Clinical Potential for Noninvasive Prenatal Diagnosis through Detection of Fetal Cells in Maternal Blood

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SUMMARY

Fetal cells circulate in maternal blood and are considered a suitable means by which to detect fetal genetic and chromosomal abnormalities. This approach has the advantage of being noninvasive. Since the early 1990s, nucleated erythrocytes (NRBCs) have been considered good target cells for a number of techniques, including fluorescence-activated cell sorting and magnetic cell sorting, using antibodies such as anti-transferrin receptor and anti-γ-hemoglobin antibodies, followed by analysis with fluorescence *in situ* hybridization or polymerase chain reaction. In the late 1990s, the National Institute of Child Health and Human Development Fetal Cell Isolation Study assessed the reliability of noninvasive prenatal diagnosis of fetal aneuploidy using NRBCs isolated from maternal circulation. This study revealed the limitations of NRBC separation using antibodies specific for NRBC antigens. A more recent study has demonstrated the efficiency and success of recovery of NRBCs using a galactose-specific lectin, based on the observation that erythroid precursor cells have a large quantity of galactose molecules on their cell surface. Thus, recent advances in this field enhance the feasibility of this diagnostic method. This review article focuses on various methods of detection of fetal cells within the maternal circulation, as well as the status of previous and current studies and the prospective view for noninvasive prenatal diagnosis using fetal cells from the maternal circulation. [*Taiwanese J Obstet Gynecol* 2006;45(1):10–20]

Key Words: fetal cells, maternal blood, nucleated erythrocytes, noninvasive prenatal diagnosis

Introduction

Reliable methods of noninvasive prenatal diagnosis have long been sought in perinatal medicine (Table 1). Current means of prenatal diagnosis, such as amniocentesis and chorionic villus sampling, carry small but significant risks of miscarriage, although these methods are highly accurate. Only a small subgroup of pregnant women, generally 35 years or older, are offered these invasive methods. However, screening based on maternal age alone misses a large number of cases, since 80% of infants with trisomy 21 are born to women 35 years of age or younger [19]. Thus, since the late 1980s, alternative noninvasive methods of screening, including serum analysis and ultrasound evaluation, have been explored to improve the efficiency of screening [20]. Although these noninvasive methods can detect approximately 85% of cases of fetal aneuploidy with a false-positive rate of 5%, they do not provide a definitive diagnosis; rather, they estimate a woman's adjusted (or posterior) risk for various chromosomal aneuploidies. In the developed world, the percentage of pregnant women 35 years or older has increased steadily since the 1980s. Therefore, the percentage of Down syndrome pregnancies involving women of advanced maternal age has increased from approximately 30% to 40% [21]. These women and their partners generally have only one or two children and therefore many are reluctant to

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Table 1. History of fetal cell discovery in maternal blood

Year	Author	Content	Target cells	Methods
1863	Schmorl [1]	Trophoblasts observed in pulmonary vessels of women dead due to preeclampsia	Trophoblasts	Autopsy microscopy Microscopy
1959	Douglas et al [2]	Trophoblasts detected in maternal blood	Trophoblasts	Culture
1969	Walknowska et al [3]	WBCs with XY karyotype detected in cultured blood from male-bearing pregnant women	WBCs	
1972	Schroder and De la Chapelle [4]	Fetal WBCs detected by Y-chromatin in the blood of pregnant women	WBCs	Y-chromatin
1984	Covone et al [5]	Trophoblast detection by flow cytometry with H315 antibody	Trophoblasts	Flow cytometry
1988	Covone et al [6]	Trophoblast detection and analysis through FISH	Trophoblasts	Flow cytometry, FISH
1989	Lo et al [7]	Fetal DNA detected in whole blood samples from male-bearing pregnant women	Whole blood	PCR
1990	Mueller et al [8]	Trophoblast enrichment and analysis by PCR	Trophoblasts	PCR
1990	Bianchi et al [9]	NRBC enrichment by FACS with transferrin receptor antibody and confirmed Y-specific sequence	NRBCs	FACS (TfR)
1991	Price et al [10]	After FACS separation, fetal trisomy 21 diagnosed by FISH analysis	NRBCs	FACS (CD71, glycophorin-A)
1992	Bianchi et al [11]	After FACS separation by transferrin receptor, fetal trisomy 21 diagnosed by FISH analysis	NRBCs	FACS (TfR), FISH
1992	Cacheux et al [12]	Separation of trophoblasts by immunobeads and diagnosis of fetal aneuploidy 47,XYY by FISH analysis	Trophoblasts	Immunobeads, FISH
1993	Ganshirt-Ahlert et al [13]	NRBC enrichment by MACS and diagnosis of trisomy 21 or 18 cells	NRBCs	MACS, anti-CD71, FISH
1993	Hamada et al [14]	FISH applied to mononuclear cells confirmed the number of fetal cells in maternal blood (early gestation: 1/100,000 mononuclear cells)	Mononuclear cells	FISH
1995	Takabayashi et al [15]	NRBCs separated by double density gradients. After confirmation of NRBCs, cell individually micromanipulated.	NRBCs	Double density, micromanipulation
1996	Sekizawa et al [16]	NRBCs isolated by Takabayashi method and cell was individually analyzed by PCR for Duchenne muscular dystrophy	NRBCs	Double density, micromanipulation, PCR
1995–1999	NIFTY study [17]	Evaluation of the prenatal diagnosis accuracy through NRBCs in maternal blood	NRBCs	FACS or MACS
2002	Kitagawa et al [18]	NRBC enrichment through galactose-specific lectin method	NRBCs	Galactose-specific lectin method

WBC = white blood cell; FISH = fluorescence in situ hybridization; PCR = polymerase chain reaction; NRBC = nucleated erythrocyte; FACS = fluorescence-activated cell sorting; TfR = transferrin receptor; MACS = magnetic-activated cell sorting.

be exposed to the risk of an invasive prenatal diagnostic test. The development of newer noninvasive methods by which to assess fetal genetic and/or chromosomal abnormalities is thus expected to improve accuracy of detection.

Recently, two methods of noninvasive prenatal diagnosis have been subject to investigation: fetal cells and cell-free DNA circulating within maternal blood (plasma or serum). The use of cell-free fetal DNA was discussed in a previous review published in this journal [22]. The present review focuses on the use of fetal cells within the maternal circulation for prenatal diagnosis.

Fetal Cells in the Maternal Circulation

Trophoblasts

It has long been known that trophoblasts circulate in maternal blood. At the turn of the 19th century, German

pathologist Schmorl demonstrated trophoblasts in the lungs of women who died from eclampsia [1]. In 1959, Douglas et al detected trophoblasts in maternal blood using light microscopy [2]. However, the limited number of trophoblasts normally present in maternal blood led to difficulty reproducing their findings. It is difficult to isolate trophoblasts since they are large multinucleated cells that become trapped in the lungs and are rapidly cleared from the maternal circulation [23], even in women with hypertension in pregnancy, from whom trophoblasts have been recovered from the uterine vein, inferior vena cava [24], and peripheral circulation [5]. In addition, trophoblast trafficking seems not to be a phenomenon common to all pregnancies [25]. Enrichment of these cells is difficult due to a lack of specific antibodies [26,6]. Furthermore, it is known that 1% of placental karyotype differs from that of the fetus due to confined placental mosaicism [27,28]. This also restricts the use of trophoblasts in maternal blood as a tool for genetic diagnosis. However, despite these obstacles, some diagnostic success has been achieved through detection of Y-chromosome sequences by polymerase chain reaction (PCR) amplification and fluorescence *in situ* hybridization (FISH) techniques using trophoblasts from maternal blood [8,29,30]. Hawes et al have also accurately detected a fetal β-globin mutation in trophoblasts from maternal blood [29].

Leukocytes

Through successful enrichment of leukocytes from the maternal circulation, it has been demonstrated that fetal leukocytes traverse the placenta. This has made noninvasive prenatal diagnosis possible. Although Schmorl discovered the presence of trophoblasts in the maternal circulation almost a century ago, the use of fetal cells for prenatal diagnosis was not considered feasible until 1969. At this time, Walknoska et al observed cells with a 46,XY karyotype in cultured lymphocytes from 21 pregnant women, 19 of whom subsequently delivered male infants [3]. This finding was later confirmed by other investigators in the early 1970s, who also demonstrated Y-chromatin within cells in the maternal circulation of women carrying male fetuses [31,4,32–36]. However, hundreds of maternal cells need to be analyzed in order to document the presence of just a few fetal cells in the maternal circulation, a labor-intensive process. Thus, these studies used methods to enrich fetal cells for detection, including a nylon wool column and selective cell culturing [37,38].

Herzenberg' group were the first to use fluorescence-activated cell sorting (FACS) to successfully enrich fetal leukocytes from the maternal circulation [39,40]. They showed a significant correlation between a male fetus at birth and human leukocyte antigen HLA-A2 with detection of a quinacrine-positive Y body in flow-sorted cells. This method, which requires HLA-typing of the father, was validated in a subsequent study using PCR amplification of Y chromosome-specific sequences after flow-sorting based on paternal HLA polymorphisms [25] However, other investigators have had limited success using this method, even following selection based on several HLA differences using monoclonal antibodies. One study found only 18 HLA-informative couples out of 78 screened [41].

All observations to date suggest persistence of fetal cells in maternal blood. Early interest in fetal leukocytes for prenatal diagnosis was based on their ability to proliferate *in vitro*, suggesting that they might also proliferate *in vivo* in maternal organs. That meant that this approach could theoretically lead to misdiagnosis in multigravida. An early study by Schroder et al determined that fetal leukocytes can still be detected in the maternal circulation up to 1 year after birth [42]. This study used mitogen stimulation of leukocytes in order to detect Y-chromatin in a woman who had previously born a son. This has been confirmed by others, in some instances up to 5 years post-partum [43–45]. Moreover, Bianchi et al used FACS enrichment of T and B lymphocytes to identify the presence of fetal progenitor cells (CD34+, 38+) in the maternal circulation 27 years post-partum [46]. In another study, T lymphocytes (CD3+, 4+, 5+) were found to persist, for as many as 6 years in one instance, after birth. These studies indicate that cell types with a short life-span should be used for prenatal diagnosis.

Fetal erythrocytes/nucleated red blood cells

In 1976, Kleihauer et al demonstrated the presence of immature erythrocytes circulating in maternal blood using a new staining method [47]. In 1964, Clayton et al observed nucleated erythrocytes (NRBCs) more frequently under certain circumstances, such as rhesus incompatibility, or following amniocentesis and termination [48]. NRBCs are one of the first hemopoietic cell lines produced during fetal development. They are abundant in the fetal circulation during early pregnancy [49]. When blood pools at the interface between fetal and maternal tissue, transfer of erythrocytes, including NRBCs, into the maternal circulation predominates over that of other cell types, including leukocytes and trophoblasts. NRBCs are mononuclear and relatively well differentiated. They also have a short life-span compared to fetal lymphocytes given their limited proliferative capacity [50], making them unlikely to persist throughout pregnancy. These characteristics make

NRBCs particularly suitable for noninvasive prenatal diagnostic testing.

In 1990, Bianchi et al discovered a way to enrich NRBCs containing fetal DNA for FACS using a monoclonal antibody against the transferrin receptor (CD71), which is highly expressed on erythoblasts [9]. The ability to do so has since been confirmed by other investigators using a variety of monoclonal antibodies and cell enrichment techniques [10,51–53]. One such method is magnetic cell sorting (MACS) using an antibody to CD71 [54]. Ganshirt-Ahlert et al have used MACS following NRBC enrichment to correctly identify fetal aneuploidy [13,24] These successful attempts at NRBC enrichment and fetal determination demonstrate the potential of using NRBCs for noninvasive prenatal diagnosis.

However, the isolation of fetal cells from the maternal circulation presents considerable challenges, given their limited numbers. Fetal cells are estimated to range from 1 in 10^5 to 1 in 10^9 cells in maternal blood [10,55]. Hamada et al used FISH on mononuclear cells isolated by density gradient separation from maternal blood to find Y chromosome-bearing cells [14] They needed to screen as many as 144,000 nuclei to find a single fetal cell containing DNA that hybridized to the Y chromosome. An increased frequency of fetal cell isolation with gestational age was observed, from less than 1 in 10^5 during the first trimester to 1 in 10^4 at term. Bianchi et al examined the number of fetal-cell DNA equivalents present in maternal blood by PCR amplification of a Y chromosome-specific sequence and found approximately one fetal cell per 1 mL of maternal blood [56]. Thus, although the presence of fetal NRBCs in maternal blood is well established, and they are currently considered the best target for noninvasive prenatal diagnosis, their detection remains problematic.

Undoubtedly, some NRBCs, even after fetal cell enrichment, are of maternal origin [49,57–59]. De Graff et al used fetal hemoglobin to differentiate maternal from fetal NRBCs, but 20% of all fetal hemoglobin-positive NRBCs were still of maternal origin [60]. Recently, Troeger et al used single-cell PCR analysis on single micromanipulated NRBCs identified by May-Grunwald Giemsa staining to demonstrate that almost half of NRBCs in maternal blood are of fetal origin [61]. These findings suggest that the origin of each cell must be confirmed for reliable clinical use when performing noninvasive prenatal diagnosis through analysis of cells recovered from maternal blood.

Another approach is to culture NRBCs. If selective induction of NRBC proliferation occurs *in vitro,* fetal genetic material can be amplified for noninvasive prenatal diagnosis. Lo et al were the first to culture mononuclear cells and to isolate fetal erythroid progenitors from the peripheral blood of pregnant women [62]. Subsequently, other investigators have successfully cultured colony-forming units, as well as erythroid and mature burst-forming units and erythroid colonies, from fetal hemopoietic progenitors enriched from maternal blood [63]. However, these results have not been replicated consistently, and thus far selective amplification of fetal over maternal hemopoietic progenitors has not been successful [64,65].

Physiologic Variation in the Maternal Circulation

Data indicating the timing of fetal cell trafficking into the maternal circulation exists. In mice, fetal cell migration is a rare event [66]. In general, it is thought that the proportion of NRBCs to non-nucleated erythrocytes diminishes in fetal blood as gestation progresses in humans. In accordance with placental growth, the interface between fetal and maternal tissue expands, such that more fetal cells may traverse the placental barrier in the early stages of gestation. In a study of two pregnant women bearing male fetuses following *in vitro* fertilization, Y chromosome-specific DNA was detected as early as 33 and 40 days gestation [67,68]. PCR amplification of a Y chromosome-specific sequence has been achieved in maternal blood at between 6 and 12 weeks in two separate studies [14,69]. Other studies using flow-sorted NRBCs have demonstrated reliable detection of fetal DNA at less than 16 weeks of gestation [70].

It has been reported that more fetal cells are recovered when the fetus is aneuploid [56,71]. This might be associated with the ultrastructure of the placenta in pregnancies affected by aneuploidy [72, 73]. It might also be associated with erythrocyte size, which differs from that in cytogenetically normal individuals of the same gestational age [74]. Aneuploid fetal cells express CD71 [75] or FB3-2 and H3-3 [76] antigens. Simpson and Elias demonstrated that, on average, 19.6% (074%) of enriched cells from maternal samples were trisomic fetal cells, using FISH analysis with chromosome-specific probes [77]. Using the same method, Ganshirt-Ahlert et al found that 10% of the final population of enriched cells were trisomic fetal cells [13].

Increased proportions of fetal cells have been detected in women with preeclampsia, which follows historical observations by Schmorl [1] and Clayton et al [48]. Holzgreve et al also noted a large increase in the number of NRBCs (38 *vs* 7) in male-bearing pregnancies

with preeclampsia [78]. Other factors that are likely to influence the degree of transfer of fetal cells into the maternal circulation are multiple gestation, feto-maternal blood incompatibilities, and other maternal complications, such as diabetes or bleeding. Another issue is the possible influence of autoimmune disease, such as scleroderma [79].

Various Methods to Enrich NRBCs from Maternal Blood

For noninvasive diagnosis using fetal cells from the maternal circulation, enrichment of NRBCs is essential. Methods include FACS, MACS, density gradient centrifugation, charged flow separation, selective erythrocyte lysis, and the lectin base method. Efficient selection of NRBCs is essential for analysis of fetal genetic abnormalities. Although a number of reports describe successful enrichment of NRBCs, a preferred method has yet to be established.

FACS and MACS

FACS and MACS were preferred methods for fetal cell enrichment in the 1990s. Both technologies rely on antigen–antibody recognition using NRBC-specific monoclonal antibodies. In order to perform FACS, the antibodies are first labeled with a fluorescent dye, while they are labeled with magnetic beads for MACS. As previously mentioned, Bianchi et al first used a monoclonal antibody against CD71 to enrich NRBCs [9]. They sorted CD71-positive cells from the blood of pregnant women at 1,217 weeks' gestation, after which they performed PCR amplification of a Y-specific sequence. They detected the Y-specific sequence in 75% of women bearing male fetuses. However, after selection using the CD71 antibody, the purity of NRBCs remained low. Subsequent to this, their group established a new NRBC marker, a monoclonal antibody against γ-hemoglobin. This marker considerably improved the purity of isolated NRBCs [80,81]. Ganshirt-Ahlert et al then used MACS separation with magnetic beads bearing antibody against CD71 to successfully identify fetal cells with chromosomal abnormalities using FISH in blood samples from 15 pregnant women bearing fetuses with chromosomal abnormalities [13, 82]. Some investigators have also used glycophorin A for enrichment of NRBCs [83]. Purity can be further enhanced by depletion of maternal cells and fetal lymphocytes with anti-CD45 antibody prior to positive selection for CD71-positive cells. Using this method, approximately 20 fetal cells are obtained from a 20-mL maternal blood sample [84]. However, even following double MACS separation, the purity of NRBCs remains low, and a prolonged interval is required for NRBC detection. The advantages of using MACS include the short time required for the procedure itself and its relatively low cost. A disadvantage is that selection is based on only one criterion, thus explaining the low purity of NRBCs obtained.

In order to improve the recovery of NRBCs, Bianchi's group primarily examined samples collected after termination procedures, which are thought to increase the number of NRBCs in maternal blood. They recovered fetal NRBCs from maternal blood in all post-termination samples [85–89]. We further observed an improvement in the recovery of NRBCs by MACS negative selection with CD45 prior to FACS separation using γ-hemoglobin antibody [81].

NIFTY Study

Based on the success of fetal NRBC detection using FACS-FISH and/or MACS-FISH analysis, NRBCs were thought to have potential for noninvasive fetal diagnosis. In the USA, to examine this possibility, a large-scale multicenter study funded by the National Institutes of Health, known as the National Institute of Child Health and Human Development Fetal Cell Isolation Study (NIFTY), was performed between 1995 and 1999 [17]. The purpose of the study was to assess the reliability of noninvasive prenatal diagnosis of fetal aneuploidy using NRBCs from maternal circulation. Subjects thought to be at risk of carrying an aneuploid fetus (> 35 years of age), or with serum screening or sonographic results suggestive of aneuploidy, about to undergo an invasive diagnostic procedure, were selected. The results were compared with the karyotype obtained following the invasive procedure as the gold standard. Fetal cell detection had very low sensitivity in this study, in which more than 2,700 maternal blood samples were examined, ranging from 13% using FACS to 44% using MACS. Thus, MACS separation yielded a better recovery of fetal NRBCs than FACS separation [17]. Although the purity of NRBCs was high following FACS separation using γ-hemoglobin antibody, fewer NRBCs were recovered. In contrast, the greater number of cells separated using MACS led to improved recovery of NRBCs, despite an overall decrease in the proportion of NRBCs to total cells collected. Thus, recovery of NRBCs using MACS was slightly better than FACS. However, fetal NRBCs were difficult to detect with both methods. This is probably due to the extremely low number and proportion of fetal NRBCs in maternal blood. Consequently, this study concluded that the utility of NRBC isolation using NRBC-specific antigens is limited.

Density gradient centrifugation

To separate NRBCs, removal of mature erythrocytes is a very important initial step. Methods include bulk separation, lysis, and various forms of selective centrifugation. Since there is no report that NRBCs are efficiently separated by cell lysis, more recent protocols begin with some form of density gradient centrifugation intended to enrich a mononuclear cell-rich population. In the early 1990s, a 1.077 g/mL density gradient was primarily used. However, in 1993, Bhat el al showed a 25-fold enhancement of NRBC isolation using a triple density gradient [90] Using this protocol, fetal NRBCs were successfully isolated from maternal blood of aneuploid pregnancies in the second and third trimesters [13].

In 1995, Takabayashi et al used discontinuous gradients to enhance enrichment [15]. They placed a 2-mL maternal venous blood sample on double density gradients of 1.075 and 1.085 g/mL Percoll solution. Following centrifugation, cells with the targeted densities were placed on slides and stained with May-Giemsa for morphologic NRBC identification. Takabayashi et al identified a large proportion of NRBCs among the nucleated cells obtained using this approach. An average of 4.1 NRBCs (122) were identified per sample analyzed, from which fetal sex could be accurately determined in 10 of 11 samples. NRBCs were detected as early as 8 weeks' gestation. Later, we evaluated the effects of various density gradients on recovery of NRBCs. We used FACS separation based on γ-hemoglobin and subsequent FISH analysis [81]. The number of NRBCs recovered using a gradient of 1.090 g/mL was three times greater than with a gradient of 1.085 g/mL. Moreover, we recovered twice as many NRBCs using 1.119 g/mL than with 1.090 g/mL [80]. Voullarie et al examined the density of NRBCs using a continuous Percoll density gradient and revealed that most NRBCs have apparently greater density than white blood cells [91]. Thus, since NRBCs are more dense than white blood cells, it is necessary to use greater density gradients to recover a high yield of NRBCs. Some investigators prefer to use Ficoll 1119 [92,93].

Other methods

Other methods used for enrichment of fetal cells include avidinbiotin columns, magnetic ferro-fluids, and cell culturing. To enhance enrichment, some investigators have sought more specific antibodies or biochemical markers, while some have relied on chemical assays for analysis, such as 2,3-biophosphoglycerate (BPG) [52], carbonic anhydrase (CA) [60,94], and thymidine kinase (TK) [95]. BPG is thought to identify fetal hemoglobin by exposing the fetal heme-iron to oxidization in a sequential peroxidase reaction, thereby forming a colored substrate-associated complex. Using a fluorescent thymidine analog, fetal cells can be differentiated from adult cells based on TK enzyme activity, which is virtually absent in adult cells. Fetal NRBCs are also less susceptible to ammonium chloride lysis than adult NRBCs since CA activity is fivefold less, and acetozolamide permeability approximately 10-fold greater. Other specific antibodies include HAE9 [96] and those developed by Genzyme (FB3-2, 2-6B/6, and H3-3) [76]. The possibility of using an erythropoietin assay has also been explored [97]. However, at the present time, most researchers still either use anti-CD71 or anti-γ-globin antibodies for enrichment of NRBCs.

Other investigators have isolated very high numbers of NRBCs by charge flow separation [98,99], which permits sorting of cells according to their characteristic surface charge densities. Using this method, several thousand NRBCs (average, 6,910) were enriched from a 20-mL maternal venous blood sample in one study, from which both fetal sex and ploidy could be accurately examined. However, this result has not been repeated.

Recent advances: the lectin-based method

A more recent study has used a galactose-specific lectin for isolation of fetal NRBCs from maternal blood [18]. The method is based on the observation that erythroid precursor cells express large numbers of galactose molecules on their cell surface, associated with development and maturation of the cell. In this study, the authors used soybean agglutinin as a galactose-specific lectin to enrich high galactose-expressing erythroid cells, from which they recovered one to several hundred NRBCs (mean ± standard deviation, 7.8 ± 8.5) from 2.3 mL of peripheral blood from 96% of pregnant women between 6 and 27 weeks' gestation. The isolated NRBCs were then analyzed using a Y chromosome-specific FISH probe in eight cases carrying male fetuses, for which Y-signals were detected in all eight cases, and more than half of all NRBCs collected were of fetal origin. Subsequent to this, Babochkina et al compared the success of this method with MACS/CD71 separation, and revealed an eightfold increase in NRBC recovery using this method [100]. In an unpublished study of this method, we evaluated how many NRBCs can be separated from the blood of normal pregnant women in early gestation. We detected NRBCs in all 32 cases examined (1–81 cells/sample). The median number of NRBCs detected in normal pregnancy was 13 cells/6 mL of maternal blood. In order to identify the NRBCs, we manually

screened two slides per case, which was very labor intensive. However, following enhancement with lectin, most contaminant cells were non-nucleated erythrocytes. The lectin method seems preferable to other methods, such as MACS, since it is easy to use with automated detection systems. Thus, the lectin method to enrich NRBCs is considered the best method so far.

Autodetection

Since enrichment of NRBCs produces a low-purity sample, the requirement for subsequent screening is labor intensive. This has created interest in automated detection systems, using laser-mediated scanning or a charge-coupled device (CCD) with a video computer-aided capture and dot-counting analysis system. An approach pioneered by Zheng et al [101] and Ferguson-Smith et al [102] has been to stain NRBCs with fluorescence-conjugated anti-fetal hemoglobin antibodies. Following detection of NRBCs, FISH analysis can be performed to diagnose fetal aneuploidies. However, this system is still associated with difficulties, since NRBCs must be morphologically identified, and optimized staining conditions have yet to be determined.

Recently, a new system has been developed in which cells enriched on the slide glass are stained, after which the entire slide is examined by microscopy and the findings read into a computer with a CCD camera, from which NRBCs are identified based on their morphology. This approach is becoming more feasible as the image-processing capacity of computers improves.

DNA Analysis

When FISH and PCR, which are sensitive enough to analyze single cells, became available in the late 1980s, noninvasive prenatal diagnosis using maternal blood became all the more possible. These two methods are now used to analyze fetal cells. FISH can analyze fetal gender and aneuploidies while PCR permits analysis of mutations in single-gene disorders, as well as fetal gender and rhesus blood type.

FISH analysis

Since Price et al reported the first case of fetal trisomy 21 diagnosed by isolation of NRBCs from maternal blood [10], the FISH method has been used for non-invasive prenatal diagnosis of fetal aneuploidies, as well as fetal gender. In our own experiments, FISH analysis of post-termination samples revealed that NRBCs detected by positive staining for γ-hemoglobin were all fetal in origin [103]. However, when blood samples from normal pregnant women were examined, most NRBCs were not suitable for FISH. We then learned that 43% of fetal NRBCs collected from maternal blood are apoptotic [87]. When NRBCs migrate into maternal blood, they circulate under relatively high oxygen tenion. As a result, they are more subject to apoptosis [104] and the size of their nuclei is diminished [105]. This might be why FISH signals are not detected in the nuclei. This problem warrants further investigation.

PCR analysis

Through PCR amplification, we can evaluate fetal DNA in NRBC-enriched samples. In fact, Bianchi et al have reported the presence of a Y chromosome-specific sequence in 75% of male-bearing maternal blood samples [9]. However, this approach is not sufficiently accurate for use in clinical practice. For diagnostic purposes, individual NRBCs of fetal origin must be examined, since half of NRBCs are maternal in origin. Unfortunately, thus far, micromanipulation remains the only method by which to isolate individual NRBCs for analysis.

In 1995, Takabayashi et al pioneered a micromanipulation method by which to isolate NRBCs based on morphology, accurately determining fetal sex in 10 of 11 cases [15]. We subsequently used this NRBC separation method to retrieve individual NRBCs and diagnose fetal single-gene disorders, such as Duchenne muscular dystrophy and ornithine transcarbamylase deficiency [16,106]. We demonstrated that PCR can be performed on the genetic material obtained from a single cell, a phenomenon made possible by whole genome random primer extension preamplification (PEP). As a result, we were able to determine whether an NRBC was of fetal origin using differences in ZFX/ZFY loci (to diagnose the gender of the cell), and also to examine a particular gene of interest. Although this was the first report of diagnosis of a fetal single-gene disorder, over 100 cycles of PCR amplification, including PEP and subsequent PCR of the target genes, were required to diagnose the origin of the cell and DNA alterations leading to one or more single-gene disorders. Thus, there are limitations to clinical use of this technology. Cheung et al have also prenatally diagnosed hemoglobinopathies using multiple singly manipulated fetal NRBCs identified by anti-fetal hemoglobin staining [107]. Using PCR, several cells should be retrieved in order to circumvent the problem of allele dropout, a phenomenon that frequently occurs when using single or low template copies for PCR.

Thus, while PCR methods are highly sensitive, PCR amplification errors at the single base sequence level and allele dropouts may still occur.

Conclusion

Fifteen years have passed since the first noninvasive approaches to diagnosis of fetal genetic and chromosomal abnormalities using NRBCs in maternal blood were developed. Although a number of noninvasive approaches have been examined, all present limitations. However, recent progress has increased the possibility of noninvasive prenatal diagnosis.

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