ORIGINAL ARTICLE

Enrichment of fetal cells from maternal blood by magnetic activated cell sorting (MACS) with fetal cell specific antibodies: One-step versus two-step MACS

Xiao Xi Zhao, Yasuhiko Ozaki, Nobuhiro Suzumori, Tsuyoshi Sato, and Kaoru Suzumori
Department of Obstetrics and Gynecology, Nagoya City University Medical School, Nagoya, Aichi, Japan

ABSTRACT We report here the results of fetal cell enrichment from maternal blood in 58 pregnant women by the use of magnetic activated cell sorting (MACS) with erythroblast-specific and/or maternal cell specific antibodies. Two approaches were compared; one-step MACS to enrich CD71+ (a membrane-bound marker) or GPA+ (another marker, glycophorin A) fetal cells versus two-step MACS to deplete CD14+ maternal cells and subsequently to enrich fetal (CD71+ or GPA+) cells. The existence of fetal cells was ensured by both FISH with Y-specific probes and karyotyping of respective amniotic and/or chorionic villus cells, the results being applied for comparison of detection rate for XY fetuses between the two MACS procedures.

In 24 (38.8%) of the 58 blood samples examined, Y-positive cells were observed by FISH, whereas there were 38 true XY fetuses later confirmed by karyotyping, including two cases of 47,XY,+21. On the other hand, in Y-negative cells by FISH, there were two cases of 47,XX,+18. The average number of cells sorted did not differ among one-step MACS procedures with anti-CD14, anti-CD71 and anti-GPA antibodies. With the latter, 12 (75%) of 16 Y-positive fetuses were detected, while only one (20%) of 5 Y-positive fetuses was detected by two-step MACS with anti-CD14/anti-GPA antibodies. The detection rate significantly varied (p = 0.0024) between the two procedures, although the numbers of cases examined were small. There was no statistical difference (p > 0.05) between one-step and two-step MACS with other combinations of antibodies. These findings indicate that one-step MACS using the anti-GPA antibody is more effective than two step MACS for enrichment of fetal cells from maternal blood.

Key words: Fetal erythroblasts, MACS, Fluorescence in situ hybridization (FISH), Male fetus detection

INTRODUCTION

The presence of fetal cells in the maternal circulation was first reported about 100 years ago (Schmorl, 1905). Although considerable efforts to isolate such fetal cells were devoted over the last several decades, applications of this phenomenon to prenatal diagnosis have only become reliable since introduction of the polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) (Ganshirt-Ahlert et al., 1994). Among possible fetal cell types in the maternal blood, trophoblasts are most easily identified because of their large size. However, they can not be used for prenatal diagnosis because of their extremely small number in the maternal blood and their multinucleated nature. Fetal lymphocytes can persist in the maternal circulation for many years after pregnancy, and thus, are not ideal for prenatal diagnosis (Bianchi et al., 1996). Since nucleated red blood cells (NRBCs) are mononuclear, abundant in the first-trimester fetal blood, and relatively well differentiated with a limited life span, they have become a target cell type for prenatal diagnosis, although their number is also small (Price et al., 1991; Hamada et al., 1993; Bianchi et al., 1997). NRBCs express membrane-bound markers, such as the fetal erythrocyte transferrin receptor (CD71) and glycophorin A (GPA), and intracellular antigens (fetal hemoglobin) that allow their isolation and identification (Loken et al., 1987; Bianchi et al., 1990). Enrichment of NRBCs from the maternal blood by fluorescence-activated cell sorting (FACS) with monoclonal antibodies has been successfully performed (Bianchi et al., 1990; Price et al., 1991), but there are problems with this procedure, e.g., the cost and a large technological effort required for FACS, which have prevented routine diagnostic use. Bianchi et al. (1993) developed a method in which maternal blood was processed by FACS with...
three different antibodies, against GPA, CD71, and/or CD36 (thrombospondin receptor). After enrichment of fetal cells, PCR was performed to detect Y-chromosome-specific sequences, and sex prediction was found to be 100% correct when using the anti-GPA antibody. Magnetic activated cell sorting (MACS) is a simple, fast and efficient method to enrich fetal erythroblasts from mononuclear cells in the maternal blood (Miltenyi et al., 1990; Holzgreve et al., 1992; Rodriguez-de Alba et al., 1999). A double-step MACS was also developed, to deplete maternal cells and then enrich CD71+ fetal cells (Busch et al., 1994). Jansen et al. (1999) compared one-step MACS for collection of CD71+ fetal cells with two-step MACS for both depletion of CD45+ and CD14+ maternal cells and enrichment of fetal cells. The one-step MACS gave a significantly higher yield of male NRBCs than the two-step method.

Here we report the results of our own study to compare efficacy for fetal cell enrichment between one-step and two-step MACS with various combinations of antibodies.

**MATERIALS AND METHODS**

**Maternal blood samples and nucleated cell collection**

Peripheral blood samples (20 ml) were obtained with informed consent from 58 pregnant women (9-20 weeks of gestation) referred to Nagoya City University Hospital for prenatal diagnosis. Fifty-four samples were obtained prior to amniocentesis, whereas 4 were obtained after aneuploidies of fetuses were diagnosed by amniocentesis or chorionic villus sampling (CVS). All samples were processed within 24 hrs. Nucleated cells collected from the samples through a Ficoll-Paque gradient were washed once with saline, and resuspended in 1 ml PBS. Then, 10μl aliquots were added to 990μl of cell mixture and the numbers of cells were counted.

**Magnetic labeling and magnetic-activated cell sorting (MACS)**

The nucleated cells collected were resuspended in 1 ml PBS and mixed with 200μl anti-CD14, anti-CD71 or anti-GPA microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation at 4°C for 15 min, the magnetic-labeled cells were washed once with PBS and applied to MACS column.

We performed one-step and two-step MACS. The one-step procedure was designed to enrich CD71+ or GPA+ fetal cells, and the two-step approach to deplete CD14+ maternal cells then enrich fetal (CD71+ or GPA+) cells. For the one-step MACS, magnetic-labeled cells were applied to a selection column (type RS for sorting with anti-CD71 and anti-GPA antibodies) placed in a Vario Magnetic field (Miltenyi Biotec, Bergisch Gladbach, Germany). After CD71- or GPA- cells were removed from the magnet, a positive cell fraction was collected by flushing out with gentle pressure using the supplied plunger, and then washed with PBS. The CD71+ or GPA+ cells were collected and their number was counted. CD14- cells sorted with anti-CD14 antibody and type BS collected and their numbers were counted.

For the two-step MACS, cells labeled with anti-CD14 microbeads were washed once with PBS, then applied to a type BS column to deplete CD14+ cells. Then, aliquots (30μl) of anti-CD71 or anti-GPA microbeads were added to the negative fraction from the first MACS, and second-labeled cells were applied to a type RS column to isolate CD71+ or GPA+ cells. Finally, CD14-/CD71+ or CD14-/GPA+ cells were eluted from the column outside the magnet, and their numbers were counted.

**Fluorescence in situ hybridization (FISH) and karyotyping**

Cells collected by MACS were analyzed with interphase FISH to ascertain whether they have Y chromosomes. In short, after the cells were centrifuged, cell pellets were treated with 75 mM KCl and fixed with methanol/acetic acid, and then interphase cell slides were made. Multicolor chromosome-specific DNA probes (Vysis Inc., Downers Grove, IL, USA) for chromosomes 18 (aqua-blue), X (green) and Y (red), or for chromosomes 13 (green) and 21 (red) were emplyed. Hybridization and photomicroscopy were conducted according to the manufacturer's protocol under a fluorescence microscope (Nikon, Tokyo, Japan) with image capture and a Microsoft computerized system (Cytovision, Santa Clara, California, USA), respectively. In each case, 100-1000 intact cells were analyzed.

The 58 pregnant women were subjected to amniocentesis and/or CVS to determine the karyotypes of the fetuses, as described previously. Male karyotypes obtained, together with FISH data, were used as references for the MACS results.

**RESULTS**

After MACS with fetal cell-specific antibodies, some 9-37 thousand cells were obtained. The average numbers did not differ among one-step MACS procedures with anti-CD14, anti-CD71 and anti-GPA antibodies (p > 0.05) (Table 1). In 24 (38.8%) of the 58 samples examined, Y-positive cells were observed by FISH with the Y specific probe (Fig. 1B), whereas true XY fetuses confirmed by karyotyping accounted for 38 cases, including two with 47,XY,+21 (Fig. 1C). The other 20 cases with the XX karyotype, including 2 cases of 47,XX,+18 (Fig. 1D), gave results consistent with their FISH data.

With the one-step MACS using the anti-GPA antibody (to enrich GPA+ cells), 12 (75%) of 16 male fetuses were detected, while only one (20%) of 5 males was detected with the two-step MACS (to enrich GPA+ cells from CD14- cells) (Table 1). The detection rate was significantly greater (p = 0.0024) with the one-step MACS, although the numbers of cases examined were small. There was no difference (p > 0.05)
Table 1 Comparison of one-step MACS and two-step MACS using various combinations of antibodies.

<table>
<thead>
<tr>
<th></th>
<th>One-step MACS</th>
<th>Two-step MACS</th>
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<tbody>
<tr>
<td></td>
<td>CD71</td>
<td>CD14</td>
</tr>
<tr>
<td>Average maternal age</td>
<td>38.3</td>
<td>36.8</td>
</tr>
<tr>
<td>(years)</td>
<td></td>
<td></td>
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<tr>
<td>Average gestational</td>
<td>12.7</td>
<td>15.5</td>
</tr>
<tr>
<td>week</td>
<td></td>
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<tr>
<td>No. of cells before</td>
<td>1.9 ± 1.5</td>
<td>2.5 ± 1.4</td>
</tr>
<tr>
<td>MACS(×10⁶)</td>
<td></td>
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<tr>
<td>No. of cells after</td>
<td>0.9 ± 0.9</td>
<td>3.7 ± 3.1</td>
</tr>
<tr>
<td>MACS(×10⁶)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cells analyzed</td>
<td>708</td>
<td>706</td>
</tr>
<tr>
<td>with FISH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of cases with Y-</td>
<td>4 (60)</td>
<td>12 (75)*</td>
</tr>
<tr>
<td>positive FISH signals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of male fetuses</td>
<td>6</td>
<td>16</td>
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<td>examined</td>
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*Welch’s t-test was used for statistical analysis. Statistically significant (p = 0.0024).

between one-step and two-step MACS with other combinations of antibodies.

**DISCUSSION**

The results of the present study confirmed the existence of fetal nucleated cells in the maternal circulation that can be enriched with MACS, also showing that the selection rate is dependent on the procedures and antibodies used. The one-step MACS using the anti-GPA antibody gave a better result than the two-step MACS using anti-CD14 / anti-GPA antibodies. The latter involves many steps or manipulations in which cells may be damaged, by the magnetic field. In fact, cell debris was frequently observed under the fluorescent microscope in FISH experiments. This may be one explanation for the difference in results between the two procedures. Among the antibodies employed, anti-GPA antibody alone led to the best results, i.e., XY cells were most significantly enriched (Table 1). Our findings are consistent with those of others (Bianchi et al., 1993; Troeger et al., 1999). Troeger et al. (1999) obtained a very high yield of fetal erythroblasts with the anti-GPA antibody, and Bianchi et al. (1993) achieved a 100% gender prediction rate using the anti-GPA antibody alone or in combination with anti-CD36 or anti-CD71 antibodies, while the rate with the latter was 57%. Since GPA is highly expressed by cells of erythroblast origin, it is likely that it is more fetal-cell-specific than other antigens. Lewis et al. (1996) reported from their FACS study that use of the anti-GPA antibody resulted in aggregation of nucleated and non-nucleated red cells. However in our present study, such cell agglutination was not observed. The discrepancy may be due to differences in the cell-sorting procedures used, MACS versus FACS, or alternatively differences in agglutinating properties of anti-GPA antibodies produced by various manufacturers.

With respect to the present FISH study using a Y-specific probe, Y-positive signals were observed in 63.2% of pregnancies having male fetuses and in 16.6% of those having female fetuses. In two other similar studies, Y-positive cells were detected in 74% of 39 pregnancies with male fetuses and 7% of 15 pregnancies with female fetuses (Ganshirt-Ahler, et al., 1993; Simpson et al., 1995). In a more recent investigation, none of 97 pregnancies featuring chromosomally normal or abnormal female fetuses, and 57% of those with male fetuses showed Y (+) signals (Al-Mufti et al., 1999).

Possible sources of male cells in pregnant woman’s blood with female fetuses include a vanished-twin gestation, a previous man-to-woman blood transfusion, or a previous pregnancy with a male fetus (Bianchi et al., 1997). Long-term persistence of fetal CD34+ and/or CD38+ cells in the maternal circulation has been reported (Bianchi et al., 1999b). In the present FISH study with the Y-probe, two of three women with Y-positive cells had previous pregnancies with male fetuses, whereas the other woman had no such history or any previous blood transfusion. The latter finding may reflect either a limitation of sex detection by single-probed FISH or an artifact. Application of poly-FISH (Zhen et al., 1998) and multi-FISH (Bischoff et al., 1998) techniques to fetal cell detection may improve the accuracy rate.

Although the isolation or enrichment of fetal cells from the maternal blood is very attractive, currently available techniques still remain relatively low in term of sensitivity and specificity. Al-Mufti et al. (1999) stated that examination of fetal cells from maternal blood should rather be used as a risk assessment method than as a technique for prenatal diagnosis. Clearly, much remain to be investigated, especially with regard to specificity.

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Fig. 1 FISH on cells in the maternal circulation. A: A cell with two chromosome 18-specific (white) and two X chromosome-specific (green) signals, consistent with a maternal or a female fetal cell. B: A cell with two chromosome 18-specific (white) signals, one each for X (green) and Y (red) chromosomes, the derivation being from a male fetus, as later confirmed by karyotyping. C: A cell with two signals (green) for chromosome 13 and three signals (red) for chromosome 21, the trisomy 21 being later confirmed by karyotyping. D: A cell with three spots (white) for chromosome 18 and two spots (green) for the X chromosome.

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