Approximately half of the erythroblasts in maternal blood are of fetal origin

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The enrichment of fetal erythroblasts from the peripheral blood of pregnant women is currently actively pursued for the development of a non-invasive means of prenatal diagnosis. Since erythroblasts in maternal blood are not all of fetal origin, and currently no reliable method exists to distinguish between the maternal and fetal erythroblasts, their use for prenatal diagnosis is not without uncertainty. The purpose of this study was to determine the percentage of fetal erythroblasts in maternal blood at the single cell level and to what extent such cells can reproducibly be used for polymerase chain reaction (PCR)-based prenatal diagnostic analyses. Erythroblasts were enriched from the peripheral blood of rhesus negative pregnant women using magnetic cell sorting (MACS). Single erythroblasts identified morphologically were individually micro-manipulated and analysed by a multiplex PCR reaction for the fetal "SRY" and "rhesus D" genes. As a control for the PCR reaction the "β-globin" gene was used. The PCR results were validated by the results obtained by invasive procedures. In all instances where single erythroblasts were examined, the correct fetal genotype for the two fetal specific loci was detected. Furthermore, our results indicate that ~50% of the enriched erythroblasts are of fetal origin.

Key words: fetal erythroblasts/maternal blood/prenatal diagnosis/single cell PCR

Introduction
Currently used methods to obtain fetal material for prenatal diagnosis are potentially dangerous as they bear a small, but significant procedure related risk. This has lead to the effort by many groups to develop methods to recover fetal cells by non-invasive means, such as their enrichment from the peripheral blood of pregnant women (Hahn et al., 1998a).

The problem, however, is that fetal cells in maternal blood are rare, with their frequency being estimated between 1 fetal cell in 10⁵ to 10⁷ maternal cells in normal primigravidae (Hamada et al., 1993; Bianchi et al., 1997). Although their frequency is proposed to be increased in fetal aneuploidies and pregnancy related diseases, e.g. pre-eclampsia (Bianchi et al., 1997; Holzgreve et al., 1998), a sophisticated technique is required for their enrichment. Currently the most commonly employed techniques are fluorescent activated cell sorting (FACS) (Bianchi et al., 1990), magnetic cell sorting (MACS) (Holzgreve et al., 1992), magnetic colloid (Steele et al., 1996), charge flow separation (Wachtel et al., 1998) or step density gradients (Oosterwijk et al., 1998). In our hands, we achieved the greatest recovery of erythroblasts with a combination of a single high density Ficoll gradient (1119 g/l) and MACS for glycophorin A (Troeger et al., 1999).

Erythroblasts in the peripheral blood of pregnant women are of both maternal and fetal origin (Ganshirt et al., 1994; Slunga Tallberg et al., 1995; Holzgreve et al., 1998). Currently it is still uncertain what percentage of them is fetal. Although previous studies using different enrichment procedures and detection methods have estimated that 30–50% of enriched erythroblasts are of fetal origin (Sekizawa et al., 1996; von Eggeling et al., 1997; Oosterwijk et al., 1998; Wachtel et al., 1998), these data were, however, not very conclusive as they were largely based on the analysis of only one locus.

Since it is particularly important to know what fraction of the erythroblasts in maternal blood is fetal when considering their use for the prenatal diagnosis of single gene disorders, we have set out to answer this question in a systematic manner by using two loci to determine fetal origin. To achieve this goal we exploited the absence of the "rhesus D" gene and the Y chromosome specific "SRY" gene in rhesus negative D pregnant women. Hence, by the use of a multiplex polymerase chain reaction (PCR) reaction we were able to rapidly and simply screen a large number of erythroblasts.

Materials and methods
Separation and enrichment of erythroblasts

Only women undergoing an invasive prenatal diagnostic test for other indications were recruited. All participants were well informed regarding the nature of the study, and signed informed consent was obtained. Peripheral blood (15 ml) was drawn prior to the invasive procedure from 19 normal singleton pregnant rhesus negative women; seven of whom were primigravidae. The mean gestational age was 14±4 weeks, ranging from 11 to 17±6 weeks. In parallel, we obtained surplus fetal cells from the chorionic villus and amniocyte cultures respectively, to enable fetal rhesus typing and sex determination for
specific amplification of the single cell PCR results, in addition to the fetal karyotype obtained by classical cytogenetic methods.

The blood samples were processed within 24 h, using the enrichment procedure we have recently described (Troeger et al., 1999). In brief, the mononucleated cells were separated with a single density gradient using Ficoll-Paque-1119® (Sigma, St Louis, MO, USA). The ring containing mononuclear cells was removed with a wide-gauge needle and washed twice with phosphate-buffered saline (PBS). The cells were resuspended in PBS containing 1% bovine serum albumin (BSA) (Sigma), incubated with anti-GPA microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated using miniMACS separation columns according to the manufacturer’s instructions. Cytospin preparations of the recovered cells were placed onto glass slides (Shandon, Frankfurt, Germany) and differentially stained with May–Grünwald–Giemsa stain (Sigma). The percentage of erythroblasts was enumerated at least 200 nucleated cells on a cytospin preparation. The number of recovered nucleated cells was determined in a haemocytometer. The yield of erythroblasts was then calculated by the number of recovered nucleated cells times the percentage of erythroblasts.

Visual detection, manipulation and digestion of erythroblasts

Erythroblasts were recognized visually using an inverse microscope at ×400 magnification (Axiovert 100; Zeiss, Jena, Germany). Only those cells that met the following criteria were considered for micromanipulation: low nucleus-to-cytoplast ratio, dense and small nucleus, orthochromatic and not granular cytoplast. Erythroblasts were detached individually from the glass slides using an extended micropipet (Drummond Scientific, Broomall, PA, USA) and a micromanipulator (Zeiss). Each erythroblast was transferred to a separate PCR tube containing 5 µl of 400 ng/µl Proteinase K and 17 µmol/l sodium dodecyl sulphate (SDS). The solution was overlaid with mineral oil and incubated at 50°C for 1 h, followed by 99°C for 30 min to extract DNA and degrade protein.

Specific amplification of the β-globin, rhesus D and SRY genes

A multiplex PCR was performed to amplify the β-globin, rhesus D and SRY genes simultaneously. The PCR for the β-globin gene was performed as a semi-nested PCR reaction using primers of the following sequences: external 5’ TCC TGA GGA GAA GTG TGC CG 3’, external and internal 5’ ACA GCA TCA GGA GTG GAC AG 3’, and internal 5’ GTG AAC GTG GAT GAA GTT GG 3’. The amplification of the SRY locus was done in a fully nested manner using the following pairs of primers: external 5’ GTG TCC TCT CGT TTT GTG AC 3’ and 5’ GAA TCA TCG TCT TTG AAT AC 3’, and internal 5’ TGG GGA TTA AGT CAA ATT CGA 3’ and 5’ CTA GTA CCC TGA CAA TGT ATT C 3’ respectively. In parallel the rhesus D gene was amplified with three primers by semi-nested PCR: external 5’ AGG GTG GTT GTA ACC GAG T 3’, and internal 5’ CCA CAT GGC ATT GGC GGC T 3’, and internal 5’ CCC CAC AGC TCC ATC ATG 3’. A total of 45 µl of PCR mix containing 25 pmol of each external primer pair, 1.5 mmol/l MgCl2, 300 mmol/l dNTPs, and 2 IU Taq Polymerase (Promega, Madison, WI, USA) was added to each reaction. Amplification was carried out following a hot start at 95°C for 5 min with 30 subsequent cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s. 1 µl aliquots were removed from the external PCR reaction tubes and placed in 20 µl PCR mix containing 10 pmol of either one of the internal primer pairs, 1.5 mmol/l MgCl2, 300 mmol/l dNTPs, and 2 IU Taq Polymerase. The amplification was performed as above. PCR products were analysed on a 2% agarose gel and visualized using the Geldoc® analysis system (Molecular Analyst 1.4; Bio-Rad Laboratories, Hercules, CA, USA). Specific amplification of the β-globin gene, SRY locus and rhesus D gene resulted in 240, 133 and 118 bp products respectively (Figure 1).

If only the β-globin gene was detected the cell was considered to be of maternal origin. If the single cell PCR revealed the same result as the fetal DNA obtained by the invasive procedures, the cell was considered as being of fetal origin. Fetal DNA obtained from cultured chorionic villus cells or amniocytes were used for confirmation of the fetal genotype.

Precautions against contamination

As the single cell PCR is a very sensitive method, strict precautions had been taken to avoid any contamination and to verify the results. The enrichment procedures were carried out under a laminar flow hood; each case being treated individually. Cytospins were stored in separate boxes. The micromanipulation was done in a separate room. Each cell was picked with an individual glass micropipet, which was discarded after the procedure. PCR was performed under a separate laminar flow hood, and PCR products were analysed in a further separate room. For all procedures disposable gloves and aerosol resistant filter tips were used. Additionally all pipettes were exposed to UV radiation prior to the experiments. Negative controls (picked erythrocyte and water blanks) were included and a positive control (human male rhesus D positive DNA) was run in parallel with each case examined.

Results

Enrichment procedures for erythroblasts were carried out on 19 blood samples obtained from rhesus-negative pregnant women. We were able to detect erythroblasts in 14 of these 19 cases. The number of fetal erythroblasts obtained differed in each case (see Table I). On average 1385 erythroblasts could be enriched from 15 ml of peripheral blood, ranging from 0 to 5200. In the 14 cases with erythroblasts a mean of 27 were determined to be of fetal origin by either the presence of the rhesus D and/or the SRY locus. Assuming that cell loss during micromanipulation and lack of complete amplification
is random, these results imply that on average half of the enriched erythroblasts are of fetal origin.

**Discussion**

The enrichment of fetal cells from the peripheral blood of pregnant women and their subsequent analysis for genetic disorders represents a challenge as they are very rare in frequency (Hamada *et al.*, 1993; Bianchi *et al.*, 1997). Most groups have selected the fetal erythroblast as their target cell type (Bianchi *et al.*, 1990; Price *et al.*, 1991; Holzgreve *et al.*, 1992). Unlike lymphocytes, the fetal erythroblast has a short life span of ~90 days, which makes it unlikely that a false diagnosis results from fetal cells which have persisted from a previous pregnancy. A further advantage is that erythroblasts express intracellular proteins, such as fetal haemoglobin and several surface antigens, such as the transferrin receptor, blood type antigens and possible erythroid specific antigens such as HAE9 or the erythropoietin receptor, which may be useful for their enrichment and detection (Bianchi *et al.*, 1993; Valerio *et al.*, 1997; Oosterwijk *et al.*, 1998; Troeger *et al.*, 1999).

A major problem, however, is that the erythroblasts present in the peripheral blood of pregnant women are of both maternal and fetal origin (Ganshirt *et al.*, 1994; Slunga Tallberg *et al.*, 1995; Holzgreve *et al.*, 1998), and that no reliable way exists to distinguish between the two groups, nor is there any clear indication as to what fraction is fetal.

In our approach to address this question, we used the rhesus *D* and the *SRY* genes to differentiate between both the maternal and fetal genome, by taking advantage of their absence in rhesus *D* negative pregnant women. As both these markers have already been analysed at the single cell level for preimplantation genetic diagnosis (Chong *et al.*, 1993; Van den Veyver *et al.*, 1995), their choice should facilitate a simple screening of numerous single cells. In addition, as both genes are present as single copies in the fetal genome and are absent in the maternal genome, single cell analysis should be easier to accomplish than by using heterozygous loci, such as micro-satellites, where allele drop-out can lead to erroneous results (El-Hashemite and Delhanty, 1997; Garvin *et al.*, 1998; Hahn *et al.*, 1998b).

It is interesting to note that, even though we have taken considerable steps to improve our enrichment procedure, erythroblasts were only detected in 14 out of the 19 cases examined (74%) and that a significant variation in the numbers recovered was noted (0–5200). These data are in accordance with our own previous observations (Ganshirt *et al.*, 1994, 1998; Holzgreve *et al.*, 1998) and those of other groups, where erythroblasts were recovered with similar frequencies even though different enrichment procedures had been used (Garcia Lloret *et al.*, 1994; Sekizawa *et al.*, 1996; von Eggeling *et al.*, 1997; Valerio *et al.*, 1997; Wachtel *et al.*, 1998).

In our PCR analysis, an informative result was obtained in 54 out of the 128 cells examined. This low degree of amplification efficiency can probably to some extent be related to cell loss during the micromanipulation, since the final transfer of the picked cell to the PCR tube is not under visual control. Several researchers have resorted to the procedure of cell pooling in order to overcome this deficiency (Cheung *et al.*, 1996; Chan *et al.*, 1998). However, this was not an option in our study as we needed to examine individual cells in order to determine what fraction of the enriched erythroblasts is fetal. It is possible that cell loss might be circumvented by laser-assisted micromanipulation and automatic transfer directly into the PCR tube (Schütze and Clement-Sengewald, 1994). Additionally, some template degradation may have occurred during the course of the enrichment and staining procedures (Reading *et al.*, 1995; Cheung *et al.*, 1996). Another possible explanation for the low amplification efficiency is that it depends to some extent on the cell type used, which may be particularly relevant for mature erythroblasts, as they are close to terminal
Fetal erythroblasts in maternal blood


Received on June 2, 1999; accepted on September 9, 1999

Acknowledgements

This work has been supported by the Swiss National Science Foundation (3200–047112.96), the National Institute of Health (Contract Number N01-HD-4–3202) and the German Research Foundation (Tr 452/1–1).

References


differentiation and nucleus extrusion, and may hence, exhibit an increased degree of DNA damage.

Despite these problems, we were nevertheless able to correctly discern fetal genotype for the two fetal (SRY and *rhesus D*) loci in all instances where single erythroblasts were examined. Furthermore, our results show that ~50% of the erythroblasts examined (27 out of 54) were of fetal origin by the presence of either one or both of the *SRY* or *rhesus D* loci.

Although we were surprised by this high value, it concurs well with some other studies where erythroblasts were either examined by single cell PCR or fluorescence in-situ hybridization (FISH), where 30–50% of the erythroblasts were also shown to be of fetal origin (Sekizawa et al., 1996; von Eggeling et al., 1997; Oosterwijk et al., 1998; Wachtel et al., 1998). A caveat of these studies was that they either used only fetal sex or highly polymorphic nucleotide repeats for fetal identification, which in combination with the error prone procedures for whole genome amplification (Hahn et al., 1998b) that they generally used, may have introduced artefacts.

Our systematic approach by the simultaneous analysis of two clearly defined fetal loci more precisely indicates the true extent of what fraction of erythroblasts in the maternal circulation is fetal.

A point which still remains to be resolved is to what extent this erythroblast ratio of fetal to maternal holds true for all pregnancies. For instance, increased numbers of fetal cells have been reported for pregnancies with aneuploid fetuses (Bianchi et al., 1997), and we have observed significant elevations in erythroblast numbers in pregnancies affected by pre-eclampsia (Gänschert et al., 1998; Holzgreve et al., 1998). In the latter instance, our results obtained by the use of FISH have indicated that ~20% of the erythroblasts in the maternal periphery are fetal (Holzgreve et al., 1998). By using a similar approach to the one we have described here, one should be able to address these issues very accurately.