Culture of fetal cells from maternal blood for prenatal diagnosis

Hélène A. Elisha Gussin and Sherman Elias

1University of Illinois at Chicago, Departments of Obstetrics and Gynecology, and 2Molecular Genetics, Chicago, Illinois, USA
3To whom correspondence should be addressed at: University of Illinois at Chicago, Department of Obstetrics and Gynecology, 820 S. Wood Street, M/C 808, Chicago IL 60612, USA; E-mail: selias@uic.edu

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.

Keywords: cell culture/maternal circulation/pregnancy/prenatal diagnosis

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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-Ahlertrt 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; Sohma 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 conven-
tional 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-spe-
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 chromo-
some 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 amplifica-
tion 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 Wohlfert, 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 glycoporphin
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), erythroblasts 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 separa-
tion 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
erthyroid 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), 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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References

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