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Maternal origin of transferrin receptor positive cells in venous blood of pregnant women

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We studied the origin of transferrin receptor (CD71) positive cells in blood from seven women pregnant with a male fetus in order to explore if fetal cells could be detected among them. We used a technique that allows direct chromosomal analysis by *in situ* hybridization on immunologically and morphologically classified cells. Enrichment was performed by magnetic activated cell sorting (miniMACS)® using an anti-CD71 monoclonal antibody. The cells were immunophenotyped by alkaline phosphatase anti-alkaline phosphatase immunostaining with the same antibody. The origin of the immunophenotyped cells was studied by *in situ* hybridization using an X cosmid Y repeat chromosome specific probe cocktail. CD71 positive cells were found in six of the seven women at the range of 4 to 43 in respective samples. Over 90% of the CD71 positive cells were nucleated erythrocytes. None of the detected positive cells were shown to be fetal. Thus, the use of transferrin receptor antigen alone in combination with the miniMACS® may not be sufficient for enrichment of fetal cells.

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Key words: immunomagnetic separation – MAC (morphology antibody chromosomes) technique – maternal venous blood – nucleated erythrocytes – transferrin receptor

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During pregnancy different types of fetal nucleated cells such as nucleated erythrocytes, leukocytes, trophoblasts, and granulocytes can be detected in maternal blood (Hamada et al. 1993, Bruch et al. 1991, Wessman et al. 1992). Fetal nucleated erythrocytes are considered to be the most promising type of cell for prenatal diagnosis of fetal genetic abnormalities because of their short lifespan and their scarcity in the venous blood of non-pregnant women. Nucleated erythrocytes can be identified on the basis of morphology and immunophenotype. Glycophorin A (GPA) is found only in erythroid cells (Loken et al. 1987a). Previous studies by us (Slunga-Tallberg et al. 1995) and others (Bianchi et al. 1994a, Büsch et al. 1994) provide evidence that GPA positive nucleated erythrocytes are mainly of maternal origin. The transferrin receptor (CD71) is another antigen found on erythroid cells (Loken et al. 1987b). This antigen

is also expressed on placental trophoblasts and on all other profile cells, such as activated lymphocytes and monocytes (Newman et al. 1982, Barclay et al. 1993). The aim of this study was to find out whether CD71 positive cells in maternal blood might be of fetal origin. The MAC (morphology, antibody, chromosomes) technique (Knuutila et al. 1994, Knuutila 1996), which allows direct chromosomal analysis on immunologically and morphologically classified cells, was used.

Material and methods

Samples

With the approval of the local ethical committees, a blood sample (20 ml) was drawn into heparinized tubes from 12 healthy women between 14 and 18 weeks of gestation; the duration of gestation was confirmed by fetal ultrasonography. The sex of the fetus was determined as described below. Only the seven women carrying a male fetus were studied further because the fetal cells could be distinguished from the maternal cells by the presence of the Y chromosome (Table 1).

Blood samples from four women (cases 1, 2, 3 and 4) (Table 1) were taken prior to amniocentesis performed for fetal karyotyping. These four women were at increased risk of trisomy 21 because of their age and/or because of their low serum levels of alpha-fetoprotein and high levels of human chorionic gonadotrophin. From three women the blood samples (cases 5, 6 and 7) were taken prior to termination of pregnancy on social grounds. The fetal sex was determined using the polymerase chain reaction (PCR) on chorionic villus tissue obtained at surgery (Witt & Erickson 1989).

Enrichment of CD71 positive cells

The mononuclear cell layer was isolated by Ficoll-Paque (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) density gradient centrifugation, and the layer of granulocytes was further isolated from the erythrocyte layer by lysis of mature red cells with 6% dextran T-500 (Pharmacia LKB). Each of the two cell layers was suspended in 1 ml PBS/BSA (0.5% BSA in PBS). Isolated cells were incubated on ice for 25 min with 7 µl of mouse IgG anti-CD71 monoclonal antibody (Boehringer Mannheim GmbH, Biochemica, Germany) per 10^7 cells. The cells were washed in PBS/BSA twice, resuspended in 80 ml of PBS/BSA per 10^7 cells, and incubated at 6–12°C for 15 min with 20 µl of magnetic microbeads conjugated to goat-anti-mouse IgG antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) per 10^7 cells. The antibody labelled cells were diluted in 0.5–1 ml PBS/BSA, and immediately separated by the magnetic activated cell-sorting (MACS) device, miniMACS® (Miltenyi), according to the manufacturer's instructions.

Slide preparation and immunophenotyping

For microscopic examinations two preparations were made from both of the labelled cell fractions using a cytocentrifuge. The slides were fixed in a buffered formaldehyde-acetone mixture (10 mg Na_2HPO_4, 15 ml distilled water, 33.75 ml acetone, 12.5 ml formaldehyde). Alkaline phosphatase anti-alkaline phosphatase (APAAP) immunostaining was performed by use of anti-CD71 monoclonal antibody. Giemsa counterstaining (Diagnostica Merck, E. Merck, Darmstadt, Germany) was used for the morphological characterization of the cells. The

Table 1. The number of CD71 positive nucleated erythrocytes in a 20-ml blood sample from seven pregnant women. The cells were detected with APAAP immunostaining, and the origin of the cells was studied by double-color FISH with X- and Y-specific probes

Subject no.	Age	No. of previous pregnancies	Gestational age at sampling (weeks)	Number of cells (10^3) in the preparations after anti-CD71 enrichment	Number of CD71 positive cells	Detection of X and Y in CD71 positive cells				Detection of X and Y in CD71 negative cells
						X	XX	XY	None*	
1	28	0	17	8	13		11	–	1	–
2	29	0	16	12	4	–	4	–	–	1
3	38	0	17	5	7	–	6	–	1	–
4	40	2	16	13	43	3	37	–	3	1
5	36	4	14	6	8	–	8	–	–	–
6	26	4	18	9	11	2	8	–	1	–
7	25	0	14	11	–	–	–	–	–	–

* Signals not detected or target cell lost after hybridization.

immunophenotyped cells were classified using a light microscope. The coordinates for the location of anti-CD71 positive cells were recorded and the cells were photographed.

Fluorescence *in situ* hybridization (FISH)

After microscopic examination of the immunophenotyped cells, the APAAP stain was removed by incubating the slides in methanol/acetic acid 3:1 for 30 min. To determine the fetal or maternal origin of the immunophenotyped cells an X cosmid, Y repeat probe cocktail (Integrated Genetics, Framingham, MA, USA), which allows simultaneous identification of both the X and the Y chromosomes in a single cell, was used. The X probe was directly labelled with a fluorochrome (Cy3) giving a red fluorescence signal. The Y probe was biotinylated and detected using fluorescein isothiocyanate (FITC), a fluorochrome giving a green fluorescence signal. Hybridization, washes and signal detection were done as described elsewhere (Slunga-Tallberg et al. 1995).

The XY probe cocktail was tested on male bone marrow preparations which had been APAAP immunostained with anti-CD71 monoclonal antibody, as described above.

To confirm that 6% dextran T-500 lyses only mature erythrocytes, but not fetal nucleated erythrocytes, we tested the method on cord blood from a 13-week-old fetus.

Results

After CD71 enrichment, the number of cells that came off the column ranged from 5000 to 13 000. CD71 positive cells were detected at the range of 4 to 43 per 20-ml blood sample in six of the seven mothers carrying a male fetus (Table 1). Most of the CD71 positive cells resembled nucleated erythrocytes. The rest of the positive cells were morphologically unclassified blast-like cells. No CD71 positive cells were detected in the CD71 negative fractions when 10 000-20 000 cells were scored.

After hybridization with the XY probe, none of the 86 CD71 positive cells showed a Y signal. Seventy-four of these cells (86%) showed two X signals, indicating their maternal origin. In six of these cells (7%), only one X signal was seen and in six of them (7%) no signals were detected, or the target cell was not found after hybridization (Table 1). In each case 800–5000 cells were screened for XY signals also in CD71 negative cells, and in two of the women one CD71 negative cell with XY signals was detected.

In normal male bone marrow preparations hybridized with the XY probe-cocktail, the hybridization frequency, showing one X and one Y signal, was 99% in CD71 negative cells and 98% in CD71 positive cells.

Fetal nucleated erythrocytes were not shown to be lysed by 6% dextran T-500.

Discussion

The CD71 positive cells found in six of the seven pregnant women were mainly nucleated erythrocytes, judged by morphological criteria. The number of nucleated erythrocytes in the blood of non-pregnant women has previously been shown to be very low (Slunga-Tallberg et al. 1994, Bianchi et al. 1994b), and therefore non-pregnant controls were not included in this study. Consequently, we cannot draw any direct conclusions from the present study, whereas previous data (Slunga-Tallberg et al. 1995) indicate that pregnancy *per se* induces transportation of maternal nucleated erythrocytes into the blood stream.

A considerable interindividual variation in the number of CD71 positive cells in the maternal circulation was observed in the present study, which is in accordance with the report of Bianchi et al. (1994b), who demonstrated a similar variation in the number of CD71 positive cells in the blood of pregnant women.

Among the CD71 positive nucleated cells no fetal cells were detected, and 86% of them were certainly shown to be maternal. This finding is in accordance with our previous data, showing that the bulk of GPA positive nucleated erythrocytes in the blood of pregnant women is of maternal origin (Slunga-Tallberg et al. 1995). Similar results have been reported by Bianchi et al. (1994a) and Büsch et al. (1994), who employed PCR to detect fetal cells in the cell fractions obtained after enrichment of nucleated erythrocytes from maternal blood with anti-CD71 monoclonal antibody alone or combined with other antibodies. Still, low amounts of fetal nucleated erythrocytes have been detected by two other groups, who used a direct technique to study the origin of enriched nucleated erythrocytes from maternal venous blood, identified by their hemoglobin content (Zheng et al. 1993, von Koskull & Gahmberg 1995). In these studies the frequency of maternal nucleated erythrocytes could not be determined. The former used an antibody to the gamma chain of fetal hemoglobin, which fails to recognize most of adult nucleated erythrocytes, and the latter applied only a Y-specific probe, which impairs reliable recognition of XX-containing cells.

As a high proportion of CD71 positive cells in the blood of pregnant women seems to be of maternal

origin, this antibody may not be one that can be used for the enrichment of cells of fetal origin in quantities sufficient to allow reliable prenatal diagnosis of fetal genetic abnormalities.

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