Leaders

Non-invasive prenatal diagnosis using fetal cells in maternal blood

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Introduction

One of the goals of modern genetics is the development of safe and reliable prenatal diagnostic tests which do not constitute a risk to the fetus. Currently, the safety of available methods is limited by the need to obtain fetal tissue for analysis by invasive means, such as amniocentesis and chorionic villus sampling (CVS), which present a finite risk to the fetus. One potentially non-invasive approach for obtaining fetal material for diagnosis is the use of fetal cells in maternal circulation. This article summarises some of the major recent developments in this rapidly expanding field.

Historical perspective

In 1893 Schmorl first suggested that nucleated fetal cells may be found in maternal circulation and that portions of the villous system of the placenta can become detached and pass into the maternal circulation.¹ He based his conclusions on the finding of multinucleate cytoplasmic fragments, which he regarded as placental in origin, in the pulmonary vessels of women who had died in eclampsia. These observations were confirmed by other investigators who found morphological evidence of syncytiotrophoblasts in lung sections of 43.6% (96 of 220) of women dying during pregnancy.² Other workers studied buffy coated smears made from maternal blood taken from broad ligament veins, ovarian veins, and the inferior vena cava and were able to detect cells with morphological syncytiotrophoblast features in a proportion of the women.³

These studies, though of historical interest, are of little relevance to the use of fetal cells for non-invasive prenatal diagnosis as, in these studies, fetal cells were only found following tissue or blood sampling through invasive means. A number of investigators have attempted to confirm the existence of fetal cells in maternal peripheral blood using a variety of methods, including cytogenetic analysis,⁴ quinacrine staining,⁵ culture,⁶ and flow cytometry.^{7,8} However, many of these methods are either non-specific or not reproducible by other groups. These issues have been discussed in a number of recent reviews and will not be discussed in this paper.^{9,10}

The development of molecular biology has provided many new and highly sensitive tools for detecting and confirming the existence of fetal cells in maternal blood. The specificity of molecular biological techniques constitutes

an important step forward as the previous criteria for identifying a given cell as of fetal origin were either subjective (morphology) or non-specific (quinacrine staining) or not easily applicable to the detection of a minority cell population (cytogenetics).

One group attempted to use Southern blotting using a Y chromosomal probe to detect Y specific sequences from peripheral blood DNA extracted from women carrying a male fetus.¹¹ Their assay was able to detect a fetomaternal ratio of 1 in 5000 but they were unable to detect any evidence of fetal specific Y sequences in any of their samples. These results were important because all the previous data using morphological, quinacrine staining, cytogenetic, and flow cytometric criteria estimated a fetomaternal ratio of much higher than 1 in 5000 (0.02%), thus suggesting that early workers might have erroneously scored many maternal cells as being of fetal origin.

Further evidence which disputed the conclusions of some of the earlier studies came from analysis of fluorescence activated cell sorter (FACS) enriched cells using molecular probes which showed that in some of these studies, the alleged "fetal cells" were actually of maternal origin.^{12,13}

The polymerase chain reaction

Further development in molecular genetics has recently rekindled excitement in the field of non-invasive prenatal diagnosis by providing an extremely powerful technique, the polymerase chain reaction (PCR).¹⁴ PCR is an elegant technique for amplifying nucleic acids *in vitro*. By repeated thermal denaturation, primer annealing and polymerase extension, PCR can amplify a single target DNA molecule to quantities which can be detected easily. This exquisite sensitivity and specificity make it an ideal tool for detecting a rare target such as the elusive fetal cell in maternal blood.¹⁵

In 1989 PCR was successfully used to detect fetal DNA in maternal circulation using a nested amplification procedure.¹⁶ In this study nested PCR was used to detect a highly repetitive Y target at the DYZ1 locus in peripheral blood DNA from 19 pregnant women. All 12 pregnant women whose samples gave rise to a positive Y signal later gave birth to boys, while all seven whose samples were negative gave birth to girls. This provides the first conclusive molecular

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Table 1 Amplification of fetal derived Y chromosomal sequences from maternal peripheral blood

Authors	Gestational age (weeks)	PCR target	Amplification regimen	Accuracy
Suzumori <i>et al</i> ¹⁷	17–20	DYZ1	40 + 20	85% (85/100)
Merel <i>et al</i> ¹⁸	13	DYZ1	40 + 17	68% (65/95)
Hamada <i>et al</i> ¹⁹	7–40	DYZ1	40 + 15–40 (semiquantitative)	80% (40/50)
Kao <i>et al</i> ²⁰	8–12	ZFY	32 + 40	92% (33/36)
Gänshirt-Ahlert <i>et al</i> ²¹	40	SRY	30 cycles followed by Southern blotting	43% (6/14)*
Adkinson <i>et al</i> ²²	11–16	DYZ1	30 + 30	96% (48/50)
Wachtel <i>et al</i> ²³	10–21	DYS14	40 + 25	65% (15/23)
Chakravarty <i>et al</i> ²⁴	6–18	DYS14	40 + 25	95% (190/200)

* Only women bearing male fetuses were studied.

evidence that fetal cells do indeed circulate in maternal blood and that genetic information regarding the fetus can be obtained from analysing maternal peripheral blood.

Amplification of fetal Y chromosomal sequences from maternal blood has now been confirmed by a number of groups.^{17–25} In all these reports fetal Y chromosomal DNA was detected by PCR from maternal peripheral blood which has not been previously enriched for fetal cells. A number of Y chromosomal targets have been used in these reports, including DYZ1,^{17–19, 22} DYS14,^{19, 23–25} ZFY,²⁰ and SRY.²¹ Data from our group^{16, 25} and Hamada *et al*¹⁹ suggest that the high copy number of the DYZ1 locus offers a higher sensitivity than the low copy number DYS14 locus. The study by Suzumori *et al*¹⁷ is interesting as it clearly demonstrates that some Y targets—for example, the amelogenin genes, which are present both on the X and Y chromosomes and produce PCR products of different sizes, are not suitable for amplification because of the low sensitivity and underlines the principle that the amplification system used for fetal cell detection should be as fetal specific as possible. However, it is also of interest to note that some investigators have consistently obtained better results than others—for example, Kao *et al*²⁰ reported an overall accuracy of 92%. There is clearly a need for investigators from different groups to compare their laboratory protocols and practice in an attempt to improve overall results. The results from these various reports are summarised in table 1.

Most of the studies mentioned in the previous paragraph used the nested PCR protocol as initially developed for the DYZ1 locus.¹⁶ While this procedure has been very useful in improving the sensitivity and specificity of the amplification step, it is relatively labour intensive and prone to contamination. Recent

development of an alternative amplification regime using the Hot Start process^{26, 27} has enabled the achievement of single molecule sensitivity without using nested PCR. This process has been shown to effectively obviate the need for nested PCR and may prove to be the method of choice in future studies involving the amplification of Y specific sequences from maternal blood.²⁸ Furthermore, the inclusion of an anti-carryover contamination measure involving the incorporation of dUTP and uracil-N-glycosylase treatment prior to amplification has improved the robustness of the resulting amplification system.²⁸

As the fetal cells in maternal circulation are surrounded by a great excess of maternal cells, the use of the PCR on maternal blood samples not previously enriched for fetal cells is limited to the detection of fetal derived, paternally inherited sequences. Apart from Y chromosome sequences of a male fetus, two autosomal fetal genes have also been successfully detected from unsorted maternal peripheral blood. The first is the detection of fetal rhesus D gene from the peripheral blood of rhesus negative women.^{29, 30} In this example the rhesus negative mother does not possess the rhesus D gene and thus the system is directly analogous to that of the Y chromosome system. The non-invasive prenatal determination of fetal rhesus D status has potential implications for the management of previously sensitised rhesus negative women with a partner heterozygous for the rhesus D gene. In this situation no further prenatal diagnostic and therapeutic procedure is necessary if the fetus can be shown to be rhesus negative.

The other system is the detection of a paternally inherited haemoglobin Lepore-Boston gene from maternal peripheral blood.³¹ The Lepore-Boston gene is caused by the fusion of the 5' half of the δ -globin gene and the 3' half of the β -globin gene, together with a seven kilobase deletion between the two fusion partners. The information on these two examples is summarised in table 2.

However, these three examples (detection of the Y chromosome, rhesus D, and Lepore-Boston sequences) represent special cases as most fetal genes which are the targets of prenatal diagnosis have maternal counterparts—for example, the β -globin gene and the cystic fibrosis transmembrane conductance regulator gene. Any PCR system which is to be used in these situations has to be much more specific than those mentioned in the previous paragraph. In these circumstances the detection system has to be able to detect the relatively small number of nucleotide differences which distinguish the fetal from the maternal sequences. This issue will be discussed in more detail later on in this paper. Assuming that this can be done, PCR may be of use in two categories of autosomal disorders namely, autosomal dominant disorders in families where the father carries the mutation and autosomal recessive disorders in which the father and mother carry different

Table 2 Detection of unique fetal derived DNA sequences from maternal blood

Authors	Fetal target	Effect of maternal background
Lo <i>et al</i> ^{16, 25, 32}	Y chromosome sequence	XX thus no amplification
Lo <i>et al</i> ²⁹	Rhesus D gene	Rhesus D negative and so does not possess the gene
Camaschella <i>et al</i> ³¹	Lepore-Boston gene	PCR will not amplify across the normal δ - and β -globin genes due to presence of seven kilobases of intervening DNA

Application of PCR for the detection of paternally inherited mutations in autosomal genes from maternal peripheral blood. In this analysis either no or a relatively low enrichment of fetal cells is assumed and PCR is used for direct mutation detection. For autosomal dominant disorders, the detection of a paternally inherited mutation indicates an affected fetus. For autosomal recessive disorders, the father and the mother must carry different mutations. Autosomal recessive disorders in which more than one mutation is found include cystic fibrosis and β -thalassaemia. The detection of a paternally inherited mutation from maternal peripheral blood indicates that there is a 50% chance that the fetus is homozygous for the disease causing mutations. Apart from direct mutation detection, PCR can also be used to detect polymorphic markers linked to disease causing genes.

mutations (figure). In certain families and in disorders in which the mutant gene has not been identified, but in which linked DNA markers are known, the method can be modified to detect paternally inherited DNA polymorphisms. Provided that the families have previously affected offspring and that the paternal chromosomes can be distinguished from one another and from the maternal ones, useful information concerning the fetal genotype can be obtained.

Detection of autosomal fetal targets for which the mother and the fetus both possess a copy of the sequence is a challenging problem. Theoretically, there are two ways to approach this problem. The first involves the use of a system which will amplify both the fetal and maternal sequences, which are then distinguished from one other by a subsequent analytical step—for example, electrophoresis. The main problem with this strategy is that the PCR plateau of 3–5 pmol³³ is then shared between the fetal and maternal PCR products. As the maternal template at the start of the PCR is in great excess compared with the fetal one, most of the resulting PCR product is of maternal origin. In other words the amplification factor for the fetal sequence is limited. The best studied examples of systems in which the majority and minority populations are co-amplified may be found in the

literature on post bone marrow transplantation chimaerism in which the typical sensitivity of these assays ranges from 0.1–1%, far below the level required to detect the rare fetal cell in maternal blood.^{15 34}

The second approach involves the design of amplification systems which will specifically amplify the fetal sequence, while remaining refractory to the related, but not identical, maternal sequence. The amplification refractory mutation system (ARMS)²⁶ is a type of PCR which is capable of achieving this aim. ARMS enables PCR to be discriminatory down to the single nucleotide level. It is based on the principle that the specificity of the PCR is conferred by the 3' end of the primers. Thus, if there is a 3' terminal mismatch between a PCR primer and the DNA target, the amplification efficiency will be greatly reduced. When using ARMS to detect paternally inherited polymorphisms of the fetus from maternal peripheral blood, ARMS primers are designed such that the last base of the primer matches the paternal allele but has a 3' terminal mismatch with the maternal alleles. It is important to realise that ARMS only confers *relative* selectivity for the detection of fetal specific sequence. In other words the mismatched allele will also be amplified, albeit with reduced efficiency compared with the matched one. Hence, one is faced with a dilemma when applying ARMS to the detection of extremely small amount of fetal genetic material from maternal blood; the small quantities of fetal material require the use of a large number of PCR cycles, but the high degree of cycling means that the extent to which the mismatched reaction takes place is also increased. With further developments in ARMS technology, such as the double ARMS,³⁵ one can achieve the goal of detecting a minority DNA population at a level of 1 in 10⁵ or below. Using double ARMS, a paternally inherited polymorphism in the β -globin locus was detected in maternal blood.³⁶ This latter system may potentially be used for the diagnosis of β -thalassaemia.

Fetal cell enrichment

Although the data published so far using PCR on unenriched samples have been encouraging, it is obvious that further improvement in diagnostic accuracy is necessary before non-invasive prenatal diagnosis using fetal cells in maternal blood can be used clinically. The main obstacle to overcome is the rarity of fetal cells in maternal blood. Many investigators have explored fetal cell enrichment techniques and, so far, have concentrated on four types of fetal cells as targets for enrichment.

NUCLEATED RED BLOOD CELLS

This is by far the most encouraging candidate cell type for enrichment. This approach is first proposed by Bianchi *et al*³⁷ and is based on the principle that nucleated red blood cells (NRBC) constitute a significant proportion of the red blood cells in fetal blood, but are very rare in peripheral adult blood. Indeed, circu-

lating NRBCs comprise about 10% of the red blood cells in the 11 week old fetus and 0.5% in the 19 week old fetus.⁹

Various markers have been employed to enrich for these NRBCs. Bianchi *et al*³⁷ initially flow sorted using an antibody against the transferrin receptor (CD71). Subsequently, further data have suggested that the combination of anti-CD71 with other antibodies—for example, with anti-glycophorin A antibody^{38,39} or anti-CD36 (thrombospondin receptor)³⁹ would further improve the results. Gänshirt-Ahlert *et al*⁴⁰ have explored the use of a triple-density gradient, followed by magnetic activated cell sorting (MACS) to isolate NRBCs and have claimed satisfactory results. Zheng *et al*⁴¹ have also reported success using MACS to deplete maternal peripheral blood of leucocytes, followed by detection of fetal erythroid cells with an antibody against fetal haemoglobin. Overall, the preliminary data on NRBCs have been encouraging but the proportion of fetal cells in the enriched samples is still low—for example, only 0.001 to 4.8% of a FACS enriched sample consists of fetal cells.⁴² Further improvement in this area will require more experimentation with more efficient systems for enrichment and possibly testing of more antibodies against NRBCs.

Very recently, a completely new approach has been proposed which involves the selective culture of fetal erythroid cells from maternal blood.⁴³ This strategy uses the fact that there are more erythroid precursors in fetal than in maternal blood, and that these fetal erythroid precursors have a shorter cell cycling time and are more sensitive to exogenously added growth factors than their adult counterparts. Further experimentation may allow the isolation of pure fetal erythroid colonies from maternal blood.

TROPHOBLAST CELLS

Trophoblast cells are the obvious cell type to be investigated because of their intimate relation with the uterine vasculature. Early work using the allegedly trophoblast specific monoclonal antibody H315⁷ was found to be invalid because H315 positive cells sorted from maternal blood were shown only to have maternal genetic markers¹³ and that H315 negative cells could adsorb the antigen *in vitro*.¹²

More recently, Mueller *et al*⁴⁴ developed and screened a panel of monoclonal antibodies against placental tissues. They found five antibodies that were specific for fetal tissue, two of which were used to isolate fetal cells using immunomagnetic techniques. Following PCR amplification using Y specific primers, fetal sex was correctly identified in seven of seven males and six of seven females. However, it has been unfortunate that so far the authors have not shared reagents with other workers and so their results have not been verified by an independent group as yet.

Although other antitrophoblast monoclonal antibodies have been generated by other groups,⁴⁵ doubts remain regarding the specificity of these antibodies.⁹

LYMPHOCYTES

The presence of fetal lymphocytes in maternal blood was suggested as early as 1969 when Walknowska *et al*⁴ performed cytogenetic analysis on phytohaemagglutinin stimulated peripheral blood lymphocyte cultures from pregnant women. Herzenberg *et al*⁸ used flow sorting to enrich for fetal nucleated cells using a paternally inherited HLA allele, which is present on the fetal, but not maternal, cells. They suggested that these cells were lymphocytes. However, it is equally likely that these cells are actually nucleated red cell precursors which have not yet lost their HLA class I antigens. Recently, this approach has been combined with PCR and has resulted in the successful amplification of paternal HLA alleles from the flow sorted cells.⁴⁶ However, the main limitation of this approach for prenatal diagnosis is the inconvenience of having to HLA type the father and the mother before flow sorting and the fact that it is impossible to predict which of the paternal HLA alleles are inherited by the fetus.

GRANULOCYTES

Weissman *et al*⁴⁷ have reported the detection of fetal granulocytes from maternal peripheral blood. Using density gradient centrifugation followed by non-isotopic *in situ* hybridisation with a Y specific probe, a proportion of Y positive cells had granulocyte morphology. However, the number of these presumed "fetal granulocytes" was unusually large (up to 0.14%) and the frequency of fetal cells was not consistent with other recent reports.

Diagnosis of fetal chromosomal aneuploidy

Several investigators have used either flow sorting or immunomagnetic separation followed by fluorescence *in situ* hybridisation (FISH) to detect aneuploid fetal cells sorted from maternal blood.^{38,40,48,49} As yet, the number of reported cases is very small and further work is necessary to assess the sensitivity and specificity of this approach.

Biological questions regarding fetomaternal cell trafficking

Relatively little is known about the biological parameters governing the passage of fetal cells into maternal circulation. For example, it is still not known whether the existence of fetal cells in maternal circulation represents a normal physiological phenomenon present in all pregnant women or whether it is merely a consequence of random fetomaternal haemorrhage. This key issue has a direct bearing on the general applicability of prenatal diagnosis using fetal cells in maternal blood.

NUMBER OF FETAL CELLS IN MATERNAL BLOOD

Data generated using PCR indicate that the frequency of fetal cells in maternal blood in most women is of the order of 1 nucleated fetal cell per 10⁵–10⁶ nucleated maternal cells.

This figure has been confirmed by data obtained using fetal cell enrichment techniques.³⁸ Thus, Price *et al*³⁸ estimated that there are between 200 and 2000 fetal nucleated red cells per 20 ml of maternal peripheral blood. There are approximately 1.6×10^8 nucleated maternal cells in 20 ml of maternal blood, resulting in a feto-maternal ratio of between 1 in 80 000 and 1 in 800 000 maternal cells.

There is also evidence in the literature that the frequency of fetal cells in the peripheral blood of women carrying aneuploid fetuses may, in some cases, be higher than that of women carrying euploid fetuses. In a case report Elias *et al*⁴⁸ estimated that 74% of cells sorted from a patient carrying a trisomy 21 fetus were of fetal origin. Apart from obvious biological interest, this observation also has direct bearing in the accuracy of non-invasive prenatal testing of fetal chromosomal aneuploidies.

The optimal time during pregnancy for detecting circulating fetal cells remains unclear. PCR data reported so far indicate that fetal DNA can be detected from maternal circulation throughout pregnancy³² starting from the fourth to sixth week of gestation.²⁵⁻⁵⁰ However, these studies have regarded nucleated fetal cells, irrespective of cell lineage, as a single group as no cell fractionation was used in these studies. A number of workers have studied one particular fetal cell population, the NRBCs, but have so far found results which are contradictory in many respects. For example, Bianchi *et al*⁵¹ reported that NRBCs were unlikely to be present after 16 weeks of gestation and attempted to explain this by referring to the reduction in the proportion of NRBCs in the fetal circulation as pregnancy progresses. However, this effect has not been observed by others.⁹⁻⁴⁰ Thus, Gänshirt-Ahlert *et al*⁴⁰ reported that NRBCs could be enriched in all three trimesters while data from the Tennessee group³⁸ suggested that NRBCs were present from at least 10 to 18 weeks of gestation. This issue will need to be resolved by sequential sampling of a large series of pregnant women.

EFFECT OF BLOOD GROUP INCOMPATIBILITY

There has been no systematic study of the effect of ABO incompatibility between the mother and the fetus and the frequency of fetal cells in maternal blood. However, recent data generated through the detection of fetal rhesus D sequences from rhesus D negative maternal blood indicates that the number of fetal cells decrease in the presence of high levels of anti-D antibody.³⁰

PERSISTENCE OF FETAL CELLS FROM PREVIOUS PREGNANCIES

Persistence of fetal cells from previous pregnancies is an issue which has implications for the diagnostic accuracy of tests involving fetal cells from maternal circulation. Data from our group suggest that this is unlikely to be an important phenomenon in most women at a detection sensitivity of 1 nucleated fetal cell

per 300 000 nucleated maternal cells.³² However, using PCR alone, it is difficult, if not impossible, to conclusively prove that this phenomenon does not exist even in a minority of women. This is because such analysis will require the study of a large number of women postpartum (for example, those who have previously delivered male babies), in which the use of PCR alone will almost certainly generate false positive results in some cases. These false positive results, if erroneously regarded as true positive, could then be interpreted as evidence for fetal cell persistence. Indeed, such interpretations have been made.⁵² Similarly, data suggesting that fetal cells persist from previous pregnancies have been presented by Bianchi *et al*⁵³ who sorted for fetal cells using anti-CD34 and were able to detect a Y chromosomal signal using nested PCR from a number of women who gave birth to boys many years before. However, the number of presumed fetal derived CD34 cells is likely to be very small because, despite prior fetal cell enrichment, the Y signal was only detected following nested PCR, with no signal being seen after one round of PCR.

RELATION TO PRE-ECLAMPSIA

Pre-eclampsia is a syndrome that develops during pregnancy, labour, or the early puerperium. Maternal and fetal clinical symptoms and signs are secondary manifestations of the disease, but its pathogenesis remains unclear. For many years, some investigators related the primary cause to the placenta, and it is becoming more apparent that the disorder is a trophoblast dependent process.⁵⁴ Many of the original morphological studies claiming the detection of trophoblasts in maternal tissue were performed on patients dying from eclampsia.^{1,2} Recently, these observations were extended by an immunocytochemical study using a monoclonal antibody against cytokeratin to label trophoblast cells in cell smears of uterine venous blood obtained at caesarean section.⁵⁵ In this study women with pre-eclampsia were found to have more trophoblast cells in uterine venous blood than women without pre-eclampsia. Further work along similar lines would therefore be of value in the understanding of the pathogenesis of this disorder.

Conclusion

In conclusion, the detection and characterisation of fetal cells in the maternal circulation is a rapidly expanding field of investigation with important implications for both prenatal diagnosis and for a better understanding of the physiology of feto-maternal interactions. It is hoped that further new concepts and technological advances will now hasten the development of this field and lead to the introduction of non-invasive prenatal diagnosis into routine clinical practice.

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