Fetal Cells in Maternal Blood: State of the Art for Non-Invasive Prenatal Diagnosis
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Abstract
In Singapore, 1 in 5 pregnancies occur in mothers >35 years old and genetic diseases, such as thalassaemia, are common. Current methods for the diagnosis of aneuploidy and monogenic disorders require invasive testing by amniocentesis, chorion villus biopsy or fetal blood sampling. These tests carry a procedure-related risk of miscarriage that is unacceptable to many couples. Development of non-invasive methods for obtaining intact fetal cells would allow accurate prenatal diagnosis for aneuploidy and single gene disorders, without the attendant risks associated with invasive testing, and would increase the uptake of prenatal diagnosis by women at risk. Isolation of fetal erythroblasts from maternal blood should allow accurate non-invasive prenatal diagnosis of both aneuploidies and monogenic disorders. Expression of $\gamma$-globin in maternal erythroblasts and the inability to locate fetal erythroblasts reliably in all pregnancies have prevented its clinical application. In the absence of a highly specific fetal cell marker, enrichment, identification and diagnosis – the 3 components of non-invasive prenatal diagnosis – have clearly defined objectives. Since fetal cells are rare in maternal blood, the sole purpose of enrichment is yield – to recover as many fetal cells as possible – even if purity is compromised at this stage. In contrast, the primary goal of identification is specificity; absolute certainty of fetal origin is required at this stage if the ultimate objective of diagnosis, accuracy, is to be achieved. This review summarises the current state of the art of non-invasive prenatal diagnosis using fetal erythroblasts enriched from maternal blood.

Key words: Epsilon globin, Erythroblasts

Introduction
Without prenatal diagnosis, 1 in 50 babies are born with serious physical or mental handicap, and as many as 1 in 30 with some form of congenital malformation.¹ These may be due to structural or chromosomal abnormalities, or single gene disorders. The diagnosis of aneuploidy and monogenic disorders requires invasive testing by amniocentesis, chorion villus biopsy or fetal blood sampling. These tests carry a procedure-related risk of miscarriage of 1% to 4%,²-⁷ which limits the uptake of these procedures by women identified at increased risk by screening tests.⁸ Observations that intact fetal cells can enter and circulate within maternal blood have raised the possibility of non-invasive access to fetal genetic material that would allow the prenatal diagnosis of chromosomal and monogenic disorders.

As early as 1893, fetal cells were thought to circulate in maternal blood; Schmorl had identified trophoblast sprouts in the lungs of a woman who died of eclampsia.⁹ Other investigators have made similar observations,¹⁰ but definitive proof that fetal cells circulate in maternal blood came only when lymphocytes bearing the Y chromosome were detected in the peripheral blood of mothers carrying male fetuses.¹¹ Since that seminal paper, research in fetal cells slowly gained momentum until the 1990s, when there was an exponential rise in the number of publications on the subject. The trigger for the escalation of interest in this field was the advent of sophisticated molecular genetic techniques, such as polymerase chain reaction (PCR)¹² and fluorescence in situ hybridisation (FISH).¹³ Current research in this area focuses not on whether these cells are present in maternal blood, but instead aims to understand their biological role in the mother and how to isolate and use these cells for non-invasive prenatal diagnosis.

Target Cells for Non-invasive Prenatal Diagnosis
Obstetricians are familiar with the phenomenon of transplacental passage of fetal anucleate erythrocytes that occurs in Rhesus (Rh) disease and potentially sensitises
informative couples out of 78 screened.\textsuperscript{19} Such a strategy may be satisfactory in a research setting, but is impractical for clinical application.

**Stem Cells and Haemopoietic Progenitors**

The rarity of fetal cells in maternal blood could be overcome if the fetal cells that are enriched readily proliferate \textit{in vitro} or can be induced to do so. This would amplify the number of cells and genetic material available for diagnosis, and potentially allow a pure source of fetal cells and deoxyribonucleic acid (DNA) by expansion of single colonies. Two cell types that could potentially achieve this aim are haemopoietic progenitors and stem cells.

Lo et al\textsuperscript{20} cultured mononuclear cells and were able to isolate fetal erythroid progenitors from the peripheral blood of pregnant women. Subsequently, Valerio et al\textsuperscript{21} successfully cultured colony-forming units, erythroid and mature burst-forming units and erythroid colonies from fetal haemopoietic progenitors enriched from maternal blood. However, these results have not been replicated by others\textsuperscript{22} and it is unclear if fetal haemopoietic progenitors require unique cytokine combinations for \textit{in vitro} multiplication.\textsuperscript{23} So far, selective amplification of fetal over maternal haemopoietic progenitors has not been successful.\textsuperscript{24,25}

A novel fetal mesenchymal stem cell has been characterised recently.\textsuperscript{26} If these cells can be enriched from maternal blood, they can be induced to proliferate \textit{in vitro} and would be an ideal fetal cell type for non-invasive prenatal diagnosis.\textsuperscript{27} However, its ability to renew itself and differentiate, which it shares with all other stem cells,\textsuperscript{28} may also limit its clinical application; they might rapidly sequester within maternal tissues after crossing the placenta\textsuperscript{29} and/or persist from previous pregnancies and continue to circulate in maternal blood in the current pregnancy.

Thus, the cell type chosen for non-invasive prenatal diagnosis should be short-lived within the mother, have no or only limited capacity to proliferate, and have unique cell surface markers to facilitate enrichment in all pregnancies.

**Erythroblasts**

Fetal nucleated erythrocytes (NRBCs; erythroblasts) are a cell type with most of the desired characteristics. They have a limited life span,\textsuperscript{28} have a distinctive morphology, are consistently present in maternal blood during pregnancy,\textsuperscript{31} carry a representative complement of fetal genes, are the abundant fetal cell type in first- and early second-trimester fetal blood,\textsuperscript{32} are mononucleated,\textsuperscript{33} and have limited proliferative capacity making them unlikely to persist across pregnancies. Furthermore, the presence of surface antigens that characterise immature erythroid cells (CD71 and CD36) allow these cells to be enriched from all Rh-negative mothers. The phenomenon can be documented \textit{in vitro} using the Kleihauer-Betke test, which stains haemoglobin F (HbF). These cells (fetal anucleate erythrocytes) are of little value for prenatal diagnosis as they lack a nucleus. In contrast, trophoblasts, leukocytes, stem cells and erythroblasts all contain nuclei and the fetal genetic material necessary for prenatal diagnosis. All 4 cell types have been explored as candidates for non-invasive prenatal diagnosis.

**Trophoblasts**

Though the first fetal cell type documented to cross into the maternal circulation,\textsuperscript{9} use of trophoblast cells for non-invasive prenatal diagnosis met with several difficulties. Trophoblast deportation into the maternal circulation does not appear to be a phenomenon common to all pregnancies.\textsuperscript{14} When it does occur, the cells are rapidly cleared by the pulmonary circulation.\textsuperscript{10} Their extraembryonic origin, as part of the placenta, implies that trophoblast cells are likely to exhibit confined chromosomal mosaicism in 1% of cases sampled,\textsuperscript{15} and any prenatal diagnostic test relying exclusively on these cells may not reflect the true fetal karyotype. Furthermore, syncytiotrophoblast cells, which are multinucleate, do not give accurate results when chromosomes are analysed by FISH. Finally, the greatest obstacle in using trophoblast cells for non-invasive prenatal diagnosis is the development of specific monoclonal antibodies against trophoblast cell surface antigens.\textsuperscript{16,17}

**Leukocytes**

Demonstration of a Y chromosome in mitogen-stimulated lymphocytes, obtained from the venous blood of pregnant women bearing male fetuses, was the first conclusive evidence that fetal cells circulate in maternal peripheral blood.\textsuperscript{11} Although one of the earlier attractions of fetal leukocytes was their ability to proliferate \textit{in vitro}, this same property now limits the development of this cell type for use in non-invasive prenatal diagnosis since it is thought that they also proliferate \textit{in vivo} in maternal organs. Bianchi et al\textsuperscript{18} found that haemopoietic stem cells, lymphoid/myeloid progenitors (CD34- and/or CD38-positive) and fetal T lymphocytes (CD3-positive) had persisted for 6 years in 1 woman. Thus, there is concern that enriched leukocytes may be the vestiges of previous pregnancies and do not represent the true fetal genetic status of the current pregnancy.

Studies of fetal white cells in the maternal circulation have also been limited by the lack of monoclonal antibodies directed against unique fetal leukocyte antigens. Fetal cell isolation based on polymorphic human leukocyte antigen (HLA) differences between fetus and mother not only requires known paternity, but also limits the test to informative couples. One study found only 18 HLA-
pregnancies, and not only from HLA-informative couples.

It was thought that NRBCs are rare in maternal blood, but recent enrichment systems have demonstrated a much larger population of NRBCs that circulate in pregnancy; unfortunately, most are maternal in origin. Fetal haemoglobin has been used to differentiate maternal from fetal NRBCs, but 20% of all HbF-positive NRBCs in maternal blood are of maternal origin and are found in all pregnancies. A more specific identification system is required if fetal erythroblasts are to be used as target cells for accurate non-invasive prenatal diagnosis. Choolani et al found all fetal primitive erythroblasts to be epsilon-globin-positive compared with none in adult NRBCs, demonstrating that first, epsilon-globin is a highly specific fetal erythroblast marker and, second, that fetal primitive erythroblasts are the ideal target fetal cell type for non-invasive prenatal diagnosis.

Enrichment, Identification and Diagnosis of Fetal Erythroblasts

Non-invasive prenatal diagnosis can be divided into 3 phases: enrichment of fetal cells from maternal blood, identification of enriched cells as fetal and making the genetic diagnosis.

Enrichment

Enriching fetal cells from maternal peripheral blood is challenging because of their rarity in the maternal circulation. Best estimates suggest that there may be 1 fetal cell per millilitre of maternal blood (10^7 nucleated cells). Several strategies to exploit the differences in physical, chemical and biological properties of individual cells have been used to enrich fetal erythroblasts from maternal blood. These include density gradient centrifugation, charged flow separation, selective erythrocyte lysis, fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). The last 2 methods exploit antigenic differences between cells.

Bhat et al demonstrated the value of density gradient centrifugation as the first enrichment step to eliminate/reduce the overwhelming abundance of maternal red blood cells and to enrich a population of mononuclear cells. They also demonstrated a 25-fold enrichment of fetal nucleated red blood cells using a discontinuous triple density gradient, a protocol adopted by Ganshirt-Ahlert et al who successfully enriched fetal NRBCs from aneuploid pregnancies in the second and third trimesters. Since then, density gradient centrifugation has been used as the first step in most enrichment protocols. Over time, denser gradients were favoured and most investigators now prefer Ficoll 1119.

The negative surface charge density on erythrocytes is due to the sialic acid molecules within their glycocalyx. Charged flow separation, which permits sorting of cells according to their characteristic surface charge densities, has been used by some investigators to enrich fetal NRBCs; they reported that up to 30% of enriched erythroblasts were fetal.

Of all the enzymes used to differentiate fetal from maternal red cells, such as 2,3-biphosphoglycerate and thymidine kinase, carbonic anhydrase (CA) is the most extensively studied. Fetal erythroblasts are less susceptible to ammonium chloride lysis than adult erythrocytes as CA activity is at least 5-fold less and acetazolamide permeability about 10-fold greater in fetal compared with adult red blood cells. Selective lysis of maternal red blood cells was first used for the diagnosis of haemoglobinopathies, where globin chains extracted from a highly purified population of fetal red blood cells were analysed by electrophoresis. Although it has been shown that ammonium chloride lysis can be used for fetal cell enrichment, the authors and other investigators have demonstrated that NRBC membrane alterations affecting downstream processing are likely.

FACS and MACS are the 2 most commonly used systems in non-invasive prenatal diagnosis.

FACS, first used over 2 decades ago for this purpose, is able to enrich cells with high purity so that slides with sorted cells can be readily scanned manually. It also allows multiparameter sorting: simultaneous analysis of several criteria on a single cell and can be adapted for use with intracytoplasmic antigens.

MACS, on the other hand, has gained popularity in this field because it is a faster and less expensive bench-top technique better suited to process larger cell numbers. It can also be performed in most laboratories without trained staff and high maintenance costs. Although both negative and positive selections can be performed on the same population of cells in the same experiment, enrichments must be performed in series because cell selection can be based on only 1 antigen at a time. When studying the absolute numbers of fetal cells recovered, it has been shown that MACS is at least as effective and more specific than FACS.

FACS and MACS exploit antigenic differences between cells to enrich target cells of interest. No surface antigens specific to fetal erythroblasts have been identified. It is likely that a combination of antibodies will be necessary to isolate fetal erythroblasts from maternal red and white blood cells present in maternal peripheral blood. Potentially useful surface antigens include CD45, glycophorin A (GPA), CD71, CD36, CD35 and CD47. GPA is expressed on all erythrocytes while CD45 is expressed on none. The expressions of CD71 and CD36 are lost beyond the reticulocyte stage, whereas CD35 is only expressed on mature erythrocytes. This inverse relationship between...
CD71 and CD36 on the one hand, and that of CD35 on the other, could help to differentiate immature from mature erythrocytes.

Identification

The accuracy of prenatal diagnosis using fetal cells enriched from maternal blood depends on the specificity of their identification. Ideally, a fetal-specific cell surface antigen could be used to isolate fetal erythroblasts from maternal blood and to identify their fetal origin. Since such a fetal marker is not yet available, most investigators use CD71 for enrichment and γ-globin for fetal cell identification. However, not all fetal erythroblasts are CD71-positive and maternal NRBCs express γ-globin.

Of all the potential fetal cell identifiers that have been studied, the 3 best candidates for NRBCs are the fetal and embryonic haemoglobins γ, ζ and ε-globins.

The “leaky” expression of γ-globin in adults prompted Cheung et al to suggest the use of the embryonic ζ-globin. However, ζ-globin expression is not completely switched off after the embryonic period. Luo et al showed that ζ-globin was present in 53% of definitive erythrocytes between 15 and 22 weeks of gestation and in 34% at term. Albitar et al had earlier demonstrated ζ-globin transcripts within the peripheral blood of healthy individuals. Chung et al identified ζ-globin chains in adults with the α-thalassaemia trait. In contrast, ε-globin is strictly confined to the embryonic period.

Although it has been known for a long time that ε-globin is not present in adult red blood cells, interest in its use for non-invasive prenatal diagnosis has emerged only in the last 4 years. Mesker et al demonstrated the presence of ε-globin-positive male fetal erythroblasts in 2 post-chorionic villus sampling (CVS) maternal blood samples. Mavrou et al compared the specificity of the 2 embryonic globins in the detection of NRBCs in CVS supernatant fluid. They found that ε-globin was more reliable and specific for the detection of embryonic NRBCs. Luo et al showed an 18-fold greater expression of ε-globin compared with ζ-globin when fetal erythroblasts were cultured in vitro, indirect evidence that ε-globin expression is more tightly regulated than that of ζ-globin. Using a sensitive reverse transcriptase-PCR (RT-PCR) method, Hogh et al found ζ-globin transcripts in the CD71-positive mononuclear cell fraction of the peripheral blood in 3 out of 20 non-pregnant women, whereas ε-globin transcripts were found in none. Chooolani et al demonstrated that ε-globin would be a suitable fetal NRBC marker before 13 weeks of pregnancy. These data suggest that ε-globin is the more suitable marker for fetal erythroblast identification in the first trimester.

Diagnosis

The 3 most important molecular techniques that have allowed genetic analysis of enriched fetal cells are the PCR, RT-PCR and FISH. The ability of PCR to amplify minute quantities of DNA (even single copy) over a billion-fold has been exploited for the prenatal diagnosis of monogenic disorders using fetal cells enriched from maternal blood. In cells that express a particular gene, there are many more copies of the ribonucleic acid (RNA) message compared with only 1 or 2 alleles within the genome. Thus, RT-PCR for fetal messenger RNA is more sensitive than PCR amplification of genomic DNA. Chromosomal FISH allows the detection of aneuploidy and chromosomal rearrangements in interphase nuclei. It has been used to detect most of the major fetal aneuploidies within fetal cells isolated from maternal blood.

Enrichment, identification and diagnosis can be regarded as separate sequential procedures. Enriched cells are labelled for fetal cell markers by immunocytochemistry and target fetal cells are investigated by cFISH or PCR, more recently after microdissection and transfer for genetic analysis. This strategy has been shown to be effective, but the technique requires expertise, is time-consuming and is associated with a cell loss of as high as 18%. Instead, methods that combine fetal cell identification with molecular genetic diagnosis in an in situ technique circumvent these limitations and are especially suited for automation. Chooolani et al recently developed such a technique that combines the fluorescent labelling of the highly specific ε-globin with cFISH diagnosis into a single, simultaneous technique.

Clinical Trials of Non-invasive Prenatal Diagnosis

The results of the only clinical trial to evaluate the accuracy of prenatal diagnosis using fetal cells in maternal blood have been recently reported. The National Institutes of Health Fetal Cell Study (NIFTY) was a phase II, non-intervention clinical investigation funded by the National Institute of Child Health and Human Development that began in 1994. It recruited almost 3,000 women considered to be at high risk for fetal aneuploidy. Isolated fetal cells were examined for aneuploidy by FISH using probes for 13, 18, 21, X and Y, and the results compared against the karyotype obtained after invasive testing. Target cell recovery and fetal cell detection were better using MACS than FACS. Blinded cFISH assessment of samples from women carrying singleton male fetuses found at least 1 cell with an X and Y signal in 41.4% of cases. In contrast, the sensitivity for fetal aneuploidy was as high as 74.4% and the false-positive rate was as low as 0.6%.

A larger trial is currently underway at the Columbia University of Health Sciences in New York. The First- and Second-Trimester Evaluation of Risk for Aneuploidy (FASTER) trial is an open-label, non-randomised, interventional study involving 11 centres that aims to...
recruit 62,000 pregnant women and to evaluate the efficacy of first- and second-trimester non-invasive screening methods for Down’s syndrome and other aneuploidies (http://clinicaltrials.info.nih.gov/). The screening modalities under investigation include maternal age, fetal nuchal translucency (NT) measurement and first- and second-trimester serum screening. Screen-positive patients (risk ≥1 in 380) are offered amniocentesis at 15 weeks of pregnancy. Those who accept invasive testing will have a tube of maternal blood drawn for enrichment and analysis of fetal NRBCs.

Conclusion

It has been argued that first-trimester non-invasive prenatal diagnosis is not ideal, partly because it would be difficult to encourage women to present that early in pregnancy, and partly because spontaneous miscarriages occur not infrequently beyond 12 weeks. It is known, however, that women readily adopt technologies that benefit their pregnancies and themselves: most women perform urine pregnancy tests at home, many have a booking scan before 14 weeks of pregnancy and 99.5% accept routine ultrasound screening for structural malformations. Furthermore, if an ultrasound scan performed at 9 weeks demonstrates fetal heart motion, 9 out of 10 mothers will carry the pregnancy to term. Thus, if it is shown that accurate non-invasive prenatal diagnosis is possible in the first trimester of pregnancy, the authors believe that most women will welcome this test.

It is likely that fetal cells derived from maternal blood could be used not only as a screening tool, either alone or (more likely) in combination with other modalities such as biochemical tests and NT scans. The high sensitivity and low false-positive rate for aneuploidy detection in the NIFTY trial support this hypothesis. Two changes in the current state of the art would allow enriched fetal cells to be used not only for screening, but also for accurate prenatal diagnosis. These improvements include reliable enrichment of fetal erythroblasts in the first trimester and specific identification of the fetal origin of these cells. The usefulness of ε-globin as a highly specific fetal cell marker needs to be verified by independent investigators. Modern micro- and nanotechnology could also be used to enhance fetal cell enrichment and to bring first-trimester non-invasive prenatal diagnosis, using fetal erythroblasts in maternal blood, closer to clinical practice.

REFERENCES

2. Tabor A, Philip J, Madsen M, Bang J, Obel E B, Norgaard-Pedersen B.


53. Von Koskull H, Gahmberg N. Fetal erythroblasts from maternal blood identified with 2,3-bisphosphoglycerate (BPG) and in situ hybridization (ISH) using Y-specific probes. Prenat Diagn 1995; 15:149-54.


QUESTIONS

1. Regarding candidate fetal cells for non-invasive prenatal diagnosis
   a) Trophoblast cells are not the ideal fetal cell type.
   b) Leukocytes are ideal as they can proliferate in vivo.
   c) If leukocytes are used, polymorphic HLA differences between fetus and mother require known paternity.
   d) Only fetal stem cells are able to proliferate in vitro.
   e) The presence of fetal trophoblast cells from previous pregnancies could hinder prenatal diagnosis.

2. Primitive fetal erythroblasts
   a) Have a characteristic morphology.
   b) Have a long life span which enables higher recovery of fetal cells from maternal blood.
   c) Are abundant in early pregnancy.
   d) Are CD34- and CD38-positive.
   e) Are multinucleated.

3. In the enrichment of fetal cells from maternal blood
   a) Density gradient centrifugation makes use of surface charge density.
   b) Ficoll 1119 is frequently used for density gradient centrifugation.
   c) 2,3-biphosphoglycerate, thymidine kinase and carbonic anhydrase are found only in maternal red blood cells.
   d) Mature erythrocytes do not contain CD71 or CD36.
   e) Glycophorin A is found on all erythrocytes.

4. In the identification of enriched fetal cells
   a) CD71 is found on all fetal erythroblasts.
   b) ζ-globin in maternal NRBCs enables fetal cell enrichment from maternal blood.
   c) Embryonic ζ-globin is occasionally expressed in both embryonic and definitive lineage erythrocytes.
   d) Adult red blood cells do not contain any ε-globin.
   e) ζ-globin is less specific for identification of fetal NRBCs.

5. In the genetic analysis of enriched fetal cells
   a) The PCR amplifies fetal genomic DNA.
   b) RT-PCR is more sensitive than PCR.
   c) cFISH detects aneuploidy and chromosomal rearrangements in PCR products.
   d) Immunocytochemistry is suitable for identification and genetic analysis of enriched fetal cells.
   e) The combination of methods for fetal cell identification and molecular genetic analysis will enable automation.