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Fetal and embryonic hemoglobins in erythroblasts of chromosomally normal and abnormal fetuses at 10-40 weeks of gestation

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ABSTRACT

Background and Objectives. **During fetal development a change in erythropoiesis from hepatic to medullary site occurs. In chromosomally abnormal fetuses this change is delayed. Hemoglobin production also undergoes developmental switches from embryonic to fetal hemoglobins in the first trimester of pregnancy. The aim of study was to determine the proportion of embryonic and fetal hemoglobins in fetal erythroblasts of chromosomally normal and abnormal fetuses at 10-40 weeks of gestation.**

Design and Methods. **Fetal blood was obtained from 93 chromosomally normal and 19 abnormal fetuses at 10-40 weeks of gestation. Fetal erythroblasts were isolated by triple density gradient centrifugation and magnetic cell sorting with CD71 antibody. Fluorescent antibodies were used to immuno-stain for zeta (ζ), epsilon (ε) and gamma (γ) hemoglobin chains.**

Results. **The percentages of the positively stained cells were calculated. In chromosomally normal fetuses the percentage of erythroblasts expressing the ζ chain was 25% at 10 weeks but this decreased exponentially with gestation to less than 1% by 17 weeks. Similarly, the percentage of cells expressing the ε chain decreased from 97% at 10 weeks to less than 1% by 25 weeks. In contrast, expression of the γ chain increased from about 30% at 10 weeks to 90% by 16 weeks and decreased thereafter to 60% at 40 weeks. In the abnormal fetuses, the percentage of erythroblasts expressing the ζ chain and the ε chain decreased to less than 1% by 23 and 28 weeks respectively, while maximum expression of the γ chain was at about 22 weeks.**

Interpretation and Conclusions. **In the chromosomally abnormal group the pattern of change in the expression of the various hemoglobin chains during gestation was similar to that in the normal fetuses but was delayed by three to six weeks. These findings suggest that in fetuses with chromosomal abnormalities there is a developmental delay in the switch from embryonic to fetal hemoglobin chains.**

Key words: Fetal blood, ζ, ε, and γ globins, gestational stages, normal and trisomic fetuses

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Hematopoiesis in the embryo and fetus can be divided conceptually into three overlapping periods: mesoblastic, hepatic, and myeloid, each corresponding to the major hematopoietic organ of the period, being the yolk sac, liver and spleen, and bone marrow, respectively.[1,2] The hepatic period extends from the 10th to the 24th gestational week, but the liver continues to produce red blood cells into the first week of postnatal life. However, from the 16th week onwards there is a rapid increase in medullary erythropoiesis.[2] In normal pregnancy, there is an exponential decrease with gestation in fetal blood mean red cell volume, erythroblast count, and CD71 expression, which is thought to be the consequence of the switch from hepatic to medullary erythropoiesis and maturation of the hematopoietic tissues, respectively.[3,4]

Hemoglobin production undergoes two developmental 'switches'; from embryonic ($\zeta_2\varepsilon_2$) to fetal hemoglobin ($\alpha_2\gamma_2$) commencing at 6-7 weeks of gestation, and subsequently from fetal to adult hemoglobin ($\alpha_2\beta_2$) at birth.[5] The hemoglobin switch, contrary to what was originally thought, is unrelated to the exact site of erythropoesis and does not coincide with the transition from yolk sac to splenic/hepatic and then medullary stages.[6] The alteration in hemoglobin chains is caused by the sequential activation of genes in the ζ and ε globin clusters on the short arms of chromosomes 16 and 11, respectively.[7]

In chromosomally abnormal fetuses mean red cell volume, erythroblast count, and CD71 expression are increased, suggesting developmental delay in the switch from hepatic to medullary hematopoiesis.[3,4] It is not known, however, whether such changes in erythropoiesis are accompanied by alterations in the expression of different types of hemoglobins.

This study examines the relative proportion of embryonic (ζ and ε) and fetal (γ) hemoglobins in fetal erythroblasts of chromosomally normal and abnormal fetuses between 10 and 40 weeks of gestation. To improve the yield of fetal erythroblasts for the study we used triple density centrifugation and magnetic cell sorting with CD71 antibody. The CD71 (transferrin receptor antigen) is an effective cell marker for erythroblasts.[8,4]

Design and methods

Subjects

Fetal blood samples were obtained antenatally by ultrasound guided cardiocentesis (n=6) or cordocen-

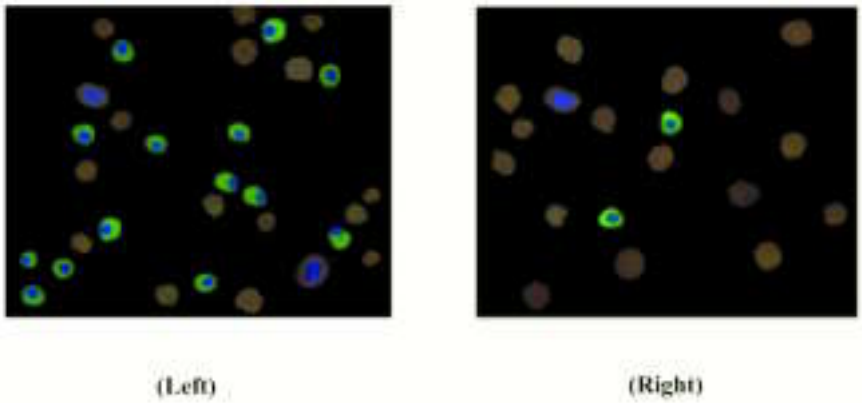

Figure 1. Photograph showing erythroblasts that are positive for ε globin chain fluorescent antibody (green) in cells after triple density centrifugation and anti-CD71 magnetic cell sorting (left) and in a whole blood film (right).

tesis (n=87) in patients undergoing prenatal diagnosis, and in 19 cases by umbilical cord puncture at elective Cesarean section. The fetal karyotype was normal in 93 cases and abnormal in 19 (47XX18, n=5; 47XY18, n=2; 47XX21, n=4; 47XY21, n=3; 47XX13, n=3; triploidy 47XXX, n=2). In all cases the Kleihauer-Betke test confirmed that the samples were fetal. Gestational age was calculated from the date of the last menstrual period and confirmed by ultrasound examination in the frist trimester. The patients gave written consent for fetal blood sampling for prenatal diagnosis and for the extra blood to be used for research. Blood samples from non-pregnant females (n=10) and males (n=10) were taken to assess the false positive rates for each of the three hemoglobin chains.

Isolation of erythroblasts and staining

Triple density gradient centrifugation with Histopaque (Sigma Aldrech, UK) and magnetic cell sorting was performed using CD71 antibody (Miltenyi Biotech, Bergisch Gladbach, Germany) for the fetal cell marker transferrin receptor antigen.[9,10] The positively selected erythroblasts were cytocentrifuged at 14.3 g for 10 minutes (Shandon, Frankfurt, Germany), and the cells were cytospun onto three glass slides. With this technique it was possible to produce slides with a high proportion of fetal erythroblasts; the alternative of using a simple blood film results in a low proportion of erythroblasts and high degree of contamination with non-nucleated cells (Figure 1).

Cells were fixed and permeabilized using a commercial 'Fix and Perm' reagents (Caltac Burlingham, CA, USA) at room temperature, and each slide was then incubated with monoclonal FITC conjugate fluorescent antibody for the ζ, ε and γ chains. The slides were washed in phosphate buffered saline solution, mounted with DAPII and then visualized under a fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Göttingen, Germany). Nucleated cells that showed specific staining above the background stain were counted as positive. At least 100 nucleated cells were counted.

Statistical analysis

For each hemoglobin chain the relation between fluorescent antibody positive erythroblasts (as a percentage of the total nucleated cells) and gestational age was determined by regression analysis. Multiple regression analysis was carried out to compare the percentage of positive cells in the three different chains between the chromosomally normal and abnormal groups, taking into account gestational age.

Results

The mean gestation for the chromosomally normal group was 27 weeks (range 10-40 weeks) and for the abnormal group was 23 weeks (range 13-38). In the chromosomally normal fetuses the percentage of erythroblasts expressing the ζ chain was 25% at 10 weeks but this decreased exponentially with gestation to less than 1% by 17 weeks (Figure 2; $Y = 474.29-110.43X+10.59X^2-0.535X^3+0.015X^4-0.0002X^5+0.00000013X^6$). Similarly, the percentage of cells expressing the ε chain decreased from 97% at 10 weeks to less than 1% by 25 weeks (Figure 2; $Y=1673.4884-373.897X+34.397X^2-1.662X^3+0.04439X^4-0.0006219X^5+0.0000036X^6$). In contrast, expression of the γ chain increased from about 30% at 10 weeks to 90% by 16 weeks and decreased thereafter to 60% at 40 weeks (Figure 2; $Y=-1563.069+412.56X-41.84X^2+2.21357X^3-0.064619X^4+0.000986X^5-0.000006139X^6$). In the chromosomally abnormal group the pattern of change in the expression of the various hemoglobin chains during gestation was similar to that in the normal fetuses but delayed by about three to six weeks (Figure 3). Thus, in the chromosomally abnormal group, the percentage of erythroblasts expressing the ζ chain or the ε chain decreased to less than 1% by 23 weeks and 28 weeks, respectively (compared to 17 weeks and 25 weeks in the normal group). Maximum expression of the γ chain was at about 22 weeks in the abnormal group, compared to 16 weeks in the normal group. Multiple regression analysis demonstrated that the percentages of all three hemoglobin chains, independent of gestation, were significantly higher in the chromosomally abnormal group than in the normal group (ζ, $p<0.0001$, $R=0.553$; ε, $p<0.0001$, $R=0.532$; γ, $p<0.0001$, $R=0.588$).

In the adult controls three of the 20 cases demonstrated positive cells for the fluorescent antibody for the γ chain and in these three cases the percentage of positive cells was 0.2%, 0.3% and 0.7%. None of the controls expressed any cells positive for the ζ or ε chains.

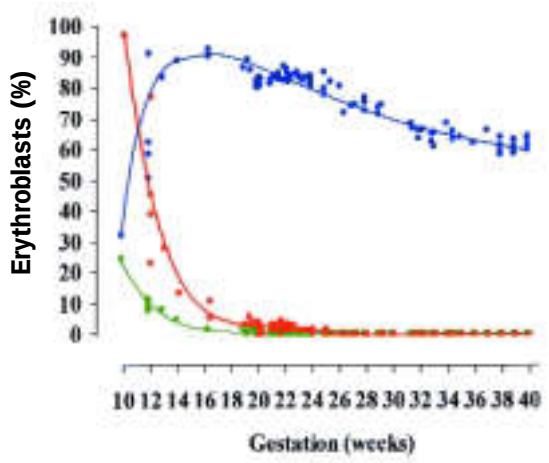

Figure 2. Percentage of erythroblasts positive for the γ, ε and ζ hemoglobin chains in relation to gestational age in chromosomally normal fetuses. The blue line = γ, the red line = ε, and the green line = ζ.

Discussion

The data of this study demonstrate that in chromosomally normal fetuses there is an exponential decrease with increasing gestation in the proportion of fetal erythroblasts expressing ζ and ε hemoglobin chains, with an initial increase and subsequent fall in the percentage of cells expressing the γ chain. These findings are compatible with the results of genetic studies reporting a gestation-related switch from embryonic to fetal hemoglobins in early pregnancy and a switch from fetal to adult hemoglobin at birth.[5,6]

In the samples from adult controls none of the cells expressed ζ or ε globin chains, but in 3 cases 0.2-0.7% of the cells demonstrated the presence of γ chains. The ζ globin chain is generally believed to be present in the embryo only during the first two months of life,[5] but it can persist beyond this gestational period in certain hematologic disorders, such as α-thalassemia and Hb Bart's hydrops syndrome.[11–13] The expression of ε globin chain usually ceases at 10-12 weeks of gestation and persistence of this gene expression is not known to occur. Fetal hemoglobin continues to be produced in adult life but its presence is confined specifically to very few erythrocytes called F-cells and the amount is 0-0.8%.[14]

The pattern of embryonic hemoglobin chains found in chromosomally abnormal fetuses suggests that in such fetuses there is a developmental delay in the switch from embryonic to fetal hemoglobin chain expression. This disturbance in normal hematopoiesis is also compatible with the results of previous hematological studies that reported persistence of macrocytosis and erythroblastosis in chromosomally abnormal fetuses.[3,4]

The findings of altered embryonic hemoglobin expression in our chromosomally abnormal fetuses cannot be explained by a gene dosage effect because the genes for the hemoglobin chains are situated on

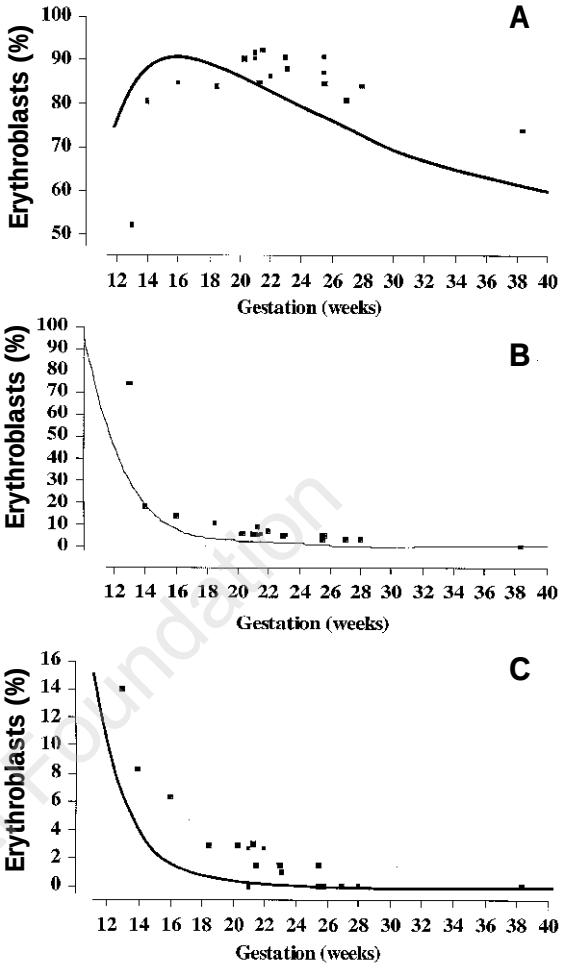

Figure 3. Percentage of erythroblasts positive in the three hemoglobin chains in chromosomally abnormal fetuses in relation to gestational age for γ (A), ε (B) and ζ (C). The curved line is the regression line of the association of these hemoglobin chains with gestation in chromosomally normal fetuses.

chromosomes 11 and 16. A possible explanation for our findings is that in fetuses with chromosomal abnormalities the process of DNA hypomethylation is altered. In eukaryotes, there is a strong direct correlation between hypomethylation and gene expression for the hemoglobin chains.[15-17] The ε gene is hypomethylated at six weeks of gestation and is fully methylated and suppressed starting from 10 weeks. Similarly the γ chain becomes hypomethylated at 10 weeks onwards. This DNA hypomethylation may be a prerequisite in the activation of a gene cluster coordinately expressed during human development.

A potential clinical application of our findings is the non-invasive diagnosis of fetal chromosomal abnormalities by examination of fetal cells in the maternal circulation. Previous studies have established that about one in 10^7 cells in maternal blood

are fetal erythroblasts and these cells can be enriched by magnetic cell sorting after attachment of magnetically labeled anti-CD71.[8,9,18,19] However, CD71 antigens are found in both fetal and maternal erythroblasts, whereas the embryonic hemoglobin chains ζ and ε are present only in fetal erythroblasts. This is particularly important in relation to screening for chromosomal abnormalities by measurement of fetal nuchal translucency at 10-14 weeks,[20] because at this gestational period there is an increased percentage of embryonic hemoglobins in the erythroblasts of chromosomally abnormal fetuses. We are currently investigating the potential value of these markers in the enrichment/isolation of fetal cells from the maternal circulation.

Potential implications for clinical practice

The findings in this paper have the potential to be used in the future in the identification of fetal cells from maternal blood as a non-invasive method for prenatal diagnosis.

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AMR: contributor of the concept and design of the study, performing the experimental work; performing data analysis and statistical work, writing the manuscript. HH: supervising the experimental work; contributing to the revision of the manuscript. FF: contributing to the revision of the manuscript. KHN: the main supervisor of the study; contributor of the concept and design of the study; supplying blood samples, reviewing data analysis; contributing to the writing and revisionof the manuscript.

Disclosures

Conflict of interest: none.

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