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Prenatal Genetic Diagnosis from Maternal Blood: Simultaneous Immunophenotyping and FISH of Fetal Nucleated Erythrocytes Isolated by Negative and Positive Magnetic Activated Cell Sorting

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Abstract

Fetal nucleated red blood cells (nRBCs) are rare in maternal circulation, but their presence constitutes a potential source of non-invasive prenatal genetic diagnosis. This study was undertaken to establish a non-invasive prenatal genetic diagnosis method using isolated fetal nRBCs. A multi-step method including triple density gradient and magnetic activated cell sorting (MACS) using CD45 and CD71, cytospin centrifugation, K-B staining, and glycophorin A-immuno fluorescence in situ hybridization (GPA-immuno FISH) was performed. The study population included 65 patients from 8 to 41 weeks of gestation, and fetal nRBC was separated from all cases. The number of fetal nRBCs retrieved was 12.8±2.7 in 8 to 11 gestational weeks, 15.2±6.5 in 12 to 18 gestational weeks, 16.4±6.5 in 19 to 23 gestational weeks, 10.6±3.2 in 24 to 28 gestational weeks, and 5.5±1.9 in 35 to 41 gestational weeks: the mean number of nRBCs collected from 20 ml of maternal peripheral blood was 13.7±6.2. The highest value of yield was 45.6% from 12 to 18 weeks gestation. The fetal sex determination confirmed by amniocentesis or chorionic villus sampling showed 100% sensitivity and 91.7% specificity for males; 91.7% sensitivity and 100% specificity for females. We showed that fetal cells can be reliably enriched from maternal blood and that they can be used for detecting specific chromosomes by FISH with a specificity superior to current non-invasive methods.

Key Words: Prenatal diagnosis, fetal nRBC, density gradient, MACS, GPA-immuno FISH

INTRODUCTION

Invasive methods such as amniocentesis, chorionic villus sampling, and cordocentesis are the currently used methods of prenatal genetic diagnosis, but they always encompass the possibility of morbidity to the mother and fetus. Triple marker testing using maternal blood is a currently used noninvasive method, but due to the lack of sensitivity and specificity, it can only be designated as a screening test. Accordingly, a noninvasive method, using fetal cells from maternal blood is under extensive investigation.

Fetal cells including lymphocyte, trophoblast, and nucleated red blood cells (nRBC) are found in maternal blood, and perfecting techniques to isolate these cells for prenatal genetic diagnosis has been an area of intensive research.[1] Fetal nRBC is considered to be the best choice for prenatal genetic diagnosis, but only extremely small amounts of nRBC are found in maternal blood, which necessitates the sorting and enrichment of fetal cells from maternal blood.[1-4] Recently, methods such as fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS) have been applied to achieve better quality fetal cell sorting and enrichment.[5-8] Polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are also successfully used in the prenatal genetic diagnosis.[9-12] This study was undertaken to establish a non-invasive prenatal genetic diagnosis method using isolated fetal nRBCs.

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MATERIALS AND METHODS

Subjects

Peripheral venous blood samples were collected from 65 women between 8 and 41 weeks of gestation and treated by heparin.

Methods

Isolation of fetal cells: For the isolation of fetal cells, we used a modified Gnshirt-Ahlert method. The maternal venous blood samples were diluted 1 : 2 with phosphate buffered saline (PBS). Then 10 ml of 1 : 2 diluted blood samples were underlaid with Ficoll-Histopaque (Sigma, St. Louis, Montana, U.S.A.) 1077, 1107, 1119 in the 50 ml tube and centrifuged for 30 minutes at 3,000 rpm. The mononuclear cells, in the second layer from the top, were isolated. Since this layer contains a large amount of maternal origin cells, we washed these mononuclear cells with 1*PBS 3 to 4 times in the 15 ml polyethylene tube. Well-washed cells were resuspended 80 μl/10 cells of anti-CD45 magnetic microbeads (DAKO, Carpinteria, California, U.S.A.) and incubated form 20 minutes in the refrigerator (8°C). For CD45 negative cell separation, separation columns were designed for optimal negative selections and a magnetic activated cell sorting (MACS) system was used. The cells adherent to the beads were removed and the negative- selected cells were washed 2 to 3 times with 1*PBS. After washing, the cells were centrifuged for 10 minutes at 1200 rpm at 4°C, the pellet was treated with 40 μl/ 10^7 cells of anti CD71 magnetic microbeads (DAKO) and incubated for 20 minutes in the refrigerator (8°C). After incubation, CD71 positive cells in the MACS separation column were isolated by using a pressure syringe in the MACS kit.

The isolated fetal cells were washed 2 to 3 times with 1*PBS and centrifuged for 5 minutes at 1,000 rpm. After the top layer was removed, the remaining layers were resuspended with PBS and centrifuged again. The cell solution which was centrifuged by using cytosin (Sandon, Germany) was taken (about 40 μl) and then made into a slide. The slide was incubated for 20 minutes at 37°C and treated by warmed 50 mM of KCl. After 20 minutes, KCl was removed. The slide was treated by 30% fresh fixative (3 : 1 v/v methanol acetic acid) and 70% 75 mM KCl. After 5 minutes, the slide was treated by new cooled fixatives for 1 minute and than dried for 2 minutes by slide warmer.

Kleihaur-Betke (KB) stain: The 37.5 ml of 0.1 M citric acid mixed with 10.5 ml of 0.2 M sodium phosphate was fixed to PH 3.2 at 37°C. The air-dried slide was fixed in 80% ethanol for 5 minutes, washed and dried. The slide was prepared in McIlvaine's buffer for 11 minutes, washed and stained by Erythrocin B solution for 5 minutes and Harris hematoxylene for 5 minutes and than examined under a microscope.

Immunochemistry: The slide which was kept in the refrigerator was warmed about 20 minutes at 37°C, refixed with cold Carnoy's solution and air-dried. After being dehydrated with 1*tris buffer saline (TBS, Sigma, St. Louis, Montana, U.S.A.), the slide was air-dried. The slide was treated with glycophorin A (GPA) monoclonal antibody and incubated in a moisture chamber for 1 hour. As soon as the slide was carried out of the chamber, the slide was washed with 1*TBS and combined with a second antibody (rabbit anti-mouse IgG (DAKO)). Washed in 1*TBS again, anti-rabbit APAAP complexes (DAKO) were added. In the moisture chamber, the slide was reacted for about 30 minutes and washed 2 to 3 times with 1*TBS until it looked clear to the naked eye. The next step was staining with alkaline phosphatase and fast red substrate (DAKO) by DAKO protocol. After counter-staining with hematoxylene (Sigma, St. Louis, Montana, U.S.A.) for 10 minutes, we washed and examined the slide by microscope. If the stained cytoplasmic membranes were examined, we confirmed DNA in the nucleus. If not, a signal amplification step was added. For signal amplification, hematoxylene stain was removed completely in the fixation solution (methanol : acetic acid=1 : 3), and we carried out the process again from the second antibody treatment step. When the signal was examined under the microscope, we caught the image by CCD camera, captured and memorized the special site and the morphology of the cells by using Immuno-FISH software (Cytovision Chromosome Auto Analyzer, Santa Clara, California, U.S.A.). The GPA immunostained slide was washed 3 times with 50°C 2× SSC/Tween 20 (each time took 5 minutes) and dried. Treated with 50°C ethanol 70%, 70%, 90%, 90% for 1 minute at each concentration, the slide was dried. Prepared in 73°C denaturation solution for 1 minute,

the slide was treated with 73°C ethanol 70%, 85%, 100% for 1 minute at each concentration. The slide was hybridized with a probe mixture (2 μl XY probe was mixed with 8 μl hybridization buffer) on the 46–50°C hotplate and then sealed with rubber cement in a 37°C humidity chamber overnight. Next morning, the cover slip was removed and then the slide was washed 3 times with 50% formamide/2×SCC. After being washed with 2×SCC for 10 minutes, 2×SCC/0.1 NP-40 for 5 minutes, the slide was air-dried in a dark room. We laid 10 l of counterstain on the target area and then examined it under a fluorescent microscope.

RESULTS

Microscopical analysis

Fetal nRBCs were identified under the microscope after the K-B staining to detect the presence of fetal hemoglobin (Fig. 1).

Number of nRBCs retrieved according to weeks of gestation

The study population included 65 patients from 8 to 41 weeks of gestation, and nRBC was separated from all cases. The number of nRBCs retrieved was 12.8 (2.7 in 8 to 11 gestational weeks), 15.2 (6.5 in 12 to 18 gestational weeks), 16.4 (6.5 in 19 to 23 gestational weeks), 10.6 (3.2 in 24 to 28 gestational weeks), and 5.5 (1.9 in 35 to 41 gestational weeks): the mean number of nRBC collected from 20 ml of maternal peripheral blood was 13.7 (6.2). The number of nRBCs retrieved increased from the beginning of the pregnancy, which reached its peak in the mid-trimester, and decreased abruptly thereafter (Table 1).

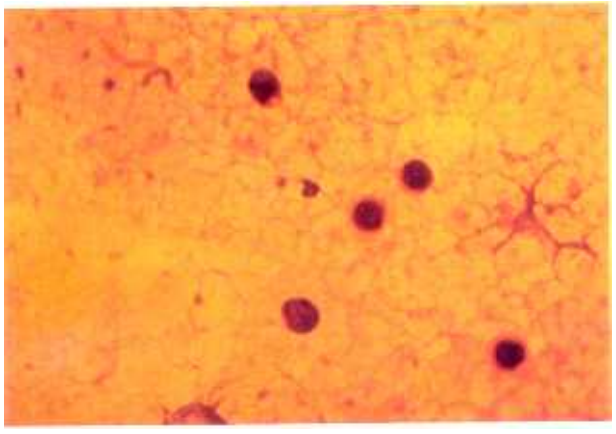

Fig. 1. Fetal nucleated red blood cells shown by Kleihaur-Betke stain.

The yield of nRBCs according to weeks of gestation

The yield of nRBCs was defined as the ratio of fetal nRBCs to the total number of nRBCs, where fetal nRBC was determined by K-B staining. The yield was 40.35% in 8 to 11 gestational weeks, 45.61% in 12 to 18 gestational weeks, 43.23% in 19 to 23 gestational weeks, 27.42% in 24 to 28 gestational weeks, and 15.28% in 35 to 41 gestational weeks. The yield was highest in 12 to 18 weeks of gestation. As the pregnancy went on, the portion of maternal nRBCs increased, and therefore the yield decreased. (Table 2, Fig. 2)

Fetal nRBC analysis using the GPA immuno-FISH

Simultaneous immunophenotyping of cells with the

Table Frequency of nRBC at Different Gestational Ages

Gestational age (wks)	Presence of nRBC	NRBC in 20 ml of maternal venous blood Range (mean±SD)
8–11 weeks (9 cases)	9/9 (100%)	12.8±2.7
12–18 weeks (25 cases)	25/25 (100%)	15.2±6.5
19–23 weeks (17 cases)	17/17 (100%)	16.4±6.5
24–28 weeks (8 cases)	8/8 (100%)	10.6±3.2
35–41 weeks (6 cases)	6/6 (100%)	5.5±1.9
Total (65 cases)	65/65 (100%)	13.7±6.2

Table 2. Frequency of nRBC at Different Gestational Ages Evaluated by K-B Stain

Gestational age (weeks)	8 11	12–18	19–23	24–28	35–41
Number of patient	9	25	17	8	6
Yields of nRBC (mean)	40.35	45.61	43.23	27.42	15.28

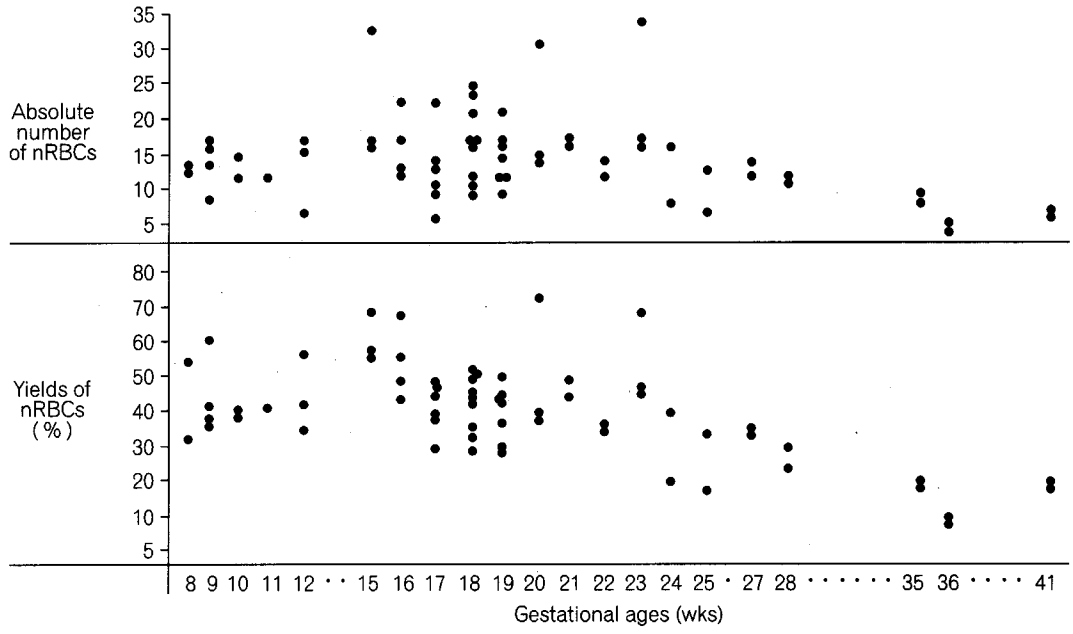

Fig. 2. The absolute number of nucleated red blood cells (nRBCs) and the yield of nRBCs at different gestational ages.

Table 3. Details of 11 Blood Samples Stained by Glycophorin A (GPA) Immuno-FISH

NO.	Gestational age	NO. of GPA (+) cells	FISH X/Y X signal 0	X signal 1	X signal 2	Y signal 0	Y signal 1	Sex by immuno-FISH	Karyotype by CVS or amnio
29	23 weeks	12	0	0	12	0	0	F	46XX
30	9 weeks	11	0	8	3	0	8	M	46XY
39	8.5 weeks	12	0	0	12	0	0	F	46XX
49	23 weeks	18	0	0	18	0	0	F	46XX
51	18 weeks	16	0	6	10	0	6	M	46XY
52	19 weeks	8	0	0	8	0	0	F	46XX
53	18 weeks	10	0	7	3	0	7	M	46XY
55	8 weeks	12	0	0	12	0	0	F	46XX
56	10 weeks	10	0	8	2	0	8	M	46XY
67	16 weeks	13	0	0	13	0	0	F	46XX
68	24 weeks	14	0	0	14	0	0	F	46XX

anti-glycophorin A antibody combined with FISH analysis using chromosome X and Y-specific DNA probes was performed. (Table 3, Fig. 3). Of the 65 samples, 96.9% (63/65) were immunostained, and in 95.2% (60/63) two signals could be concomittantly identified under fluorescent microscopy. As shown in Table 3, the XX signals were identified in 3 out of 11 nRBCs in case 30, 10 out of 16 in case 51, 3 out of 10 in case 53, and 2 out of 10 in case 56, but all these cells were from maternal origin. From the rest of the cells, XY signals were identified which confirmed the sex of the fetus to be male, and this was confirmed using the CVS or amniocentesis. In the other 7 cases, XY signals were not identified, and all the GPA-stained cells gave XX signals which confirmed the sex of the fetus to be female, and this result was also confirmed using the CVS or amniocentesis. Among the 65 cases, 53 patients undertook amniocentesis or CVS before 24 weeks of gestation according to indications such as advanced maternal

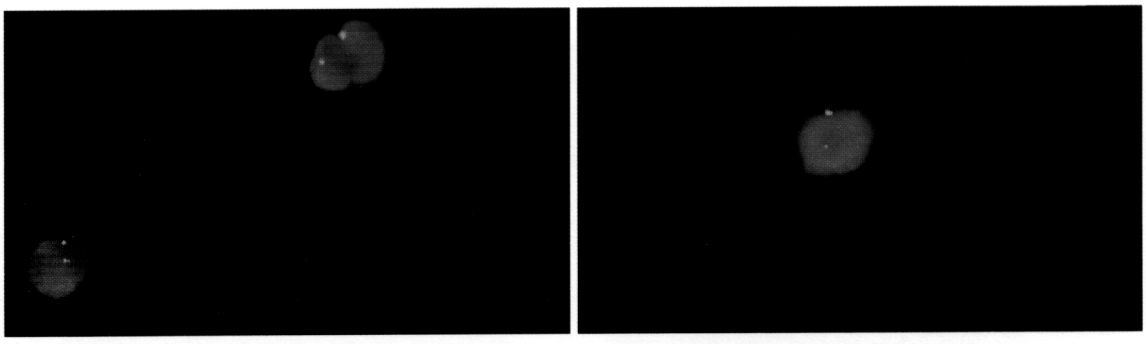

Fig. 3. Fluorescence in situ hybridization (FISH) of dual X/Y probes to fetal nucleated red blood cells purified by magnetic activated cell sorting (MACS) and glycophorin A (GPA) immunostaining. DNA (dual X/Y) probes were CEPX and LSI®SRY.

age, abnormal maternal serum markers, or previous history of giving birth to a chromosomally anomalous child. The fetal sex of the remaining 12 cases were confirmed after the child's birth. Thirty-eight cases were confirmed to be female and 27 were male. In 5 cases, GPA immuno-FISH could not be applied (2 male sex, 3 female sex), and there were 3 cases in which the result of CVS or amniocentesis differed from the GPA immuno-FISH result. The sex differentiation of the GPA immuno-FISH method showed 100% sensitivity and 91.7% specificity in the male sex, and 91.7% sensitivity and 100% specificity in female sex, which confirmed the high value of fetal cell separation from the maternal circulation in the clinical application.

DISCUSSION

The first report of fetal cell identification in the maternal body came from Schomorl; he found trophoblasts in the maternal lung of a patient who died because of eclampsia.[13] Fetal cells in the maternal blood are a potential source for the non-invasive prenatal genetic diagnosis, but only a small number of them are present in the maternal blood.[4] Estimates of the frequency of fetal cells in maternal blood vary, ranging from 1 in 10^5 to 1 in 10^9, and this rarity of fetal cells presents a technical challenge with regard to their isolation from maternal blood.[11]

The possible cell types that can be isolated from maternal blood and used for prenatal diagnosis include trophoblasts, lymphocytes, and erythroblasts.[1-3] The rarity of trophoblasts found in maternal blood during the first trimester and the lack of identity in representing the chromosome complement of the fetus makes them unsuitable for prenatal genetic diagnosis.[14]

Herxenberg et al. demonstrated the existence of fetal lymphocytes at 15 weeks gestation in pregnant women.[15] Although lymphocytes sorting using HLA-A2 and HLA-DR4 has been actively investigated, the formation of antibody is practically impossible due to the polymorphism of the HLA genomic locus.[16] The long life span of the lymphocyte being about 5 years, and the abundance of lymphocytes from maternal origin, makes the separation of fetal lymphocytes even harder.[14]

The focus is lately being given to fetal nRBC. The presence of fetal nRBCs was initially reported by Greger and Steele.[17] The almost perfect enclosure of genetic information, the existence of unique antigens such as the transferrin receptor, the short life span reaching about 30 days, and the absence of nRBCs originating from the mother in usual circumstances makes nRBC adequate for prenatal genetic diagnosis.[14]

The triple density gradient centrifuge separates the Ficoll-Histopaque in 1077, 1107, and 1119 layers, isolating nRBC from leukocytes and lymphocytes by forming a separate layer.[6] Nevertheless, most of the cells after triple density gradient centrifugation were still of maternal origin, therefore additional methods such as MACS or FACS were warranted.[18-21]

Among the numerous methods of cell separation, FACS and MACS are most commonly used. Herzenberg et al. were the first group to use FACS in the

cell separation process, but FACS is time consuming and too expensive.[15] MACS is a less expensive technique than FACS and allows a large volume of blood to be separated in a short period of time.[8]

Various monoclonal antibodies were employed to aid in the sorting process using MACS. Zheng et al. used CD45 and CD32 in MACS to exclude unnecessary lymphocytes and leukocytes in the process of fetal nRBC sorting.[22] Use of monoclonal antibody to CD 71 enabled the separation of dividing cells that are actively incorporating iron, which is a characteristic of fetal cells.[8] However, CD 71 is not uniquely expressed in fetal cells; erythrocytes express CD 71 on their cell surfaces from the burst-forming unit and erythroid stage, up to and including the reticuloyte stage.[5,8] Thilaganathan et al. reported almost 95% of nRBC to have CD71.[23] Gnshirt-Ahlert et al. concluded that the use of CD71 alone is not likely to enable a reliable identification of fetal cells in maternal circulation.[8] Holzgreve, Bianchi, and Wachtel used CD71, CD 36, and GPA successfully in the concentration process of nRBC.[19-21,24]

Significant maternal contamination was still present even after MACS enrichment preventing the accurate analysis of fetal cells by fluorescence in situ hybridization (FISH).[22] To overcome this problem, we performed simultaneous immunophenotyping of cells with the Glycophorin A combined with FISH analysis using chromosome X and Y specific DNA probes. Glycophorin A (GPA) is the major sialoglycoprotein of the erythroid cell membrane and it is only found on the surface of erythrocytes.[25] The potential problem of GPA is its lack of ability in distinguishing between fetal and maternal erythroid cells.[26] Bianchi et al. reported that the results when the red cell specific marker GPA was employed (alone or in combination with CD71 or CD36) were highly significant (100% accuracy in 13 cases).[5] Zheng et al. applied simultaneous mouse anti-HbF antibody (UCH) immunophenotyping-FISH analysis to MACS processed peripheral blood samples: 5 out of 6 pregnant blood samples revealed nucleated cells, where no nucleated cells were detected in 3 nonpregnant blood samples.[22] In the work of Troeger et al., the combination of 1119 density centrifugation and MACS with anti-GPA was most efficient, as this lead to the greatest yield of erythroblasts; while with anti-GPA, the yield of erythroblasts from the artificial mixture was highest compared to anti-CD36, anti-CD71, HAE9 and anti i.[27]

The method that we have used was a modification of the method used by Gnshirt-Ahlert et al. who performed triple density gradient, MACS, and cytospin: in this study we used triple density gradient, negative-MACS using CD45, positive-MACS using CD71, cytospin centrifugation, K-B stain, and GPA-immuno FISH.[28] The results of our study revealed a 100% success rate in the retrieval of fetal nRBCs in 65 pregnancies: in male sex, 100% sensitivity and 91.7% specificity was achieved, and in female sex, sensitivity was 91.7% and specificity 100%. Our results were better than Price et al. and Wachtel et al., which can be attributed to GPA immuno-FISH.[11,24] The 3 unsuccessful results can be attributed to the contamination of nucleated cells from maternal origin, or to a too-heavily concentrated nucleus of fetal cells and apoptosis leading to the failure of hybridization.[29] Nevertheless, the results could be considered outstanding, augmenting the possibility of this multi-step concentration system in the separation of fetal nRBCs.

K-B stain was used to analyze the morphology of nRBC.[30] Using the K-B stain, adult hemoglobin was washed from red cells of peripheral blood smears without removing the fetal hemoglobin, therefore identifying the presence of fetal hemoglobin.[29,30,33] The ratio of fetal nRBCs depicted by K-B stain from all the nRBCs retrieved is defined as the yield, and this parameter was used in comparing the concentration status.[31] According to the results of our study, the yield was highest (45.61) between 12 to 18 weeks gestation, and it decreased after 24 weeks gestation.

The fetal nRBC retrieval according to gestational weeks was high from 12 to 23 gestational weeks, being 15.2 ± 6.5 to 16.4 ± 6.5, and it decreased thereafter. This result implies that an important proportion of nRBCs in maternal blood are of fetal origin before 23 weeks gestation, while afterwards the majority of nRBCs may be of maternal origin. However, fetal nRBCs cannot be readily retrieved after 20 gestational weeks, and the reason might be explained based on the developmental process of fetal hematopoiesis; after 20 gestational weeks, most of the fetal blood components are formed from bone marrow, as in adults, and rather than nRBCs, leukocytes become the main components.[32] The result of Kuo et al. and Hamada et al. was also in agreement with the above results;

fetal nRBC increased in maternal peripheral blood with advancing gestation, from less than 1 in 100,000 nucleated cells in the first trimester to around 1 in 10,000 at term.[18,33] According to the study of Gnshirt-Ahlert et al., using more than 600 samples, the mean yield and purity of nRBCs increased with increasing gestational age, ranging from 100 to 1,000 cells per 40 ml blood sample and from 0.1 to 1%, respectively, from the 6th week of gestation to term.[28]

So far, fetal cell separation has been in use in two categories: fetal sex determination using Y-specific DNA sequence PCR or DNA aneuploidy analysis using FISH.[9,11] Fetal sex determination was successfully undertaken by Lo et al. and Yang et al., in maternal blood by applying PCR without fetal cell sorting and enrichment.[9,10] Recently, Sekizawa et al. introduced a suitable density gradient centrifugation method using 1.090 g/ml Percoll density gradient, where the result produced a mean of 13.3 fetal nRBCs out of 10ml of maternal serum.[34] The triple density gradient method that we have used in the enhancement of concentrated fetal cells can increase the purification ability, but comparatively more cells are destroyed, decreasing the number of nRBCs retrieved. Whether to use a triple or single-density gradient has not yet been determined.

So far, the prenatal genetic diagnosis of trisomy 18 and 21 were held by Elias et al. using FACS, Bianchi et al. using FACS and CD71, and Ganshirt-Ahlert et al. using MACS with the application of FISH to the separated fetal cells.[35-37] With advances in technology, the application of simultaneous multicolor-FISH to the immunophenotyped isolated fetal cells from maternal blood would provide the potential for the detection of common fetal aneuploidies. Further extension upon this study should allow the detection of Mendelean disorders by examining single enriched fetal cells isolated by micromanipulation by single cell PCR.

In conclusion, we have shown that fetal cells can be reliably sorted and enriched from maternal blood and that they can be used for detecting specific chromosomes by FISH with specificities superior to current non-invasive methods.

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