Isolation of fetal cells from maternal blood

Technical and clinical aspects

Isolatie van foetale cellen uit moederlijk bloed

Technische en klinische aspecten

Maria Wilhelmina Johanna Christina Jansen

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Vooruitgang

Soms gaat vooruitgang zo snel
dat we niet alles wat mooi is en goed
met ons mee kunnen dragen.
Daarom moeten we van tijd tot tijd even pauzeren
en achterom zien om de nagestuurde bagage in ontvangst te nemen.

Kadé Bruin uit "Uitsmijters van scharreleieren"

Voor Xris & Wilh
Voor Ingmar

Contents

Preface

Traditional approaches towards prenatal diagnosis of fetal abnormalities are ultrasonography and the invasive techniques of amniocentesis, chorionic villus sampling (CVS) and cordocentesis. Although the invasive methods reach virtually 100 % accuracy, there is a small procedure-related risk for the fetus. Fetal loss rate following an invasive procedure is estimated at 0.5 – 1 %, depending on the technique employed. In the Netherlands, invasive prenatal testing is offered to women for various reasons: (1) advanced maternal age (≥ 36 years); (2) a parental carrier status of a balanced chromosomal anomaly; (3) a previous child with a chromosomal abnormality and/or multiple congenital malformations; (4) increased risk of a neural tube defect; (5) increased risk of a monogenic disease demonstrated by biochemical or DNA analysis; and (6) fetal congenital defects seen on ultrasound examination.

During the last 15 years, high resolution ultrasound equipment has made possible the identification of a host of fetal congenital anomalies as early as the early second trimester of pregnancy (Wladimiroff, 1994; den Hollander *et al.*, 1998). Late first trimester and early second trimester sonographic markers for aneuploidy have been developed of which nuchal translucency has been shown to be the most effective one (Pajkrt *et al.*, 1998; Snijders *et al.*, 1998). Biochemical testing for chromosome anomalies includes first trimester and early second trimester maternal serum screening (Wald *et al.*, 1997; de Graaf, 1999; van Rijn, 1999). Both sonographic markers and biochemical tests represent a risk assessment with emphasis on Down syndrome. Depending on the nature of these tests, detection rates of 70-80 % for Down syndrome at a 5 % false positive rate have been claimed. Risk assessment by means of maternal serum screening and/or fetal nuchal translucency screening is currently subject to debate in the Netherlands (Discussion Document Dutch Society of Obstetrics and Gynecology, 1997).

An alternative non-invasive approach of potential diagnostic significance is the isolation of fetal cells from maternal blood, which is receiving increasing attention during the last two decades. This may lead to the elimination of fetal cell sampling by invasive techniques such as CVS or amniocentesis. To date, it is not yet clear whether the technique of fetal cell isolation from maternal blood will be accurate enough for fetal diagnosis. This thesis will give an overview of the current state of the art of fetal cell isolation.

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Chapter 1

General introduction

Feto-maternal cell traffic in pathological conditions was first recognized in 1893, when Schmorl identified trophoblast cells in lung capillaries of women dying of eclampsia (Schmorl, 1893). In 1969, Walknowska *et al.* identified male metaphases in cultured lymphocytes isolated from blood of healthy pregnant women, who subsequently gave birth to a boy. They were the first to demonstrate that fetal cells enter the maternal circulation in normal pregnancy and they suggested that these cells might be used for chromosome analysis. Since then, many investigators have focussed on the development of a safe and reliable test to perform non-invasive prenatal diagnosis, as an alternative for chorionic villus sampling and amniocentesis. Before a non-invasive prenatal diagnostic test using fetal cells in maternal blood will be available, a number of questions need to be addressed, such as: (1) at what period of gestation does feto-maternal transfer of cells take place; (2) what is the best fetal cell type to isolate; (3) are fetal cells detectable in the blood of all pregnant women; (4) do chromosomally abnormal pregnancies result in increased or decreased transfer of fetal cells into the maternal circulation; and (5) may fetal cells from prior pregnancies persist in the maternal circulation, and hence interfere with a reliable prenatal diagnosis.

1.1. Biological basis of passage of fetal cells into the maternal circulation

Passage of fetal cells into the maternal circulation will occur at the feto-maternal interface. Knowledge of embryonic development and the formation of the feto-maternal interface (placentation) will gain insight into the process of feto-maternal cell trafficking. Furthermore, knowledge of fetal hematopoiesis will lead to more information about the fetal cell types that circulate in maternal blood during pregnancy.

1.1.1. Embryonic development and placentation

After fertilization of an oocyte by a sperm cell, the formed zygote undergoes a series of rapid mitotic cell divisions known as cleavage. The zygote is cleaved into a number of blastomeres and subsequent cell divisions of these blastomeres result in the formation of the morula (32-cell stage; day 3). Subsequently, the morula develops into the blastocyst (64-cell stage; day 4-5) which contains an outer cell layer (trophoblast) which gives rise to part of the placenta, and a group of centrally located cells, the inner cell mass (embryoblast) which gives rise to the embryo. After the blastocyst has attached to the endometrial epithelium (day 6), the trophoblast differentiates into two cell types: (1) the cytotrophoblast, which is mitotically active and forms new cells that migrate into the increasing mass of syncytiotrophoblast; and (2) the syncytiotrophoblast. The latter rapidly becomes a large, thick, multinucleated

protoplasmic mass in which no cell boundaries are distinguishable (syncytium). Subsequently, a lacunar network develops in the syncytiotrophoblast, and maternal capillaries near the syncytiotrophoblast expand to form maternal sinusoids establishing a primitive uteroplacental circulation, in which the primary chorionic villi develop (day 13). In the further maturation of the villous tree, fetal blood vessels invade this villous connective tissue and connect the vessels to the embryonic circulation. When the fetal heart begins contracting on day 22 or 23, a primitive fetal placental circulation establishes (Moore, 1982).

One of the important placental structures that assures the transfer of nutrients to enhance fetal growth is the so-called 'placenta membrane', a thin layer of fetal tissues separating the maternal and fetal circulation. This membrane consists of syncytiotrophoblast, cytotrophoblast, the connective tissue core of the villus, and the endothelium of the fetal capillaries. As pregnancy advances, the placental membrane becomes progressively thinner while simultaneously fetal blood flow and blood pressure increase as the villous tree enlarges (Sutton *et al.*, 1990).

Until now, it is not known at what period during placentation a feto-maternal transfusion may occur. One can readily imagine that a disruption of the relatively thin placental membrane would lead to fetal bleeding into the intervillous space at the time when this membrane thins with advancing pregnancy. Another theoretical possibility may be that feto-maternal cell trafficking takes place during the formation of villi, at the time when fetal capillaries are formed and the pumping action of the fetal heart begins (Benirschke, 1994).

1.1.2. Hematopoiesis

Fetal hematopoiesis is one of the first processes established following implantation of the blastocyst, and can be divided into three main overlapping periods: mesoblastic, hepatic, and myeloid (figure 1). It was originally assumed that the mesoblastic hematopoiesis starts in the yolk sac between days 16 and 19 (Huyhn *et al.*, 1995), followed by hepatic hematopoiesis at approximately 5 weeks after fertilization (Migliaccio *et al.*, 1986). The final phase of hematopoiesis takes place in the bone marrow, starting at 10 weeks after fertilization in the long bones (Metcalf and Moore, 1971; Kollmann *et al.*, 1994; Charbord *et al.*, 1996). Recently, it has been shown that in developing mammals stem cells can be derived from an intraembryonic site called the aorta-gonad-mesonephros (AGM) region (Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996; Tavian *et al.*, 1996). However, the site of origin of definitive hematopoietic stem cells in the developing fetus remains controversial. Evidence from some studies indicates that hematopoietic stem cells from the yolk sac are responsible for transient primitive hematopoiesis, but they appear to lack the ability to reconstitute the hematopoietic system in adult animals (Cumano *et al.*, 1996; Medvinsky and Dzierzak,

1996). Instead, stem cells derived from the AGM region have been shown, both in mice and man (Tavian *et al.*, 1996), to be responsible for definitive hematopoiesis (Godin *et al.*, 1993; Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996) by first colonizing the fetal liver and later the bone marrow (Delassus and Cumano, 1996). On the other hand, evidence from other studies suggests that hematopoietic stem cells with the capacity to contribute to definitive hematopoiesis are present both in the yolk sac and in the AGM region of murine embryos prior to fetal liver colonization (Yoder *et al.*, 1997a, b).

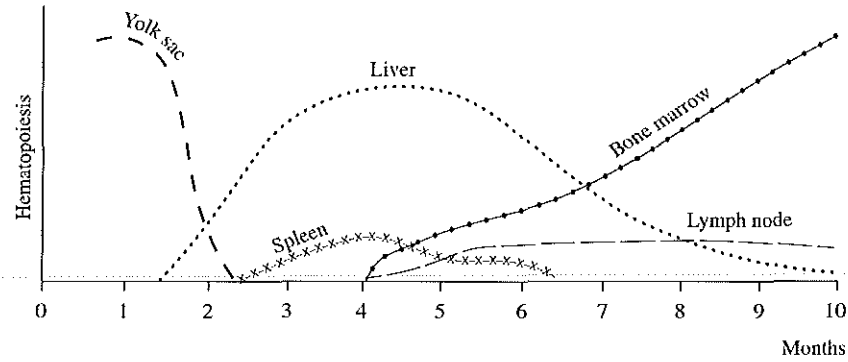

Figure 1 Hematopoiesis in the fetus (after Kelemen *et al.*, 1979)

Hematopoiesis in the yolk sac is distinct from that of fetal liver and adult marrow hematopoiesis in that it appears to be restricted to the generation of two lineages: embryonic erythrocytes, which represent the major hematopoietic component of the blood islands in the yolk sac, and macrophages that are dispersed throughout the yolk sac (Metcalf and Moore, 1971; Russell, 1979). These early erythroid cells, known as primitive erythrocytes, are large and remain nucleated. These primitive erythroid cells predominantly express the ε-globin and ζ-globin genes (the embryonic forms of hemoglobin) and low levels of α- and β-globin genes (Barker, 1968; Brotherton *et al.*, 1979; Russell, 1979). The transition from yolk sac to fetal liver defines the switch from primitive to definitive hematopoiesis and the replacement of the primitive erythroid cells by multilineage hematopoiesis including definitive erythropoiesis, myelopoiesis, and lymphopoiesis (Metcalf and Moore, 1971). Definitive erythroid cells generated in the fetal liver differ from primitive erythrocytes in the yolk sac in that they are small and that they enucleate. This coincides with a switch from ε- to γ-globin gene expression and from ζ- to α-globin gene expression (Peschle *et al.*, 1985). During the fetal

period, the site of erythropoiesis gradually switches from the liver to the spleen and finally the bone marrow. Gamma-globin gene expression decreases during this period with a reciprocal increase in the expression of the adult β-globin gene and the appearance of low levels of adult δ-globin gene expression (figure 2) (Barker, 1968; Brotherton *et al.*, 1979).

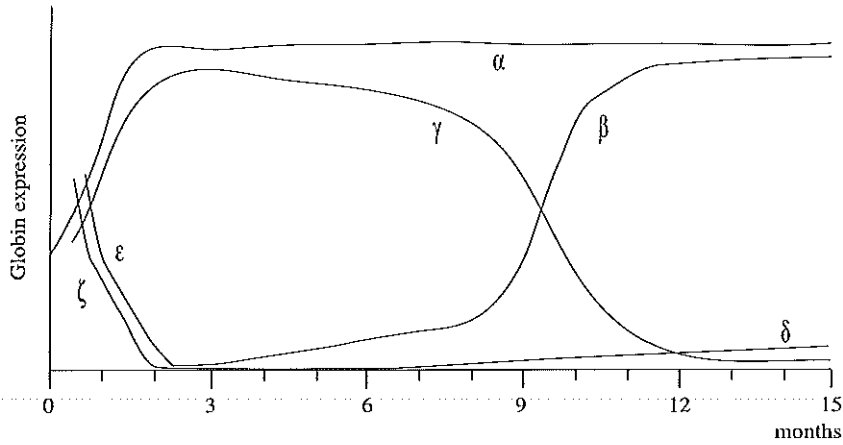

Figure 2 Expression of globins during fetal development (after Barker, 1968; Brotherton *et al.*, 1979; Peschle *et al.*, 1985)

With the development of cordocentesis, access to the fetal circulation has provided an opportunity to study fetal hematological profiles. However, it is technically difficult to obtain fetal blood before 18-20 weeks of gestation. In the developing fetus, the number of erythrocytes increases linearly, whereas the number of erythroblast cells decreases with advancing gestation. In contrast, the number of fetal white blood cells increases with gestation (table 1) (Millar *et al.*, 1985; De Waele *et al.*, 1988; Nicolaides *et al.*, 1989; Forestier *et al.*, 1991; Thilaganathan *et al.*, 1992, 1994).

For the development of a non-invasive prenatal diagnostic test, fetal erythroblast cells appear to be the fetal cell type to target, since these cells are the first cells that are formed during fetal hematopoiesis in the yolk sac. In addition, erythroblast cells are abundantly present in the fetus during the first trimester of pregnancy, when compared with lymphoid and myeloid cells.

Table 1 Mean values for the number of white blood cells, red blood cells and erythroblast cells in fetal, neonatal and adult blood

Gestational age	White blood cells ($x10^9$/l)	Red blood cells ($x10^{12}$/l)	Erythroblast cells ($x10^9$/l)
16 – 17 weeks	2.00	2.62	2.70
18 – 21 weeks	2.98	2.82	1.75
22 – 25 weeks	4.51	3.00	0.95
26 – 29 weeks	5.16	3.46	0.99
neonate	14.1	4.60	0.50
adult	6.00	4.70	<0.01

Data are adapted from Millar *et al.*, 1985; De Waele *et al.*, 1988; Forestier *et al.*, 1991.

1.2. Fetal cell types

The next issue concerns the types of fetal cells that have been found in the maternal circulation. The fetal cell types that have been studied by numerous investigators worldwide include fetal leukocytes, i.e. fetal lymphocytes and granulocytes, fetal nucleated red blood cells (NRBCs) and trophoblast cells.

1.2.1. Fetal leukocytes

Lymphocytes

The presence of fetal lymphocytes in the maternal circulation was first described in 1969 by Walknowska *et al.*. These investigators demonstrated the presence of a Y chromosome in mitogen-stimulated lymphocytes obtained from pregnant women carrying a male fetus. These results were confirmed by others using similar techniques or by investigating quinacrine-stained interphase nuclei for the presence of fluorescent Y chromosome signals (Schindler and Martin-du-Pan, 1972; Schröder and de la Chapelle, 1972).

Another breakthrough was the isolation of fetal lymphocytes by fluorescence-activated cell sorting (FACS) using a monoclonal antibody against the human leukocyte antigen HLA-A2, which was only expressed on fetal lymphocytes and not on maternal cells (Herzenberg *et al.*, 1979; Iverson *et al.*, 1981). Unfortunately, until now the isolation of fetal lymphocytes is considered impractical due to the necessity of performing HLA typing of both parents and the lack of other specific markers that distinguish fetal from maternal lymphocytes. Moreover,

the fact that fetal lymphocytes might persist from an earlier pregnancy makes it difficult to establish whether isolated lymphocytes derive from the current pregnancy (Schröder *et al.*, 1974). This is especially problematic for the detection of trisomic cells in women with prior spontaneous abortions, given the high likelihood (50%) that these abortions were associated with a chromosomally abnormal fetus (Sargent *et al.*, 1994).

Fetal granulocytes

This fetal cell type has received little attention. In 1975, Zilliacus *et al.* detected fetal granulocytes in the circulation of pregnant women. Several years later, Wessman *et al.* (1992) isolated granulocytes from maternal peripheral blood samples using density gradient centrifugation and they correctly identified the Y chromosome in these granulocytes by *in situ* hybridization.

1.2.2. Trophoblast cells

Trophoblast cells are particularly attractive for the development of a non-invasive prenatal test because of their unique morphology, which permits microscopic identification. Goodfellow and Taylor (1982) were the first to demonstrate trophoblast cells in peripheral blood samples from pregnant women using differential centrifugation and indirect immunofluorescence detection. Covone *et al.* (1984) identified fetal trophoblast cells in the peripheral blood from pregnant women using FACS and a monoclonal antibody against a syncytiotrophoblast-specific antigen, H315. However, subsequent work showed that the H315-positive cells were of maternal origin (Covone *et al.*, 1988). It was suggested that the results were due to adsorption of the H315 onto maternal cells and no fetal cells had been isolated either in this study or the previous study. These problems may be overcome by the use of new markers, such as HASH-2, human placental lactogen hormone (hPL) (Latham *et al.*, 1996) or HLA-G (Moreau *et al.*, 1994; van Wijk *et al.*, 1996).

Another difficulty is the fact that trophoblast cells appear to be rare in the maternal circulation, probably because they are able to form large multi-nucleated giant cells which are filtered out by the maternal pulmonary circulation so that they do not reach the peripheral circulation (Attwood and Park, 1960). Especially, when the pregnancy develops normally, trophoblast cells do not appear to be present in great numbers in maternal peripheral blood (Sargent *et al.*, 1994). However, trophoblast cells are detectable in increased numbers in cases of preeclampsia, although it is not yet known whether these increased numbers are a cause or an effect of preeclampsia (Chua *et al.*, 1991; Sargent *et al.*, 1994).

A further concern using trophoblast cells is the fact that they are part of the placenta and potential problems may arise when chromosome abnormalities are present in the placenta but

not in the fetus (Henderson *et al.*, 1996; Goldberg and Wohlferd, 1997; van Opstal, 1998). This confined placental mosaicism has been documented to occur in 1 % of all cases of chorionic villus sampling. Therefore, genetic analysis of a placental trophoblast cell might not be representative of the fetal karyotype.

1.2.3. Fetal nucleated red blood cells

The last few years, most attention has been focussed on the isolation of fetal NRBCs from maternal blood. Fetal NRBCs are the predominant nucleated cell type in the fetal circulation in the first trimester of pregnancy, during the yolk sac and liver phases of hematopoiesis (Metcalf and Moore, 1971). In blood of 10- to 20-week fetuses, NRBCs make up approximately 10 % of the total population, whereas in adults they are quite rare (<0.1 %). In addition, red cell development in the fetus is more advanced than white cell development during the first trimester (see paragraph 1.1.2., table 1). If fetal cell trafficking occurs, they are likely to be the major cell type in the maternal circulation.
NRBCs have been isolated using antibodies against membrane-bound markers like the transferrin receptor (CD71) and glycophorin A (GPA) or intracellular antigens like fetal and embryonic hemoglobin (Loken *et al.*, 1987; Bianchi *et al.*, 1990; Zheng *et al.*, 1995; Mesker *et al.*, 1998) (see paragraph 1.3.1.).
Another reason why the isolation of fetal NRBCs is attractive is that fetal NRBCs have a limited life span of about 120 days (Pearson, 1967), and are therefore unlikely to persist between pregnancies, unlike fetal lymphocytes (Simpson and Elias, 1994).

1.3. Isolation and identification strategies for fetal cells in maternal blood

1.3.1. Isolation strategies

The number of fetal cells in the maternal circulation is limited, and therefore, most efforts have been concentrated on the development of a highly efficient enrichment procedure. Enrichment can be achieved by either positive selection of target cells using unique fetal characteristics, or by depletion of contaminating maternal cells. For the isolation of rare cell populations two considerations should be taken into account: yield and purity. Yield is very important because the number of fetal cells in maternal blood is very low and loss of fetal cells should be avoided. Purity will be determined by the relative number of fetal and maternal cells remaining after enrichment. A relative increase in the absolute number of fetal cells after enrichment allows the reduction of the amount of maternal background cells, and

thereby, fetal cell identification by fluorescence *in situ* hybridization (FISH) or polymerase chain reaction (PCR) analysis is facilitated.

To distinguish fetal cells from the vast majority of their maternal counterparts a specific marker is needed. Until now, no single marker antigen is known that is specific for fetal cells. An overview of isolation approaches that have been designed to recover fetal cells from the maternal circulation is given in table 2.

Table 2 Isolation strategies used for the isolation of fetal cells from the maternal circulation

Isolation strategy	Fetal cell type	References
Fluorescence activated cell sorting (FACS)	lymphocytes NRBCs trophoblast cells	Herzenberg *et al.*, 1979; Iverson *et al.*, 1981; Bianchi *et al.*, 1990; Price *et al.*, 1991; Wachtel *et al.*, 1991; Tse *et al.*, 1994; Johansen *et al.*, 1995; Lewis *et al.*, 1996; Sohda *et al.*, 1997
Magnetic activated cell sorting (MACS)	NRBCs trophoblast cells	Ganshirt-Ahlert *et al.*, 1992, 1993; Zheng *et al.*, 1993; Busch *et al.*, 1994; Durrant *et al.*, 1996; Ganshirt *et al.*, 1998
Charge flow separation (CFS)	NRBCs	Wachtel *et al.*, 1996, 1998; Shulman *et al.*, 1998
Density gradient centrifugation	lymphocytes granulocytes trophoblast cells NRBCs	Bhat *et al.*, 1993; Oosterwijk *et al.*, 1996; Sitar *et al.*, 1997; Ganshirt *et al.*, 1998; Sekizawa *et al.*, 1999
Immunomagnetic beads separation	NRBCs trophoblast cells	Wessman *et al.*, 1992; Johansen *et al.*, 1995; Bianchi *et al.*, 1996b
Immunomagnetic colloid system	NRBCs trophoblast cells	Steele *et al.*, 1996; Martin *et al.*, 1997; Lim *et al.*, 1999
Avidin-conjugated columns with biotinylated antibodies	NRBCs	Hall *et al.*, 1994
Micromanipulation of individual cells	NRBCs	Takabayashi *et al.*, 1995; Sekizawa *et al.*, 1996a, b, 1998; Watanabe *et al.*, 1998
Carbonic anhydrase inhibition	NRBCs	de Graaf *et al.*, 1999a

Fluorescence activated cell sorting (FACS)

FACS or flowcytometry is used for positive selection of target cells as well as for depletion of contaminating maternal cells (Herzenberg *et al.*, 1979; Iverson *et al.*, 1981; Bianchi *et al.*, 1990; Price *et al.*, 1991; Wachtel *et al.*, 1991; Tse *et al.*, 1994; Johansen *et al.*, 1995; Lewis *et al.*, 1996; Sohda *et al.*, 1997). The sample for sorting is incubated with a fluorescent-

labeled antibody specific for the target cell. The FACScan identifies the cells labeled with the antibody which are then collected into a tube or onto a slide for further analysis. Although it is possible to achieve a high purity of target cells making microscopic analysis easier and more accurate, this method of fetal cell isolation requires considerable operator expertise, is time-consuming and the expense of the equipment also limits its application on a wide scale.

Magnetic activated cell sorting (MACS)

MACS is the most widely used method of fetal cell isolation and can be used for positive selection of fetal cells as well as depletion of maternal cells (Ganshirt-Ahlert *et al.*, 1992, 1993; Zheng *et al.*, 1993; Busch *et al.*, 1994; Durrant *et al.*, 1996; Ganshirt *et al.*, 1998). Target cells are labeled with an antibody attached to magnetic beads. The cell suspension is passed over a separation column with a magnetizable matrix that is placed into a magnetic field of extreme strength. Unlabeled cells flow through the matrix, and labeled cells stick to the column and can be eluted after being taken away from the magnetic field. MACS isolation is less expensive and time-consuming than FACS, and requires less expertise to perform. The major disadvantage of MACS is that target cells are contaminated by maternal cells, resulting in low purity, and hence, complicating fetal cell identification.

Charge flow separation (CFS)

Charge flow separation, an alternative approach for fetal cell enrichment requiring no antibody for cell selection has recently been described (Wachtel *et al.*, 1996, 1998; Shulman *et al.*, 1998). The method is based on the migration of cells in an electric field. It permits differentiation of cell types according to their characteristic surface charge densities using a cross-flow fluid gradient without the need of an antibody. This technology results in a significantly higher recovery of NRBCs than observed by other groups using conventional methods for fetal cell isolation, like FACS and MACS. However, it is presently unclear as to whether all NRBCs isolated by this technique are of fetal origin.

Antibodies used for the isolation of fetal NRBCs from maternal blood

A variety of monoclonal antibodies have been used for the isolation of fetal NRBCs from maternal blood samples. The majority of researchers have utilized a monoclonal antibody to the transferrin receptor (CD71) for the isolation of fetal NRBCs (Bianchi *et al.*, 1990; Ganshirt-Ahlert *et al.*, 1992; Lewis *et al.*, 1996; Sohda *et al.*, 1997). CD71 is expressed on all cells actively incorporating iron, and on nearly all first-trimester fetal nucleated blood cells (Price *et al.*, 1991; Wachtel *et al.*, 1991; Bianchi *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1992; Durrant *et al.*, 1994; Zheng *et al.*, 1997). Its expression declines with gestational age but is increased in fetuses with an abnormal karyotype (Thilaganathan *et al.*, 1995; Zheng *et al.*, 1999). The disadvantage of CD71 is that it is also expressed on a subpopulation of maternal

cells, such as activated lymphocytes, which results in low purity (Bianchi *et al.*, 1994; Zheng *et al.*, 1997).

Monoclonal antibodies against GPA, present on maturing erythrocytes but not on lymphocytes, have been used in combination with anti-CD71 in an attempt to increase the specificity of recovery (Price *et al.*, 1991). Unfortunately, anti-GPA causes agglutination of the target red cells, preventing efficient sorting (Simpson *et al.*, 1995).

Other monoclonal antibodies used for fetal NRBCs isolation are those that recognize the embryonic (HbE) and fetal (HbF) hemoglobin (Zheng *et al.*, 1993, 1995; Reading *et al.*, 1995; Cheung *et al.*, 1996; Demaria *et al.*, 1996; Lewis *et al.*, 1996; Oosterwijk *et al.*, 1996, 1998b, c; Mesker *et al.*, 1998). HbE (ε/ζ-chain), although unique to fetal cells, is expressed only during a narrow window of time during gestation and ceases during the first trimester of pregnancy (Zheng *et al.*, 1999). HbF (γ-chain) is expressed in most fetal cells over a wide range of gestational ages. Unfortunately, HbF is not fetal-specific since about 1% of adult erythroid cells also contain HbF (Turpeinen and Stenman, 1992). Moreover, increased erythropoiesis during pregnancy will stimulate the synthesis of fetal hemoglobin in the female adult, thereby limiting the usefulness of the HbF antibody (Pembrey *et al.*, 1973; Slunga-Tallberg *et al.*, 1995).

Additional monoclonal antibodies used for the isolation of fetal NRBCs are presented in table 3.

1.3.2. Identification of fetal cells in maternal blood

Genetic analysis of fetal cells in maternal blood has relied primarily on two techniques: FISH using chromosome-specific probes and PCR to amplify unique fetal gene sequences enabling subsequent DNA analysis of gene mutations.

Fluorescence in situ hybridization

The major fetal cell conditions associated with an abnormality in chromosome number can be easily detected by FISH. Since sorted samples are in interphase, counting the chromosomes by direct visualization is impossible using standard cytogenetic methods. FISH offers this possibility by using chromosome-specific labeled probes, which bind to regions of the target chromosome. Before FISH can be applied for fetal cell identification, enrichment of fetal cells is necessary to avoid fetal signals being overruled by signals from maternal cells and to decrease the time of microscopic examination to analyse a sufficient number of fetal cells. In cases of low purity, identification of fetal cells can be improved through better and faster

Table 3 Cell markers used for the enrichment of fetal NRBCs and depletion of maternal cells

Cell marker	Expressed on	Used for	References
CD71(transferrin receptor)	erythroid cells activated lymphocytes	enrichment	Bianchi *et al.*, 1990; Ganshirt-Ahlert *et al.*, 1992; Lewis *et al.*, 1996; Solida *et al.*, 1997; Ganshirt *et al.*, 1998
GPA (glycophorin A)	erythroid cells	enrichment	Price *et al.*, 1991; Wachtel *et al.*, 1991; Simpson *et al.*, 1995; Lewis *et al.*, 1996
HbE/HbF (embryonic/fetal hemoglobin)	erythroid cells	enrichment	Zheng *et al.*, 1993, 1995; Reading *et al.*, 1995; Cheung *et al.*, 1996; Demaria *et al.*, 1996; Lewis *et al.*, 1996; Oosterwijk *et al.*, 1996, 1998b, c; Mesker *et al.*, 1998
Blood group antigens	erythroid cells	enrichment	Savion *et al.*, 1997; Troeger *et al.*, 1999
Erythropoietin receptor	erythroid cells	enrichment	Valerio *et al.*, 1996, 1997a, b
CD36 (thrombospondin receptor)	monocytes platelets erythroid cells	enrichment	Bianchi *et al.*, 1993; Troeger *et al.*, 1999
HAE9; FB3-2, 2-6B/6, H3-3 (fetal liver surface antigens)	erythroid cells	enrichment	Savion *et al.*, 1997; Zheng *et al.*, 1997, 1999; Troeger *et al.*, 1999
CD45	leukocytes	depletion	Bianchi *et al.*, 1991; Busch *et al.*, 1994; Jansen *et al.*, 1997, 1999; Lim *et al.*, 1999
CD32	granulocytes	depletion	Zheng *et al.*, 1993; Ferguson-Smith *et al.*, 1994
CD14	monocytes	depletion	Lewis *et al.*, 1996; Steele *et al.*, 1996; Jansen *et al.*, 1997, 1999

recognition of fetal cells through automated scanning (Oosterwijk *et al.*, 1996, 1998a; Tanke *et al.*, 1996; de Graaf *et al.*, 1999a). By combining FISH and immunocytochemical staining for HbF, fetal cells can be identified by automated image analysis consisting of computerized microscopy, combining bright field and fluorescence microscopy, and subsequent visual evaluation of image memories. Automated image analysis appeared to be more sensitive than manual identification of fetal cells (Oosterwijk *et al.*, 1998a).

It has now been possible to detect almost all of the significant fetal aneuploidies using fetal cells isolated from maternal blood (Price *et al.*, 1991; Bianchi *et al.*, 1992; Cacheux *et al.*,

1992; Elias *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1993; Simpson and Elias, 1993; Zheng *et al.*, 1995; Pezzolo *et al.*, 1997; Oosterwijk *et al.*, 1998d; Al-Mufti *et al.*, 1999; de Graaf *et al.*, 1999b; Rodriguez de Alba *et al.*, 1999). These comprise all of the major autosomal trisomies including trisomy 13, trisomy 18, trisomy 21, some of the sex chromosome abnormalities like 47, XXY and 47, XYY, and triploidy. Recent technical advances in FISH analysis of fetal cells include the capability to detect aneuploidies using multicolor FISH (Bischoff *et al.*, 1998) and repeated hybridization of cells that permit analysis of all chromosome pairs (poly-FISH) (Zhen *et al.*, 1998). Poly-FISH is a technique of sequential FISH analysis that involves removal of the previous hybridized probe and rehybridization with different probes to improve FISH efficiency. This technique facilitates the analysis of multiple chromosome-specific probes on the same nuclei, and thereby, permits analysis of all chromosome pairs.

Interestingly, fetal NRBCs isolated from maternal blood may be more representative of the fetal karyotype than chorionic villi obtained through the traditional invasive technique. In a case report, Bischoff *et al.* (1995) identified a 46, XY/47, XXY mosaicism in fetal cells flow-sorted from maternal blood. In cultured chorionic villi obtained from the same woman, only four 47, XXY nuclei were identified out of 500 nuclei analyzed.

Polymerase chain reaction

The development of PCR (Saiki *et al.*, 1985, 1988) provides a sensitive method for DNA analysis of fetal gene sequences in maternal peripheral blood samples. PCR has been successfully applied for the detection of rare cells such as in cases of minimal residual disease in cancer by picking up few malignant cells expressing a genetic marker susceptible of amplification among a large multitude of negative normal cells (Lee *et al.*, 1987, 1989; van Dongen *et al.*, 1998). The presence of fetal cells in maternal blood can be investigated by a similar approach provided that a suitable genetic marker is available in the fetus and absent in the mother, i.e. the Y chromosome and paternally inherited markers.

Lo *et al.* (1989) were the first to identify the Y chromosome in the peripheral blood of pregnant women using nested PCR for a Y chromosome-specific sequence. They correctly identified fetal sex on all 19 cases tested, of which 12 women were carrying a male fetus and 7 women a female fetus. Thereafter, several groups identified the existence of fetal cells in maternal blood by nested PCR analysis (Bianchi *et al.*, 1990; Lo *et al.*, 1990, 1993; Kao *et al.*, 1992; Suzumori *et al.*, 1992; Adkison *et al.*, 1994). However, almost all groups reported false-positive as well as false-negative results. The main drawback of nested PCR is its susceptibility to exogenous contamination necessitating more stringent precautions during the technical process to minimize false-positive results. On the other hand, false-positive results may be obtained after cross-reactivity with maternal sequences or residual fetal cells from previous conceptuses (see paragraph 1.5.3.). False-negative results can be explained by

absence of fetal cells in the maternal circulation or by removal of fetal cells by the maternal immune system due to feto-maternal blood group incompatibility. Alternatively, false-negative results could simply reflect technical failure because the number of fetal cells was below the limit of sensitivity to detect fetal DNA.
Other uniquely fetal gene sequences that have been detected by PCR include various mutations in beta globin genes, such as hemoglobin Boston-Lepore (Camaschella *et al.*, 1990) or mutations associated with β-thalassemia (Hawes *et al.*, 1994), the HLA-DR and DQ alpha genes (Yeoh *et al.*, 1991; Geifman-Holtzman *et al.*, 1995), and Rhesus D and Rhesus C (Lo *et al.*, 1994b; Geifman-Holtzman *et al.*, 1996, 1998; Toth *et al.*, 1998).

Micromanipulation and single cell PCR
Using PCR for amplification of fetal sequences that are contaminated with maternal cells, only sex determination and paternally derived disease marker analysis are feasible. In approximately 50% of pregnancies, i.e. those with a female fetus, the cell samples can not be confirmed to be of fetal origin. Therefore, the development of a new method that can distinguish between fetal and maternal cells is necessary. By the development of micromanipulation of single cells, fetal cells can be distinguished from maternal cells by PCR amplification of unique fetal sequences, and thereby, the identification of other inherited diseases has become available in a laboratory setting. Takabayashi *et al.* (1995) were the first to report the use of micromanipulation to remove single fetal cells isolated from maternal blood. They correctly identified fetal sex in ten of eleven cases of which five of six were male, with no false positives. The same technique was applied to the diagnosis of Duchenne muscular dystrophy, Rhesus D (RhD), HLA-DQ alpha genotype by Sekizawa *et al.* (1996a, b, 1998), diagnosis of spinal muscular atrophy by Chan *et al.* (1998), and ornithine transcarbamylase deficiency by Watanabe *et al.* (1998). Cheung *et al.* (1996) used antibodies to fetal and/or embryonic hemoglobin to label fetal cells for isolation by micro-dissection from slides. They studied 10 pregnancies including one at risk of β-thalassemia and another of sickle cell disease. In both cases, they correctly predicted the fetal genotype as confirmed by chorionic villus sampling.
The use of single cell PCR requires a high number of amplification cycles with a risk of exogenous DNA contamination resulting in false-positive results. Before single cell PCR can be applied for fetal diagnosis, fetal cells have to be identified by cytological/immuno-cytochemical staining requiring cell fixation on slides. This staining procedure and subsequent manipulation of fetal cells may cause breakage of DNA leading to false-negative results. Therefore, amplification of multiple fetal cells should be performed in order to obtain a reliable diagnosis. So far, micromanipulation and single cell PCR of fetal cells has only been described in a laboratory setting.

1.4. Feto-maternal cell trafficking

1.4.1. Timing and proportion

Another issue involves the timing of feto-maternal transfer. Due to the very small volume of blood in the fetus and placenta, it was originally assumed that too few cells were transferred from the fetus to the mother during the first trimester of pregnancy. However, it was demonstrated by several investigators that Y chromosomal DNA was present in maternal blood samples as early as 5 weeks of gestation (Lo *et al.*, 1990; Hamada *et al.*, 1993; Liou *et al.*, 1993; Thomas *et al.*, 1994). It is most likely that early in gestation trophoblast cells will be the first to enter the maternal circulation due to the ongoing process of placentation. During this stage of embryonic development the numbers of fetal NRBCs and leukocytes are expected to be very low.

In several studies, it has been investigated at what time in pregnancy the number of fetal cells in the maternal circulation reaches its maximum. Relevant information regarding the frequency of fetal NRBCs in maternal blood is contradictory, and the frequency of fetal NRBCs was reported to vary significantly among individuals and throughout the three trimesters of pregnancy ranging from 10^{-5} to 10^{-8} (Price *et al.*, 1991; Hamada *et al.*, 1993; Slunga-Tallberg *et al.*, 1995; Smid *et al.*, 1997; Ganshirt *et al.*, 1998; Kuo, 1998). Using both FISH and PCR on unsorted maternal blood, Hamada *et al.* (1993) reported frequencies of fetal NRBCs in the maternal blood ranging from 10^{-4} to 10^{-5}. Sohda *et al.* (1997) estimated fetal NRBCs frequencies at 8.1×10^{-5} and 1.6×10^{-5} in the first and second trimester, respectively. Kuo *et al.* (1998) recently demonstrated an increase in the total number of NRBCs in maternal blood as gestation advanced. The frequency increased from 2.4×10^{-7} in early gestation (6-10 weeks) to 4.2×10^{-6} near term. However, the variations in the frequency of male DNA equivalents measured by PCR were different, increasing from 2.7×10^{-7} (6-10 weeks) to a peak of 1.48×10^{-6} (15-20 weeks) and then slightly decreased to 1.31×10^{-6} (33-39 weeks). This implies that before 24 weeks of gestation a significant proportion of NRBCs in maternal blood is of fetal origin whilst in late gestation the majority of NRBCs may be of maternal origin.

The fetal cell frequency in maternal blood is influenced by a number of biological parameters. Factors that may influence these frequencies include the type of fetal cell analyzed, gestational age at the time of sampling and the accuracy of methods to enrich, identify and quantify the fetal target population. The occurrence of fetal cells in maternal blood has been reported to be increased after chorionic villus sampling (Jansen *et al.*, 1997), in women with preeclampsia (Chua *et al.*, 1991; Ganshirt *et al.*, 1994; Holzgreve *et al.*, 1998; Lo *et al.*, 1999b, Chapter 5), and in pregnancies in which the fetal and placental karyotypes

were abnormal (Elias *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1993; Simpson and Elias, 1993; Bianchi *et al.*, 1997). For chromosomal abnormal pregnancies, it has previously been suggested that feto-maternal cell trafficking may be the result of altered placental structures (Kuhlmann *et al.*, 1990; Simpson and Elias, 1994; Genest *et al.*, 1995; Jauniaux and Hustin, 1998).

1.4.2. Clearance versus persistence of fetal cells from the maternal circulation

Disappearance of fetal cells from the maternal circulation after delivery is an important consideration because of the implications for prenatal diagnosis of subsequent pregnancies. Clearance of fetal cells from the maternal circulation requires that there are mechanisms by which fetal cells are continuously removed from the circulation. There are virtually no data on the fate of fetal cells in maternal blood, at least not in humans. Such data could lead to a better understanding of the feto-maternal immune relationship, and of the purpose of feto-maternal cell traffic in general. The main mechanisms of fetal cell clearance are removal by the maternal immune system, apoptotic cell death due to an inappropriate environment, and retention in maternal tissues.

The fetus is a semi-allograft and it is well established that the paternal antigens elicit a response from the maternal immune system (Wood, 1994). The fetus is protected by an unknown mechanism and it is not known whether this protection is also applied to individual fetal cells in the maternal circulation. In mice, a rapid clearance of fetal cells by the maternal immune response has been demonstrated and this clearance mechanism would likely affect most types of fetal cells (Bonney and Matzinger, 1997). Another mechanism may be apoptosis of fetal cells. Proliferating progenitor cells in their various stages of differentiation need specific cytokines for survival, otherwise they will apoptose rapidly (Williams *et al.*, 1990). These cytokines are available in the hemopoietic tissues and probably in fetal blood, but may not be sufficiently supplied in the maternal peripheral blood, leading to fetal cell death. On the other hand, fetal cells can leave the maternal circulation to settle in maternal tissues. These cells are most likely progeny from fetal stem cells that have lodged in the maternal hemopoietic tissues or trophoblast cells being trapped in the maternal lungs.

The possible persistence of fetal cells in maternal blood after delivery is of concern because of the chance that diagnostic error might occur from genetic analysis of circulating cells that originated from a previous pregnancy. Long term persistence of male fetal cells in maternal blood has been described by several investigators (Schröder *et al.*, 1974; Ciaranfi *et al.*, 1977; Hsieh *et al.*, 1993; Hamada *et al.*, 1994; Liou *et al.*, 1994; Bianchi *et al.*, 1996a). Schröder *et al.* (1974) originally described the persistence of fetal leukocytes in the maternal circulation after delivery. In this study, interphase Y body fluorescence was used to determine the

frequency of male fetal cells in maternal blood and the kinetics of their subsequent disappearance. In a group of 20 primigravidae sampled postpartum, quinacrine fluorescent signals were detected up to 1 year after delivery. In a related study, Ciaranfi *et al.* (1977) analyzed samples from women 5-7 years after delivery and detected male lymphocytes 2 years after birth in more than half of the 62 samples analyzed. However, these studies were performed in the 1970s using techniques that were less sensitive and less accurate than those available today. In 1994, Liou *et al.* investigated the presence of Y chromosome containing cells using PCR in maternal blood samples from 28 pregnant women. These samples were obtained up to 10 months after delivery. In 11 women, fetal cells were detected up to four months after delivery but in one woman, the Y sequence was still detectable 10 months after delivery. Bianchi *et al.* (1996a) isolated mononucleated cells by FACS using antibodies to CD antigens 3, 4, 5, 19, 23, 34 and 38, from 32 pregnant women and 8 nonpregnant women who had given birth to males 6 months to 27 years earlier. In 4 out of 13 pregnancies with a female fetus, male DNA was detected by PCR, whereas in 6 out of 8 nonpregnant women the presence of male DNA was demonstrated in isolated CD34+CD38+ cells, even in a woman who had her last son 27 years prior to blood sampling. These isolated cells that contained male DNA may either have been lymphocytes from that time of pregnancy, or may be a false-positive result since no blood samples were included in this study that were derived from women who never had been pregnant at all. The occurrence of false-positive results is not unlikely with the currently used sensitive PCR methods used for Y chromosome determination (see paragraph 1.3.2.).

1.5. New research areas

1.5.1. Fetal DNA in maternal plasma and serum

Almost all prior studies in the past have focused on complete and intact fetal cells in the maternal circulation, suitable for either cell culture or FISH or DNA analysis. Recently, however, Lo *et al.* (1997, 1998a) demonstrated the presence of fetal DNA in maternal plasma and serum by using a quantitative PCR assay for the sex-determining region Y (SRY) gene on the Y chromosome, as a marker for male fetuses. These fetal DNA levels gradually increased in the course of pregnancy, especially towards the end of pregnancy. They demonstrated that significantly more fetal DNA was present in the serum and plasma than prior studies using intact fetal cells would indicate. A mechanism that could explain these findings is continuous leakage of fetal cells across the placenta which are rapidly destroyed by the maternal immune system, leaving DNA remaining in the plasma. This would imply that investigators who isolated fetal cells from maternal blood only detected a limited fraction

of what had entered the maternal circulation. An alternative explanation is that there is active remodeling of the placenta at the feto-maternal interface, with continuous cell lysis and release of fetal DNA into the maternal circulation.

High concentrations of fetal DNA have also been detected in maternal plasma before spontaneous preterm delivery, which may be used as a marker for preterm labour (Leung *et al.*, 1998). Another clinical application of fetal DNA in maternal plasma/serum is the detection of RhD-specific sequences. Knowledge of fetal RhD genotype is important in the management of rhesus allo-immunisation during pregnancy. Fetal RhD has been successfully demonstrated in maternal plasma and serum by several investigators (Faas *et al.*, 1998; Lo *et al.*, 1998b; Bischoff *et al.*, 1999).

Recently, Lo *et al.* (1999c) analyzed plasma samples of women 1 to 42 days after delivery of a male baby and found that circulating fetal DNA was undetectable by day 1 after delivery, whereas most maternal plasma samples showed undetectable levels of circulating fetal DNA by 2 hours postpartum. Moreover, they demonstrated a rise in plasma fetal DNA concentrations shortly after delivery, i.e. 5 minutes, compared with the predelivery fetal DNA levels, indicating that a feto-maternal transfusion may occur at time of delivery. The observation of rapid clearance of fetal DNA from maternal plasma suggests that analysis of circulating fetal DNA is more powerful than analysis of intact fetal cells, because of the lower risk to detect fetal DNA from previous pregnancies. However, technical limitations to distinguish fetal DNA from maternal DNA might limit application of this technique.

The same group (Lo *et al.*, 1999a) recently demonstrated high concentrations of cell-free fetal DNA in plasma samples in a proportion of women carrying a fetus with trisomy 21. However, PCR amplification of Y chromosomal sequences was used and DNA concentrations of normal pregnancies overlapped these of trisomic pregnancies. In order to quantitatively analyse the DNA concentration in trisomy 21 pregnancies, additional markers on chromosome 21 will be necessary. For a definitive karyotypic diagnosis, the isolation of circulating nucleated fetal cells still remains the best candidate technology for the development of non-invasive prenatal diagnosis of fetal aneuploidies.

1.5.2. *In vitro* expansion of fetal cells

If fetal cells could be stimulated to proliferate in culture, the technical limitations of working with very small numbers of cells could be overcome. The idea to increase the numbers of fetal cells from maternal blood by amplification of progenitor cells has been discussed for a long time. A hemopoietic clonogenic cell can produce hundreds to thousands of progeny, so that a culture with even just one fetal clonogenic cell could yield a sufficient number of fetal cells for the diagnosis of genetic abnormalities.

The first attempt to culture fetal cells was reported in 1994. Alter (1994) cultured red cells by exploiting the difference in sensitivity of fetal and maternal red cells to erythropoietin in culture. The technique was successfully tested in a model system, confirming that there is a growth differential in favor of neonatal cells of up to tenfold. Lo *et al.* (1994a) used a similar method in a study on five maternal blood samples where the fetus was known to be male. Using PCR, they identified the Y chromosome in all five cases after seven days of culture. Valerio *et al.* (1996) utilized a magnetic cell sorting method to separate fetal erythroid progenitor cells from maternal blood and successfully cultured them for 10-12 days, followed by the detection of Y chromosomal sequences using PCR and FISH.
More recently, Bohmer *et al.* (1998, 1999) described a novel method to distinguish fetal from maternal cells in culture based on differences in fetal hemoglobin production. During the first week of culture, fetal erythroid cells exclusively expressed HbF, whereas the majority of maternal cells contained high levels of adult hemoglobin (HbA) alone or a combination of HbF and HbA. However, this preferential growth was not observed by others. Two recent reports demonstrated that culturing of fetal erythroid cells derived from contaminating maternal blood mainly produced erythroid colonies derived from maternal erythroid progenitors (Chen *et al.*, 1998; Han *et al.*, 1999).
So far, most attention has been focussed on the amplification of fetal erythroid progenitors. In 1997, Little *et al.* cultured FACS-sorted CD34+ hemopoietic progenitor cells derived from 10-13 week maternal blood samples. They showed a slight expansion of fetal CD34+ cells after 5 days of culture, but in most cases (10 out of 18 (55 %)) no male fetal cells could be detected.
These studies suggest that *in vitro* expansion of fetal cells is not yet suitable for clinical application since the extent of expansion of the different fetal cell types is contradictory and because of the small numbers of analyzed cases in the different studies.

1.5.3. Fetal cell microchimerism

As described above, the possible persistence of fetal cells in maternal blood after delivery is a concern because of the chance that diagnostic error might occur from genetic analysis of circulating cells that originated from a previous pregnancy. Long term persistence of male fetal cells in maternal blood has been described by several investigators (Schröder *et al.*, 1974; Ciaranfi *et al.*, 1977; Hsieh *et al.*, 1993; Hamada *et al.*, 1994; Bianchi *et al.*, 1996a). This led to the speculation that normal pregnancy can lead to a physiological state of low-grade microchimerism in a woman. It has been suggested that persistence of fetal cells after birth may be related to the etiology of autoimmune disorders that have a higher incidence in women and have an onset after the child-bearing years. Evidence for this

hypothesis came in 1998, when Nelson demonstrated significantly increased amounts of male fetal DNA in peripheral blood of women who suffered from the disease scleroderma, as compared to their healthy sisters and normal controls. In addition, Arlett *et al.* (1998) demonstrated male lymphocytes in skin biopsies of women with scleroderma.
At the time of delivery a feto-maternal transfusion might occur including some fetal cells with proliferative potential. These fetal cells can migrate to lymphopoietic organs and proliferate. Subsequently, a graft-versus-host response may occur, which may result in the development of an autoimmune disease.

1.6. Conclusions and objectives of the thesis

The last few decades, many investigators have focussed on the isolation of fetal cells from the maternal circulation in order to develop a non-invasive prenatal diagnostic test. The optimal fetal cell type to target appears to be the fetal NRBCs since they are present most abundantly in the fetus during the first trimester of pregnancy, they have a limited life-span and may not persist from prior pregnancies. However, the number of fetal NRBCs in the maternal circulation remains very low and extensive enrichment and purification strategies are necessary to increase the detectability of these cells. If fetal cells can eventually be isolated, possible clinical applications include screening for fetal chromosome abnormalities by FISH and for gene abnormalities by PCR.
Before the isolation of fetal cells can be used for diagnostic purposes, several biological questions have to be answered and technical obstacles have to be overcome. From a biological point of view, we need to know more about the number of fetal cells, fetal cell types and their properties to distinguish these cells from maternal cells, as well as the biological consequences of their presence in the maternal circulation. Data on the frequency of fetal cells in maternal blood are contradictory as the frequency of fetal NRBCs appears to vary among individuals and throughout the three trimesters of pregnancy. Under some circumstances, the number of fetal cells in maternal blood is increased, i.e. in cases with a chromosomally abnormal fetus, in pregnancies complicated with preeclampsia and after chorionic villus sampling. From a technical point of view, we need to maximize both yield and purity of the isolation procedure to improve the identification of fetal cells. In order to optimize isolation strategies, different model systems have been described using artificial mixtures of male neonatal cord blood cells or male fetal liver cells, and adult female peripheral blood cells. In these model systems, different isolation protocols were evaluated.
A new research area concerns the *in vitro* expansion of fetal cells. The question as to whether fetal cells can be clonally expanded in order to increase their detectability has partly been

resolved by several recent studies. Erythroid as well as other hemopoietic progenitor cells have been successfully expanded.

According to these biological and technical questions the following objectives of this thesis were defined:

1. The development of a model system using *in vitro* expanded erythroid cells derived from umbilical cord blood samples for the evaluation of different isolation strategies for the enrichment of fetal NRBCs in maternal blood. This part of the study is presented in Chapter 2.
2. Examination of the preferential expansion of hemopoietic progenitor cells derived from male umbilical cord blood samples diluted into female progenitor cells. According to this expansion protocol, the usefulness of *in vitro* expansion of fetal hemopoietic progenitor cells isolated from maternal blood for diagnostic purposes was evaluated. Results of this part of the study are presented in Chapter 3.
3. Determination of the effect of chorionic villus sampling on the number of fetal cells isolated from maternal blood and on maternal serum alpha-fetoprotein levels. Data of this study are shown in Chapter 4.
4. The impact of maternal preeclampsia on the incidence of fetal cells in the maternal circulation. These results are presented in Chapter 5.

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Chapter 2

The use of in vitro expanded erythroid cells in a model system for the isolation of fetal cells from maternal blood

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The isolation of fetal cells from maternal blood is hampered by the low frequency of these cells in maternal blood requiring extensive enrichment and purification procedures before any detection techniques for fetal cells can be applied. A considerable amount of effort has been concentrated on the improvement of different isolation techniques. The currently used isolation strategies, like MACS and FACS have been optimized using model systems in which male umbilical cord blood cells or fetal liver cells were diluted into adult female peripheral blood mononuclear cells. This chapter describes the use of *in vitro* expanded erythroid cells in a model system for the isolation of fetal cells from maternal blood. These cells were immunophenotypically identical to fetal nucleated red blood cells isolated from maternal blood expressing high levels of CD71 and were used to compare two different MACS isolation procedures: isolation of CD71+ cells after depletion of lymphocytes and monocytes, and the direct enrichment of CD71+ cells.

The use of in vitro expanded erythroid cells in a model system for the isolation of fetal cells from maternal blood

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ABSTRACT

The development of a non-invasive prenatal diagnostic test using fetal nucleated red blood cells (NRBCs) isolated from the maternal circulation is hampered by the low frequency of these cells in maternal blood requiring extensive enrichment procedures before any analytical procedure can be performed. In order to improve and simplify these procedures, we have used *in vitro* expanded erythroid cells derived from male umbilical cord blood in a model system for the isolation of fetal NRBCs from maternal blood. Erythroblast cells were *in vitro* expanded to high cell numbers and were immunophenotypically identical to fetal NRBCs isolated from maternal blood. Magnetic activated cell sorting (MACS) isolation procedures were optimized using *in vitro* expanded male NRBCs diluted up to 1 in 400,000 with female peripheral blood mononucleated cells. The number of recovered male cells was determined using two-color fluorescence *in situ* hybridization with X and Y chromosomal probes. Using this model system, an NRBC isolation technique is described. It is based on a one-step MACS enrichment protocol for CD71+ cells, which showed a significant (Wilcoxon signed ranks test, $p<0.05$) two-fold higher yield of male NRBCs than previously described MACS methodologies, in which CD71+ cells were enriched after depletion of other cell types. Application of these isolation strategies to maternal blood samples resulted in a similar improved enrichment of male fetal cells after the direct enrichment of CD71+ cells.

INTRODUCTION

Fetal blood cells that leak through the placenta into the maternal circulation provide a potential source of fetal material for the development of a non-invasive prenatal diagnostic test. Four different fetal cell types have been detected in maternal blood: trophoblast cells (Goodfellow and Taylor, 1982), lymphocytes (Herzenberg *et al.*, 1979), granulocytes (Wessman *et al.*, 1992) and nucleated red blood cells (NRBCs) (Bianchi *et al.*, 1990). Most

attention has been focused on the NRBCs which have been isolated using antibodies against membrane-bound markers (CD71, glycophorin A) or intracellular antigens (hemoglobin F) (Loken *et al.*, 1987; Bianchi *et al.*, 1990; Zheng *et al.*, 1995).
Since the number of fetal NRBCs in maternal blood is very low, isolation of these cells requires extensive enrichment and purification procedures. Two major methods of cell separation enable fetal cell isolation from maternal blood: fluorescence activated cell sorting (FACS) (Bianchi *et al.*, 1990) and magnetic activated cell sorting (MACS) (Ganshirt-Ahlert *et al.*, 1992).
In order to obtain an optimal isolation procedure, different model systems have been described using artificial mixtures of male neonatal cord blood cells or male fetal liver cells, and adult female peripheral blood mononuclear cells (Andrews *et al.*, 1995; Bianchi *et al.*, 1996). In these model systems, different isolation protocols were evaluated using density gradient centrifugation, MACS, FACS or immunomagnetic beads for the isolation of CD71+ NRBCs, after depletion of female peripheral blood mononuclear cells.
In the present study, we describe a MACS isolation protocol, in which we used *in vitro* expanded NRBCs derived from male umbilical cord blood mixed with female peripheral blood mononucleated cells as a model system for the isolation of fetal NRBCs from maternal blood. We were able to expand these NRBCs to high cell numbers and to maintain these cells in an erythroblastic cell stage. These *in vitro* expanded NRBCs also showed a high level of CD71 expression. In the described model system, CD71+ cells were isolated using a one-step MACS isolation protocol based on the direct enrichment of CD71+ cells. This isolation procedure is compared with a previously published and commonly used two-step technique based on depletion of monocytes (CD14) and lymphocytes (CD45) followed by the enrichment of CD71+ cells (Busch *et al.*, 1994; Jansen *et al.*, 1997). It is suggested that these depletion procedures may cause cell damage and cell loss, leading to lower levels of recovery of fetal NRBCs. Isolation efficiencies for both MACS isolation procedures were also compared for the isolation of male CD71+ fetal cells from maternal blood samples. The number of recovered male CD71+ cells was analyzed by two-color FISH for X and Y chromosomes.

MATERIALS AND METHODS

In vitro expansion of erythroblast cells
Male umbilical cord blood samples (1-5 ml) were collected immediately after birth into vacutainers containing ethylenediaminetetra-acetic acid (EDTA). Blood samples were diluted 1:1 with phosphate buffered saline (PBS) and mononucleated cells were isolated by Ficoll-Paque-Plus (1.077 g/ml; Pharmacia Biotech, Uppsala, Sweden) density gradient

centrifugation at 750 G for 20 minutes. Cells were washed once with Iscove's modified Dulbecco's medium (Life Technologies B.V., Breda, The Netherlands) containing 10 % fetal calf serum (FCS) and centrifuged at 370 G for 5 minutes. Cord blood cells were cultured as recently described by von Lindern *et al.* (1998). Briefly, cells were cultured at 37 °C and 5 % CO_2 at a density of 10^6 cells/ml in CFU-E medium as described previously (Hayman *et al.*, 1993; Schroeder *et al.*, 1993), with minor modifications: conalbumin was replaced by human transferrin-holo (Intergen, Toronto, Canada), chicken serum was omitted and only FCS (12 %) was used. Human stem cell factor (100 ng/ml), human recombinant erythropoietin (0.5 U/ml; Boerhinger Mannheim GmbH, Germany), and dexamethasone (10^6 M; D-1756, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were added every second day. After two days, remaining erythrocytes were removed by Ficoll-Paque-Plus density gradient centrifugation at 750 G for 20 minutes. After 7-10 days, erythroblast cells (NRBCs) were isolated using Percoll (1.072 g/ml; Pharmacia Biotech). The number of cells and cell size distribution were determined in an electronic cell counter (Casy-1; Schärfe system, Germany), followed by analysis of CD71 and CD45 expression using a FACScan (Becton Dickinson, San José, CA, USA). On day 12, unutilized *in vitro* expanded erythroid cells were aliquoted and stored in liquid nitrogen until further usage.
To analyze cell morphology, cells were cytocentrifuged onto slides and stained with hematological dyes and neutral benzidine for hemoglobin (Beug *et al.*, 1982).

Model system
Two different MACS isolation procedures were compared using a mixture of male *in vitro* expanded NRBCs and female peripheral blood mononuclear cells. To this end, 50 or 500 male CD71+/CD45- cells were diluted in 10-20x10^6 female cells. The one-step method involved the direct enrichment of CD71+ cells, whereas the two-step method included depletion of CD45+ and CD14+ cells followed by the enrichment of CD71+ cells.

Patient samples
Peripheral venous blood samples (13-20 ml) were obtained from pregnant women at 12-14 weeks of gestation immediately before chorionic villus sampling (CVS), and collected into vacutainers containing ethylenediaminetetra-acetic acid (EDTA). In 31 cases, the fetal karyotype was 46,XY as was demonstrated by cytogenetic analysis on semi-direct villus preparations. The one-step MACS isolation procedure was used in 14 of these maternal blood samples, whereas 17 cases were subjected to the two-step method. All blood samples were obtained with the patients' informed consent.

Antibody labeling, FACS analysis and MACS separation

In vitro expanded NRBCs derived from male umbilical cord blood were labeled with CD71-FITC (5 µl/10^6 cells; 100 µg/ml; LO1.1, IgG2a, Becton Dickinson) and CD45-PE (5 µl/10^6 cells; CLB, Amsterdam, The Netherlands) in 200 µl MACS buffer (PBS containing 1 % BSA, 0.01 % sodium azide (NaN_3) and 5 mM EDTA) supplemented with 5 % human serum and the CD71 and CD45 expression was measured using a FACSscan. In every experiment, only NRBCs with an expression of CD71 in 95-99 % of the cells and a CD45 expression in less than 5 % of the cells were used.

Maternal blood samples and peripheral venous blood samples of non-pregnant female volunteers were diluted 1:1 with PBS and mononucleated cells were isolated by Ficoll-Paque-Plus density gradient centrifugation at 750 G for 10 minutes. Cells were washed twice in MACS buffer and centrifuged at 250 G for 10 minutes. The number of viable cells was calculated using a Bürker counting chamber.

Mononuclear cells derived from maternal blood and mixtures of 50 or 500 male CD71+ cells with 10-20 x 10^6 female mononuclear cells were processed for the different MACS isolation procedures. For the one-step procedure, cell suspensions were labeled on ice with CD71-FITC (10 µl/10^6 cells) in 200 µl MACS buffer with 5 % human serum for 15 minutes. Cells were washed in MACS buffer and labeled with IgG2a+b-conjugated microbeads (20 µl/10^7 cells; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4 °C. Cells were washed in MACS buffer, resuspended in 1 ml MACS buffer and applied via a pre-separation filter (30 µm; CLB) onto a miniMACS column (type MS; Miltenyi). The non-attached fraction was collected and applied again to the column in order to achieve an optimal depletion. After removing the column from the magnet, CD71+ cells were eluted with 10 ml MACS buffer. For the two-step procedure, cell labeling and MACS separation were performed as described previously (Jansen *et al.*, 1997). Briefly, cells were labeled for 15 minutes on ice with CD45-PE (50 µl/20 x 10^6 cells) in 200 µl MACS buffer with 5 % human serum, washed once in MACS buffer and labeled with CD14-conjugated microbeads and rat anti-mouse IgG1-conjugated microbeads (20 µl/10^7 cells each; Miltenyi Biotec) at 4 °C for 15 minutes. Labeled cells were resuspended in 1 ml MACS buffer and applied to an AS-depletion column (Miltenyi) using a 26G needle as flow resistor. The negative fraction was applied again to the column in order to achieve an optimal depletion. Antibody labeling and MACS separation for the negative fraction obtained after depletion was performed as described for the one-step isolation procedure. The number of viable cells in each fraction was calculated using a Bürker counting chamber.

Fluorescence in situ hybridization (FISH)

Cell fractions were treated with 75 mM KCl, incubated for 18 minutes at 37 °C, fixed in methanol: acetic acid (3:1) and stored at -20 °C until further analysis. Cells were dropped onto Vectabond™ (Vector Laboratories, Inc, Burlingame, CA, USA) coated slides and air-dried. Slides were heated for 10 minutes at 80 °C and pre-treated with pepsin (100 µg/ml) in 0.01 N HCl at 37 °C for 15 minutes, followed by postfixation in 3.7 % formaldehyde in PBS for 15 minutes. Subsequently, slides were denatured for 5 minutes in 70 % formamide (pH 7.5) in 2X SSC at 75 °C, followed by dehydration in 70 %, 90 % and 100 % ethanol for 1 minute each. Slides were prewarmed at 45 °C until the probe was applied.

Two-color FISH was performed using a SpectrumOrange labeled alpha-satellite probe (DXZ1) for centromere region Xp11.1-q11.1 and a SpectrumGreen labeled satellite III probe for the Yq12 region (Vysis, Downers Grove, IL, USA). The probes were denatured for 5 minutes at 75 °C and hybridization was allowed to continue overnight at 42 °C in a humidified chamber.

Slides were post-washed at 70 °C in 0.4X SSC/0.3 % NP-40 (pH 7.2), followed by 5-60 seconds in 2X SSC/0.1 % NP-40 (pH 7.2) at room temperature. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Inc.) containing DAPI (4', 6-diamidino-2-phenylindole) and analyzed under a Leica Aristoplan fluorescence microscope using a triple band-pass filter block. Images were captured using a Xybion CCD 24-bit color camera with a Genetiscan ProbeMaster system and MacProbe 2.5 image analysis software (PSI, Chester, UK).

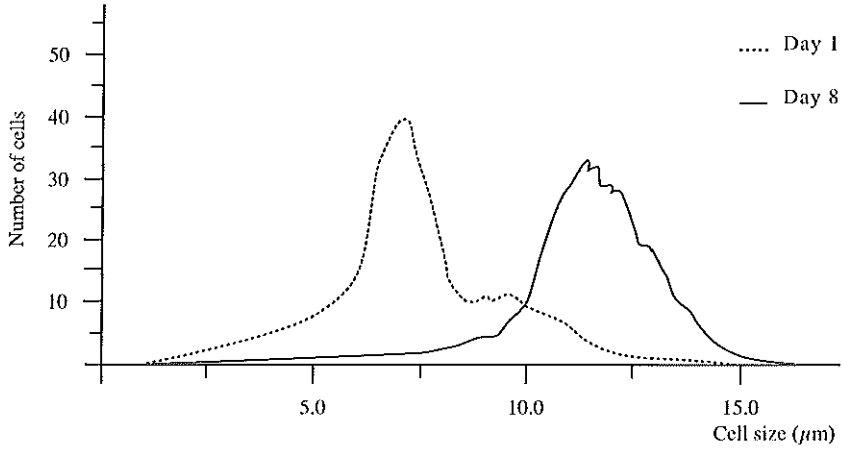

Figure 1 Cell size distribution before (day 1) and after (day 8) *in vitro* expansion of erythroid cells

RESULTS

Model system

Male neonatal NRBCs were *in vitro* expanded by exposure to stem cell factor (SCF), erythropoietin and dexamethasone. After eight days in culture, an increase in cell size (figure 1), a decreased expression of CD45 and an increased expression of CD71 were noted (figure 2). The expanded cell population was characterized by an expression of CD45 in less than 5 % of the cells and a CD71 expression in 95 % to 99 % of the cells. Morphologically, these cells predominantly resemble proerythroblast cells and basophilic erythroblast cells (figure 3).

Cell mixtures of 50 or 500 expanded male NRBCs and 10-20 x 10^6 female peripheral blood mononuclear cells were used for MACS isolation of NRBCs. Two MACS isolation procedures were compared: a one-step protocol based on the enrichment of CD71+ cells alone, and a two-step isolation procedure in which CD71 enrichment was combined with an initial depletion of CD45+ and CD14+ cells.

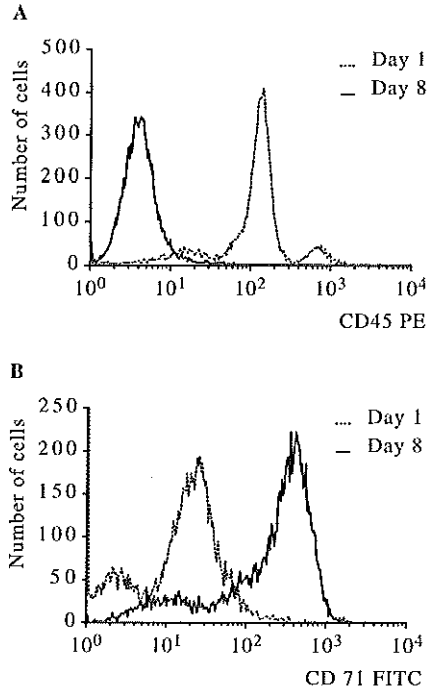

Figure 2 CD45 (A) and CD71 (B) expression during *in vitro* expansion of erythroid cells derived from male umbilical cord blood

The number of XY cells in the MACS-enriched fractions was determined by two-color FISH using X and Y chromosome-specific probes (figure 4; table 1). The total number of cells in the CD71+ fraction that was analyzed by FISH ranged between 1-25 x 10^4 (mean 10.8 x 10^4) after the one-step isolation procedure and between 1-11 x 10^4 (mean 5.3 x 10^4) after the two-step isolation procedure (table 1). Using the one-step isolation procedure, the number of XY positive cells recovered after addition of 50 male CD71+ cells, ranged from 2-25 XY cells (4-50 %) with a mean of 8 XY cells (16 %), whereas after addition of 500 male CD71+ cells, the number of XY positive cells varied between 32 and 195 XY cells (6-39 %), with a mean number of XY cells of 79.8 (16 %). The number of XY positive cells in the CD71+ fraction recovered after the two-step isolation procedure ranged from 2-9 XY cells (4-19 %) after addition of 50 male CD71+ cells, with a mean number of cells of 4.2 XY cells (8.3 %). After addition of 500 male CD71+ cells, between 5 and 130 XY cells (1-26 %) could be identified in the CD71+ fraction, with a mean number of XY cells of 39.7 (7.9 %).

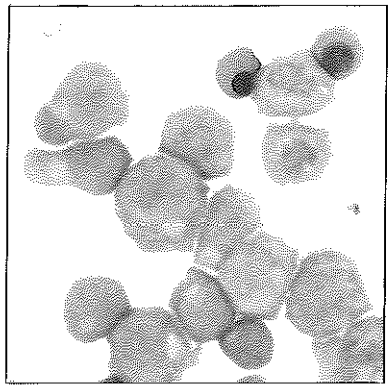

Figure 3
Combination of a hematological and neutral benzidine staining for hemoglobin of *in vitro* expanded erythroid cells derived from male umbilical cord blood. More mature erythroid cells stained yellow to brownish due to the presence of hemoglobin.

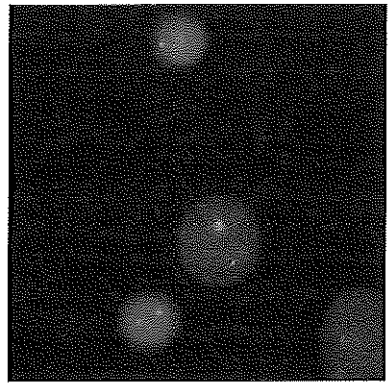

Figure 4
In situ hybridization on a MACS-enriched fraction of female peripheral blood mononuclear cells mixed with a known number of *in vitro* expanded male erythroid cells. One male erythroid cell is shown characterized by both a Y (green) and an X (red) chromosomal signal, and several female peripheral blood mononuclear cells are shown with two X chromosomal signals (red). (magnification x1000)

After addition of 50 as well as 500 male CD71+ cells, the one-step isolation procedure showed a mean number of XY positive cells twice as high as the number of XY positive cells recovered after the two-step isolation procedure. This higher level of recovery was significant in the condition in which 500 male CD71+ cells were added (Wilcoxon Signed Ranks Test, $p<0.05$).

Table 1 Number of XY cells recovered after MACS isolation using the one-step and two-step procedure

	One-step procedure		Two-step procedure	
	Number of cells analyzed ($x10^4$)	Number XY cells in CD71+ fraction	Number of cells analyzed ($x10^4$)	Number XY cells in CD71+ fraction
Experiment 1				
50 XY + 12 x 10^6 XX	1	25 (50 %)	9	7 (14 %)
500 XY + 12 x 10^6 XX	5	195 (39 %)	6	130 (26%)
Experiment 2				
50 XY + 10 x 10^6 XX	4	5 (10 %)	1	3 (6 %)
500 XY + 10 x 10^6 XX	4	77 (15 %)	11	12 (2.4 %)
Experiment 3				
50 XY + 20 x 10^6 XX	25	4 (8 %)	6	9 (18 %)
500 XY + 20 x 10^6 XX	20	91 (18 %)	3	31 (6 %)
Experiment 4				
50 XY + 12 x 10^6 XX	7	3 (6 %)	3	2 (4 %)
500 XY + 12 x 10^6 XX	7	32 (6 %)	8	34 (7 %)
Experiment 5				
50 XY + 15 x 10^6 XX	6	2 (4 %)	1	2 (4 %)
500 XY + 15 x 10^6 XX	7	45 (9 %)	7	26 (5 %)
Experiment 6				
50 XY + 15 x 10^6 XX	21	9 (18 %)	5	2 (4 %)
500 XY + 15 x 10^6 XX	22	39 (8 %)	4	5 (1 %)
Mean values				
50 XY	10.7	8.0 (16 %)	4.2	4.2 (8.3 %)
500 XY	10.8	79.8 (16 %)	6.5	39.7 (7.9 %)

Maternal blood samples

The two different MACS isolation procedures were also used for the isolation of fetal NRBCs from maternal blood. Maternal blood samples, from pregnancies from which the fetal karyotype was 46,XY, were obtained before chorionic villus sampling (CVS) from 31 pregnant women at 12-14 weeks of gestation. In 14 cases, cells were isolated using the one-step MACS isolation procedure, whereas in 17 cases the two-step isolation procedure was used (table 2). The number of mononuclear cells before MACS isolation

Table 2 Number of XY cells recovered from maternal blood after MACS isolation using the one-step and two-step procedures

Karyotype	Gestational age (weeks)	Number of cells before MACS isolation ($x10^6$)	Number of cells analyzed ($x10^4$)	Number of XY cells
One-step procedure				
46, XY	12	24.0	5	0
46, XY	13	32.4	88	0
46, XY	14	24.0	20	0
46, XY	12	32.4	30	0
46, XY	12	36.6	48	0
46, XY	12	30.9	16	0
46, XY	12	26.3	21	0
46, XY	13	23.4	15	0
46, XY	12	33.3	19	2
46, XY	13	24.9	22	2
46, XY	12	49.8	25	2
46, XY	12	23.4	23	1
46, XY	12	16.3	24	0
46, XY	13	30.9	49	2
Mean values	12	29.2	28.9	0.6
Two-step procedure				
46, XY	12	24.3	11	0
46, XY	12	28.5	9	0
46, XY	12	38.7	27	0
46, XY	12	38.7	18	1
46, XY	12	31.3	8	1
46, XY	12	49.0	29	0
46, XY	12	29.0	25	2
46, XY	12	35.1	11	1
46, XY	13	42.0	80	0
46, XY	12	17.0	6	0
46, XY	12	55.0	1.5	0
46, XY	12	41.7	14	0
46, XY	13	13.2	8	0
46, XY	12	31.2	21	0
46, XY	13	26.4	20	0
46, XY	12	23.0	14	0
46, XY	12	30.3	17	0
Mean values	12	32.6	18.8	0.3

ranged from 16.3-49.8 x 10^6 (mean 29.2 x 10^6) for the one-step isolation procedure and from 13.2-55.0 x 10^6 (mean 32.6 x 10^6) for the two-step isolation procedure. The number of cells analyzed in the CD71+ fraction ranged between 5-88 x 10^4 (mean 28.9 x 10^4) after the one-step isolation procedure and between 1.5-80 x 10^4 (mean 18.8 x 10^4) after the two-step isolation procedure. The number of XY positive cells that was found after the one-step and

two-step isolation procedures varied between 0 and 2 XY cells, with a mean of 0.6 and 0.3 XY cells, respectively. In 5 out of 14 cases (35.7 %) and in 4 out of 17 cases (23.5 %) XY positive cells could be detected using the one-step or two-step isolation protocol, respectively. This better enrichment of male cells after the direct enrichment of CD71+ cells did not reach statistical significance.

DISCUSSION

In this paper, we describe a model system for the isolation of fetal cells from maternal blood using *in vitro* expanded NRBCs derived from male umbilical cord blood. Umbilical cord blood was prefered above adult peripheral blood because of the larger amount of immature erythroid cells. Although fetal blood samples contain many immature erythroid cells, they are more difficult to obtain and are frequently contaminated with significant amounts of maternal blood.

Two different MACS isolation procedures were compared by analysis of recovery of known numbers of male cells mixed into female cells. In contrast to previously reported model systems for fetal cell isolation from maternal blood (Andrews *et al.*, 1995; Bianchi *et al.*, 1996), in which cord blood cells or fetal liver cells were used, we were able to obtain a homogenous erythroid cell population derived from *in vitro* expanded umbilical cord blood cells. These erythroid cells could be *in vitro* expanded to high cell numbers (10^7-10^8 erythroid cells/ml cord blood, after 10-15 days of culturing) and could be maintained in an erythroblastic cell stage. The expanded erythroid cells were immunophenotypically identical to fetal NRBCs derived from 10-20 week fetal liver (Bianchi, 1994), expressing high levels of CD71, a cell surface marker frequently used for the isolation of fetal NRBCs from maternal blood. This might indicate that the *in vitro* expanded erythroid cells resemble fetal NRBCs circulating in maternal blood.

Using these CD71+ cells, we were able to quantitatively compare two different MACS isolation procedures: a one-step isolation procedure which includes the enrichment of CD71+ cells alone, without depletion of other cell types, and a previously described (Busch *et al.*, 1994; Jansen *et al.*, 1997) and widely employed two-step isolation procedure which includes the depletion of monocytes (CD14) and lymphocytes (CD45) followed by the enrichment of CD71+ cells. Although the results of our experiments demonstrated a marked variation in the number of male cells recovered, the MACS isolation based on the enrichment of CD71+ cells alone was found to be two-fold more efficient and less time consuming than the combined depletion/enrichment protocol.

The two different MACS isolation strategies were also used for the isolation of fetal CD71+ cells from maternal blood samples. Although the number of male cells that could be

isolated from maternal blood was very low (0-2 cells per sample), it was shown that the one-step procedure resulted in a tendency towards a better recovery of male cells compared with the two-step protocol.

The one-step MACS isolation procedure was also shown to result in a higher absolute number of cells in the CD71+ fraction, in the model system as well as in maternal blood samples. This increase was due to the presence of $CD45^+$ $CD71^+$ and $CD14^+CD71^+$ cells, likely representing activated lymphocytes and monocytes of maternal origin, respectively, which are depleted after the two-step MACS isolation procedure. Alternatively, the CD71+ fraction obtained after the one-step isolation procedure may comprise cells of the erythroid lineage that still express CD45 at a low level and which were not depleted using the combined depletion/enrichment protocol. This may explain the two-fold higher yield of male cells observed after the one-step isolation protocol.

An interesting question that arises is, whether the *in vitro* expansion protocol for erythroid cells described in this paper can also be used for the expansion of fetal NRBCs derived from maternal blood. It has previously been described that fetal committed erythroid progenitors (CFU-E, M-BFU-E) derived from maternal blood were successfully proliferated *in vitro* after their prior enrichment by biotin-labeled human erythropoietin ligand and MACS (Valerio *et al.*, 1996). Also the expansion of CD34+ hematopoietic progenitor cells isolated from maternal blood by FACS has been reported (Little *et al.*, 1997). However, Chen *et al.* (1998) recently showed that culturing of fetal erythroblast cells derived from maternal blood mainly produced erythroid colonies derived from maternal erythroid progenitors. In general, expansion of fetal cells for analytical purposes is highly desirable given the rarity of these cells in the maternal circulation. Amplification of the number of fetal NRBCs using expansion protocols like the one described in this paper, might facilitate non-invasive prenatal detection of genetic abnormalities in the future. However, fetal cell enrichment protocols should be improved first in order to acquire higher recovery levels of fetal erythroid cells.

In summary, a more efficient and less time-consuming one-step MACS isolation procedure is described that improves the isolation of NRBCs in both the model system as well as in maternal blood samples. In addition, the *in vitro* expanded NRBCs used in the model system may not only be used to optimize different isolation strategies, but may also be of help in the development of new detection and analysis techniques for fetal erythroid cells isolated from maternal blood.

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Chapter 3

How useful is the in vitro expansion of fetal CD34+ progenitor cells from maternal blood samples for diagnostic purposes?

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The idea to increase the number of fetal cells from maternal blood by amplification of fetal hemopoietic progenitor cells has been discussed for a long time. If fetal cells could be stimulated to proliferate in culture, the technical limitations of working with very small numbers of cells could be overcome. This chapter describes the evaluation of the usefulness of *in vitro* expansion of fetal hemopoietic progenitor cells from maternal blood for diagnostic purposes. In order to determine whether limiting numbers of fetal CD34+ cells present in an excess of maternal cells are able to overgrow the maternal component, we used a model system in which limiting numbers of male CD34+ umbilical cord blood cells were diluted into female CD34+ peripheral blood mononuclear cells and *in vitro* expanded in liquid culture. The same culture protocol was applied to CD34+ cells isolated from maternal blood samples obtained at 7-16 weeks of gestation and the number of XY positive cells was determined using FISH for X and Y chromosomes.

How useful is the in vitro expansion of fetal CD34+ progenitor cells from maternal blood samples for diagnostic purposes?

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ABSTRACT

Fetal cells present in the maternal circulation are a potential source of fetal DNA that can be used for the development of a prenatal diagnostic test. Since their numbers are very low, amplification of fetal cells has been discussed for a long time. So far, most studies have focussed on culturing fetal erythroid cells. In this study, we evaluated whether limiting numbers of fetal hemopoietic progenitor cells present in an excess of maternal cells were able to overgrow the maternal component. Therefore, we used a model system in which limited numbers of male CD34+ umbilical cord blood cells were diluted in 400,000 female CD34+ peripheral blood cells. The number of XY positive cells derived from umbilical cord blood was determined using two-color *in situ* hybridization with X and Y chromosomal probes. We demonstrated a 1500-fold relative expansion of male umbilical cord blood cells over the peripheral blood component after 3 weeks of liquid culture, which also corresponded to the extent of expansion of CD34+ cells derived from 20-week fetal blood. However, application of the same culture protocol to maternal blood samples obtained at 7-16 weeks of gestation showed no preferential growth of fetal hemopoietic progenitor cells. This study, therefore, suggests that fetal primitive hemopoietic progenitor cells do either not circulate in maternal blood before 16 weeks of gestation, or require different combinations/concentrations of cytokines for their *in vitro* expansion.

INTRODUCTION

The development of a non-invasive prenatal diagnostic test using fetal blood cells that leak through the placenta into the maternal circulation would eliminate the small but significant risk to the fetus associated with more traditional procedures like chorionic villus sampling and amniocentesis. Fetal cell types that have been isolated from maternal blood include nucleated red blood cells (Bianchi *et al.*, 1990), lymphocytes (Herzenberg *et al.*, 1979),

granulocytes (Wessman *et al.*, 1992), trophoblast cells (Goodfellow and Taylor, 1982), and hemopoietic progenitor cells (Bianchi *et al.*, 1996b; Little *et al.*, 1997). However, frequencies of these cell types are very low. Factors that may influence these frequencies include the type of fetal cell analyzed, gestational age at the time of sampling and the accuracy of methods to enrich, identify, and quantify the fetal target population. The incidence of fetal cells in maternal blood has been reported to increase after chorionic villus sampling (Jansen *et al.*, 1997), in patients with preeclampsia (Holzgreve *et al.*, 1998; Lo *et al.*, 1999; Jansen *et al.*, submitted), and in pregnancies in which the fetal and placental karyotype were abnormal (Elias *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1993).

Despite all efforts to develop enrichment procedures such as fluorescence-activated cell sorting (FACS) (Bianchi *et al.*, 1990, 1996a) and magnetic cell sorting (MACS) (Ganshirt-Ahlert *et al.*, 1992; Jansen *et al.*, 1999) that would increase the detectability of fetal cells in a maternal blood sample, the number of fetal cells recovered still remains very low. Considerable progress has been made in the qualitative assessment of fetal cell populations found in maternal peripheral blood. However, more information is needed regarding the enumeration and characterization of fetal cell populations that circulate during pregnancy. The question as to whether fetal cells present in maternal blood samples can be *in vitro* expanded has been partially resolved by several investigations. Lo *et al.* (1994) were the first who were able to culture fetal erythroid cells from maternal peripheral blood by exploiting the growth advantage of fetal cells over maternal cells. Also, the successful proliferation of fetal committed erythroid progenitors (CFU-E, M-BFU-E) derived from maternal blood has been described (Valerio *et al.*, 1996, 1997). However, two recent reports (Chen *et al.*, 1998; Han *et al.*, 1999) have cast doubt on the proposition that maternal blood reliably contains fetal clonogenic erythroid cells. Recently, Little *et al.* (1997) were able to expand FACS-sorted CD34+ hemopoietic progenitor cells derived from maternal blood samples. They showed a 2-5 fold expansion of CD34+ fetal cells after 5 days of culture.

So far, most attention has been focussed on the amplification of fetal erythroid progenitors. In the present study, we evaluated the usefulness of the expansion of fetal hemopoietic progenitor cells derived from maternal blood samples for the development of a non-invasive prenatal test. Since it is known that after MACS separation fetal cells are often contaminated with an excess of maternal cells, we aimed at selectively expanding few fetal cells as compared to the maternal component. Therefore, limiting numbers of CD34+ cells derived from male umbilical cord blood (UCB) were spiked in an excess of CD34+ cells derived from female peripheral blood (PB). Cells were cultured for up to three weeks, and the number of XY cells was determined every week by FISH analysis using X and Y chromosome-specific probes. In order to evaluate whether the expansion capacity of hemopoietic progenitor cells derived from umbilical cord blood was comparable with growth profiles of fetal cells in maternal blood, we also examined the culture characteristics of CD34+ cells

derived from fetal blood (FB) samples. FB samples were obtained through cordocentesis at 20 weeks of gestation. Below that age, the success rate of this technique becomes markedly reduced. However, in order to develop a non-invasive prenatal diagnostic test using fetal cells in maternal blood, it is important to obtain maternal blood samples as early as possible. Fetal hemopoiesis starts in the yolk sac between days 16 and 19 followed by hepatic hemopoiesis at approximately 6 weeks, indicating that fetal hemopoietic progenitor cells may be present in the maternal circulation from week 3 of gestation (Metcalf and Moore, 1971). In the present study, maternal blood samples were obtained during the first and early second trimester of pregnancy, i.e. at 7-16 weeks of gestation, and the same protocol was used for the expansion and detection of fetal hemopoietic progenitor cells.

MATERIALS AND METHODS

Blood samples

Male umbilical cord blood (UCB) samples (n=5) were obtained from placentas of full-term normal pregnancies, while peripheral venous blood (PB) samples (n=4) were collected from female non-pregnant volunteers (nulligravidae). For spike experiments, two to three UCB or PB samples were pooled in order to obtain enough CD34+ cells after MACS separation.

Fetal blood (FB) samples (n=2) were obtained from discarded material after medically indicated cordocentesis at 20 weeks of gestations. FB samples at the target fetal age of between 7 to 16 weeks were unavailable because of the technical impossibility of umbilical cord sampling at this fetal age.

A total of 100 pregnant women who came to the Department of Obstetrics and Gynecology for prenatal diagnosis because of advanced maternal age (≥ 36 years) were enrolled in this part of the study. Thirty-five of these 100 pregnant women were carrying a female fetus and were used as negative controls for FISH analysis. The other 65 women were carrying a male fetus. The sex of the fetus was determined by cytogenetic analysis on semi-direct villus preparations or on amniocytes. Pregnancy duration varied between 7 and 16 weeks of gestation, as calculated from the first day of the last menstrual period. Maternal blood samples were taken according to a cross-sectional study design at: 7-11 weeks of gestation (group A; n=20) at the time of genetic counseling; 11-14 weeks of gestation, either before (group B; n=14) or after chorionic villus sampling (group C; n=21); or at 15-16 weeks of gestation prior to amniocentesis (group D; n=10). The study protocol was approved by the local Ethics Review Board, and all blood samples were obtained with patients' informed consent.

Blood samples were collected in vacutainers containing ethylenediamine-tetra-acetic acid (EDTA) and were diluted 1:1 with phosphate-buffered saline (PBS). Mononuclear cells were

isolated by Ficoll-Paque-Plus (1.077 g/ml; Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation at 750 g for 20 minutes. Cells were washed twice in Hanks' balanced salts (HBSS; Life Technologies B.V., Breda, The Netherlands). The number of viable cells was calculated using a Bürker counting chamber. Cells isolated from umbilical cord blood, fetal blood and maternal blood samples were cryopreserved in Iscove's modified Dulbecco's medium (IMDM; Life Technologies B.V., Breda, The Netherlands) containing 20 % fetal calf serum (FCS) and 10 % dimethylsulphoxide (DMSO) in liquid nitrogen until further analysis.

Magnetic Activated Cell Sorting (MACS)

Isolation of CD34+ cells was performed as described by the manufacturer using a CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

The number of viable cells in the CD34+ fraction was calculated using a Bürker counting chamber. Part of this fraction was used for liquid culture as well as for fluorescence *in situ* hybridization (FISH). For the spike experiments, 3, 10, 36 or 71 male CD34+ UCB cells were diluted in 400,000 female CD34+ PB cells. CD34+ derived from FB samples were only *in vitro* expanded and the number of nucleated cells was determined every week.

The percentage of CD34+ cells in the unseparated sample as well as the purified CD34+ fraction was determined by fluorescence-activated cell sorting (FACS) analysis. The percentage of CD34+ cells in the purified fraction varied between 60-90 %.

Liquid culture of CD34+ cells

The CD34+ cell fractions were seeded in standard six-well plates and cultured as described previously, with some modifications (Piacibello *et al.*, 1997; Rappold *et al.*, 1999). Briefly, CD34+ cells were cultured at 37 °C and 10 % CO_2 for 1 to 5 weeks in Stem Cell Growth Medium (SCGM; Boehringer Ingelheim, Heidelberg, Germany) containing 20 % FCS, thrombopoietin (tpo; 10 ng/ml; a generous gift from Genentech, South San Francisco, CA, USA), flt3-ligand (50 ng/ml) and stem cell factor (SCF; 100 ng/ml; both kindly provided by Amgen, Thousand Oaks, CA, USA) and IL-6 (100 ng/ml; a gift from Genetics Institute, Cambridge, MA, USA). Both tpo and flt3-ligand were added twice a week. Every week, the number of viable nucleated cells was determined using a Bürker counting chamber and part of the cells were treated for FISH analysis, whereas rest of the cells were cultured further. All CD34+ cell fractions were analyzed for the presence of X and Y chromosomes without prior knowledge of the fetal karyotype or donor origin to avoid sampling bias.

Fluorescence in situ hybridization (FISH)

Cells were treated with 75 mM KCl, incubated for 18 minutes at 37 °C, fixed in methanol:acetic acid (3:1) and stored at –20 °C until further analysis. Cells were dropped onto Vectabond™-coated slides (Vector Laboratories, Inc, Burlingame, CA, USA) and

air-dried. The number of nucleated cells analyzed per sample per slide varied from 10-20x10^4. Slides were pre-treated with pepsin (100 µg/ml) in 0.01 N HCl at 37 °C for 15 minutes, followed by postfixation in 3.7 % formaldehyde in PBS for 15 minutes. Subsequently, slides were denatured for 5 minutes in 70 % formamide (pH 7.5) in 2X SSC at 75 °C, followed by dehydration in 70 %, 90 % and 100 % ethanol for 1 minute each.

Two-color FISH was performed using a SpectrumOrange-labeled alpha-satellite probe (DXZ1) for centromere region Xp11.1-q11.1 and a SpectrumGreen-labeled satellite III probe for the Yq12 region (Vysis, Downers Grove, IL, USA). Samples were hybridized overnight at 37 °C in a humidified chamber.

Slides were post-washed at 70 °C in 0.4X SSC/0.3 % NP-40 (pH 7.2), followed by 5-60 seconds in 2X SSC/0.1 % NP-40 (pH 7.2) at room temperature. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) containing DAPI (4', 6-diamidino-2-phenylindole) and analyzed under a Leica Aristoplan fluorescence microscope using a triple band-pass filter block. Images were captured using a Xybion CCD 24-bit color camera with a Genetiscan ProbeMaster system and MacProbe 2.5 image analysis software (PSI, Chester, UK).

Since only part of the cultured cells was used for FISH analysis, the absolute number of XY cells during 1-5 weeks of liquid culture was calculated.

RESULTS

In vitro expansion of CD34+ cells derived from umbilical cord blood, peripheral blood and fetal blood

CD34+ sorted cells derived from male umbilical cord blood (UCB), female peripheral blood (PB) and fetal blood (FB) at 20 weeks of gestation were analyzed for their growth in liquid culture (figure 1). CD34+ cells derived from PB samples did not show any significant growth during 3 to 5 weeks of liquid culture. In contrast, CD34+ cells derived from UCB as well as FB showed an up to 4-log fold increase of nucleated cells after 5 weeks of liquid culture, indicating that UCB and FB CD34+ cells exhibit similar expansion capacities, and therefore, fetal cells derived from maternal blood may show the same growth profiles.

Spike experiments of male umbilical cord blood cells in female peripheral blood cells

In order to investigate whether extremely small numbers of fetal cells can be selectively expanded in maternal blood, we used a model system in which 3, 10, 36 or 71 male CD34+ UCB cells were diluted into 400,000 female CD34+ PB cells and cultured in liquid culture. Every week, the number of nucleated cells and the number of XY cells present in every

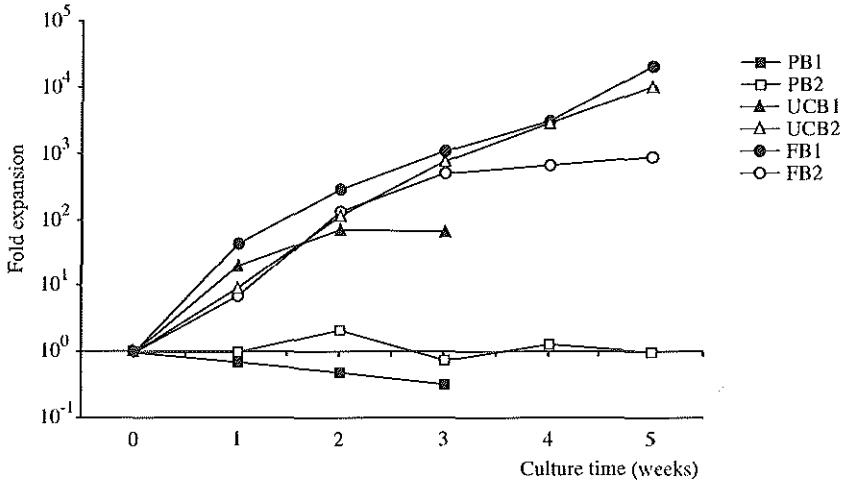

Figure 1 Fold expansion of CD34+ cells from peripheral blood (PB), umbilical cord blood (UCB) and fetal blood (FB) during liquid culture. The starting concentration of PB, UCB and FB were $40x10^4$, $25x10^4$ and $5x10^4$ CD34+ cells per milliliter of culture, respectively. Each line for UCB and PB corresponds with two to three pooled samples.

mixture was determined using FISH for X and Y chromosomes (figure 2). For each sample, the number of nuclei analyzed by FISH before and after liquid culture ranged between $10\text{-}20x10^4$. All four samples showed at least a 250-fold increase in the absolute number of XY cells (UCB) per 10^4 XX cells (PB), which was most abundant during the first week of liquid culture. Even 3 male CD34+ UCB cells (dilution 1) were able to expand up to 1500-fold after 3 weeks of culture. This suggests that even very low numbers of fetal CD34+ cells if present in maternal blood can be selectively expanded over adult female CD34+ PB cells, which would facilitate their detection. This is further supported by the fact that we observed comparable levels of expansion of CD34+ cells derived from 20-week FB (figure 1).

In vitro culture and detection of fetal hemopoietic progenitor cells in maternal blood

On the basis of the above described findings, we investigated the possibility to detect male fetal hemopoietic progenitor cells in peripheral blood from pregnant women by expanding their relative numbers during liquid culture. Maternal blood samples from pregnancies of which the fetal karyotype was 46, XY, were obtained from 65 pregnant women at 7-16 weeks of gestation. CD34+ cells were isolated and cultured in liquid culture during 0 to 5 weeks (table 1). For each sample, the number of nuclei analyzed by FISH ranged between

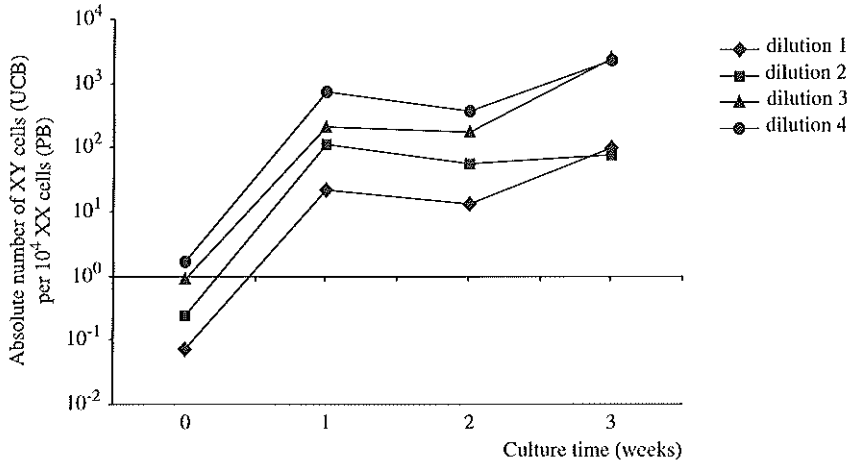

Figure 2 Number of XY positive cells per 10^4 XX cells during liquid culture of CD34+ cells in cell mixtures of umbilical cord blood (UCB) and peripheral blood (PB). Dilution 1, 2, 3 and 4 correspond to 3, 10, 36 and 71 male CD34+ UCB cells, respectively, diluted into 400,000 female CD34+ PB cells before liquid culture.

$10\text{-}20x10^4$. In group A, B, C and D, XY positive cells could be detected either before or during liquid culture in 11 out of 20 (55 %), 3 out of 14 (21 %), 12 out of 21 (57 %) and 2 out of 10 (20 %), respectively. In all other samples in the four different groups no XY positive cells could be detected either before or during liquid culture. The number of XY cells before liquid culture varied from 0 to 11.1 and in most of the samples no XY cells could be detected. In group A, B, C and D, XY positive cells could be detected before liquid culture in 8 out of 20 patients (40 %), 2 out of 14 (14 %), 2 out of 20 (10 %) and 1 out of 10 (10 %) patients, respectively. After 1 week of cell culture, the number of XY cells ranged from 0 to 16.7 XY positive cells in the four different groups, whereas in group B no XY positive cells could be detected. In group A, C and D, XY positive cells were found in 4 out of 7 (57 %), 7 out of 8 (88 %) and 1 out of 4 (25 %) patients, respectively. There was a significant difference for the presence of XY cells between group B and C after 1 week of liquid culture (Fisher's exact test, p=0.005). The number of XY positive cells varied from 0 to 22.2 XY positive cells after2 weeks of culture. XY positive cells were detected in group A, B and C in 1 out of 8 (12.5 %), 1 out of 5 (20 %) and 3 out of 9 (33 %) patients, respectively. No XY cells were found in the four patients analyzed in group D. After 3 weeks of culture of CD34+ cells, the number of male cells varied from 0 to 22.2, with the highest number in a patient from group A. In group A and C, 2 out of 3 (66 %) and 1 out of 4 (25 %) patients showed

Table 1 FISH analysis of CD34+ cells before and during liquid culture

Group/ patient number	Number of cultured CD34+ cells ($x10^4$)	Number of XY cells at week					
		0	1	2	3	4	5
A1	14.0	1.0					
A2	48.0	1.0					
A3	13.0	6.5	3.2				
A4	17.5	5.4	0.0				
A5	6.0	8.0	0.0				
A6	17.0	0.0	5.1				
A7	2.0	2.2	n.d.	0.0			
A8	15.0	2.5	n.d.	0.0			
A9	11.0	0.0	5.6	11.1	0.0		
A10	38.0	0.0	0.0	0.0	10.0	0.0	0.0
A11	23.0	5.9	3.0	0.0	22.2	20.0	20.0
XYneg [a]	23.3 (6-56)*	9	0	3	0	0	0
B1	7.0	6.9	0.0				
B2	7.0	0.0	n.d.	3.4	0.0		
B3	37.0	11.1	0.0	0.0	0.0	0.0	0.0
XYneg [a]	19.7 (9-55)*	11	3	3	1	0	0
C1	28.9	4.3					
C2	31.0	1.0					
C3	3.0	0.0	6.0				
C4	19.0	0.0	4.8				
C5	15.0	0.0	5.7				
C6	3.3	0.0	6.6				
C7	17.0	0.0	n.d.	7.0			
C8	9.0	0.0	n.d.	7.1			
C9	60.0	0.0	16.7	22.2	0.0		
C10	14.0	0.0	5.6	0.0	0.0		
C11	37.0	n.d.	n.d.	0.0	2.6	0.0	
C12	32.0	0.0	2.4	0.0	0.0	0.0	0.0
XYneg [a]	16.1 (4-24)*	9	1	3	0	0	0
D1	10.0	3.3	n.d.	0.0			
D2	37.0	0.0	2.4	0.0	0.0	0.0	0.0
XYneg [a]	12.8 (2-20)*	8	3	2	1	0	0

CD34+ cells were isolated from 7-22 ml maternal blood and cultured for 1 to 5 weeks. Weekly, part of the cells were collected and the absolute number of XY positive cells was determined. For each sample, the number of nuclei analyzed by FISH ranged between $10\text{-}20x10^4$. Only samples with detectable XY cells either before or during liquid culture are presented. Group A maternal blood samples were collected at 7-11 weeks of gestation; group B and C samples at 11-14 weeks of gestation, before or after chorionic villus sampling, respectively; and group D samples at 15-16 weeks of gestation. All maternal blood samples were derived from women carrying a male fetus.

[a] number of samples without detectable XY positive cells; * mean number of cultured CD34+ cells (range); n.d. not determined.

XY positive cells, respectively, whereas no male cells were found in group B and D. After 4 and 5 weeks of liquid culture, only in group A, in one out of two patients 20.0 XY positive cells were found. These results indicate that fetal hemopoietic progenitor cells, if present at all, may not preferentially expand over the maternal CD34+ component, which is in contrast with the observations in the spike experiments.

As negative controls to determine the background level for FISH analysis, a total of 39 peripheral blood samples from 35 control patients carrying a female fetus and 4 female non-pregnant volunteers (nulligravidae) were analyzed for the presence of male cells. From all 39 blood samples CD34+ cells were isolated. CD34+ cell fractions from 20 pregnant and 4 non-pregnant women were cultured during 0 to 3 weeks, whereas from the other 15 samples only FISH analysis was performed after MACS isolation. In none of the non-pregnant samples male cells could be detected, whereas in 2 out of 35 pregnant women (5.7 %) XY positive cells were found; 3.3 XY cells were detected on week 0 in one patient and 5.2 XY cells were detected after 2 weeks of liquid culture in an other patient. In both cases a prior male pregnancy was ascertained. These data suggest that the presence of male cells in female pregnancies may be caused by aspecific binding of the Y chromosomal probe or might be the result of persisting fetal cells from earlier pregnancies.

DISCUSSION

The development of a non-invasive prenatal diagnostic test using fetal cells present in the maternal circulation is hampered by the low frequency of these cells in maternal blood. The idea to increase the number of fetal cells by amplification of progenitor cells has been discussed for a long time. In most recent studies, the expansion of fetal erythroid cells is described (Lo *et al.*, 1994; Valerio *et al.*, 1996, 1997; Chen *et al.*, 1998; Han *et al.*, 1999), whereas less is known about the amplification of fetal CD34+ progenitors (Little *et al.*, 1997). In the present study, we evaluated the usefulness of *in vitro* expansion of CD34+ fetal cells isolated from maternal blood samples for diagnostic purposes. Two major methods of cell separation enable fetal cell isolation from maternal blood: fluorescence activated cell sorting (FACS) (Bianchi *et al.*, 1990) and magnetic activated cell sorting (MACS) (Ganshirt-Ahlert *et al.*, 1992). Both isolation techniques have the disadvantage that the purified fraction still contains many maternal cells. In order to evaluate whether limited numbers of fetal CD34+ cells present in an excess of maternal cells are able to overgrow the maternal component, we used a model system in which CD34+ cells derived from male umbilical cord blood (UCB) were diluted up to 3 in 400,000 with CD34+ cells derived from non-pregnant female volunteers (nulligravidae) and *in vitro* expanded in liquid culture. We showed that even very low numbers of CD34+ cells derived from UCB (3 XY positive cells) diluted into

an excess of PB cells were able to expand to high cell numbers, corresponding to the *in vitro* expansion of CD34+ nucleated cells derived from undiluted UCB samples. As the magnitude of *in vitro* expansion of CD34+ cells derived from 20-week FB samples corresponded to the growth of hemopoietic progenitor cells derived from UCB, it is suggested that the growth capacities of fetal hemopoietic progenitor cells of between 7 and 16 weeks of gestational age may show similar expansion patterns. Consequently, the limited numbers of fetal hemopoietic progenitor cells present in maternal blood samples may show the same growth profiles. In contrast to the findings in the model system, we were not able to show abundant growth of fetal male CD34+ cells isolated from maternal blood samples. In only a few samples in group A and C, cell growth of male fetal cells could be detected. These results are in concordance with the observations described by Little *et al.* (1997). Using an other cytokine combination, they also showed a limited expansion of CD34+ fetal cells derived from 10-13 week maternal blood samples after 5 days of culture, but in most cases (10 out of 18 (55 %)) no XY cells could be detected.

In many studies it has been investigated at what time in pregnancy the number of fetal cells has reached its maximum. Relevant information regarding the frequency of fetal nucleated red blood cells (NRBCs) in maternal blood is contradictory, and the frequency of fetal NRBCs was reported to vary significantly among individuals and throughout the three trimesters of pregnancy (Hamada *et al.*, 1993; Slunga-Tallberg *et al.*, 1995; Smid *et al.*, 1997; Kuo, 1998). Fetal cell frequency in maternal blood is influenced by a number of biological parameters that are mainly unknown. Gestational age seems to be one of the factors involved. The optimal period during pregnancy for detecting circulating fetal cells remains unclear. Therefore, we have analyzed maternal blood samples of different gestational ages, varying between 7 and 16 weeks of gestation (group A, B and D). In most cases no male fetal cells could be detected, whereas in 28 out of 65 maternal blood samples of women carrying a male fetus XY positive cells could be observed. However, there was no statistically significant increase in the number of patients with one or more XY positive cells between 7 and 11 weeks of gestation (group A) compared to patients of later gestational ages (group B and D). This suggests that the number of fetal hemopoietic progenitor cells present in blood from first trimester pregnant women might not be high enough or that these cells are even absent in most pregnant women.

Another question concerns the stability of fetal cell properties. Are fetal progenitor cells present in the maternal circulation clonal and possess unchangeable characteristics or are they transient and respond to a changing environment? Until now, it is not known whether fetal cells continue to express the same marker antigens in the new maternal environment after crossing the placenta, and respond to similar cytokines. The cytokine combination used in this study has previously been shown to be capable of extensive amplification and self-renewal of human primitive hemopoietic progenitor cells derived from umbilical cord

blood (Piacibello *et al.*, 1997; Rappold *et al.*, 1999). However, other, perhaps yet unidentified (combinations or concentrations of) growth factors may induce a more vigorous proliferative response, promoting the *in vitro* expansion of fetal cells isolated from maternal blood samples.

Another factor that influences the incidence of fetal cells in maternal blood is chorionic villus sampling (CVS), which has previously been reported to cause a feto-maternal transfusion of fetal NRBCs (Jansen *et al.*, 1997). The question arises as to whether it may also have an effect on the number of fetal hemopoietic progenitor cells in the maternal circulation. Although there was a significant increase of male cells after one week of liquid culture in samples obtained after chorionic villus sampling (CVS)(group C) compared to before CVS (group B), in most of the post-CVS samples the number of male fetal hemopoietic progenitors does not markedly exceed the number of male cells in blood samples obtained before any invasive procedure (group A, B and D).

Male cells were found in 2 out of 35 pregnant women carrying a female fetus. In both cases a prior male pregnancy was confirmed. Therefore, these cells may either represent false-positives due to non-specific binding of the Y chromosomal probe to non-target sequences, or represent residual fetal cells persisting from prior male pregnancies, since fetal progenitor cells have been reported to circulate in maternal blood as long as 27 years after birth (Bianchi *et al.*, 1996b). Furthermore, a feto-maternal transfusion may occur at the time of birth. This may establish fetal microchimerism in the mother, which has been reported to be implicated in the subsequent development of diseases, such as scleroderma that are common in women (Artlett *et al.*, 1998; Nelson, 1998; Evans *et al.*, 1999). Therefore, it is important to know whether fetal cells might persist from prior pregnancies, not only for the development of a non-invasive prenatal diagnostic test, but also to evaluate the immunological consequences of feto-maternal transfusion.

Until now, most attention has been focused on fetal NRBCs which have been isolated using antibodies against membrane-bound markers (CD71, glycophorin A) or intracellular antigens (hemoglobin F) (Loken *et al.*, 1987; Bianchi *et al.*, 1990; Zheng *et al.*, 1995). The number of fetal NRBCs in maternal blood is very low, and expansion of these cells might enhance the detectability of this cell type. A previously reported cell culture protocol for erythroid cells (Jansen *et al.*, 1999; von Lindern *et al.*, 1999) was used for CD71+ cells in a spike experiment similar to that used for hemopoietic progenitor cells described in this paper (data not shown). CD71+ cells derived from male UCB were diluted up to 1 in 400,000 in CD71+ cells derived from female PB samples. This cell mixture was cultured according to the previously described erythroid cell culture protocol (Jansen *et al.*, 1999; von Lindern *et al.*, 1999) and the number of XY positive cells was determined weekly. However, preferential expansion of CD71+ UCB cells as shown for CD34+ UCB cells could not be observed, suggesting that the expansion potential of fetal NRBCs in maternal blood samples is not

comparable with the expansion potential of hemopoietic progenitor cells, and therefore, fetal NRBCs may not be able to overgrow the excess of maternal cells. These results were similar to those reported by others (Chen *et al.*, 1998; Han *et al.*, 1999), who recently showed that culturing of fetal erythroblasts derived from contaminating maternal blood mainly produced erythroid colonies derived from maternal erythroid progenitors.
In summary, expansion of fetal cells isolated from maternal blood samples for analytical purposes is highly desirable given the rarity of these cells in the maternal circulation. Amplification of the number of fetal cells using cell culture protocols might facilitate non-invasive prenatal detection of genetic anomalies. In the current study, the described protocol for the expansion of hemopoietic progenitor cells resulted in a dramatic expansion of even very few CD34+ UCB cells spiked into PB samples, which also corresponded to a similar marked increase in the number of CD34+ cells from 20-week fetal blood. However, the expansion of fetal CD34+ cells derived from 7-16 week maternal blood samples did not result in significant growth of these rare cells and therefore is not yet suitable for diagnostic purposes.

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Chapter 4

The effect of chorionic villus sampling on the number of fetal cells isolated from maternal blood and on maternal serum alpha-fetoprotein levels

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The presence of fetal cells in maternal blood is supposed to be the result of a feto-maternal transfusion at the placental interface. It has previously been reported that the introduction of a biopsy needle into placental tissue to aspirate chorionic villi via the transabdominal route might induce a feto-maternal transfusion. Such a transfusion has been demonstrated by elevated levels of maternal serum alpha-fetoprotein after chorionic villus sampling. In this chapter, the effect of an invasive procedure like chorionic villus sampling was investigated by analyzing the number of fetal nucleated red blood cells in the maternal circulation before and after transabdominal chorionic villus sampling.

The effect of chorionic villus sampling on the number of fetal cells isolated from maternal blood and on maternal serum alpha-fetoprotein levels

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ABSTRACT

Fetal cells are present in the circulation of pregnant women and can be isolated using density gradient centrifugation and magnetic cell sorting. In the present study, maternal cell preparations were depleted for CD45- and CD14-positive cells and enriched for CD71-positive cells. The number of fetal nucleated cells was determined using fluorescence *in situ* hybridization for X and Y chromosomes. Analysis of maternal blood samples taken before and after transabdominal chorionic villus sampling (TA-CVS) showed an increase in the number of fetal cells in 10 out of 19 male pregnancies after the invasive procedure. This cellular transfusion was found to correlate with elevated maternal serum alpha-fetoprotein levels. TA-CVS-induced cellular transfusion may form a good *in vivo* system to optimize fetal cell isolation procedures and to study fetal cell dynamics and characteristics.

INTRODUCTION

Cells of fetal origin have been isolated from blood of pregnant women and can be used for non-invasive prenatal detection of genetic abnormalities. Cell types isolated from maternal blood include nucleated red blood cells (NRBCs) (Bianchi *et al.*, 1990), lymphocytes (Herzenberg *et al.*, 1979), granulocytes (Wessman *et al.*, 1992), and trophoblast cells (Goodfellow and Taylor, 1982). Attention has been focused primarily on NRBCs as they express both membrane-bound markers (CD71, glycophorin A) and intracellular antigens (hemoglobin F) which allow their isolation and identification (Loken *et al.*, 1987; Bianchi *et al.*, 1990; Zheng *et al.*, 1995).

The occurrence of fetal NRBCs in maternal blood is a rare event, and extensive enrichment and purification procedures are necessary to detect these cells. Isolation techniques currently

available have succeeded in the isolation of only a very small number of fetal cells in some of the pregnancies investigated (Reading *et al.*, 1995; Lewis *et al.*, 1996). Improvements in isolation technology that will result in a larger number of fetal cells are necessary to allow the use of these cells for diagnostic purposes.

The presence of fetal cells in the maternal circulation is supposed to be the result of a feto-maternal transfusion (FMT) at the placental interface (Price *et al.*, 1991; Bianchi *et al.*, 1992). This FMT has also been postulated to occur as a consequence of transabdominal chorionic villus sampling (TA-CVS), which was shown to induce an increase in maternal serum alpha-fetoprotein (MSAFP) levels (Los *et al.*, 1989, 1993; Smidt-Jensen *et al.*, 1993; Brezinka *et al.*, 1995). In the present study, we investigated whether the transfer of fetal plasma components after TA-CVS is accompanied by a concurrent increase in the number of fetal nucleated cells in the maternal circulation.

MATERIALS AND METHODS

Patient samples

Peripheral venous blood samples (13-18 ml) were obtained from 32 pregnant women (11-14 weeks of gestation) referred for prenatal diagnosis because of advanced maternal age (n=29), familial Down syndrome (n=2), or a previous child with congenital abnormalities (n=1). Mean maternal age was 37 years. Two blood samples were taken from each patient, the first one immediately before TA-CVS, the second 5-20 minutes after the invasive procedure. Blood samples were collected into vacutainers containing ethylenediaminetetra-acetic acid (EDTA). TA-CVS was performed according to Jahoda *et al.* (1990). In all patients a single needle puncture was needed to obtain 5-30 mg of chorionic villi. Fetal karyotyping was performed on semi-direct villus preparations. All samples were obtained with the patient's informed consent.

Cell preparation

Venous blood samples were diluted to 30 ml with phosphate-buffered saline (PBS) and mononucleated cells were isolated by Ficoll-Paque-Plus (1.077 g/ml; Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation at 750 G for 15 minutes. Cells were washed twice with PBS and centrifuged at 250 G for 10 minutes. For each sample, human serum was collected and stored at - 80 °C until further analysis.

Alpha-fetoprotein measurement

Maternal serum AFP (MSAFP) was measured with Amerlex M second-trimester radio-immuno-assay (RIA) kits for AFP (Kodak) and was expressed in kIU/l.

Magnetic activated cell sorting (MACS)

Fetal cells were isolated according to a procedure described by Büsch *et al.* (1994) with modifications. Briefly, cells were labeled for 15 minutes on ice with CD45-PE (50 µl/20x10^6 cells; KC 56, T-200 (IgG1), Coulter, Krefeld, Germany) in 200 µl PBNHS (PBS containing 1 % bovine serum albumin (BSA), 0.01 % sodium azide (NaN_3), and 10 % autologous serum), washed once in PBNHS, and labeled with 30 µl of CD14-conjugated microbeads and 30 µl of rat anti-mouse IgG1-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in 200 µl of PBNHS at 4 °C for 15 minutes. Labeled cells were washed once in PBNHS, resuspended in 500 µl wash buffer (PBS containing 1 % BSA, 0.01 % NaN_3, and 5 mM EDTA), and applied to a pre-separation filter (30 µm; CLB, Amsterdam, The Netherlands) in order to obtain a single cell suspension. The labeled cell suspension was applied to an AS-depletion column (Miltenyi) using a 26G needle as flow resistor. The negative fraction was applied again to the column in order to achieve an optimal depletion, followed by elution with 3 ml wash buffer. After an additional wash using a 24G needle, the column was removed from the magnetic device and CD45- and CD14-positive cells were collected.

The negative fraction was labeled for 15 minutes on ice with CD71-FITC (10 µl/10^6 cells; 100 µg/ml; LO1.1, IgG2a, Becton Dickinson, San José, CA, USA) in 200 µl of PBNHS. Cells were washed once in PBNHS and labeled for 15 minutes at 4 °C with 30 µl of rat anti-mouse IgG2a+b-conjugated microbeads (Miltenyi) in 200 µl of PBNHS, and washed again in PBNHS. Labeled cells were resuspended in 500 µl wash buffer and applied to a pre-separation filter (30 µm; CLB). The cell suspension was added to a mini MACS column (type MS; Miltenyi) and the non-magnetic cell fraction was collected. After removing the column from the magnet, the CD71+ cell fraction was eluted.

The number of viable cells in each fraction was calculated before and after loading the MACS column using a Bürker counting chamber.

Fluorescence in situ hybridization (FISH)

Cell fractions were treated with 75 mM KCl, incubated for 18 minutes at 37 °C, fixed in methanol: acetic acid (3:1), and stored at -20 °C until further analysis. Cells were dropped onto 3-aminopropyltriethoxysilane (2 % in acetone) -coated slides and air-dried. Slides were treated with 70 % formamide (pH 7.0) in 2X SSC, washed in PBS, and dehydrated in 70 %, 90 % and 100 % ethanol for 5 minutes each. After heating the slides for 10 minutes at 80 °C, cells were pretreated with pepsin (100 µg/ml) in 0.01 N HCl at 37 °C for 15 minutes, followed by post-fixation in 3.7 % formaldehyde in PBS for 15 minutes. Subsequently, slides were denatured for 5 minutes in 70 % formamide (pH 7.5) in 2X SSC at 75 °C, followed by

dehydration in 70 %, 90 % and 100 % ethanol for 1 minute each. Slides were prewarmed at 45 °C until probe was applied.

Two-color FISH was performed using a SpectrumOrange labeled alpha-satellite probe (DXZ1) for centromere region Xp11.1-q11.1 and a SpectrumGreen labeled satellite III probe for the Yq12 region (Vysis, Downers Grove, IL, USA). The probes were denatured for 5 minutes at 75 °C and hybridization was allowed to continue overnight at 42 °C in a humidified chamber.

Slides were post-washed three times at 46 °C in 50 % formamide in 2X SSC (pH 7.5) for 10 minutes each, followed by one wash in 2X SSC (pH 7.0) for 10 minutes and one wash in 2X SSC/0.1 % NP-40 (pH 7.0) for 5 minutes, both at 46 °C. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) containing DAPI (4', 6-diamidino-2-phenylindole) and analyzed under a Leica Aristoplan fluorescence microscope using a triple band-pass filter block. Images were captured with a Genetiscan ProbeMaster system (PSI, Chester, UK) using a Xybion CCD 24-bit color camera.

RESULTS

Blood samples were obtained from 32 pregnant women at 11-14 weeks of gestation (table 1). Cytogenetic analysis of the villus sample showed a female karyotype in 13 cases and a male karyotype in 19 cases. Two chromosomal abnormalities (47,XXY; 47,XY, +13) were encountered in two pregnancies referred for advanced maternal age.

CD71-positive cells were isolated from maternal blood taken before and after TA-CVS using MACS separation. First, monocytes (CD14) and lymphocytes (CD45) were depleted, followed by enrichment of CD71-positive cells. The number of XY cells in the CD71-positive fractions was determined by FISH using X and Y chromosome-specific probes. The number of cells analyzed in the CD71-positive fractions ranged from 0.3 to 80 x 10^4 (table 1).

In 3 out of the 13 female cases (23 %), one XY positive cell per sample could be detected, either before or after TA-CVS. In two out of these three cases, a prior male pregnancy was either ascertained or could not be excluded due to a previous spontaneous abortion. In the third case, only prior female pregnancies were reported. The number of XY-positive cells before TA-CVS in the male cases ranged from 0 to 4 XY cells, with the highest amount of X- and Y-positive cells in the 47,XXY case (table 1 and figure 1A). The number of XY-positive cells after TA-CVS in the male cases ranged from 0 to 187, with the highest amount of cells in the case with trisomy 13 (table 1 and figure 1B). A total of ten out of 19 male cases (53 %) displayed an increase in the number of XY positive cells after TA-CVS compared with the number of XY cells before TA-CVS.

Table I Number of XY cells and alpha-fetoprotein values before and after TA-CVS

Case no.	Fetal karyotype	Gestational age (weeks)	Number of cells analyzed* ($\times 10^4$)		Number of XY cells		AFP values (kIU/l)	
			Before CVS	After CVS	Before CVS	After CVS	Before CVS	After CVS
1	46, XX	12	17	22	0	0	7.9	14.6
2	46, XX	12	15	15	0	1	3.0	8.8
3	46, XX	11	13	15	0	0	1.0	1.0
4	46, XX	12	13	14	0	0	1.6	3.3
5	46, XX	12	11	12	0	0	1.0	32.2
6	46, XX	12	10	17	0	0	14.3	13.8
7	46, XX	12	21	24	0	0	2.5	3.4
8	46, XX	12	13	12	1	0	8.0	10.3
9	46, XX	12	28	41	0	0	8.7	8.4
10	46, XX	12	29	25	0	0	7.4	12.0
11	46, XX	12	7	11	0	0	2.0	9.9
12	46, XX	12	8	20	0	1	1.4	3.3
13	46, XX	12	28	17	0	0	14.7	19.3
14	46, XY	12	11	12	0	0	3.0	2.3
15	46, XY	12	9	11	0	0	4.4	7.8
16	46, XY	12	27	8	0	0	1.0	9.0
17	46, XY	12	18	18	1	1	7.2	17.9
18	46, XY	12	8	14	1	175	1.4	332.6
19	46, XY	12	29	32	0	185	3.8	720.0
20	46, XY	12	25	24	2	15	29.9	63.9
21	46, XY	12	11	9	1	0	2.9	16.8
22	46, XY	13	80	40	0	1	5.8	11.5
23	46, XY	12	6	0.8	0	0	10.8	11.1
24	46, XY	12	1.5	0.3	0	7	10.8	18.1
25	46, XY	12	14	25	0	0	4.6	6.3
26	46, XY	13	8	8	0	2	6.5	164.3
27	46, XY	12	21	11	0	1	1.4	2.5
28	46, XY	13	20	10	0	4	13.8	34.0
29	46, XY	12	14	10	0	0	4.5	28.8
30	46, XY	12	17	22	0	0	6.4	10.9
31	47, XXY	12	19	25	4†	23†	4.4	26.4
32	47,XY,+13	13	28	21	2	187	4.2	72.2

* All cells obtained after CD71 enrichment were analyzed by FISH using X and Y chromosome specific probes; † number of XXY cells

MSAFP levels before and after TA-CVS were determined and compared with the number of XY cells before and after TA-CVS (table 1). The mean level of MSAFP before TA-CVS was 6.3 kIU/l (combination of all male and female pregnancies) and 53.0 kIU/l after TA-CVS, indicating a significant 8.4-fold increase in MSAFP levels due to the TA-CVS procedure (two-sample Wilcoxon test; $p < 0.01$). For the male cases, MSAFP levels were found to be positively correlated with the number of XY cells after TA-CVS (Spearman rank correlation coefficient $r = 0.78$; $n = 19$; $p < 0.001$). No significant difference could be observed in MSAFP levels after TA-CVS between male and female cases (Mann-Whitney U-test, $p=0.47$). There was no correlation between the amount of villi taken (range 5-30 mg) and increases in fetal cell counts or MSAFP levels.

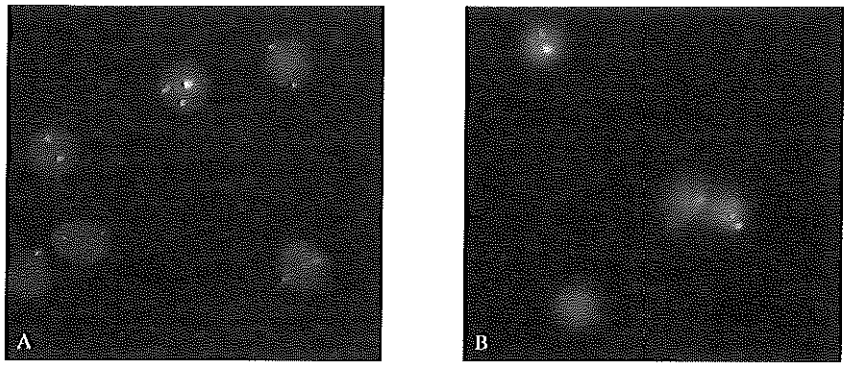

Figure 1 *In situ* hybridization on nucleated cells isolated from maternal blood showing X (red) and Y (green) chromosomal signals. Maternal cells present with two X signals per cell, while fetal cells present with two X and one Y signal in the case of a fetal 47, XXY karyotype (A) and with one X and one Y signal in the case of a fetal 47,XY, +13 karyotype (B). (magnification 1000x)

DISCUSSION

In this paper, we have shown that TA-CVS not only leads to a FMT of fetal plasma components, but also results in the transfer of fetal nucleated cells into the maternal circulation. The extent of plasma FMT was found to be positively correlated with the amount of fetal nucleated cells that could be isolated from maternal blood.

Previously, it has been reported that the introduction of a biopsy needle into placental tissue to aspirate chorionic villi via a transabdominal route might induce a FMT. This was demonstrated by elevated levels of MSAFP measured after TA-CVS. Estimates of the extent of the FMT have indicated cases in which up to 40 % of the fetal plasma volume has leaked into the maternal circulation (Los *et al.*, 1989; Rodeck *et al.*, 1993; Smidt-Jensen *et al.*, 1994; Brezinka *et al.*, 1995). In spite of this extensive trauma, fetal demise as a result of TA-CVS is rare and has been documented only in sporadic cases (Los *et al.*, 1993). In the present study, we have shown that the transfer of plasma components after TA-CVS is accompanied by a transfusion of fetal nucleated cells. However, the extensive plasma FMT estimated on the basis of MSAFP may not reflect a transfusion of a comparable volume of whole blood, because the number of fetal nucleated cells detected after TA-CVS is relatively low. If the size of the FMT is estimated on the basis of the assumption that a rise in MSAFP corresponds to an equivalent transfusion of whole blood, it can be calculated that a rise of 650 kIU/l

corresponds to a FMT of 1 ml (Los *et al.*, 1989). A transfusion of this size will lead to a leakage of at least 4 x 10^6 fetal nucleated cells into the maternal circulation (Millar *et al.*, 1985). This estimated level does not correspond to the observed number of fetal nucleated cells in the present study, which is at least an order of magnitude lower. This lower level of fetal nucleated cells might be explained by removal of these fetal cells by the maternal immune system, by retention of fetal cells in maternal tissues, or by an inefficient isolation procedure. Alternatively, it is possible that an extensive plasma FMT as indicated by a large rise in MSAFP is not accompanied by a proportional cellular transfusion. In the present study, the extent of plasma FMT after TA-CVS significantly correlates with the extent of cellular FMT, although in individual cases an increase in plasma FMT was found that was not accompanied by a demonstrable cellular FMT. Retention of the fetal blood cellular component compared with the plasma FMT after TA-CVS might explain why relatively few cases with extreme plasma FMT are accompanied by fetal death.

The number of fetal cells that could be isolated from maternal blood before TA-CVS was found to be very low in the male cases (0-4 cells per sample). This is in agreement with other recent reports in which similar levels of fetal cells were found (Reading *et al.*, 1995; Lewis *et al.*, 1996). In the two cases with a chromosome abnormality (47,XXY; 47,XY, +13), a relatively high number of fetal nucleated cells were isolated. It has previously been suggested that a higher level of fetal cells can be found in maternal blood in cases with chromosome abnormalities, possibly as a result of an altered placental structure (Simpson and Elias, 1994). More studies are needed to investigate whether higher levels of cellular FMT occur in abnormal pregnancies, as this will facilitate prenatal diagnostic procedures.

Some XY-positive cells were found in female pregnancies; these cells may either represent false positives due to non-specific background staining of the Y-chromosome probe, or represent cells persisting from prior male pregnancies, since fetal lymphocytes were found to circulate in maternal blood as long as 27 years after birth (Bianchi *et al.*, 1996).

Elevated MSAFP levels induced by TA-CVS were found in both male and female pregnancies; although the three cases with the largest FMT were all male pregnancies, no significant difference between the two groups could be observed.

The increase in fetal cell number after TA-CVS, resulting in the detection of XY cells in ten out of 19 male pregnancies, represents an interesting experimental *in vivo* system. *In vitro* model systems based on artificial mixtures of neonatal cord blood NRBCs and adult female peripheral blood cells have been described (Andrews *et al.*, 1995), but they have the disadvantage that cell characteristics of erythroblasts at the 20 to 40-week stage may differ from those at 12 weeks of gestation. The present *in vivo* model of TA-CVS-induced cellular FMT has the important advantage that fetal cells are derived from the correct gestational stage and may be used to improve isolation procedures and to study fetal cell dynamics and characteristics.

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Chapter 5

Significantly higher number of fetal nucleated red blood cells in the maternal circulation of women with preeclampsia

Submitted

Preeclampsia is a common, pregnancy-specific disease defined by clinical findings of elevated blood pressure combined with proteinuria and edema. Although the etiology of preeclampsia is not known, there are indications that abnormal placentation and endothelial dysfunction are involved in the pathogenesis of preeclampsia. In this chapter, we investigated whether this abnormal placentation results in a transfusion of increased numbers of fetal nucleated red blood cells in the maternal circulation.

Significantly higher number of fetal nucleated red blood cells in the maternal circulation in women with preeclampsia

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ABSTRACT

Although the pathophysiology of preeclampsia is unknown, several studies have described that abnormal placentation early in pregnancy might play a key role. Therefore, we investigated whether this abnormal placentation results in a feto-maternal transfusion of fetal nucleated red blood cells in women with preeclampsia. Male fetal nucleated red blood cells were isolated using magnetic activated cell sorting from 20 women with preeclampsia and 20 controls, matched for gestational age and parity. The number of XY cells was determined using two-color fluorescence in situ hybridization for X and Y chromosomes. Significantly more XY cells could be detected in women with preeclampsia (0.61 ± 1.2 XY cells/ml blood) compared to women with uncomplicated pregnancies (0.02 ± 0.04 XY cells/ml blood) (Mann-Whitney U test, $p<0.001$). These results suggest that fetal cell trafficking is enhanced in women with preeclampsia and may contribute to the understanding of the disease.

INTRODUCTION

Preeclampsia is a pregnancy-specific syndrome particularly manifested in late pregnancy. Preeclampsia is clinically characterized by hypertension and proteinuria. Although the etiology of preeclampsia is unknown, in the last decade it has been proven that preeclampsia is related to abnormal placentation early in pregnancy. Abnormal placentation might be due to incomplete trophoblast invasion. The cause of failure of trophoblastic invasion is unknown, but there appears to be a combination of different factors, including genetic and environmental factors (Roberts and Redman, 1993).

Abnormal placentation has been suggested to play an important role in feto-maternal cell traffic, which was first recognized in 1893, when Schmorl identified trophoblast cells in the lung capillaries of women dying of eclampsia. For the last 20 years, investigators have used the presence of fetal cells in maternal blood in an attempt to develop a non-invasive prenatal diagnostic test (Simpson and Elias, 1994; Bianchi, 1998). Trafficking of fetal cells into the

maternal circulation have raised several questions, particularly regarding the types of cells that are able to pass the placental barrier, the number of fetal cells, and the consequences of semi-allogeneic cells in the new host.

In several recent studies, the exchange of fetal and maternal blood cells in women with preeclampsia has been investigated (Chua *et al.*, 1991; Holzgreve *et al.*, 1998; Knight *et al.*, 1998; Lo *et al.*, 1999). Recently, Holzgreve *et al.* (1998) showed that a substantial proportion of erythroblast cells present in the blood of women with preeclampsia (n=8) were of fetal origin, and that a significant increase in cross-placental traffic of fetal cells occurred in women with preeclampsia compared to controls. In addition, Lo *et al.* (1999) recently demonstrated a similar feto-maternal transfusion on the basis of abundance of fetal DNA in maternal serum of patients suffering from preeclampsia (n=20).

To investigate whether abnormal placentation associated with preeclampsia results in a feto-maternal transfusion, we isolated fetal nucleated red blood cells (NRBCs) from women with preeclampsia compared to controls, both carrying a male fetus, using a very efficient magnetic activated cell sorting (MACS) protocol and two-color fluorescence *in situ* hybridization (FISH) for X and Y chromosomes.

MATERIALS AND METHODS

Patients

The study protocol was approved by the local Ethics Review Board. After patients had given informed consent, maternal blood samples (8-24 ml) were collected in vacutainers containing ethylenediaminetetra-acetic acid (EDTA). Blood samples were obtained from 20 singleton pregnancies associated with preeclampsia and 20 women with uncomplicated pregnancies, both carrying a male fetus. Samples were matched for gestational age (± 1 week) and parity. The gender of the fetus was confirmed after delivery. Preeclampsia was defined as an absolute diastolic bloodpressure ≥90 mmHg, combined with proteinuria, which was defined as ≥0.3 g/l in 24 hours urine (Davey and MacGillivray, 1988). HELLP (hemolysis, elevated liver enzymes, and low platelets) was defined as thrombocytes $<100x10^9$/l, ASAT and ALAT both >30 U/l, and haptoglobin < 0.28 g/l.

Eighteen healthy pregnant controls carrying a female fetus were recruited as negative controls. These controls were included in order to determine the background level for FISH analysis.

Magnetic activated cell sorting (MACS)

Maternal blood samples were diluted 1:1 with phosphate-buffered saline (PBS) and mononucleated cells were isolated by Ficoll-Paque-Plus density gradient centrifugation at

750 G for 10 minutes. Cells were washed twice in MACS buffer (PBS) containing 1 % bovine serum albumin (BSA), 0.01 % sodium azide (NaN_3), and 5 mM EDTA) and centrifuged at 250 G for 10 minutes. The number of viable cells was calculated using a Bürker counting chamber.

Mononuclear cells derived from maternal blood were labeled on ice with CD71 (10 µl/10^6 cells) in 200 µl MACS buffer for 15 minutes. Cells were washed in MACS buffer and labeled with IgG2a+b-conjugated microbeads (20 µl/10^7 cells; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4 °C. Cells were washed in MACS buffer, resuspended in 1 ml MACS buffer and applied via a pre-separation filter (30 µm; CLB; Amsterdam, The Netherlands) onto a miniMACS column (type MS; Miltenyi). After removing the column from the magnet, CD71+ cells were eluted with 15 ml MACS buffer. The number of viable cells in each fraction was calculated using a Bürker counting chamber.

Fluorescence in situ hybridization (FISH)

Cell fractions were treated with 75 mM KCl, incubated for 18 minutes at 37 °C, fixed in methanol: acetic acid (3:1) and stored at -20 °C until further analysis. Cells were dropped onto Vectabond™ (Vector Laboratories, Inc, Burlingame, CA, USA) coated slides and air-dried. Slides were pre-treated with pepsin (100 µg/ml) in 0.01 N HCl at 37 °C for 15 minutes, followed by postfixation in 3.7 % formaldehyde in PBS for 15 minutes. Subsequently, slides were denatured for 5 minutes in 70 % formamide (pH 7.5) in 2X SSC at 75 °C, followed by dehydration in 70 %, 90 % and 100 % ethanol for 1 minute each.

Two-color FISH was performed using a SpectrumOrange labeled alpha-satellite probe (DXZ1) for centromere region Xp11.1-q11.1 and a SpectrumGreen labeled satellite III probe for the Yq12 region (Vysis, Downers Grove, IL, USA). Samples were hybridized overnight at 37 °C in a humidified chamber.

Slides were post-washed at 70 °C in 0.4X SSC/0.3 % NP-40 (pH 7.2), followed by 5-60 seconds in 2X SSC/0.1 % NP-40 (pH 7.2) at room temperature. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Inc.) containing DAPI (4', 6-diamidino-2-phenylindole) and analyzed under a Leica Aristoplan fluorescence microscope using a triple band-pass filter block. Images were captured using a Xybion CCD 24-bit color camera with a Genetiscan ProbeMaster system and MacProbe 2.5 image analysis software (PSI, Chester, UK).

All slides were analyzed without prior knowledge of the gender of the fetus.

Statistic analysis

Clinical data of women with preeclampsia and their controls are presented in table 1 as mean values ± SD. The unpaired Student t-test was used for a normal distribution, tested by

Kolmogorov-Smirnov and Shapiro-Wilk tests. For non-normal distributions the Mann-Whitney U test was applied. Intra-uterine growth restriction is defined as a birth weight below the fifth-percentile according to Kloosterman (1970). The exact Pearson chi-square test was used to test the fifth-percentile. Two-tailed tests with $p<0.05$ were accepted as significant differences.

RESULTS

Patients characteristics

As expected, women with preeclampsia displayed a significantly higher mean systolic and diastolic blood pressure and a lower gestational age at delivery and fetal birth weight than women with uncomplicated pregnancies (table 1). Preeclampsia was also associated with a higher incidence of intra-uterine growth restriction. Unexpectedly, women with preeclampsia had lower levels of hematocrit.

Table 1 Clinical characteristics

	Women with preeclampsia N=20	Control group N=20	P- value
Maternal age (years)	29.6 ± 5.4	29.7 ± 4.7	0.95
Nulliparae (N)	14 (70 %)	15 (75 %)	n.d.
Systolic blood pressure (mmHg)	169.2 ± 28.8	117 ± 8.0	<0.001
Diastolic blood pressure (mmHg)	107.0 ± 13.3	75 ± 9.0	<0.001
Proteinuria (g/l)	5.07 ± 4.5	n.d.	n.d.
Gestational age at blood sampling (days)	207.9 ± 21.3	207.7 ± 20.5	0.88
Gestational age at delivery (days)	213.3 ± 21.8	276.5 ± 14.6	<0.001
Birthweight (gram)	1244 ± 530.2	3206 ± 670.9	<0.001
Intra-uterine growth restriction (N)	6	0	0.02
Hematocrit at blood sampling (vol/vol %)	32.7 ± 1.3	35.1 ± 3.6	0.014

n.d. not determined

Pregnancies with a male fetus

XY positive cells were found in 70 % of women with preeclampsia (n=20, 0.61±1.2 XY cells/ml blood), whereas this was in 10% of controls (n=20, 0.02 ± 0.04 XY cells/ml blood) (figure 1). This difference in the number of XY cells is statistically significant (Mann-Whitney U test, $p<0.001$). The total number of cells analyzed in the CD71+ fraction ranged

between 23–2000x10^4 (216.5x10^4 ± 440.4x10^4) in women with preeclampsia and 14–375x10^4 (mean 145.1x10^4±440.4x10^4) in controls. This difference was not statistically significant (Mann-Whitney U test; p=0.95).

In women with preeclampsia, no correlation was observed between the number of XY-positive cells and the severity of the disease, i.e. HELLP (n=12), proteinuria (n=20) and hypertension (diastolic blood pressure ≥90 mmHg; systolic blood pressure ≥140; n=19) (Spearman's rank correlation coefficient, p>0.22).

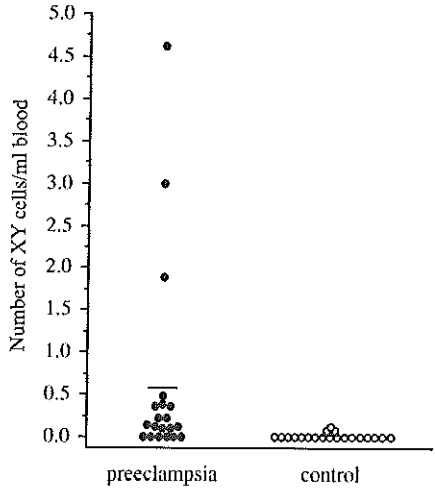

Figure 1 Number of XY positive cells per milliliter maternal blood in 20 women with preeclampsia (●) and 20 women with uncomplicated pregnancies (○). Each symbol corresponds to an individual woman, whereas the horizontal line represents the mean number of XY cells per ml blood.

Pregnancies with a female fetus

As negative controls to determine the background level for FISH analysis of XY cells, 18 control patients carrying a female fetus (mean gestational age 209 days) were analyzed for the presence of male cells after MACS isolation of CD71+ cells. In 3 out of 18 (16.7 %) control patients 0.07 XY cells per ml maternal blood were found.

COMMENT

We demonstrated an increased transfusion of fetal nucleated red blood cells in women with preeclampsia compared with women with uncomplicated pregnancies. The number of male fetal cells found in the maternal circulation of women with preeclampsia was up to 30-fold higher than in controls, indicating a six times higher level of fetal cells compared to previously reported results (Holzgreve *et al.*, 1998; Lo *et al.*, 1999). Holzgreve *et al.* (1998) isolated fetal nucleated red blood cells using the same MACS protocol as described in this study, although they used directly labeled CD71 beads. This might explain our promising higher recovery of male cells since we have previously demonstrated in a model system using *in vitro* expanded erythroid cells (Jansen *et al.*, 1999) that the isolation of nucleated red blood cells was more efficient using indirect beads labeling (unpublished results). In the study of Lo *et al.* (1999), fetal DNA concentrations appear to be fivefold higher in maternal serum samples derived from women with preeclampsia compared to controls, indicating that the amount of DNA in maternal blood samples may not correspond with the number of cells that enter the maternal circulation in women with preeclampsia.

Some XY-positive cells were found in pregnancies carrying a female fetus. In 2 out of 3 of these pregnancies a previous pregnancy carrying a male fetus could not be excluded due to a previous spontaneous abortion, whereas in one case a previous male pregnancy was ascertained. No XY-positive cells were found in primigravidae carrying a female fetus. These male cells in female pregnancies may either represent false positives due to non-specific background staining of the Y chromosome probe, or represent fetal cells persisting from previous pregnancies carrying a male fetus, since fetal cells have been found in the maternal circulation as long as 27 years after birth (Bianchi *et al.*, 1996).

The increased feto-maternal transfusion observed in women with preeclampsia might be explained by a decreased intravascular volume due to vasoconstriction and leading to an increased absolute cell concentration and consequently a higher number of fetal cells. However, unexpectedly hematocrit values were significantly lower in women with preeclampsia compared to controls. These lower levels can be explained by treatment of women with preeclampsia with plasma volume expansion medication before the time of blood sampling.

The mechanism of fetal cell escape into the maternal circulation is unknown. Preeclampsia is related to poor placental transfusion probably due to abnormal placentation early in pregnancy. This might result in the release of unknown factors, which in turn will lead to destruction of the maternal vascular endothelium. The abnormal placentation may explain the increased transfusion of fetal products in preeclampsia. Furthermore, feto-maternal transfusion has been observed in patients with chromosomal anomalies and after chorionic

villus sampling, possibly as a result of disrupted or altered placental morphology (Simpson and Elias, 1994; Jansen *et al.*, 1997).

The clinical findings of preeclampsia become manifested late in pregnancy, usually after the 20th week of gestation and can develop in a life-threatening disease. Preeclampsia is a major cause of fetal and maternal morbidity and mortality. Therefore, it is important to develop a predictive screening test early in pregnancy. Relevant information regarding the frequency of fetal nucleated red blood cells in maternal blood is contradictory, and the frequency of fetal nucleated red blood cells varies significantly among individuals and throughout the three trimesters of pregnancy (Hamada *et al.*, 1993; Slunga-Tallberg *et al.*, 1995; Smid *et al.*, 1997; Kuo, 1998). The question arises as to whether fetal cells are present in maternal blood of women with preeclampsia early in pregnancy and whether they can be used for the development of a prognostic test. Longitudinal studies are needed to investigate the frequency of fetal cells in maternal blood throughout gestation and in particular in those patients at risk for preeclampsia.

The presence of fetal cells in blood of women with preeclampsia may contribute to the disease process. Although we did not demonstrate a significant correlation between the number of male cells in the circulation of women with preeclampsia and the severity of their clinical symptoms, fetal cells are foreign to the maternal host and may have immunological consequences for mother and fetus. Furthermore, a feto-maternal transfusion may occur at the time of labor and delivery. This may establish fetal cell microchimerism in the mother, which may be implicated in the subsequent development of diseases such as scleroderma that are more common in females (Artlett *et al.*, 1998; Nelson, 1998; Evans *et al.*, 1999).

In summary, we demonstrated an increased number of male fetal nucleated red blood cells in pregnancies complicated with preeclampsia, which could not be observed in their matched controls. This suggests that fetal cell trafficking is enhanced in women with preeclampsia and may contribute to our understanding of the disease.

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Chapter 6

General discussion and conclusions

The isolation of fetal cells from the maternal circulation may have implications in prenatal diagnosis since it would eliminate the small but significant risk to the fetus associated with more traditional procedures like chorionic villus sampling and amniocentesis. If true fetal cells are conclusively isolated, clinical applications could include screening for fetal chromosome abnormalities by FISH and gene abnormalities by PCR. Most attention has been focussed on the isolation of fetal NRBCs. A small number of NRBCs occur in the peripheral venous blood of women during normal pregnancy, but they are less common in the blood of healthy non-pregnant women (Bianchi *et al.*, 1991; Ganshirt-Ahlert *et al.*, 1992; Slunga-Tallberg *et al.*, 1994). It was, therefore, believed that these cells were of fetal origin and could be a promising source of fetal material for non-invasive prenatal diagnosis, especially since the short lifespan of erythroid cells eliminates the possibility of such cells to persist from previous pregnancies. However, most of these NRBCs in pregnant women appeared to be of maternal origin (Bianchi *et al.*, 1994a; Busch *et al.*, 1994; Slunga-Tallberg *et al.*, 1995, 1996), leaving a very small number of fetal NRBCs which may not be sufficient for a reliable prenatal diagnostic test on a large scale. To establish high purity and yield of these rare fetal cells, more specific and/or more efficient enrichment techniques are needed, which have been developed using different model systems (Andrews *et al.*, 1995; Bianchi *et al.*, 1996a; Troeger *et al.*, 1999).

6.1. Model systems used for the isolation of fetal cells from maternal blood

Attempts to isolate fetal cells from maternal blood has been frustrated due to very low yields of NRBCs or even no detectable fetal cells in many maternal blood samples. Absence of fetal cells has been reported in numerous papers and might be caused by inefficient isolation strategies or low sensitivity of the used identification methods. In order to improve the efficiencies of isolation techniques, different model systems have been described. In most of these studies, artificial mixtures of male umbilical cord blood cells, fetal blood cells or male fetal liver cells and adult female peripheral blood mononuclear cells were used (Andrews *et al.*, 1995; Bianchi *et al.*, 1996a; Troeger *et al.*, 1999). In these model systems, different isolation protocols were evaluated using density gradient centrifugation, MACS, FACS or immunomagnetic beads for the isolation of CD71+ NRBCs, after partial depletion of contaminating maternal cells. In addition, Troeger *et al.* (1999) also compared the retrieval of NRBCs using antibodies against GPA, CD36 and the fetal liver surface antigen HAE9.
In the study described in Chapter 2, we have used *in vitro* expanded erythroid cells derived from male umbilical cord blood mixed with female peripheral blood mononuclear cells as a model system for the isolation of fetal cells from maternal blood. In contrast to the previously reported model systems (Andrews *et al.*, 1995; Bianchi *et al.*, 1996a; Troeger *et al.*, 1999),

we were able to obtain a homogenous erythroid cell population, that could be expanded *in vitro* to high cell numbers, i.e. 10^7-10^8 erythroid cells/ml cord blood after 10-15 days of culture, and these cells could be maintained in an erythroblastic cell stage. More importantly, the expanded erythroid cells were immunophenotypically identical to fetal NRBCs derived from 10-20 week fetal liver (Bianchi, 1994b), expressing high levels of CD71. This might indicate that the *in vitro* expanded erythroid cells resemble fetal NRBCs circulating in maternal blood. Nevertheless, it should be mentioned that the most ideal source of fetal material used in any model system for the isolation of fetal cells from maternal blood would be fetal blood of 8-15 weeks of gestation. However, it is technically difficult to obtain fetal blood via cordocentesis before the gestational age of 20 weeks. To overcome this problem, a more physiological model system, which is the isolation of fetal cells after chorionic villus sampling, can be used to optimize fetal cell isolation procedures. We demonstrated, as described in Chapter 4, a significant increase in the number of detectable fetal NRBCs in the maternal circulation directly after chorionic villus sampling. This indicates that maternal blood samples obtained shortly after chorionic villus sampling are more likely to reveal fetal NRBCs, and that these fetal cells are derived from the correct gestational age, i.e. at 12 weeks of gestation, which may be an optimal time in pregnancy for the development of a non-invasive prenatal diagnostic test. This *in vivo* model system has been used by several investigators (Oosterwijk *et al.*, 1996, 1998a, b; de Graaf *et al.*, 1999).

For the isolation of fetal NRBCs, many investigators have used antibodies against CD71, the transferrin receptor (Bianchi *et al.*, 1990; Ganshirt-Ahlert *et al.*, 1992; Lewis *et al.*, 1996; Sohda *et al.*, 1997). CD71 is not only expressed on the entire erythroid lineage (Loken *et al.*, 1987), but also on activated lymphocytes, monocytes, trophoblast cells and any cells incorporating iron (Krantz, 1991). To improve overall efficiency for fetal cell isolation using the CD71 antigen, a strategy based on prior depletion of total white blood cells has recently been devised using monoclonal antibodies against CD45 and CD14 for depletion of lymphocytes and monocytes, respectively (Bianchi *et al.*, 1991; Busch *et al.*, 1994; Reading *et al.*, 1995; Lewis *et al.*, 1996). In this thesis (Chapter 2), we show a significant two-fold higher yield of male NRBCs derived from *in vitro* expanded erythroid cells using a direct enrichment protocol for CD71+ cells compared to the combined depletion/enrichment protocol. This suggests that depletion of lymphocytes and/or monocytes may also lead to loss of fetal cells, which may comprise cells of the erythroid lineage that express CD45 at a low level. Consequently, this will result in a lower yield of fetal NRBCs. A disadvantage of the direct enrichment of CD71+ fetal cells is that the isolated fraction has a low purity due to the presence of a high number of maternal cells. This might hamper the subsequent identification of fetal cells by FISH or PCR analysis.

6.2. *In vitro expansion of fetal cells*

For selective amplification of the target cell population for prenatal genetic diagnosis, a simple conceptual approach may be the *in vitro* cultivation of fetal cells. So far, the number of fetal cells that have been isolated from maternal blood samples is very low, and therefore, it is generally desirable that fetal cells can be selectively expanded *in vitro*. In order to develop a non-invasive prenatal diagnostic test, it is important to obtain maternal blood as early as possible. Fetal hemopoiesis starts in the yolk sac between days 16 and 19 followed by hepatic hemopoiesis at approximately 6 weeks, indicating that fetal hemopoietic progenitor cells may be present in the maternal circulation from week 3 of gestation (Metcalf and Moore, 1971).

Several studies have suggested that fetal progenitor cells differ from those of adults (Forestier *et al.*, 1991; Weinberg *et al.*, 1992). This urges for an approach to exploit these differences for the selective expansion of fetal progenitor cells. It may be possible to use cultures of mononuclear cells obtained from maternal blood to improve the proportion of fetal to adult progeny by manipulation of the culture conditions, and thereby exploiting the growth advantages of fetal cells over maternal ones. This may result in an enhancement of both the relative and absolute number of fetal cells.

The most suitable candidates among the various types of fetal cells that cross the placenta are the hemopoietic progenitor cells. These cells are characterized by the expression of the CD34 cell surface antigen. Previous studies using *in vitro* colony-forming assays as a measure of fetal hemopoietic progenitors have demonstrated that both early (12-24 weeks of gestation) and pre-term (25-32 weeks of gestation) fetal blood samples have a higher frequency of hemopoietic progenitors compared with term umbilical cord blood, adult peripheral blood, and bone marrow, and this frequency declines with advancing gestation (Linch *et al.*, 1982; Clapp *et al.*, 1989; Andreux *et al.*, 1991; De Bruyn *et al.*, 1995). Moreover, Shields and Andrews (1998) recently demonstrated that early second trimester fetal blood produced a significantly greater number of erythroid burst-forming units (BFU-E) compared with umbilical cord blood from term gestations. This higher number of BFU-E may be related to a large number of hemopoietic progenitors committed to red blood cell differentiation because the fetal red blood cell mass is expanding rapidly early in gestation. These early gestation erythroid progenitors also appear to have a greater proliferative response to a given growth factor stimulus relative to those in term umbilical cord blood. Recently, Campagnoli *et al.* (2000) investigated the number and cell characteristics of circulating progenitor cells in first trimester fetal blood and demonstrated a higher number of CD34+ cells compared to term cord blood. This suggests that circulating CD34+ cells are likely to contribute significantly to hematopoiesis in early fetal life. Moreover, short-term

liquid culture of first trimester fetal blood cells revealed extensive *in vitro* proliferation in response to various cytokines.

In our study, described in Chapter 3, we evaluated whether fetal hemopoietic progenitor cells derived from maternal blood samples can be selectively expanded *in vitro* in order to develop a non-invasive prenatal diagnostic test. Although to date some success has been reported (Valerio *et al.*, 1996), most groups have found the degree of expansion insufficient against the higher number of background maternal cells (Little *et al.*, 1997; Chen *et al.*, 1998; Han *et al.*, 1999). To mimic this situation, i.e. low number of fetal cells in an excess of maternal cells, we first used a model system in which limiting numbers of CD34+ umbilical cord blood (UCB) cells were diluted in up to 1 in 400,000 with CD34+ adult peripheral blood cells to determine whether these limited numbers can be selectively expanded. We demonstrated that even very low numbers of CD34+ UCB cells could be expanded 1500-fold, which also corresponded to the expansion of CD34+ cells derived from 20-week fetal blood. Unfortunately, application of the same culture protocol to maternal blood samples obtained at 7-16 weeks of gestation did not show preferential growth of fetal cells. This suggest that primitive fetal cells might either not circulate in maternal blood or may require different combinations and/or concentrations of cytokines for their *in vitro* expansion.

Another question concerns the stability of fetal cell properties. If fetal hemopoietic progenitor cells are present in the maternal circulation, the question arises whether they are clonal and possess unchangeable characteristics or whether these cells are transient and respond to a changing environment. When fetal cell properties are determined, for example surface antigens, this is performed on cells recruited directly from fetal blood or umbilical cord blood samples. However, it is not known whether these cells keep the same marker antigens expressed in the new environment, i.e. the maternal circulation, after crossing the placenta. Therefore, the question arises, especially for cells in culture, as to whether fetal cells after a few rounds of proliferation and differentiation maintain or acquire properties that make them distinguishable from the abundant maternal counterparts.

Further development of *in vitro* expansion approaches will involve detection of differences in optimal growth factor requirements between maternal and fetal hemopoietic progenitor cells in order to exploit them for selective fetal cell expansion. Furthermore, properties of fetal cells circulating in maternal blood and after *ex vivo* cultivation should be investigated.

6.3. *Clinical implications of fetal cells in the maternal circulation*

A major point of interest about fetal cells in the maternal circulation is whether their existence may have an effect on the maternal immune system, especially in cases with high numbers of fetal cells. The incidence of fetal cells in maternal blood has been reported to

increase in pregnancies in which the fetal and placental karyotype was abnormal (Elias *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1993; Simpson and Elias, 1993; Bianchi *et al.*, 1997), after chorionic villus sampling (Chapter 4), and in women with preeclampsia (Chapter 5; Chua *et al.*, 1991; Ganshirt *et al.*, 1994; Holzgreve *et al.*, 1998; Lo *et al.*, 1999).

6.3.1. Aneuploidy

Almost all numerical chromosomal disorders have been detected in fetal cells isolated from maternal blood, including trisomy 13, trisomy 18, trisomy 21, some of the sex chromosome abnormalities and triploidy. In most aneuploid pregnancies, it has been shown that the number of fetal cells in the maternal circulation is increased compared to normal pregnancies (Elias *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1993; Simpson and Elias, 1993; Bianchi *et al.*, 1997). This increased feto-maternal cell trafficking could be the result of altered placental structures, i.e. destruction of the placental feto-maternal barrier (placenta membrane; see Chapter 1, paragraph 1.1.1.), reduced vascularization or disturbed development or function of placental villi (Kuhlmann *et al.*, 1990; Simpson and Elias, 1994; Genest *et al.*, 1995; Jauniaux and Hustin, 1998). This elevated number of fetal cells in chromosomally abnormal pregnancies can also be explained by an increased isolation efficiency of fetal NRBCs, since it has previously been reported that both CD71 expression as well as the number of NRBCs is increased in fetal blood samples of aneuploid fetuses (Thilaganathan *et al.*, 1995; Zheng *et al.*, 1999). Therefore, it might be expected that the isolation of enough fetal cells is facilitated in most aneuploid pregnancies, and hence, may be applicable for prenatal diagnosis in the near future.

6.3.2. The effect of chorionic villus sampling

It has previously been reported that the introduction of a biopsy needle into placental tissue to aspirate chorionic villi via the transabdominal route might induce a feto-maternal transfusion. This was demonstrated by elevated levels of maternal serum alpha-fetoprotein levels measured before and after transabdominal chorionic villus sampling (Los *et al.*, 1989; Rodeck *et al.*, 1993; Smidt-Jensen *et al.*, 1994; Brezinka *et al.*, 1995). Although estimates of the extent of the feto-maternal transfusion indicated that up to 40 % of the fetal plasma volume may have leaked into the maternal circulation, fetal demise is a rare occurrence and has been documented only in sporadic cases (Los *et al.*, 1993). We were interested in the question whether this transfer of fetal plasma was accompanied by a concurrent increase in the number of fetal nucleated cells in the maternal circulation (Chapter 4). We isolated fetal

NRBCs from maternal blood before and after chorionic villus sampling and were able to demonstrate an increase in the number of fetal cells in 10 out of 19 male pregnancies following the invasive procedure. This feto-maternal transfusion not only leads to the transfer of fetal nucleated cells but also results in the leakage of alpha-fetoprotein. However, feto-maternal plasma transfusion estimated on the basis of alpha-fetoprotein concentrations does not reflect a transfusion of a comparable volume of whole blood, because the number of fetal NRBCs detected after chorionic villus sampling is relatively low. This lower level of fetal NRBCs might be explained by removal of these fetal cells by the maternal immune system, by retention of fetal cells in maternal tissues, or by an inefficient isolation procedure. The question remains what the consequence is of these semi-allogeneic cells in the new host.

6.3.3. Feto-maternal cell trafficking in preeclampsia

In Chapter 5, we demonstrated a 30-fold higher number of fetal NRBCs in patients with preeclampsia compared to women with uncomplicated pregnancies. The mechanism of this fetal cell escape is not known. Preeclampsia is related to a poor placental transfusion probably due to an abnormal placentation early in pregnancy. This might result in the release of unknown factors, which in turn will lead to destruction of the maternal vascular endothelium. The abnormal placentation may explain the increased transfusion of fetal products in preeclampsia.
Although we did not demonstrate a significant correlation between the number of fetal cells in the circulation of patients with preeclampsia and the severity of their clinical symptoms, fetal cells are foreign to their maternal host and may have immunological consequences for mother and fetus. Together with a feto-maternal transfusion occurring at the time of labor and delivery, these fetal cells may establish microchimerism in the mother, which may be implicated in the subsequent development of diseases such as scleroderma that are more common in women (Artlett *et al.*, 1998; Nelson, 1998a; Evans *et al.*, 1999). It would, therefore, be of interest to investigate whether autoimmune diseases will occur more often in patients with a previous history of preeclampsia.

6.4. Fetal-maternal immunology: tolerance versus autoimmune disease

The application of molecular biological techniques to the study of human pregnancy has resulted in the recognition of two-directional cell trafficking of nucleated cells between the fetus and the mother (Lo *et al.*, 1996). It has, therefore, been suggested that pregnancy may establish as a long-term, low-grade chimeric state in the human female. Chimera is

mythologically described as having the head of a lion, the body of a goat, and the tail of a serpent. In medicine, the term "chimera" is used to indicate a body that contains cell populations derived from (a) different individual(s).

Fetal cells have been detected in the maternal circulation as early as 4 weeks and 5 days postconception (Thomas *et al.*, 1994). The origins of these cells are presently unknown. It has been suggested that active cellular traffic across the placenta early in gestation is important and perhaps necessary for inducing tolerance to the human fetus. Recently, Bianchi *et al.* (1996b) demonstrated fetal progenitor cells in maternal blood as long as 27 years after birth of a male infant. The establishment of fetal progenitor cells in maternal lymphoid organs or bone marrow may help to maintain tolerance of the fetal graft in a manner analogous to allogeneic organ transplantation. Starzl *et al.* (1993a, b) have demonstrated chimerism resulting from widespread seeding of donor dendritic and hemopoietic cells that were derived from whole organs being transplanted, such as kidney, liver, or intestine. They have postulated that bi-directional cell migration and repopulation is the first step in the acquisition of donor-specific tolerance, and, ultimately, successful graft acceptance. The human pregnancy may therefore benefit from similar one-way or even two-way traffic of fetal and/or maternal cells.

On the other hand, it has previously been hypothesized that microchimerism may contribute to the pathogenesis of some autoimmune diseases, such as scleroderma, systemic lupus erythematosus (SLE), Sjögren's syndrome, Hashimoto's thyrioditis, and primary biliary cirrhosis (Furszyfer *et al.*, 1970; Hochberg, 1985; Danielsson *et al.*, 1990; Kelly *et al.*, 1991; Silman, 1991). Autoimmune diseases are thought of as disorders in which a body's cells inexplicably attack its own tissues. The exposure to fetal cells during pregnancy represents an immunological event because these cells express gene products that are inherited from the father, and are thus foreign to the pregnant woman.

An example of an autoimmune disease in which microchimerism may be involved in the pathogenesis is scleroderma. Scleroderma has a strong predisposition to women with a female-to-male ratio ranging from 3:1 to 8:1, and the highest incidence of scleroderma in women occurs between the age of 35 and 54, generally after the childbearing years (Silman *et al.*, 1988). Scleroderma also has clinical similarities to a known condition of human chimerism: chronic graft-versus-host disease that occurs after allogeneic transplantation of hematopoietic tissue (Furst *et al.*, 1979).

Recently, microchimerism has been studied in women with scleroderma who had previously given birth to at least one son prior to the disease onset. This patient group was compared with healthy controls with at least one son (Nelson *et al.*, 1998a). Male DNA was found more frequently and in greater amounts in women with scleroderma than in healthy controls. This observation was extended in a study by Artlett *et al.* (1998) addressing the important issue as to whether microchimerism could be detected in the primary target organ of scleroderma,

which is the skin. In their study, a Y-chromosome-specific sequence was found more often in DNA extracted from skin biopsy specimens and peripheral blood samples in women with scleroderma than in controls. The majority of women with positive results had previously given birth to a male child, although for some women with positive results the pregnancy history was unknown or included miscarriage but not the birth of a son.

Pregnancy presents an immunological challenge to a woman since half of the genes of the fetus are derived from the father. HLA genes are of particular interest since they encode for molecules that are known to function as classical transplantation antigens and also govern immune responses. It has been suggested that HLA compatibility of a previously born child might constitute a risk factor for the subsequent development of scleroderma in the mother (Nelson, 1996). Accordingly, a strong association between HLA-DRB1 compatibility and scleroderma has recently been demonstrated (Artlett *et al.*, 1997; Nelson *et al.*, 1998b). No association was observed for HLA class I antigens.

The finding of persistent microchimerism of fetal cells, however, does not explain the occurrence of scleroderma in men or in women who have never been pregnant. Alternative sources of microchimerism in those patients include the engraftment of donor cells after a blood transfusion, or from a twin (Nelson, 1996). Another possibility is that microchimerism is derived from maternal cells, since these cells have been detected in cord blood samples, indicating that the traffic of cells during pregnancy is bi-directional (Hall *et al.*, 1995; Lo *et al.*, 1996).

A mechanism by which microchimerism might contribute to the pathogenesis of scleroderma is unknown, but insight can be gained through knowledge acquired from studies of microchimerism in transplantation biology. It is also not known how a small degree of microchimerism might result in either tolerance, breaking of tolerance or graft-versus-host disease. Although fetal cells could be primarily sequestered in the affected tissues, the low concentration of fetal cells argue against a role for these cells as direct effectors of damage to host tissues. Another possibility is that a small population of non-host cells (or peptides) could start a process in which subsequent damage is caused by these host cells. Alternatively, a small population of non-host cells could downregulate host immunoregulatory cells, which would allow damage by autoreactive host cells (Fink *et al.*, 1988).

In conclusion, it seems that microchimerism may account for a balance between a host-versus-graft reaction and a graft-versus-host reaction, leading to acceptance of the allogeneic fetus. Under some circumstances this balance has been disturbed and an autoimmune disease might develop. The reason for disturbance of this balance has not yet been discovered.

6.5. *Conclusions and future research*

The isolation of fetal cells from the maternal circulation has the potential to allow early non-invasive genetic analysis without endangering the fetus. Targeting these cells has proved to be technically challenging because of their low frequency and the absence of suitable unique fetal cell markers and corresponding monoclonal antibodies. To be useful in prenatal diagnosis, fetal cells must be distinguished from the vast majority of maternal cells, they must be enriched to an acceptable level of purity, and must then be identified as cells of fetal origin. A major concern is the identification of female fetal cells necessitating unique fetal markers in combination with FISH or PCR.

Fetal cell detection and genetic analysis by PCR or FISH is hampered by the presence of maternal sequences excluding the detection of maternally inherited and X-linked disorders. Thereby, detection of aneuploidy and paternally inherited disorders is most accessible for prenatal diagnosis using fetal cells isolated from maternal blood. In order to differentiate between maternal and fetal sequences single fetal cells should be retrieved by microdissection.

Despite some promising results from investigations in this field, many of the basic questions regarding the circulation of fetal cells in the maternal blood remain unanswered. Specifically, numerous questions have to be addressed concerning the frequency and the type of fetal cells that circulate in the maternal blood during pregnancy. Especially in normal pregnancies, the possibility of clonal expansion of fetal cells needs to be further explored. Furthermore, techniques that facilitate the identification and isolation of fetal cells needs to be optimized before this technique can be used for prenatal diagnosis.

It has previously been reported by various groups that aneuploid pregnancies are associated with an increased feto-maternal transfusion as a result of altered placental structures. Therefore, it may be expected that a non-invasive prenatal diagnostic test using fetal cells in maternal blood may only be applicable in cases of fetal chromosomal abnormalities. In our opinion, it will not fully replace currently used invasive procedures like chorionic villus sampling and amniocentesis. Instead, the isolation of fetal cells may provide an additional screening test adjunct to the current non-invasive tests, like ultrasound screening and maternal serum screening.

Another question concerns the immunological consequences of the presence of fetal cells in the circulation of pregnant women and after pregnancy. Fetal cells may establish microchimerism in the mother after the occurrence of a feto-maternal transfusion, e.g. after chorionic villus sampling, in patients with preeclampsia, or in pregnancies with a chromosomally abnormal fetus, and even in normal pregnancies. These fetal cells could be engrafted in maternal lymphoid organs or bone marrow. Alternatively, fetal progenitor cells

could remain in the circulation and continue to divide for years. It is also conceivable that in subsequent pregnancies, in the presence of appropriate stimuli, these cells might begin active cell cycling and feed cells of other hemopoietic lineages, such as granulocytes, erythrocytes, macrophages and megakaryocytes. The next question is whether these (persistent) fetal cells might be implicated in the pathogenesis of preeclampsia and/or in the development of autoimmune disorders.

In conclusion, the use of fetal cells isolated from maternal blood remains a promising approach for the development of a non-invasive test. Nevertheless, after more than two decades of research numerous questions remain unsolved. Most of these questions can be answered once technical problems have been overcome, and sensitivity of detection methods has increased. Only then, fetal cell detection in maternal blood may be applied as a screening test for prenatal diagnosis.

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Summary

Summary

Current methods for prenatal diagnosis of genetic abnormalities include chorionic villus sampling and amniocentesis. Although these invasive methods are accurate, there is a small procedure-related risk for the fetus. The development of an equally reliable non-invasive prenatal diagnostic test using fetal blood cells that leak through the placenta into the maternal circulation would eliminate this small but significant risk.

Chapter 1 comprises a literature appraisal about the current knowledge of fetal cells in maternal blood. The fetal cell types present in maternal blood that have been studied by numerous investigators include fetal lymphocytes, granulocytes, trophoblast cells and nucleated red blood cells (NRBCs). Most attention has been focussed on the isolation of fetal NRBCs, since they are abundantly present in the fetus during the first trimester of pregnancy, they have a limited life span and may not persist from previous pregnancies. Fetal NRBCs have been isolated using antibodies against both membrane-bound markers, including the transferrin receptor (CD71) and glycophorin A (GPA) and intracellular antigens, like embryonic (HbE) and fetal (HbF) hemoglobin. Despite all efforts to develop enrichment and purification strategies that would increase the detectability of fetal cells in a maternal blood sample, the number of fetal cells recovered still remains very low. Two major methods of cell separation enable fetal cell isolation from maternal blood: fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). If fetal cells are conclusively isolated, genetic analysis of these cells include fluorescence *in situ* hybridization (FISH) using chromosome-specific probes for the detection of chromosomal abnormalities, and polymerase chain reaction (PCR) to amplify unique fetal gene sequences for the detection of gene abnormalities.

Our research has primarily been focussed on the technical and biological aspects of fetal cell isolation. Since the number of fetal cells in maternal blood is very low, their isolation is technically challenging and requires extensive enrichment procedures before any analytical procedure can be performed. In Chapter 2, we describe the use of *in vitro* expanded erythroid cells in a model system for the isolation of fetal cells from maternal blood. Erythroblast cells derived from male umbilical cord blood cells were *in vitro* expanded to high cell numbers. These cells could be maintained in an erythroblastic stage and expressed high levels of CD71, a marker frequently used for the isolation of fetal cells from maternal blood. Two approaches of MACS isolation were evaluated using a mixture of *in vitro* expanded male NRBCs diluted in up to 1 in 400,000 female peripheral blood mononucleated cells. The first strategy was based on the direct enrichment of CD71+ cells using a one-step MACS isolation protocol. This isolation procedure was compared with a previously published and commonly used two-step technique based on depletion of monocytes (CD14) and lymphocytes (CD45)

followed by the enrichment of CD71+ cells. The number of recovered male cells was determined using two-color FISH with X and Y chromosomal probes. We demonstrated a significant two-fold higher yield of male NRBCs using the one-step enrichment protocol. Application of these isolation strategies to maternal blood samples resulted in a similarly improved enrichment of male fetal cells after the direct enrichment of CD71+ cells. An interesting question remains as to whether the described expansion protocol for erythroid cells can also be used for expansion of fetal NRBCs derived from maternal blood samples.

The idea to increase the number of fetal cells from maternal blood by amplification of fetal hemopoietic progenitor cells has been discussed for a long time. If fetal cells could be stimulated to proliferate in culture, the technical limitations of working with very small number of cells could be overcome. In Chapter 3, we evaluated the usefulness of the *in vitro* expansion of fetal hemopoietic progenitor cells (CD34+) from maternal blood samples for diagnostic purposes. After MACS isolation, fetal cells are often contaminated with an excess of maternal cells and the question was whether fetal cells are able to overgrow the maternal component. Therefore, a model system was used in which limiting numbers of male CD34+ umbilical cord blood (UCB) cells were diluted in up to 400,000 female CD34+ peripheral blood cells. We demonstrated a 1500-fold increase of male UCB cells over the female peripheral blood component after 3 weeks of liquid culture, which also corresponded to the extent of expansion of CD34+ cells derived from 20-week fetal blood. Unfortunately, we were not able to confirm these results using fetal CD34+ cells isolated from maternal blood samples obtained at 7-16 weeks of gestation, indicating that hemopoietic progenitor cells do either not circulate in maternal blood before 16 weeks of gestation, or require different combinations and/or concentrations of cytokines for their *in vitro* expansion.

The presence of fetal cells in maternal blood is supposed to be the result of a feto-maternal transfusion at the placental interface. The incidence of fetal cells in maternal blood has been reported to increase in chromosomally abnormal pregnancies, after chorionic villus sampling (Chapter 4), and in women with preeclampsia (Chapter 5). It has previously been reported that the introduction of a biopsy needle into placental tissue to aspirate chorionic villi via the transabdominal route might induce a feto-maternal transfusion. Such a transfusion has been demonstrated by elevated levels of maternal serum alpha-fetoprotein levels after chorionic villus sampling (CVS). In Chapter 4, we investigated whether this transfusion of alpha-fetoprotein was accompanied by a concurrent transfusion of fetal NRBCs in the maternal circulation. We isolated fetal CD71+ cells from maternal blood samples obtained before and after chorionic villus sampling, after depletion of maternal lymphocytes (CD45+ cells) and monocytes (CD14+ cells). Analysis of these CD71+ fractions revealed an increase in the number of fetal cells in 10 out of 19 male pregnancies after the invasive procedure, which also correlated with elevated maternal serum alpha-fetoprotein levels. Moreover, this

CVS-induced cellular transfusion may form an interesting *in vivo* model system, which can be used to improve isolation procedures and to study fetal cell dynamics and characteristics.
Several recent reports suggest that fetal cell trafficking into the maternal circulation is disturbed in preeclampsia. Preeclampsia is a common, pregnancy specific disease defined by clinical findings of elevated blood pressure combined with proteinuria and edema. Although the etiology of preeclampsia is not known, there are indications that abnormal placentation and endothelial dysfunction are involved in the pathogenesis of preeclampsia. In Chapter 5, we investigated whether this abnormal placentation results in a transfusion of fetal NRBCs in the maternal circulation of women with preeclampsia. We demonstrated a 30-fold higher number of fetal NRBCs in women with preeclampsia compared to women with uncomplicated pregnancies, suggesting that fetal cell trafficking is enhanced in women with preeclampsia. Furthermore, this feto-maternal transfusion may provide insight into the pathophysiology of the disease.
In conclusion, the isolation of fetal cells from maternal blood has proved to be technically challenging because of the low frequency of these cells in the maternal circulation requiring extensive enrichment procedures for their subsequent isolation. To be useful in prenatal diagnosis, fetal cells must be distinguished from the vast majority of maternal cells, they must be enriched to an acceptable level of purity, and must then be identified as cells of fetal origin. One promising technique may be the *in vitro* expansion of fetal cells in order to overcome the technical limitations of working with very small numbers of cells. Erythroid as well as other hemopoietic progenitor cells have been successfully expanded, but the technique has to be further improved.
A major point of concern remains the possibility that fetal cells from previous pregnancies persist in the maternal circulation, not only in view of the development of a non-invasive prenatal diagnostic test, but also because of their immunological consequences. Especially in cases with an increased feto-maternal transfusion, fetal cell microchimerism may have immunological consequences and may subsequently result in the development of an (auto)immune disease in these women.
In conclusion, the use of fetal cells isolated from maternal blood remains a promising approach for the development of a non-invasive prenatal diagnostic test. Nevertheless, after more than two decades of research numerous questions remain unsolved. Most of these questions can be answered once technical problems have been overcome, and the sensitivity of the detection methods has been improved. Only then, fetal cell detection in maternal blood may be applied as a screening test for prenatal diagnosis.

Samenvatting

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De huidige methoden voor prenatale diagnostiek voor de detectie van genetische afwijkingen omvatten met name de vlokkentest en vruchtwaterpunctie. Hoewel deze invasieve methoden zeer nauwkeurig zijn, is er toch een klein risico voor de foetus gerelateerd aan de procedure. De ontwikkeling van een gelijkwaardig betrouwbare niet-invasieve prenatale diagnostische test zal dit kleine, maar significante risico elimineren. Bij deze test wordt gebruik gemaakt van foetale cellen die via de placenta het perifere bloed van de moeder bereiken.

Hoofdstuk 1 omvat een literatuurstudie over de huidige kennis van de isolatie van foetale cellen uit moederlijk bloed. Verschillende foetale celtypen circuleren in het moederlijke bloed, zoals foetale lymfocyten, granulocyten, trofoblastcellen en kernhoudende rode bloedcellen (KRBCs). De meeste aandacht wordt besteed aan de foetale KRBCs, omdat zij in grote aantallen voorkomen in het moederlijke bloed, met name in het eerste trimester. Bovendien hebben zij een korte levensduur waardoor de aanwezigheid van KRBCs van een eerdere zwangerschap vrijwel uitgesloten is. Foetale KRBCs worden geïsoleerd door gebruik te maken van antilichamen gericht tegen zowel membraan-gebonden merkers, zoals de transferrine receptor (CD71) en glycoforine A (GPA), als tegen intracellulaire antigenen, zoals embryonaal (HbE) en foetaal (HbF) hemoglobine. Ondanks alle inspanningen om verrijkings- en zuiveringsstrategieën te ontwikkelen die het aantal foetale cellen in moederlijk bloed zouden vergroten, blijft het aantal geïsoleerde foetale cellen erg klein. Twee technieken die veel gebruikt worden voor de isolatie van foetale cellen uit moederlijk bloed zijn: Fluorescent-Activated Cell Sorting (FACS) en Magnetic-Activated Cell Sorting (MACS). Indien foetale cellen daadwerkelijk geïsoleerd zijn, worden deze cellen op genetisch niveau geïdentificeerd. Chromosomale abnormaliteiten worden gedetecteerd middels fluorescentie *in situ* hybridisatie (FISH) door gebruik te maken van chromosoom-specifieke probes. De detectie van afwijkingen op genniveau geschiedt door middel van amplificatie van unieke foetale gensequenties met behulp van de polymerase ketting reactie (PCR).

Het onderzoek beschreven in dit proefschrift richt zich met name op de technische en biologische aspecten van de isolatie van foetale cellen uit moederlijk bloed. Aangezien het aantal foetale cellen in moederlijk bloed bijzonder klein is, wordt de isolatie van deze cellen bemoeilijkt, en zijn geavanceerde verrijkingsmethoden noodzakelijk voordat foetale cellen genetisch geïdentificeerd kunnen worden. In Hoofdstuk 2 wordt het gebruik van *in vitro* geëxpandeerde erythroide cellen in een modelsysteem voor de isolatie van foetale cellen uit moederlijk bloed beschreven. Erythroblast cellen geïsoleerd uit mannelijk navelstrengbloed werden *in vitro* geëxpandeerd tot grote aantallen. Deze cellen konden behouden worden in een erythroblast stadium en ze expresseren een hoog niveau van CD71, een merker die vaak gebruikt wordt voor de isolatie van foetale cellen uit moederlijk bloed. Twee MACS

isolatiestrategieën werden geëvalueerd door gebruik te maken van een mengsel van *in vitro* geëxpandeerde mannelijke KRBCs die 400.000 keer verdund werden in kerndragende cellen afkomstig van perifeer bloed van vrouwelijke, niet-zwangere vrijwilligers. De eerste isolatiestrategie was gebaseerd op de directe verrijking van CD71+ cellen door gebruik te maken van een één-staps MACS isolatieprotocol. Deze isolatieprocedure werd vergeleken met een eerder beschreven en een vaak gebruikte methode, namelijk een twee-staps techniek waarbij eerst de monocyten (CD14+) en lymfocyten (CD45+) verwijderd worden, gevolgd door de verrijking van CD71+ cellen. Het aantal verkregen mannelijke CD71+ cellen na MACS isolatie werd bepaald met behulp van een twee-kleuren FISH met X- en Y-chromosomale probes. Het één-staps MACS protocol bleek een twee keer zo grote opbrengst van mannelijke KRBCs op te leveren dan de twee-staps strategie. Toepassing van beide isolatieprotocollen op moederlijke bloedmonsters resulteerde in een vergelijkbaar verbeterde verrijking van mannelijke foetale cellen na de directe verrijking van CD71+ foetale cellen (één-staps protocol). Een interessante vraag is of het beschreven expansieprotocol voor erythroide cellen ook gebruikt kan worden voor de expansie van foetale KRBCs geïsoleerd uit moederlijke bloedmonsters.

Het idee om het beperkte aantal foetale cellen uit moederlijk bloed te vermenigvuldigen door foetale hemopoietische stamcellen (CD34+ cellen) te kweken, wordt al een lange tijd besproken. Als foetale cellen gekweekt kunnen worden, dan zouden de technische beperkingen door te werken met hele kleine aantallen cellen omzeild kunnen worden. In Hoofdstuk 3 wordt geëvalueerd of de *in vitro* expansie van foetale hemopoietische stamcellen uit moederlijke bloedmonsters toegepast kan worden voor de ontwikkeling van een niet-invasieve prenatale diagnostische test. Aangezien na MACS isolatie van foetale cellen uit moederlijk bloed vrijwel altijd een overmaat aan moederlijke cellen aanwezig is, was de eerste vraag of de geïsoleerde foetale cellen in staat zijn om boven de moederlijke component uit te groeien. Om dit aan te tonen hebben we gebruik gemaakt van een modelsysteem waarbij hele kleine aantallen CD34+ stamcellen uit mannelijk navelstreng bloed 400.000 maal verdund zijn in CD34+ stamcellen afkomstig uit perifeer bloed van een vrouwelijke, niet zwangere, volwassen vrijwilliger. Na drie weken kweken bleken de mannelijke CD34+ stamcellen uit navelstrengbloed 1500 maal vermenigvuldigd te zijn in vergelijking tot de vrouwelijke volwassen CD34+ stamcellen. Deze expansie van mannelijke CD34+ navelstrengbloed cellen correspondeerde met de mate van expansie van CD34+ cellen afkomstig uit foetaal bloed van 20 weken. Expansie van foetale hemopoietische stamcellen (CD34+) verkregen uit moederlijke bloedmonsters afgenomen tussen 7 en 16 weken van de zwangerschap was echter niet succesvol. Dit betekent enerzijds dat foetale hemopoietische stamcellen niet aanwezig zijn in het bloed van zwangeren voor 16 weken van de zwangerschap, of anderzijds dat andere combinaties of concentraties van

groeifactoren noodzakelijk zijn voor de *in vitro* expansie van foetale stamcellen aanwezig in moederlijk bloed.

De aanwezigheid van foetale cellen in moederlijk bloed is mogelijk het gevolg van een cellulaire transfusie tussen moeder en kind (feto-maternale transfusie) op het niveau van de placenta. Het is eerder beschreven dat de frequentie van foetale cellen in de moederlijke circulatie verhoogd is wanneer het een zwangerschap betreft met een chromosomaal afwijkend kind. Ook na vlokkentest (Hoofdstuk 4) en bij vrouwen met pre-eclampsie (Hoofdstuk 5) wordt een verhoogd aantal foetale cellen gevonden in het moederlijk bloed. Recentelijk is gebleken dat de introductie van een biopticnaald in placentaweefsel, om chorionvlokken op te zuigen via een transabdominale route, mogelijk een feto-maternale transfusie veroorzaakt. Dit is aangetoond door de aanwezigheid van verhoogde concentraties van het alfa-foetoproteïne in moederlijk serum na het uitvoeren van een vlokkentest. In Hoofdstuk 4 onderzoeken we of deze transfusie van foetale eiwitten samen gaat met een transfusie van foetale KRBCs in de moederlijke perifere circulatie. Foetale CD71+ cellen zijn geïsoleerd uit moederlijke bloedmonsters die afgenomen zijn zowel voor als na de vlokkentest. Allereerst zijn de moederlijke lymfocyten (CD45+ cellen) en monocyten (CD14+ cellen) gedepleteerd, gevolgd door de verrijking van CD71+ cellen middels MACS-isolatie. Analyse van de CD71+ celfracties toonde aan dat na de invasieve ingreep een verhoogd aantal foetale cellen aanwezig was in 10 van de 19 zwangerschappen. Deze verhoogde transfusie van foetale cellen correleerde met een verhoogde concentratie van alfa-foetoproteïne in het moederlijke serum. Bovendien vormde deze door de vlokkentest geïnduceerde cellulaire transfusie een goed *in vivo* modelsysteem, dat gebruikt kan worden voor verbetering en optimalisatie van isolatieprocedures, en voor het bestuderen van de dynamiek en karakteristieken van foetale cellen in het moederlijk bloed.

In verschillende recente studies is onderzocht of de uitwisseling van foetale en moederlijke cellen verstoord is bij patiënten met pre-eclampsie (zwangerschapsvergiftiging). Pre-eclampsie is een ernstige, veelvoorkomende aandoening in de zwangerschap, die gekenmerkt wordt door verhoogde bloeddruk en eiwitverlies in de urine. De ontstaanswijze van pre-eclampsie is onbekend, maar er zijn aanwijzingen dat abnormale ontwikkeling van de placenta en vaatwandbeschadiging (endotheel) bij de moeder een belangrijke rol spelen. In Hoofdstuk 5 wordt onderzocht of deze abnormale ontwikkeling van de placenta resulteert in een transfusie van foetale cellen in de perifere circulatie. In het bloed van vrouwen met pre-eclampsie bleken 30 maal zoveel foetale KRBCs aanwezig te zijn in vergelijking met bloed van vrouwen met een ongecompliceerde zwangerschap. Dit suggereert dat er bij vrouwen met pre-eclampsie een verhoogde transfusie plaatsvindt van foetale cellen. Mogelijk zou deze verhoogde feto-maternale transfusie meer inzicht kunnen geven in de ontstaanswijze van pre-eclampsie.

Concluderend kan gesteld worden dat de isolatie van foetale cellen uit moederlijk bloed ernstig bemoeilijkt wordt door de zeer lage frequentie van deze cellen. Om deze zeer zeldzame foetale cellen te isoleren zijn effectieve verrijkingsmethoden noodzakelijk. Voor de ontwikkeling van een niet-invasieve prenatale diagnostische test moeten foetale cellen onderscheiden worden van moederlijke cellen, vervolgens moeten ze geïsoleerd worden tot een acceptabel zuiverheidniveau voor analyse, en tenslotte moeten ze geïdentificeerd worden als zijnde foetaal. Een veelbelovende techniek is de *in vitro* expansie van foetale cellen waarmee de technische beperkingen van het werken met zeer kleine aantallen foetale cellen omzeild kunnen worden. Zowel foetale erythroïde cellen als hemopoietische stamcellen zijn recentelijk succesvol geëxpandeerd. Het blijft echter noodzakelijk om deze klonale expansie verder te evalueren en te verbeteren.

Een groot probleem bij de isolatie van foetale cellen uit moederlijk bloed blijft echter de mogelijkheid dat foetale hemopoietische cellen van een eerdere zwangerschap blijven circuleren in de moederlijke circulatie. Dit is niet alleen belangrijk met het oog op de ontwikkeling van een betrouwbare niet-invasieve prenatale diagnostische test, maar zeker ook met betrekking tot de immunologische reactie die de aanwezigheid van foetale cellen mogelijk veroorzaakt. Met name in gevallen van een verhoogde feto-maternale transfusie kan microchimerisme van foetale cellen immunologische consequenties hebben, en mogelijk resulteren in de ontwikkeling van een (auto)immuunziekte bij deze vrouwen.

De ontwikkeling van een niet-invasieve prenatale test door gebruik te maken van foetale cellen uit moederlijk bloed blijft een veelbelovende techniek. Desalniettemin zijn na meer dan 20 jaar onderzoek nog steeds een aantal belangrijke vragen onbeantwoord. De meeste vragen zullen beantwoord worden wanneer de technische problemen van foetale celisolatie opgelost zijn, en de sensitiviteit van de detectiemethoden verbeterd is. Hopelijk zal daardoor in de toekomst foetale celdetectie gebruikt kunnen gaan worden als screeningstest voor prenatale diagnostiek.

List of abbreviations

AGM	Aorta-gonad-mesonephros
BFU-E	Erythroid burst-forming unit
CD71	Transferrin receptor
CFS	Charge flow separation
CFU-E	Erythroid colony-forming unit
CVS	Chorionic villus sampling
FACS	Fluorescence activated cell sorting
FB	Fetal blood
FISH	Fluorescence *in situ* hybridization
FMT	Feto-maternal transfusion
GPA	Glycophorin A
HbA	Adult hemoglobin
HbE	Embryonic hemoglobin
HbF	Fetal hemoglobin
HELLP	Hemolysis, elevated liver enzymes, and low platelets
HLA	Human leukocyte antigen
KRBCs	Kernhoudende rode bloedcellen
MACS	Magnetic activated cell sorting
MSAFP	Maternal serum alpha-fetoprotein
NRBCs	Nucleated red blood cells
PB	Peripheral blood
PCR	Polymerase chain reaction
RhD	Rhesus D
SRY	Sex-determining region Y
TA-CVS	Transabdominal chorionic villus sampling
UCB	Umbilical cord blood

Curriculum Vitae

1968	Geboren te Spijkenisse
1981-1988	VWO-B, OSG "De Ring van Putten" te Spijkenisse
1988-1993	Studie Biomedische Wetenschappen, Rijksuniversiteit Leiden Keuzevakken: - Kindergeneeskunde, Immunologische Laboratorium, Academisch Ziekenhuis Leiden (Dr. M.J.D. van Tol) - Klinische Oncologie, Academisch Ziekenhuis Leiden (Dr. P.I. Schrier) - Anthropogenetica, Sylvius Laboratorium, Leiden (Dr. E. Bakker; Mw. Dr. M. Skraastad) - Inleiding beleid en organisatie (RUU) - Inleiding management en organisatie (RUU) Hoofdvak: Moleculaire en cellulaire immunologie, Immunohaematologie en Bloedbank, Academisch Ziekenhuis Leiden (Dr. P.E. van den Elsen; Mw. Dr. L. Struijk)
1994-1995	Wetenschappelijk medewerker bij de afdeling Immunohaematologie en Bloedbank, Academisch Ziekenhuis Leiden
1995-2000	Assistent in Opleiding (AIO) bij de afdeling Verloskunde en Vrouwenziekten aan de Erasmus Universiteit, o.l.v. Prof. Dr. J.W. Wladimiroff. Cursussen: Strategies for purification, sequencing, and structural analysis of proteins, gene expression and intracellular localization of gene products; Oncogenesis and tumor biology; The Oxford examination in English as a foreign language, higher level
2000-heden	Post-doc bij de afdeling Verloskunde en Vrouwenziekten aan de Erasmus Universiteit, o.l.v. Prof. Dr. J.W. Wladimiroff en Mw. Dr. C.J.M. de Groot

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