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Detection of Fetal Erythroid Cells from Maternal Blood Using Fluorescence In Situ Hybridization and Liquid Culture

Fetal nucleated erythrocytes circulating in maternal blood are a potential source of fetal DNA for noninvasive prenatal genetic diagnosis. However, the estimated ratio of fetal to maternal cells is extremely small. In order to enrich these cells, we performed direct culture using a two-phase liquid system. Mononuclear cells were obtained from maternal blood samples at 8-10^{+3} weeks of gestation and cultured in the first phase. After 4-5 days, the nonadherent cells were harvested and recultured with erythropoietin in the second phase for another 3-5 days. We examined cellular morphology, and counted the number of benzidine-positive cells and the percentage of glycophorin A/CD71 positive erythroid cells. We also did Kleihauer-Betke stain for Hb F, polymerase chain reaction (PCR) for SRY/DYZ1, chromosome analysis, and fluorescence in situ hybridization (FISH). The number of total erythroid cells reached about 0.1×10^6-1.0×10^6/mL with a purity of 84.0-97.3%. Hb F stain showed total erythroid cells of approximately 0.4×10^4-9.8×10^4/mL. Male DNA was detected in one case by PCR. In this case, the XY karyotype was confirmed by FISH and amniocentesis. This approach provides enriched source of fetal cells for further prenatal genetic analysis without complicated separation or sorting procedures.

Key Words: *Fetal Blood; Liquid Culture; Maternal Blood; Kleihauer-Betke Stain; Glycophorin A/CD71; In Situ Hybridization; Prenatal Diagnosis*

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INTRODUCTION

Fetal nucleated cells circulating in maternal blood are important candidate sources for noninvasive prenatal diagnosis. It can eliminate the small but significant risk to the fetus associated with traditional prenatal diagnostic approaches such as amniocentesis and chorionic villus sampling. Although a number of cell types have been investigated for potential use in prenatal diagnosis, fetal erythroblasts have received the most attention as they comprise about 10% of all red blood cells in the 11-week-old fetus and have a short life span (1-3).

The small number of fetal cells obtained, with ranges of $1/10^5$-$1/10^9$ fetal to maternal cells, however, has been the significant limitation for this kind of analysis. In these regards, if it is possible to culture the small number of fetal cells, more genetic material would be available for analysis. Lo et al. (4) cocultured male fetal liver-derived hematopoietic cells with 100-fold excess of mononuclear cells from a nonpregnant woman and after 7 days, they detected 0.01% to 0.25% male cells. Valerio et al. (5) separated fetal erythroid cells from maternal blood by magnetic sorting and then identified fetal BFU-E and CFU-E after 6-10 days of semisolid culture.

In this study, we employed a two-phase liquid culture system (6, 7) to enrich the fetal nucleated erythroid cells. The liquid culture consists of erythropoietin-independent (phase I) and erythropoietin-dependent (phase II) phases, and this method has the advantages in manipulation of culture conditions and components without terminating the culture. We successfully cultured the fetal erythroid cells up to 0.4×10^4-9.8×10^4/mL, which is enough amount for a variety of prenatal genetic studies.

MATERIALS AND METHODS

Subjects

We obtained maternal blood samples from the five pregnant women at 8-10^{+3} weeks of gestation with informed consent. Only primigravidas were included to rule out the influence from the previous pregnancies. In all cases, 15-20 mL of peripheral blood was collected in

Table 1. Summarized results of five maternal blood samples studied by a two-phase liquid culture

Case No.	Gestational weeks	Benzidine (+) cells (/mL)	GPA/CD71 (+) cells (%)	Hb F (+) cells (%)	Fetal erythroid cells (/mL)	Chromosome analysis [No.]	SRY/DYZ1 PCR	FISH XY/XX	Karyotype of amnios
1	10^{+3}	0.4×10^6	95.2	2.3	0.9×10^4	46, XX [33]	−/−	0/200	
2	9^{+4}	0.3×10^6	84.0	1.3	0.4×10^4	46, XX [18]	−/−	0/200	
3	8	1.0×10^6	87.6	9.8	9.8×10^4	46, XX [57]	−/−	0/270	
4	10^{+2}	0.5×10^6	97.3	5.8	2.9×10^4	46, XX [45]	−/−	0/200	
5	9^{+2}	0.1×10^6	93.7	3.6	0.4×10^4	46, XX [28]	+/+	6/212	46, XY

GPA, glycophorin A

sodium heparin tubes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). One pregnancy had amniocentesis later during the second trimester because of abnormal triple marker study.

Two-phase liquid culture

Mononuclear cells were separated from the maternal peripheral blood by Ficoll-Hypaque (density 1.077 g/cm^2) density gradient centrifugation and cultured in the first phase in alpha minimal essential medium (α-MEM) with 10% fetal calf serum, 1.5 mM/L glutamine, 1% penicillin-streptomycin, 1 μg/mL cyclosporin A, 10% conditioned medium collected from cultures of 5637 bladder carcinoma cell line at 37℃, 5% CO_2. After 4-5 days, the nonadherent cells were harvested and recultured in the same amount of α-MEM containing 30% fetal calf serum, 1% deionized bovine serum albumin, 10^{-5} M/L β-mercaptoethanol, 10^{-6} M/L dexamethasone, 0.3 μg/mL transferrin, 10 ng/mL stem cell factor (Sigma, St. Louis, MO, U.S.A.), and 1 U/mL erythropoietin (Cilag AG, Schaffhausen, Switzerland) for another 3-4 days.

Identification of fetal erythroid cells

We examined cellular morphology on cytospin-prepared slides at the second phase of the liquid culture. The number of hemoglobin-containing erythroid cells was counted by acid-benzidine stain (8) and the percentage of glycophorin A/CD71 positive erythroid cells was estimated using dual color flow cytometry on FACSort (Becton Dickinson, San Jose, CA, U.S.A.). We also did Kleihauer-Betke stain to identify fetal hemoglobin (Hb F) positive cells and calculated the number of fetal erythroid cells as the number of benzidine positive cells×the percentage of Hb F positive cells.

Five mL suspension from the second phase of liquid culture was used for chromosome analysis. Following conventional G-banding, standard metaphase preparations were made. Male fetal sex was determined by PCR amplification of sex-determining region of Y (SRY) gene and DYZ1 family encompassing 270 bp and 154 bp segments of the Y chromosome, respectively (9). Finally, air-dried metaphase slides were hybridized for fluorescence in situ hybridization (FISH) analysis with chromosomes X- and Y-specific centromere probes (Vysis, Inc., Downers Grove, IL, U.S.A.). Slides were counterstained and examined under a standard epifluorescent microscope equipped with a CCD camera, ChromoFluor System (Applied Imaging, Santa Clara, CA, U.S.A.). For each case analyzed, a minimum of 200 nuclei were counted.

RESULTS

Maternal blood samples produced erythroid progenitor cells in liquid culture. The most frequent cell type was pronormoblasts after 3-4 days of second phase (Fig. 1). Table 1 summarizes the clinical data, the total number of erythroid and fetal cells, and cytogenetic, molecular,

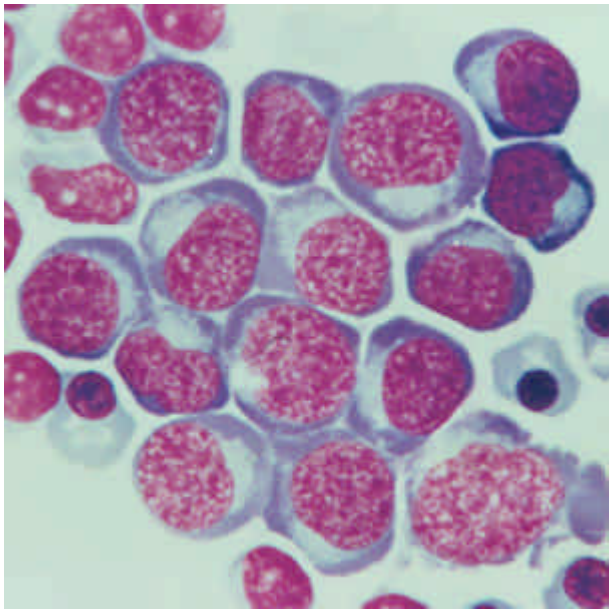

Fig. 1. Cytospin smear from the second phase of liquid erythroid culture. Pronormoblasts showed synchronized development around day 3 to 4 following exposure to erythropoietin (Wright-Giemsa, ×1,000).

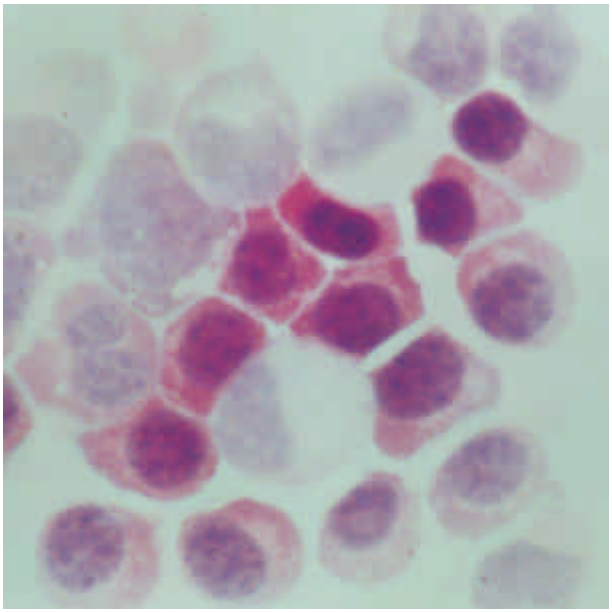

Fig. 2. Kleihauer-Betke staining of cultured erythroblasts in the second phase. Fetal erythroid cells stained positive due to the presence of Hb F (Kleihauer-Betke, ×1,000).

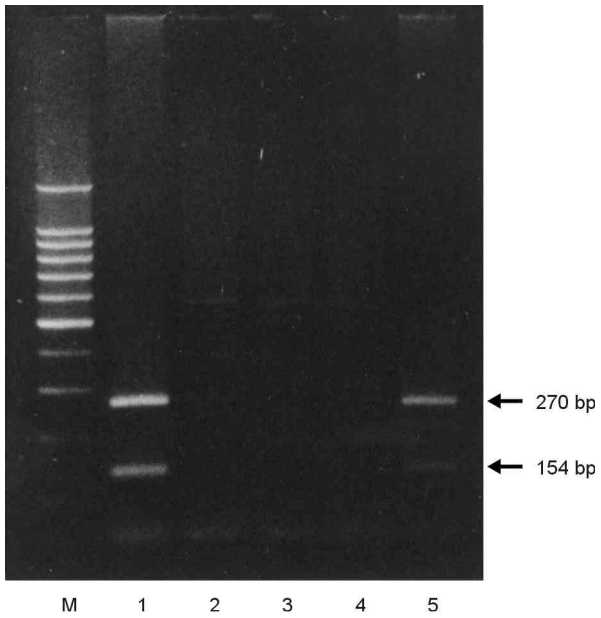

Fig. 3. Coamplification of SRY and DYZ1 loci from Y chromosome. The predicted size of the PCR products (270 bp for SRY and 154 bp for DYZ1) is indicated by the arrows (lane 1, normal male; lane 2, normal female; lanes 3-5, cases 1, 3, 5, respectively).

and FISH results of the cases studied.

The number of hemoglobin-containing total erythroid cells based on acid-benzidine stain reached approximately 0.1×10^6-1.0×10^6/mL. Dual color flowcytometry of glycophorin A/CD71 staining showed positive erythroid cells of 84.0-97.3% purity. The percentage of Hb F positive fetal cells (Fig. 2) ranged from 1.3% to 9.8% of total erythroid cells, and therefore the number of fetal erythroid cells was about 0.4×10^4-9.8×10^4/mL.

While counting 18-57 metaphases, only XX female karyotypes were observed. Male DNA was detected in one case by PCR amplification of SRY and DYZ1 sequences (Fig. 3). In this case, FISH analysis with X- and Y-specific probes (Fig. 4) showed Y-specific signals in six out of 212 nuclei (2.8%) and the XY karyotype of the fetus was confirmed by amniocentesis during the second trimester of the pregnancy.

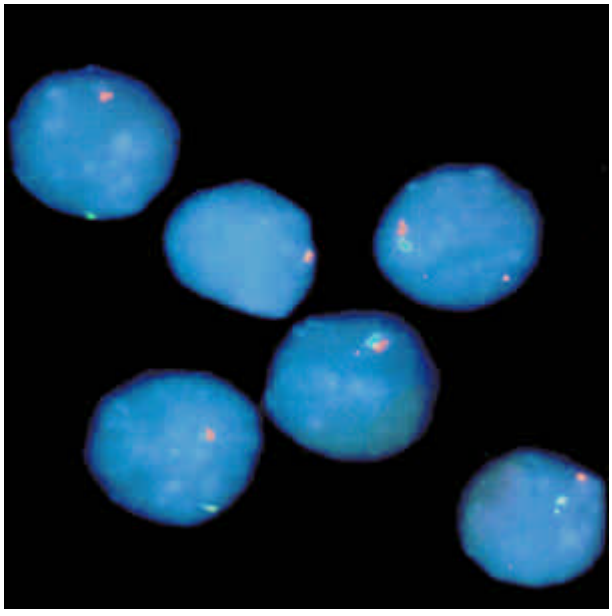

Fig. 4. FISH with X-(green) and Y-specific (red) probes show both X and Y signals in 6 interphase nuclei from the case 1 (×1,000).

DISCUSSION

Isolating fetal cells from maternal blood for genetic analysis is the least invasive method currently being evaluated. A number of cell types have been investigated as candidate fetal cells for use in prenatal diagnosis. The optimal cell type obviously must have a nucleus with DNA available for genetic analysis, be consistently present in the maternal circulation, be differentiated from maternal cells, and originate from the current pregnancy (1-3, 10, 11).

Fetal nucleated red blood cells have been the focus of some of the most successful work in this area. Although erythroid cells are very rare in peripheral adult blood,

they represent a much higher percentage of the nucleated cell population in the peripheral blood of the fetus. Because they have a well defined half-life, the circulating fetal nucleated erythroid cells in a pregnant woman would not be expected to persist in the next pregnancy. In addition, they express specific cell surface antigens like glycophorin A, transferrin receptor (CD71), and thrombospondin receptor (CD36). Thus, monoclonal antibodies against these antigens have been used for isolation of these cells in conjunction with various sorting techniques (1-3, 10-12).

Because of the rarity of fetal cells in maternal circulation, most investigators have utilized a variety of enrichment techniques (12-15). These techniques have included fluorescence-activated cell sorting, magnetic activated cell sorting, immunomagnetic beads, and antibody-conjugated columns. However, there is no clear consensus, at present, on which technique gives the best yield as judged by the total number of fetal cells recovered and the ratio of fetal/maternal cells obtained. One of the strategies may be the in vitro cultivation of the fetal erythroid cells (4, 5, 14, 16-18).

In this study, to enrich the fetal erythroid cells, we employed a two-phase liquid culture which supports the growth and differentiation of earlier human erythroid progenitor cells (6, 7). During the phase I, early committed progenitors proliferate and differentiate, and in the phase II, following exposure to erythropoietin, they continue to proliferate and mature into hemoglobin-containing normoblasts. We could directly culture mononuclear cells from five maternal blood samples. The number of total erythroid cells reached approximately 0.1×10^6-1.0×10^6/mL with a purity of 84.0-97.3% and the number of fetal erythroid cells isolated was 0.4×10^4-9.8×10^4/mL. Because we usually extract DNA and perform ordinary genetic analysis from 3-5 mL whole blood, the total number of fetal erythroid cells as much as 0.4×10^4-9.8×10^4/mL would suffice for the prenatal genetic diagnosis especially if we compare the number of cells recovered from the 3-5 mL whole blood and 20-30 mL culture suspension. In one case, male DNA was detected by PCR amplification of SRY/DYZ1 sequences, although chromosome study showed only XX karyotypes. In this case, the male karyotype was confirmed by FISH and subsequent amniocentesis.

The majority of the previous in vitro studies employed semisolid culture rather than liquid after separation of erythroid progenitor cells using various sorting techniques (4, 5, 14, 16-18). In the semisolid medium, cells are immobilized while growing, leading to several disadvantages or making it technically difficult or even impossible to carry out quantitative analysis of growth kinetics and/or biochemical and immunologic characterization of the developing cells. Valerio et al. (5) and Chen et al. (16) directly cultured mononuclear cells from the maternal blood specimens and could identify 18% and no fetal cells, respectively. Other studies (4, 14, 17, 18) were not designed for direct, prospective culture of fetal erythroid cells from maternal blood, but performed coculture of fetal and adult cells and only suggested that the methods could identify and isolate fetal nucleated red cells from the maternal erythroid cells.

Hb F, the marker which we used to identify fetal cells, is expressed in the most of fetal erythroid cells over a wide range of gestational ages. Although some maternal cells may produce Hb F, Bianchi (3) has found that most Hb F-positive cells in maternal blood to be fetal origin by molecular cytogenetic studies. As shown in Table 1 and Fig. 2, 3, and 4, our results clearly reveal that fetal erythroid cells can be cultured in vitro with reliable reproducibility. This liquid culture system permitted morphologic, cytogenetic, and molecular biologic studies without terminating the cultures during the study period. Although the maternal erythroid progenitor cells outnumbered those of fetal origin in culture, PCR, FISH, and amniocentesis results strongly suggest that there exist the increase of numbers of fetal cells in cultured blood.

In conclusion, this study showed that fetal erythroid cells were successfully cultured using a two-phase liquid culture system and the absolute number reached approximately 0.4×10^4-9.8×10^4/mL, which is enough amount for the majority of successive cytological and molecular researches. These promising results combined with thorough knowledge about biologic characteristics of fetal erythroid cells suggest that it may be eventually possible to obtain sufficient numbers of uncontaminated fetal cells in culture even for the prenatal analysis of chromosome abnormalities.

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