythropoietin correlates with umbilical plasma erythropoietin in normal and abnormal pregnancy. Obstet Gynecol 69:710–716
9. Zanjani ED, Gordon AS 1971 Erythropoietin production and utilization in fetal goats and sheep. Isr J Med Sci 7:850–856
10. Zanjani ED, Peterson EN, Gordon AS, Wassermann LR 1974 Erythropoietin production in the fetus: role of the kidney and maternal anemia. J Lab Clin Med 83:281–287
11. Zanjani ED, Banisadre M, Ascensao JL 1982 Evidence for independent regulation of erythropoiesis in the fetus. Clin Res 30:333 (abstr)
12. Matoth Y, Zaizov R 1971 Regulation of erythropoiesis in the fetal rat. Isr J Med Sci 7:839–845
13. Jacobson LO, Marks EK, Gaston EO 1959 Studies on erythropoiesis, XII. The effect of tranfusion-induced polycythemia in the mother on the fetus. Blood 14:644–653
14. Koury MJ, Bondurant MC, Graber SE, Sawyer ST 1988 Erythropoietin messenger RNA levels in developing mice and transfer of ^{125}I-erythropoietin by the placenta. J Clin Invest 82:154–159
15. Krystal G, Eaves CJ, Eaves AC 1981 A quantitative bioassay for erythropoietin, using mouse bone marrow. J Lab Clin Med 97:144–157
16. Mladenovic J, Eschback JW, Koup JR, Garcia JF, Adamson JW 1985 Erythropoietin kinetics in normal and uremic sheep. J Lab Clin Med 105:659–663
17. Finne PH 1967 On placental transfer of erythropoietin. Acta Paediatr Scand 56:233–242
18. Brace RA 1983 Blood volume and its measurement in the chronically catheterized sheep fetus. Am J Physiol 244:H487–H494
19. Rich IN, Kubanek B 1976 Erythroid colony formation (CFU-e) in fetal liver and adult bone marrow and spleen from the mouse. Blut 33:171–180
20. Johnson PM, Brown PJ 1981 Fcγ receptors in the human placenta. Placenta 2:355–370
21. Pekonen F, Rosenlöf K, Rutanen E-M, Fyhrquist F 1988 Erythropoietin binding sites in human foetal tissues. Acta Endocrinol (Copenh) 116:561–567
22. Sibley CP, Bauman KF, Firth JA 1982 Permeability of the foetal capillary endothelium of the guinea-pig placenta to haem proteins of various molecular sizes. Cell Tissue Res 223:165–178
23. Gitlin D, Kumate J, Urrusti J, Morales C 1964 The selectivity of the human placenta in the transfer of plasma proteins from mother to fetus. J Clin Invest 43:1938–1950
24. Enders AC 1965 A comparative study of the fine structure of the trophoblast in several hemochorial placentas. Am J Anat 116:29–68
25. Wilbur WG, Power GG, Longo LD 1979 A mathematical model: comment on the placental water exchange model of Wilbur, Power, and Longo. Am J Physiol 237:R90–R91