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INVITED EDITORIAL
Fetal Cells in Maternal Circulation: Progress in Analysis of a Rare Event

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Amniocentesis and, recently, chorionic villus sampling (CVS) provide fetal cells for genetic testing and allow at-risk couples the option of having unaffected offspring. The most common indication for prenatal diagnosis is advanced maternal age, but a significant number of procedures are also performed to identify single-gene disorders. Unfortunately, with these sampling procedures comes a small (0.5%–1.0%) procedure-related risk of miscarriage. Because of this, efforts have been directed toward less invasive approaches for prenatal diagnosis. Currently, the most common strategy for detecting aneuploidy (primarily trisomies 21 and 18) is midtrimester maternal serum biochemical marker analysis, followed by amniocentesis in screen-positive cases. This approach detects ~60%–90% of cases of Down syndrome in pregnant women. Much ongoing research in this area aims to improve detection efficiency by using new markers, but cases of aneuploidy will still be missed, and this approach will not be useful for the diagnosis of single-gene disorders. Thus, an ideal strategy would entail identifying fetal cells in the maternal circulation and making them available for a range of diagnostic tests.

Walknowska et al. (1969) first reported the detection of fetal lymphocytes in the circulation of pregnant women. After mitogen stimulation, they found rare lymphocytes having a small acrocentric chromosome consistent with a Y chromosome. These findings were highly correlated with women who were carrying a male fetus. This report stimulated a wide range of investigations focusing on the identification and enrichment of fetal cells in the maternal circulation and on the analysis of these cells.

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Several fetal cell types have been reported to exist in the maternal circulation. These include fetal trophoblasts, lymphocytes, granulocytes, nucleated erythrocytes, and platelets. Except for platelets (which lack genomic DNA), all of these cell types have been investigated as a source of fetal cells for prenatal diagnosis.

Few investigators have used trophoblast cells as a source of fetal cells in maternal circulation. However, Mueller et al. (1990) reported isolating trophoblasts from maternal circulation by using murine monoclonal antibodies (mAb) to human trophoblast antigens. Hawes et al. (1994) identified a paternal β-globin mutation in a trophoblast preparation enriched from maternal blood by affinity to a cocktail of mAbs. Durrant et al. (1996) used a different mAb and were able to correctly identify male gender in only 39% of cases. The diagnosis by FISH analysis of a 47,XYY fetus by enrichment using anti-trophoblast antibodies GB25 and GB17 has been reported (Cacheux et al. 1992). A concern about the use of trophoblasts for noninvasive prenatal diagnosis is the fact that these cells are frequently multinucleate, which complicates interphase FISH. In addition, extensive experience with CVS has demonstrated the presence of confined placental mosaicism (CPM) (Goldberg and Wohlferd 1997). Thus, cytogenetic results obtained from trophoblast cells might not truly reflect the fetal status.

Many studies have confirmed the report by Walknowska et al. regarding the presence of fetal lymphocytes in the maternal circulation. Herzenberg et al. (1979) were the first to attempt to enrich for the population of fetal lymphocytes. Using flow sorting, they identified fetal cells with an antibody against a paternally inherited HLA antigen, followed by quinacrine staining and Y chromatin identification. Unfortunately, this approach requires an informative polymorphic couple and known paternity, which makes this approach not universally applicable. Another potential problem with this approach is the persistence of fetal lymphocytes in maternal circulation for long periods (discussed below). Fetal granulocytes have also been evaluated in a limited number of cases, but their presence in maternal blood is uncertain.

Fetal nucleated red blood cells (NRBCs) have been the most commonly studied cell type, for several reasons. On the basis of studies of staining for fetal hemoglobin, it has been known for many years that fetal red blood cells cross into the maternal circulation (Clayton et al. 1964), and the frequency of NRBCs in the fetus early in gestation is relatively high (Thomas and Yoffey 1962). These cells are also fairly well differentiated and likely to have a limited life span in the maternal circulation.

The presence of fetal NRBCs was first described by Bianchi et al. in 1990 (Bianchi et al. 1990). These cells were identified by morphologic features, by positive staining for fetal hemoglobin, and, in females with male fetuses, by identification of Y chromosome–specific sequences. In this study, fetal NRBCs were identified by flow sorting after binding to a mAb to the transferrin receptor, an antigen expressed on all cells that take up iron, including all NRBCs in maternal blood. Unfortunately, evidence suggests that a significant proportion of NRBCs in maternal blood are of maternal origin, having increased during pregnancy, as compared with nonpregnant controls (Slunga-Talberg et al. 1995).

It is clear that fetal cells in the maternal circulation are few, but the exact frequency is controversial, with ranges of $1/10^5$–$1/10^9$ maternal cells reported. Most of the studies that have attempted to answer this question have used fetal cell–enrichment techniques and have had an unknown rate of loss because of the enrichment steps. Hamada et al. (1993) used a direct approach on unsorted cells. They selected cells that were Y-sequence positive on FISH analysis and reported that the frequency of Y-positive cells increased from $<1/10^5$ in the first trimester to $1/10^4$ at term. The paper by Bianchi et al. (1997) in this issue of the *Journal* uses quantitative PCR to determine the number of male fetal-cell DNA equivalents in the blood of pregnant women. They find the mean number of male fetal-cell DNA equivalents amplified from 16 ml of maternal blood to be 19 (range 0–91), with male DNA detected in 99.3% of pregnant women carrying a male fetus. There was a sixfold increase in the number of DNA cell equivalents in pregnancies where the fetus had trisomy 21. Equally interesting was the finding that, in 25.7% of women carrying a female fetus, male DNA was detected. As pointed out by the authors, this could reflect a vanishing-twin gestation, previous maternal blood transfusion from a male donor, or persistent cells from a prior male pregnancy. This finding emphasizes both the need to use a cell type with a limited life span and the need for methods to specifically identify cells of fetal origin.

Another important issue is the timing of passage of fetal cells into the maternal circulation. Investigators have demonstrated by PCR in mothers carrying male fetuses the presence of Y sequences as early as 33 and 40 d of gestation in dated pregnancies following in vitro fertilization (Thomas et al. 1994). The cell type is unlikely to be fetal NRBCs, since fetal vessels are not present in the villous stroma until ~8 wk gestation. A more likely candidate would be circulating trophoblasts. As mentioned above, Hamada et al. (1993) found the concentration of fetal cells in nonenriched samples to be <1/100,000 in the first trimester, but other investigators, working with enriched samples, found fetal NRBCs within the first trimester (Cheung et al. 1997), suggesting that a first-trimester approach might be feasible.

Because of the rarity of fetal cells in maternal circulation, most investigators have utilized a variety of enrichment techniques. There is no clear consensus, at present, on which techniques produce the best separation as judged by the total number of fetal cells recovered and the ratio of fetal/maternal cells obtained. Most investigators begin with ~20 ml of maternal venous blood from maternal nonnucleated erythrocytes which are removed, usually by density gradient centrifugation. After this step, various positive and negative selection techniques have been used after specific antibody conjugation. These techniques have included fluorescence-activated cell sorting, magnetic activated cell sorting, immunomagnetic beads, and antibody-conjugated columns. Recently several investigators have performed immunostaining with fetal hemoglobin antibodies to specifically identify fetal cells in sorted samples of NRBCs. Zheng et al. (1993) utilized a mouse anti–fetal hemoglobin antibody (i.e., UCHγ) and FISH analysis to identify fetal gender from sorted cells. There is some concern with this approach, because some mothers will have elevated fetal hemoglobin levels, leading to misidentification. To avoid this, Cheung et al. (1996) utilized an anti-embryonic (i.e., ζ) antibody to identify sorted fetal cells.

The small number of fetal cells obtained has been a significant limitation with this type of analysis. If the small number of cells could be cultured, more material would be available for analysis. Lo et al. (1994) have reported the successful culture of fetal erythroid cells, resulting in a purity rate of 0.001%–0.25%. Valerio et al. (1996) used magnetic sorting with biotin-labeled erythropoietin preenrichment and were able to culture fetal CFU-E (colony forming unit–erythroid) and M-BFU-E (mature burst-forming unit–erythroid) cells, achieving 18% purity. This approach holds great promise for providing increased numbers of fetal cells for analysis.

Many investigators have reported a number of cases of false-positive results in finding cells with Y sequences from blood of women carrying female fetuses. Although this may have resulted from nonspecific amplification/binding or contamination, it has also raised the possibility, as mentioned above, that cells from past pregnancies may remain in the maternal circulation. Bianchi et al. (1996) reported the presence of male DNA in flow-

sorted $CD34^+CD38^+$ cells from six of eight nonpregnant women who had previously given birth to a son. The interval from birth to testing was 6 mo–27 years. This finding suggests that there might exist a low-level chimerism from past pregnancies. The biological significance of this is unknown, but Bianchi (1997) has reported an association between the persistence of fetal cells and the later development of autoimmune disorders such as Sjogren syndrome and scleroderma.

The analysis of fetal cells in maternal circulation has primarily relied on PCR and FISH techniques. PCR has been used for the amplification of sequences in both nonenriched and enriched maternal blood. Lo et al. (1989) showed the amplification of Y sequences in nonenriched maternal blood from women carrying male pregnancies. They identified all 12/19 women who later gave birth to boys. The diagnosis of single-gene disorders is also possible if the father carries a mutation or polymorphism that the mother lacks. For example, both the detection of fetal hemoglobin Lepore-Boston and the detection of the Rh D gene have been reported using this strategy (Camaschella et al. 1990; Lo et al. 1993). At present, the accuracy of this approach is unknown.

FISH technology has been used for the diagnosis of aneuploidy in maternal blood samples enriched for fetal cells. Simpson and Elias (1993) reported that, after enrichment by flow sorting with mAbs to the transferrin receptor and glycophorin A, they could diagnose 47,XXY, trisomy 18, and trisomy 21. The percentage of fetal aneuploid cells detected was 0%–74%. Gänshirt-Ahlert et al. (1993) have reported 15 cases of fetal aneuploidy detected by FISH analysis following triple density–gradient separation and magnetic sorting of transferrin receptor–labeled cells. Other groups have reported similar results. Because of the significant background of maternal cells, this current approach will probably only be useful as an improved population screen with the use of CVS or amniocentesis as a diagnostic test. Only if pure fetal cells could be obtained reliably would this become a primary diagnostic test.

An approach to obtaining pure fetal cells has been described by Takabayashi et al. (1995). They retrieved single cells by use of a micromanipulator under a microscope and analyzed them for Y sequences. Cheung et al. (1996) applied this technique to transferrin receptor–positive cells that had been stained for embryonic hemoglobin. The retrieved cells were then pooled and used for the PCR-based diagnosis of sickle-cell anemia and thalassemia. These investigators identified 7–22 fetal cells from 16–18 ml of maternal blood.

Although significant advances have been made in the isolation and analysis of fetal cells from maternal blood, very few data address the sensitivity or specificity of this approach. The main reason for this has been that separation and analysis techniques are still evolving. Until a large prospective study is performed, this approach will not be ready for clinical application. The National Institute of Coronary Heart Disease is currently sponsoring a phase II clinical trial to evaluate the accuracy of cytogenetic diagnosis based on fetal cells in maternal blood, as compared with traditional amniocentesis or CVS (de la Cruz et al. 1995).

Not surprisingly, several important ethical and social issues need to be resolved before widespread or universal testing of fetal cells in maternal circulation is undertaken. The first issue is whether this type of screening should be offered to all pregnant women. A comprehensive cost-effectiveness evaluation should be performed to help answer this question. Related to this is the question of whether women will feel coerced, by health-care providers, family, or friends, into being tested. An equally important issue that has never been resolved with prenatal diagnosis is the issue of gender selection. With the availability of an essentially noninvasive approach to diagnose fetal gender, this technique could be widely used by families that have a gender preference. The medical genetics community will need to develop broad-based consensus guidelines in this area.

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