Relationship Between α-Fetoprotein and Fetal Erythropoiesis

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OBJECTIVE: To evaluate the relationship of fetal erythropoiesis to both maternal and fetal serum α-fetoprotein (AFP) levels.

STUDY DESIGN: We evaluated the relationships between maternal serum AFP levels and fetal hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration in 63 fetuses, 19 of them by prenatal cordocentesis and 44 at elective cesarean section before labor. We also evaluated the relationships between fetal AFP and fetal hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, erythroblasts, erythropoietin, serum iron, transferrin, ferritin, bilirubin, total proteins and albumin in 51 umbilical cords immediately after singleton childbirth, in healthy, pregnant women at term. Statistical analysis was performed by linear regression.

A significant negative correlation between fetal RBC, hemoglobin, hematocrit, erythropoitin and AFP was demonstrated....

RESULTS: After adjustment according to gestational age, significant correlations were found between maternal AFP and fetal hemoglobin (r = −.30, P = .017) and hematocrit (r = −.25, P = .044). We also found a significant negative correlation between fetal serum α-fetoprotein and fetal red blood cells (r = −.55, P = .000026), hemoglobin (r = −.40, P = .0035), hematocrit (r = −.46, P = .00052), erythropoietin (r = −.40, P = .005) and transferrin (r = −.33, P = .016). Erythropoietin also positively correlated with fetal red blood cells (r = .47, P = .001), hemoglobin (r = .45, P = .001), hematocrit (r = .47, P = .001) and erythroblasts (r = .42, P = .003).

CONCLUSION: Since a significant negative correlation was demonstrated between fetal red blood cell count, hemoglobin, hematocrit, erythropoietin and AFP, it is reasonable to speculate that AFP could play a role in fetal erythropoiesis. (J Reprod Med 1999;44:689–697)

Keywords: alpha-fetoproteins, erythropoiesis, fetus.

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Introduction

Very little is known about the biologic role of α-fetoprotein (AFP) in normal fetal development. It appears that AFP might serve as a modulator/modifier of various cell growth regulatory pathways during embryonic and fetal development in vertebrates.[1,2]

Several findings lead us to hypothesize about a relationship between AFP and fetal hemoglobin. First, occasionally patients with hepatocellular carcinoma present with erythrocytosis, and high serum levels of both AFP and erythropoietin are associated with this paraneoplastic syndrome,[3-5] whereas elevations of erythropoietin alone are not enough to cause this syndrome.[5] Light microscopic immunohistochemistry has shown that AFP is located in hepatocellular carcinoma cells that are erythropoietin positive in serial sections.[4] If a high AFP concentration is related to erythrocytosis in this pathologic condition, it can be hypothesized that AFP plays a physiologic role in fetal erythropoiesis.

Second, AFP is synthesized mainly by the fetal liver and yolk sac. Both sites are hematopoietic organs in the fetus, and both tumors of the yolk sac and hepatocellular carcinoma can produce AFP. Finally, increased maternal serum AFP is commonly found in women bearing fetuses with acquired anemia due to different causes, such as infectious diseases[6] and Rh immunization,[7] although, until now, this phenomenon was attributed only to placenta-associated anomalies.

The aim of this study was to evaluate the relationships between AFP and various parameters of fetal erythropoiesis.

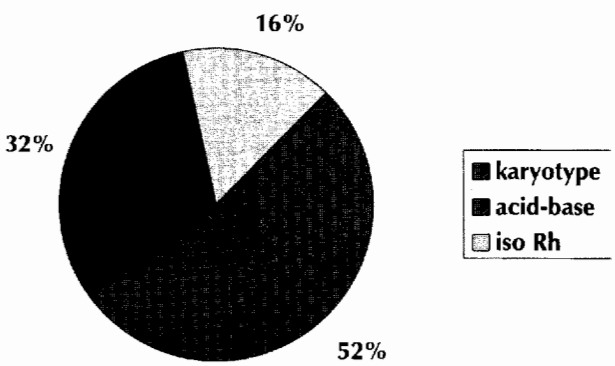


Figure 1 Indications of cordocentesis. Study of fetal karyotype, acid-base status and Rh isoimmunization.

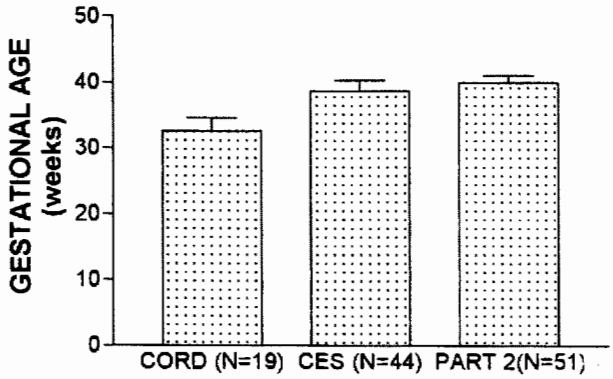


Figure 2 Gestational age at the time of obtaining the blood sample in the cordocentesis group (CORD) and cesarean section groups (CES) and in the patients in part 2 of the study (birth at term).

Subjects and Methods

Part 1 of the Study

We began by evaluating the relationships between maternal serum AFP levels and fetal hemoglobin, hematocrit, red blood cell count (RBC), mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration.

Blood was obtained from 63 fetuses, 19 of them by prenatal cordocentesis and the other 44 at elective cesarean section before labor. The reasons for cordocentesis (Figure 1) were: 10 cases for fetal karyotype (3 cases of duodenal atresia, 2 cases of holoprosencephaly, 2 cases of posterior urethral valves, 1 case of microcephaly, 1 case of congenital renal tumor and 1 of pulmonary hypoplasia), 6 for fetal acid-base status (4 cases of severe fetal growth restriction and 2 cases of fetal atrioventricular block) and 3 cases for fetal red cell isoimmunization, 1 of them prior to fetal transfusion. α–Fetoprotein was measured from maternal serum immediately before cordocentesis or cesarean section. Cases that presented with uterine contractions or maternal bleeding were systematically excluded from the study because in such cases there may be disruptions of the matenal-fetal interface, leading to greater-than-normal transfer of AFP from the fetal to maternal circulation. Cases with open structural defects in the neural tube or abdominal wall of the fetus were also excluded because maternal serum AFP is higher in pregnant women bearing fetuses with these defects. The average maternal age was 26.53 ± 3.32 years (range, 18–35), 28.52 ± 2.28 for the

Table I *Relation Between MSAFP Level and Fetal Hematologic Parameters Before and After Adjustment for Gestational Age Using Delta Values*

	Total (n = 63)				Cordocentesis (n = 19)				Cesarean section (n = 44)			
	AV		ΔV		AV		ΔV		AV		ΔV	
MSAFP	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
RBC	−.48	.00004	−.16	NS	−.53	.01	−.59	.007	−.10	NS	.02	NS
HB	−.48	.00004	−.30	.017	−.45	.04	−.51	.02	−.24	NS	−.19	NS
HTO	−.44	.0002	−.25	.044	−.44	.05	−.48	.03	−.20	NS	−.13	NS
MCV	.14	NS	−.13	NS	.23	NS	.29	NS	−.34	.02	−.34	.02
MCH	−.02	NS	−.17	NS	.21	NS	.17	NS	−.32	.03	−.32	.03
MCHC	−.14	NS	−.12	NS	.20	NS	.06	NS	−.21	NS	−.19	NS

MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, Hb = hemoglobin, HTO = hematocrit, AV = absolute values, ΔV = delta values.

cordocentesis group and 24.56 ± 2.32 for the cesarean section group. Forty-five percent were nulliparous (47% for the cordocentesis group and 43% for the cesarean section group). Gestational age was assessed by reliable menstrual history and scan. The average gestation at the time of blood collection was 36.82 weeks (range, 29–42, SD 3.36), 32.58 ± 1.98 for the cordocentesis group and 38.70 ± 1.67 for the cesarean section group (Figure 2). Both groups were analyzed together and separately.

Part 2 of the Study

The relationships between fetal AFP and fetal hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, erythroblasts, erythropoietin, serum iron, transferrin, ferritin, bilirubin and albumin were evaluated in the second half of the study. The relationships between the above-mentioned fetal parameters and fetal erythropoietin were also analyzed. Moreover, we studied the differences in fetal AFP levels between fetuses with hematocrit ≤ 45% and those with hematocrit > 45%.

Blood was obtained from 51 umbilical cords immediately after singleton childbirth in healthy, pregnant women at term. The average maternal age was 28.75 years (range, 18–39, SD 5.16), and 54.90 (28/51) were nulliparous. Gestational age was assessed by reliable menstrual history and previous scans. The average gestational age at the time of blood collection was 39.96 weeks (range, 38–42, SD 1.13) (Figure 2).

Maternal and fetal hematologic measurements were analyzed in the five minutes following venipuncture. Sera were kept frozen at −80°C, and biochemical parameters were obtained on the same day.

RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were measured by standard, automated techniques (Coulter, Coulter Inc., Miami, Florida).

An erythroblast blood count was taken with an automated instrument (H*3, Technicom, Miles Diagnostics Swords, Dublin, Ireland). Blood smears were examined after panoptic staining (Wright); 500 nucleated cells were identified to obtain the erythroblast percentage in the sample. The absolute number of erythroblasts was obtained by the formula erythroblasts/mL:leukocytes/mL × % erythroblasts/100.

Total proteins, serum iron and bilirubin were calculated using a colorimetric assay with a Hitachi 917 autoanalyzer (Boehringer Mannheim, Mannheim, Germany). Unconjugated bilirubin was obtained by the formula unconjugated bilirubin = total bilirubin − conjugated bilirubin.

Transferrin was measured by immunoturbidimetric assay. Albumin was calculated using nephelometry using a Behring nephelometer analyzer II (Dade Behring, Marburg, GmbH, Germany).

AFP was measured in maternal and fetal serum by enzyme immunoassay (ELISA) using the Enzymun-Test AFP kit (Boehringer Mannheim). Ferritin was also measured by ELISA, and erythropoietin was measured using radioimmunoassay.

Statistical Analysis

Statistical analysis was performed with a specialized software program (SYSTAT, version 2.0, SYSTAT Inc., Evanston, Illinois), by linear regres-

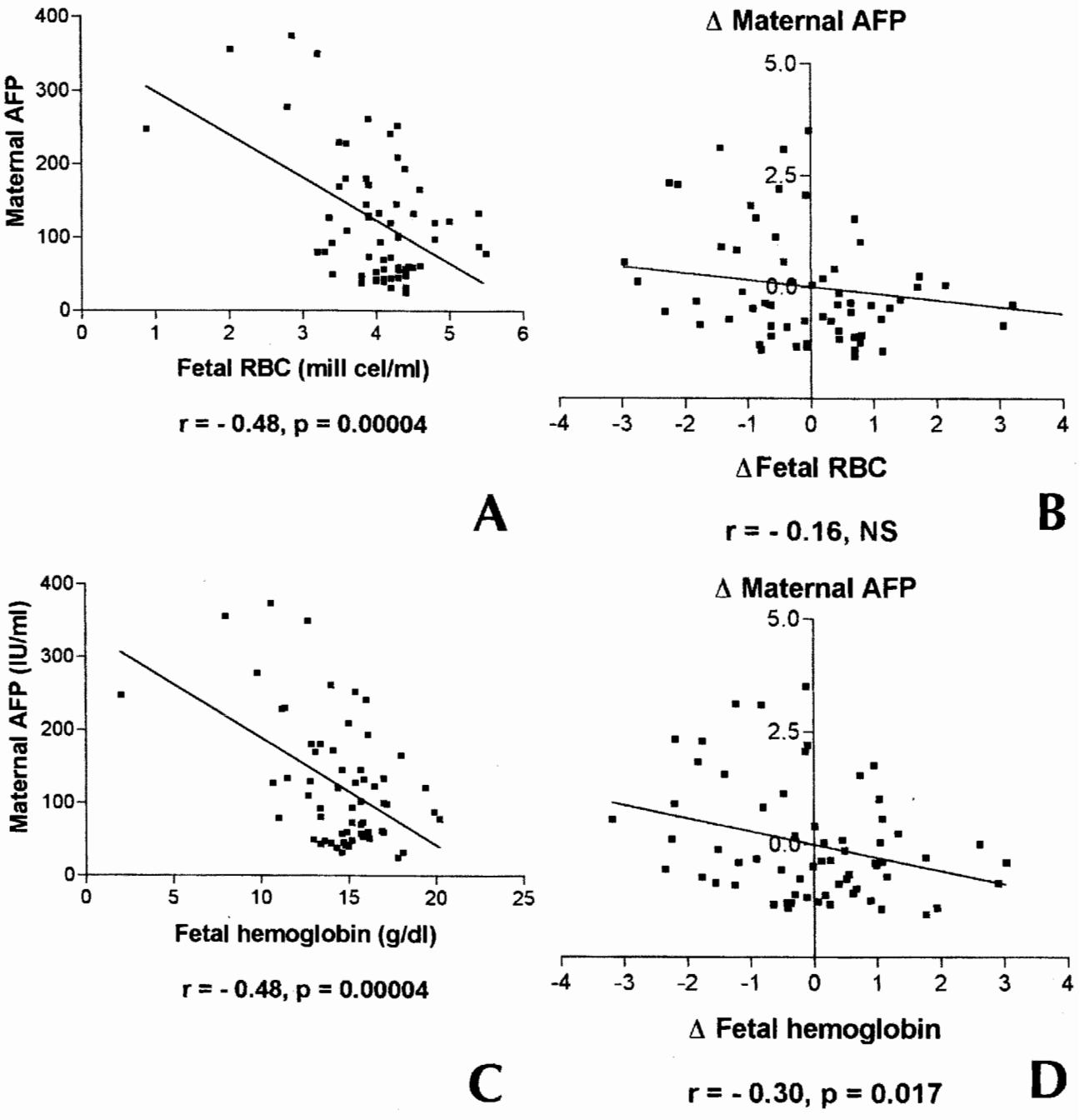
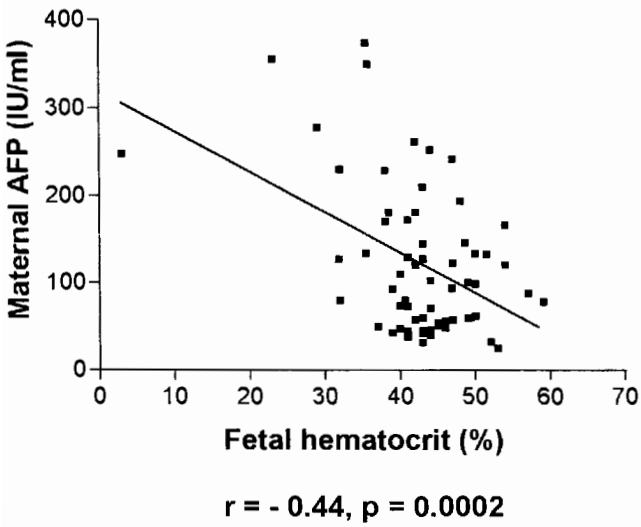


Figure 3 Correlations between MSAFP and (A and B) fetal RBC, (C and D) fetal hemoglobin and (E and F) fetal hematocrit, using absolute values (A and B) and delta values (C and D). Delta values expressed the number of SDs by which the individual values differed from the appropriate normal mean for gestation. mill cel = 1,000,000 cells.

sion. Student's *t* test was used for comparisons of fetal AFP between fetuses with hematocrit ≤ or >45%.

Since all measured parameters change with gestation, individual values in part 1 were expressed as the number of SDs by which the measurements differed from the appropriate normal mean for gestation (delta values).



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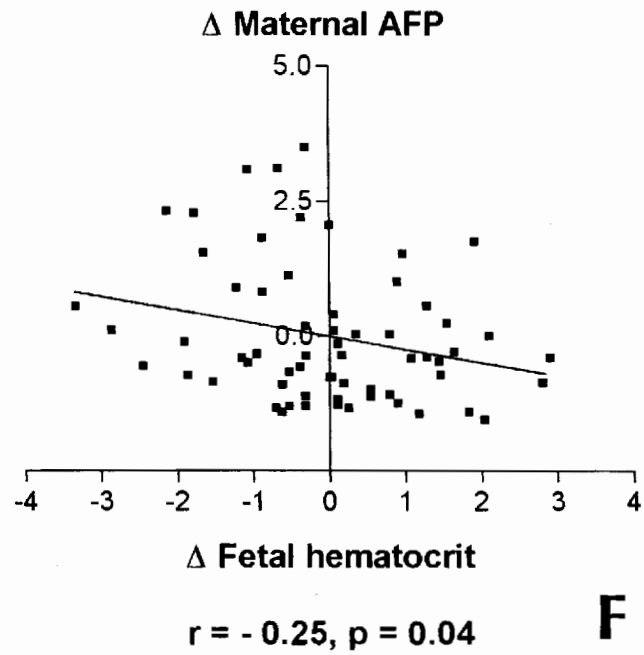

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Although some measured parameters changed with gestational age within the narrow gestational range in part 2, no parameter was significantly associated with gestation. Since AFP, erythropoietin and erythroblasts are distributed in a nonparametric manner, we used log-transformed values for analysis.

Results

Part 1 of the Study

As shown Table I, before adjustment by gestational age, RBC ($r = -.48$, $P = .0004$), hemoglobin ($r = -.48$, $P = .00004$) and hematocrit ($r = -.44$, $P = .0002$) had a significant negative correlation with maternal serum AFP (MSAFP). After adjustment by gestational age using delta values, these correlations were kept for hemoglobin ($r = -.30$, $P = .017$) and hematocrit ($r = -.25$, $P = .044$) but not for RBC (Figure 3). There were no significant correlations between MSAFP and mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) or mean corpuscular hemoglobin concentration.

After analyzing the cordocentesis and cesarean section groups separately, we found that the correlations between MSAFP and RBC, hemoglobin and

Table II *Correlations Between AFP, Erythropoietin and Fetal Hematologic Parameters*

Hematologic parameters	AFP		Erythropoietin	
	r	*P*	*r*	*P*
RBC	−.55	.000026	.47	.00065
Hemoglobin	−.40	.0035	.45	.0013
Hematocrit	−.46	.00052	.47	.00082
MCV	.12	NS	.01	NS
MCH	.24	NS	−.03	NS
MCCH	.17	NS	−.02	NS
IDH	−.06	NS	−.03	NS
Erythroblasts	−.15	NS	.42	.003

MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration.

Table III *Correlations Between AFP, Erytropoietin and Fetal Biochemical Measurements*

Measurement	AFP		Erytropoietin	
	r	*P*	*r*	*P*
Iron	.07	NS	−.22	NS
Ferritin	.19	NS	−.12	NS
Tranferrin	−.33	.016	.23	NS
Total bilirubin	.14	NS	.17	NS
Conjugated bilirubin	.19	NS	−.23	NS
Unconjugated bilirubin	.09	NS	.23	NS
Protein	−.22	NS	.20	NS
Albumin	.13	NS	.17	NS
Erythropoietin	−.40	.005	—	—

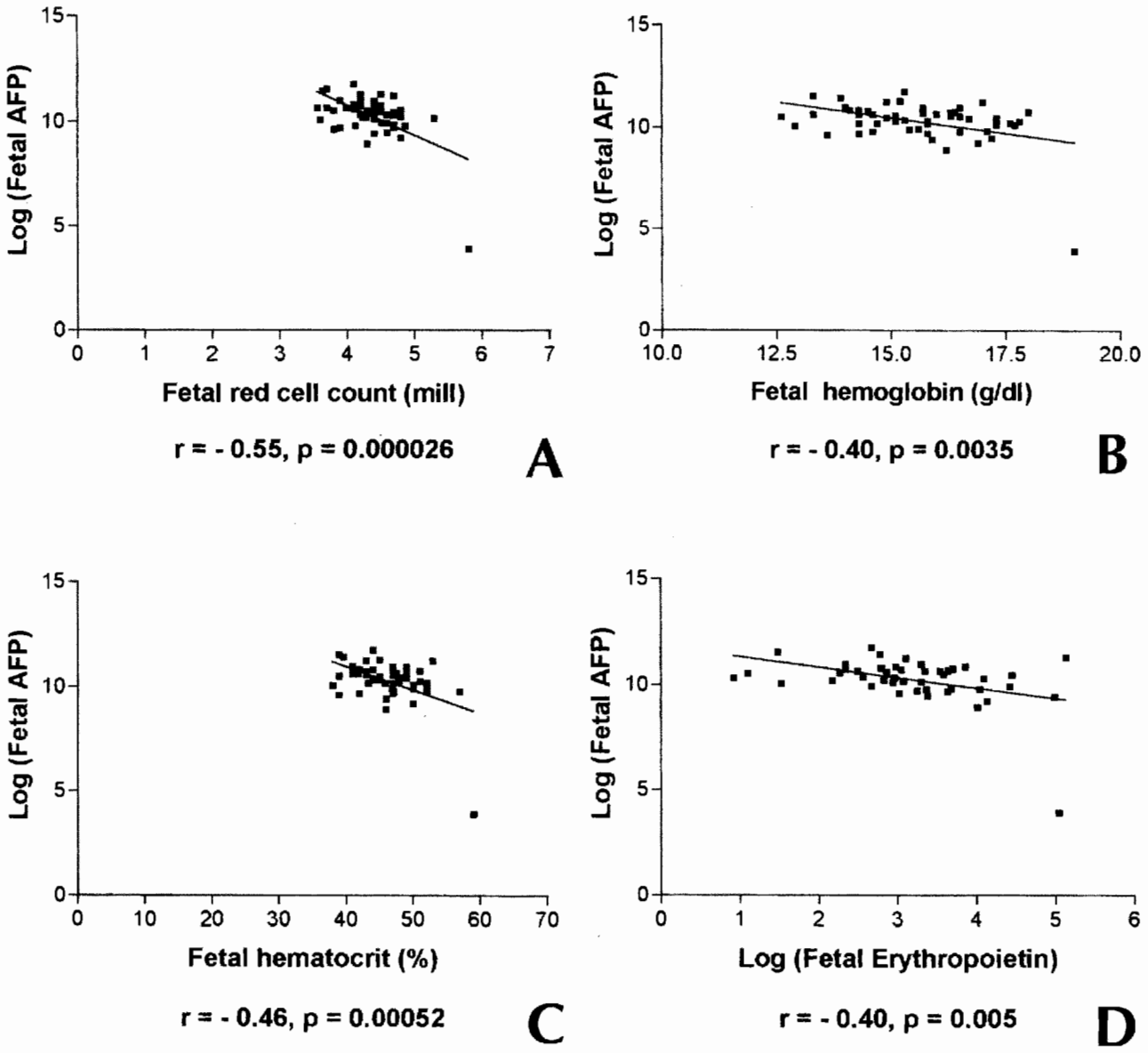


Figure 4 Correlations between AFP and (A) fetal RBC, (B) fetal hemoglobin, (C) fetal hematocrit, (D) fetal erythropoietin and (E) fetal transferrin. mill = 1,000,000 cells.

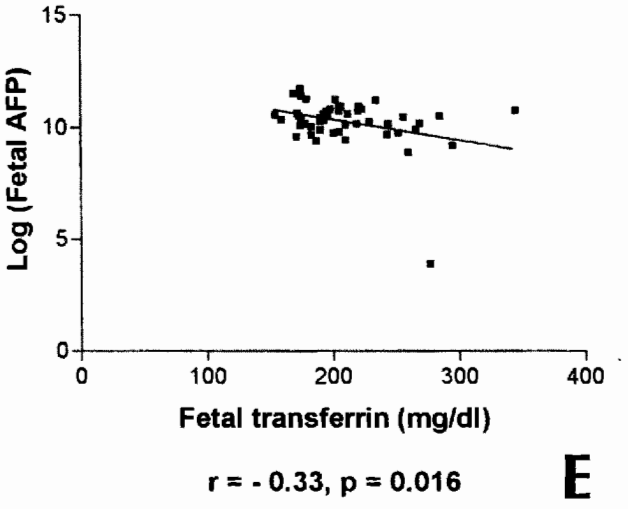
hematocrit were significant only in the cordocentesis group. Significant correlations were found in the cesarean section group between MSAFP, MCV and MCH (Table I).

Part 2 of the Study

A significant negative correlation was found between fetal serum AFP and RBC ($r = -.55$, $P = .000026$), hemoglobin ($r = -.40$, $P = .0035$), hematocrit ($r = -.46$, $P = .00052$) (Table II), erythropoietin ($r = -.40$, $P = .005$) and transferrin ($r = -.33$, $P = .016$) (Table III, Figure 4). No significant correlations were found with erythroblasts or any other biochemical or hematologic parameters.

Erythropoietin positively correlated with fetal RBC ($r = .47$, $P = .00065$), hemoglobin ($r = .45$, $P = .0013$), hematocrit ($r = .47$, $P = .00082$) and erythroblasts ($r = .42$, $P = .003$) (Table II).

Fetuses with hematocrit ≤ 45% (n = 22) had higher MSAFP levels (50,629.31 ± 28,762.60) than those



with hematocrit >45% (n=29) (28,762.60 ± 17,023.43). The difference was statistically significant even when log-transformed values were used (10.68 ± 0.55 vs. 9.97 ± 1.29) ($P = .01$) (Figure 5).

Discussion

AFP, serum albumin and vitamin D binding protein are members of a multigene family of proteins showing high structural homology. The physiologic role of AFP would be to mediate the transfer of fatty acids into cells and, as such, AFP would serve to modulate various growth regulatory pathways during fetal development.[1,2] Moreover, it has been suggested that an AFP/receptor autocrine system might operate in normal and malignant blood mononuclear cells.[8] However, the main biologic role of this abundant protein in fetal development is still unclear.

When a negative correlation between AFP and fetal red cell values exists, one should expect a high AFP level in anemic fetuses and a low one in situations of hemoconcentration. This could prove useful in explaining some poorly understood clinical facts. Increased levels of MSAFP have been found in two conditions associated with fetal anemia: parvovirus B19 infection and Rh immunization. In the first case, increased maternal serum AFP levels[6] have been attributed to viral hepatic injury or to placental leak secondary to immunologic damage at the maternal-fetal interface, but the increase in AFP has also been related to fetal aplastic crisis. In Rh immunization,[7] it is thought that the associated large placenta may allow a greater surface area for AFP transport from fetus to mother, although it is also known that in these cases, anemia is the main manifestation.

Decreased levels of MSAFP have been observed in clinical situations associated with fetal hemoconcentration. For example, decreased MSAFP is a screening method of detecting women at risk of bearing fetuses with chromosomal abnormalities, above all trisomy 21,[9,10] and these fetuses show increased fetal hemoglobin, hematocrit and RBC.[11] In diabetic pregnancies, decreased AFP in both amniotic fluid and maternal serum[12] and fetal hemoconcentration are common findings.[13]

Unexplained maternal serum AFP elevations have been associated with an increased risk of poor perinatal outcome, but the explanation for this phenomenon is unclear.[14,15] Several authors[14-17] have reported that elevated levels of MSAFP in the presence of a structurally normal fetus appear to result from some breakdown in the placental-maternal interface, leading to a greater-than-normal transfer of AFP from the fetal to maternal circulation, but the poor perinatal outcome found in these women cannot be explained by these conditions alone. Fetal anemia leading to higher hepatic hematopoietic activity could be involved in these cases.

We found a negative correlation between AFP and transferrin. Transferrin and AFP are proteins synthesized by hepatocytes, and it has been demonstrated that cellular interactions act as mod-

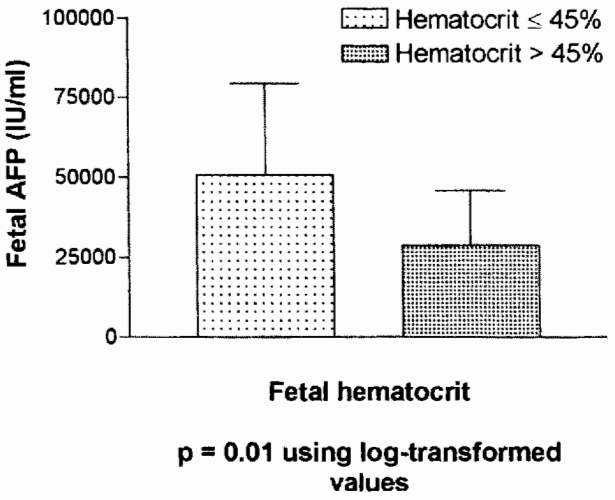


Figure 5 Comparison of fetal AFP between fetuses with hematocrit ≤ and >45%.

ulators, modifying gene expression of these proteins.[18] However, both proteins enter the cells via small vesicles and endosomes and move to multivesicular bodies and tubular vesicular elements located in the Golgi centrosphere region to finally be recycled back into the medium.[19] Therefore, we suggest that the finding of a negative correlation between both proteins could be due to a competition between them in gene expression and/or cellular action.

The exact role of AFP in fetal erythropoiesis has yet to be clarified.

We did not find a correlation between fetal AFP and fetal erythroblasts. Moya et al[20] studied erythropoietin in human fetuses with immune hemolytic anemia and hydrops fetalis and found that at mid to late gestation these fetuses responded with increases in plasma erythropoietin but that the changes were substantially attenuated before 24 weeks' gestation. Thus, gestational age seems to play a role in determining the magnitude of the erythropoietin response to anemia. It has been speculated that other growth factors might also contribute to erythropoietin in human fetuses. Thus, it may be that erythropoietin plays a less-important role in the regulation of fetal hematopoiesis at earlier developmental stages. In part 1 of our study, we found significant correlations between MSAFP and fetal hemoglobin only in the cordocentesis group, which had an earlier gestational age than the cesarean section group. In part 2 of the study, fetal serum AFP was studied only in term fetuses. Within this narrow gestational age range, 38–42 weeks' gestation, AFP highly correlated with hematologic parameters and erythropoietin, but failure of correlation between AFP and erythroblasts could probably be explained by the fact that in this period of pregnancy, erythropoietin is the main regulator of fetal erythropoiesis. The hypothetical role of AFP at that time could be much more limited. Further studies on preterm fetuses could be useful to clarify the relationships between fetal AFP and hematopoietic parameters in early pregnancy.

The exact role of AFP in fetal erythropoiesis has yet to be clarified. However, several connections can be found between AFP and hematopoiesis in the medical literature. It has been reported that interleukin 6 amniotic fluid levels during the second trimester of pregnancy significantly correlate with AFP.[21] The stimulatory activities of IL-6 on hematopoietic progenitor cells have been found in mice *in vivo*[22] and in both adult[23] and fetal[24] human cells *in vitro*. However, when administered to mice *in vivo*, IL-6 suppressed erythropoietin-induced erythropoiesis[25]; that effect was similar to the finding in this study of a negative correlation between AFP and erythropoietin. Therefore, AFP could be connected to the autocrine system, which regulates hematopoiesis and works with several growth factors and cytokines, including IL-6.

In conclusion, a significant negative correlation between fetal RBC, hemoglobin, hematocrit, erythropoietin and AFP was demonstrated but further clinical and experimental studies are needed to clarify the role of AFP in fetal erythropoiesis.

References

1. Mizejewski GJ: Alpha-fetoprotein binding proteins: Implications for transmembrane passage and subcellular localization. Life Sci 1994;56:1–9
2. Geuskens M, Torres JM, Esteban C, et al: Endocytosis of three serum proteins of a multigene family and of arachidonic acid in human lectin-stimulated T lymphocytes. Microsc Res Tech 1994;28:297–307
3. Hwang SJ, Lee SD, Wu JC, et al: Clinical evaluation of erythrocytosis in patients with hepatocellular carcinoma. Chuang Hua Hsueh Tsa Chih Tapei 1994;53:262–269
4. Sakisaka S, Watanabe M, Tateishi H, et al: Erythropoietin production in hepatocellular carcinoma cells associated with polycythemia: Immunohistochemical evidence. Hepatology 1993;18:1357–1362
5. Kew MC, Fisher JW: Serum erythropoietin concentrations in patients with hepatocellular carcinoma. Cancer 1986;58: 2485–2488
6. Carrington D, Gilmore DH, Whittle MJ, et al: Maternal serum alpha-fetoprotein: A marker of fetal aplastic crisis during intrauterine human parvovirus infection. Lancet 1982;1:433–434
7. Seppälä M, Ruoshlati E: Alpha-fetoprotein in Rh-immunized pregnancies. Obstet Gynecol 1973;42:701–705
8. Esteban C, Trojan J, Macho A, et al: Activation of an alpha-fetoprotein/receptor pathway in human normal and malignant peripheral blood mononuclear cells. Leukemia 1993;7: 1807–1816
9. Wald NJ, Cuckle HS, Densem JW, et al: Maternal serum screening for Down syndrome in early pregnancy. Br Med J 1988;297:883–887
10. Mac Donald ML, Wagner RM, Slotnick RN: Sensitivity and specificity of screening for Down syndrome with AFP, hCG, uE3 and maternal age. Obstet Gynecol 1991;77:63–68
11. Extermann P, Nicolini U, Rodeck CH: Acid-base and hema-

tological values at blood sampling in the evaluation of trisomic fetuses: A case-control study. Obstet Gynecol 1993;81: 958–962

12. Henriques CU, Damm P, Tabor A, et al: Decreased alpha-fetoprotein in amniotic fluid and maternal serum in diabetic pregnancy. Obstet Gynecol 1993;82:960–964
13. Salvesen DR, Brudenell JM, Snijders RJM, et al: Fetal plasma erythropoietin in pregnancies complicated by maternal diabetes mellitus. Am J Obstet Gynecol 1993;168:88–94
14. Katz VL, Chescheir NC, Cefalo RC: Unexplained elevations of maternal serum alpha-fetoprotein. Obstet Gynecol Surv 1990;45:719–726
15. Boyd PA: Why might maternal serum AFP be high in pregnancies in which the fetus is normally formed? Br J Obstet Gynaecol 1992;99:93–95
16. Robson M, Hamid R, McParland P, et al: Doppler ultrasound of the uteroplacental circulation in the prediction of pregnancy outcome in women with raised maternal serum alpha-fetoprotein. Br J Obstet Gynaecol 1994;101:477–480
17. Bartha JL, Comino-Delgado R, Arce F: Maternal alpha-fetoprotein in placental abruption associated with preterm labor. Int J Gynecol Obstet 1997;56:231–236
18. Lesciat G, Pasdeloup N, Kneip B, et al: Modulation of alpha-fetoprotein, albumin and transferrin gene expression by cellular interactions and dexamethasone in cocultures of fetal rat hepatocytes. Eur J Cell Biol 1987;44:128–134
19. Geuskens M, Torres JM, Esteban C, et al: Endocytosis of three serum proteins of a multigene family and of arachidonic acid in human lectin–stimulated T lymphocytes. Microsc Res Tech 1994;28:297–307
20. Moya FR, Grannum PAT, Widness JA, et al: Erythropoietin in human fetuses with immune hemolytic anemia and hydrops fetalis. Obstet Gynecol 1993;82:353–358
21. Weimann E, Reisbach G, Reinsberg J, et al: IL-6 and G-CSF levels in amniotic fluid during the second trimester in normal and abnormal pregnancies. Arch Gynecol Obstet 1995;256:125–130
22. Pojda Z, Tsuboi A: In vivo effect of human recombinant interleukin 6 on hematopoietic stem cells and progenitor cells and circulating blood cells in normal mice. Exp Hematol 1990;18:1034–1037
23. Leary AG, Ikebuchi K, Hirai Y, et al: Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: Comparison with interleukin-1α. Blood 1988;71:1759–1763
24. Gardner JD, Liechty KW, Christensen RD: Effects of interleukin-6 on fetal hematopoietic progenitors. Blood 1990;75: 2150–2155
25. Kyoizumi S, Murray LJ, Namikawa R: Preclinical analysis of cytokine therapy in the SCID-hu mouse. Blood 1993;81: 1479–1488