

Culture of fetal cells from maternal blood for prenatal diagnosis

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The isolation and analysis of fetal cells from maternal blood would allow non-invasive prenatal genetic screening and diagnosis. Over the past decade, progress has been made towards this goal using various enrichment strategies and analysis by fluorescence in-situ hybridization with chromosome-specific probes and PCR. One method that is currently being explored involves culturing fetal cells. Developing conditions which allow the number of fetal-derived cells to expand in culture and the number of maternally derived cells to be suppressed in culture may lead to a new selection process for obtaining fetal cells. Culturing of fetal cells from maternal blood could make possible conventional metaphase analysis of fetal cells for diagnosis of chromosomal abnormalities.

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Introduction

Reliable prenatal diagnosis for fetal aneuploidy and single gene disorders currently involves invasive procedures, i.e. chorionic villus sampling [at gestational age (GA) 10–12 weeks], amniocentesis (GA 15–20 weeks) or umbilical blood sampling (GA >18 weeks). The performance of these procedures requires special skills, and each has a low but finite risk to the mother and fetus. These procedures are generally not offered to all pregnant women, but only those at high risk (e.g. women aged ≥ 35 years, abnormal maternal serum marker screening, ultrasound detection of fetal abnormalities, or increased likelihood of a single gene disorder). The availability of a low-cost and low-risk reliable non-invasive method for prenatal diagnosis would be an important improvement over current prenatal diagnostic procedures (Simpson and Elias, 1994).

Presence of fetal cells in maternal blood

The presence of fetal cells in the maternal circulation during pregnancy has been observed since the end of the 19th century, as

evidenced by autopsy findings of pregnant women whose lungs showed trapped fetal squames (Schmorl, 1893; Douglas *et al.*, 1959). These fetal cells are likely to result from leakage at the placenta–uterine interface (Price *et al.*, 1991; Beer *et al.*, 1994). However, fetal cells in maternal blood are very rare, occurring at a frequency of one fetal cell per 10^5 – 10^9 maternal cells (Price *et al.*, 1991; Hamada *et al.*, 1993; Bianchi *et al.*, 1997; Little *et al.*, 1997; Sohda *et al.*, 1997). Increased leakage of fetal cells into maternal blood has been reported to occur in cases of fetal aneuploidy (Valerio *et al.*, 1996; Bianchi *et al.*, 1997; Fisher, 1997; Parano *et al.*, 2001), as well as pre-eclampsia (Jansen *et al.*, 2001). Because fetal cells in the maternal circulation are rare, enrichment from maternal cells is necessary before analysis is made possible.

Numerous methods for isolating fetal cells from maternal peripheral blood have been devised, including density gradient centrifugation (Oosterwijk *et al.*, 1996; Sekiwaza *et al.*, 1999; Samura *et al.*, 2000), magnetic-activated cell separation (MACS) (Gänshirt-Ahlert *et al.*, 1993, 1998; Busch *et al.*, 1994; Jansen *et al.*, 1999), ferrofluid suspension with magnetic separation (Steele *et al.*, 1996), flow cytometry (Bianchi *et al.*, 1990; Price *et al.*, 1991; Lewis *et al.*, 1996; Little *et al.*, 1997; Sohda *et al.*, 1997), micromanipulation of individual cells (Takabayashi *et al.*, 1995; Cheung *et al.*, 1996; Sekiwaza *et al.*, 1999; Vona *et al.*, 2002), selective lysis (de Graaf *et al.*, 1999) and charge flow separation (Wachtel *et al.*, 1996). These methods require high levels of technical expertise and, even when successful, yield few fetal cells. Moreover, each purification step involved in the separation of fetal cells from maternal blood results in loss of a significant proportion of the target fetal cells. Thus, developing conditions to

allow the number of fetal-derived cells to expand in culture and at the same time suppressing maternally derived cell in a culture system may lead to a new selection process to obtain fetal cells, reduce fetal cell loss, and simplify the enrichment process. Additionally, culture of fetal cells could make possible conventional metaphase analysis of fetal cells, and clonal expansion of fetal cells could be used as a source of pure fetal DNA for prenatal diagnosis.

In-vitro expansion of fetal cells: studies, results and trends

The main issues involved in culturing fetal cells are the selection of fetal cell type, the expansion of fetal cells over maternal cells, and the detection and analysis of the fetal cells.

Detection and analysis

The detection and analysis of fetal cells is currently performed by fluorescence in-situ hybridization (FISH) using chromosome-specific probes and by PCR. The sensitivity and specificity of the FISH method depends on sample preparation, type of DNA probes used (for prenatal diagnosis, probes specific for chromosome X, Y, 13, 18, or 21 are most commonly applied), hybridization efficiency, and expertise of laboratory personnel (Klinger, 1994). In the research setting, FISH analysis is widely used for fetal sex identification by correlating the presence of Y-signals in women carrying male fetuses and the detection of fetal aneuploidy. Detection of single gene disorders requires amplification of a DNA sequence by PCR. As with FISH, the most widely used primers in research are for detection of Y-sequence signals and correlation with fetal sex (Lo *et al.*, 1994a, 1998). However, because of its sensitivity and its ability to amplify very small quantities of DNA, PCR is very sensitive to contamination.

Selection of a type of fetal target cells

The types of fetal cells that circulate in maternal circulation include—but may not be limited to—haematopoietic progenitors and stem cells (Tilesi *et al.*, 2000; Coata *et al.*, 2001), nucleated red blood cells (NRBC) or erythroblasts (Bianchi *et al.*, 1990; Bigbee and Grant, 1994; Lo *et al.*, 1994a), leukocytes (Sargent *et al.*, 1994a) and trophoblasts (Mueller *et al.*, 1990; Sargent *et al.*, 1994b; Vona *et al.*, 2002). Trophoblasts are interesting cells because they are specific to pregnancy; however, their large size (which causes them to be sequestered in maternal lung), the fact that they are frequently multinucleated or anucleated, placental mosaicism (Goldberg and Wohlferd, 1997), and the difficulty of separating them from maternal blood (Sargent *et al.*, 1994b) make them problematic target cells. Leukocytes are long-lived cells, which may potentially cause culture contamination with fetal cells from prior pregnancies (Bianchi *et al.*, 1996). Stem cells, which express CD34 surface antigen, are a possible target for culture; however, the specific selection of fetal cells is rendered difficult by the fact that CD34 is also present on adult stem cells, and in general on haematopoietic stem and progenitor cells, as well as endothelial precursor cells (Lanza *et al.*, 2001). NRBC are an appealing candidate for in-vitro expansion. NRBC are abundant and may be identified through their expression of surface markers such as CD71, CD36 (thrombospondin receptor) and glycophorin A (Loken *et al.*, 1987; Bigbee and Grant, 1994), and intracellular

antigens such as fetal haemoglobin (HbF) (Bianchi *et al.*, 1990; Park *et al.*, 1994; Zheng *et al.*, 1995). NRBC also have a limited lifespan, which increases the likelihood that they originate from the current pregnancy, rather than from previous pregnancies. This is an important consideration since fetal cells may be detected in maternal peripheral blood up to 27 years after pregnancy (Bianchi *et al.*, 1996). Additionally, fetal erythroblasts and fetal erythroid progenitors have been reported to be more sensitive to erythropoietin (EPO) than adult erythroid progenitors; maximal growth of fetal cultures occurs with an in-vitro concentration of EPO <2 U/ml, whereas adult cultures require >2 U/ml (Weinberg *et al.*, 1992). Therefore, selection is potentially possible through variation of the EPO concentrations *in vitro*.

Preferential in-vitro expansion of fetal cells over maternal cells: promises and results

In a promising report (Lo *et al.*, 1994b), erythroid cells of fetal origin were grown in an EPO-enriched medium. Spiking experiments, as well as culture of maternal peripheral blood in five samples (male fetus), showed preferential expansion of fetal cells over maternal cells as determined by quantitative PCR. A series of promising results was reported (Valerio *et al.*, 1996) relating to eight samples from pregnant women carrying male fetuses (GA 14–16 weeks). In these samples, fetal erythroid progenitor cells were enriched using MACS with biotin-labelled human EPO ligand, cultured in a semi-solid medium, and colonies were assessed by PCR for Y-specific sequences and FISH. The colony forming unit (CFU)-erythroid, the mature burst forming unit-erythroid and occasionally CFU-GEMM colonies containing fetal cells were found.

Enthusiasm was tempered however by the results of a larger-scale culture study of erythroblasts and other cells of erythroid lineage (Chen *et al.*, 1998). Two methods of fetal cell selection were tested on 27 samples from pregnant women (GA 9–17 weeks) and five non-pregnant controls; one method was based on Weinberg *et al.*'s approach (1992) using different concentrations of EPO in culture, the other on conditions similar to Valerio *et al.*'s report (1996). Peripheral blood mononuclear cells (isolated by density centrifugation) were cultured, and cells from individual colonies were analysed by PCR (locus: ZFY/ZFX) for sex determination, FISH and immunophenotyping (HbF antibodies). These authors concluded that the erythroid colonies obtained under either culture conditions were from maternal progenitors, and not of fetal origin.

A two-phase liquid culture system for fetal erythroid cells was subsequently developed (Han *et al.*, 1999) that involves separation of EPO-dependent and EPO-independent phases (based on a concept from Fibach *et al.*, 1989), followed by HbF staining. Under these culture conditions, one blood sample from a woman who was pregnant with a male fetus (GA 10 weeks) showed the presence of fetal cells by PCR, but no XY cells were observed by metaphase analysis. A study of liquid-phase culture of fetal erythroid cells led to comparable results (Han *et al.*, 2001). When the peripheral blood mononuclear cells from 10 pregnant women (five male and five female fetuses, GA 8–14 weeks) were cultured under such conditions, PCR showed the presence of Y-specific signals in four of the five male pregnancies; however, metaphase analysis showed only XX cells.

The successful culture and isolation of single clones of fetal progenitor cells from maternal blood has also been reported (Tutschek *et al.*, 2000). Culture of mononuclear cells obtained by gradient centrifugation of 12 samples from pregnant women (GA 14–20 weeks), followed by PCR (locus: amelogenin AmgXY), showed the presence of male fetal cells. Some of the colonies were mixed (fetal/maternal cells), and some contained purely fetal cells. However, the existence of mixed clonal colonies and the use of PCR rather than FISH were questioned (Campagnoli *et al.*, 2001a). In addition, an independent group did not confirm the data of Tutschek *et al.* (2000) who used similar conditions and PCR for the SRY locus; culture of progenitor cells from 16 male pregnancies did not show Y-bearing clones. It was suggested that Tutschek *et al.*'s results were due to allele drop-out (Zimmermann *et al.*, 2002).

In an effort to achieve consistent results, several groups have attempted to optimise fetal cell selection methods and culture conditions using maternal blood models spiked with a known number of fetal cells. One group (Bohmer *et al.*, 1999) reported the optimization of serum content conditions for culture of fetal NRBCs in a co-culture model. However, when applied to maternal blood, fetal cells were not detected by FISH, PCR and sorting for HbF in 24 samples, including known male pregnancies and trisomies (GA 10–20 weeks) (Bohmer *et al.*, 2002). Others (Jansen *et al.*, 2000) used a spiking model with fetal cord blood CD34+ cells and showed a 1500-fold expansion of fetal cells in culture. The same culture conditions and cytokine combination were applied to 100 maternal samples, 65 of which were from women carrying male fetuses (GA 7–16 weeks). There was no preferential growth of fetal cells (Jansen *et al.*, 2000). In a similar study (Huber *et al.*, 2000), a spiking model was used of fetal erythroid cells which were cultured and subjected to high-performance liquid chromatography and FISH analysis. The results were promising, but application of the same method to 26 pregnant samples (including 13 confirmed male fetuses) of maternal blood did not show any Y-positive cells.

In a subsequent study (Valerio *et al.*, 2000), total nucleated cells from maternal blood, isolated by double-gradient centrifugation, were subjected to a short culture period (3 days), followed by FISH analysis. In one sample (GA 19 weeks) these authors detected 47,XY, +18 cells from an affected fetus. The value of CD34 enrichment has also been investigated. It was recently reported that first-trimester CD34+ fetal haematopoietic progenitors could be selectively expanded *in vitro* in model cultures (Campagnoli *et al.*, 2002). However, in a recent report comparing culture of CD34+ enriched and non-enriched progenitor cells from 17 samples of maternal blood (10 confirmed male pregnancies, GA 5–22 weeks), followed by PCR (AmgXY), no XY cells were detected (Manotaya *et al.*, 2002).

Therefore, despite the development of seemingly optimal fetal cell culture conditions, and identification of optimal *in-vitro* cytokine combinations that favour the proliferation of fetal over adult progenitor cells, the culture of fetal haematopoietic cells from maternal blood samples has not yet been achieved in a reproducible manner. The inability to expand fetal erythroid progenitors successfully from the maternal circulation *in vitro* might be explained by the fact that the occurrence of expandable fetal haematopoietic progenitor cells in the maternal blood are rare to very rare events (Little *et al.*, 1997; Jansen *et al.*, 2000;

Campagnoli *et al.*, 2002), or that they require some as-yet unidentified culture conditions. Based on these results, the *in-vitro* expansion of fetal cells of different lineages, such as endothelial cells and endothelial cell progenitors, is being explored.

Because embryonic and fetal development is characterized by very active vasculogenesis and angiogenesis, and because vasculogenesis involves the differentiation of endothelial cells from circulating fetal precursors (Risau, 1991; Risau and Flamme, 1995), our group has hypothesized that endothelial precursor cells of fetal origin might enter the maternal circulation and give rise to endothelial cell colonies of fetal origin when cultured *in vitro* (Gussin *et al.*, 2002). Based on a proposed model of post-natal angiogenesis and vasculogenesis—the former involving recruitment of pre-existing endothelial cells and the latter bone marrow-derived circulating endothelial precursor cells that do not normally circulate (Shi *et al.*, 1998; Rafii, 2000)—an attempt was made to differentiate maternal (pre-existing endothelial cells) from fetal cells (endothelial progenitors) by means of their time of appearance in culture. Colonies from pre-existing endothelial cells (presumed to be of maternal origin) are present after 1 week of *in-vitro* culture ('early outgrowth'). By contrast, endothelial precursor cells (presumed to be of fetal origin) mature into 'late outgrowth' endothelial cells after 4–6 weeks of culture (Rafii, 2000). Following gradient density centrifugation, peripheral blood mononuclear cells isolated from 13 maternal blood samples (GA 15–20 weeks) were cultured in conditions which promoted endothelial cell growth. The results indicated that endothelial cell progenitors were observed only in cultures from the blood of pregnant women and not in controls, but based on FISH analysis and the failure to detect XY cells in women carrying male fetuses, they appeared to be of maternal origin.

Conclusions

Fetal erythroid progenitors from maternal blood do not appear to be a good target for expanding fetal cells and suppressing maternal cells in culture. A current focus is on endothelial cells and endothelial progenitor cells. Alternatively, fetal mesenchymal stem cells, which were identified in fetuses during the first trimester, may prove to be a more optimal target for identification of fetal cells in maternal blood (Campagnoli *et al.*, 2001b). If successful, these target cells will provide a new selection method for fetal endothelial cells from maternal blood and a means of increasing the number of dividing fetal cells for the possibility of conventional cytogenetic analysis.

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