in vivo *18*: 629-632 (2004)

Use of Annexin V for the Identification of Fetal Cells in Maternal Circulation

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Abstract. *MACS with Annexin V-conjugated microbeads was used to isolate cells in apoptosis from the peripheral blood of 112 women at different weeks of gestation and from 15 women (60 samples) sequentially tested postpartum. FISH using X/Y probes was applied to quantitate fetal apoptotic cells. The mean apoptosis rate in the 16th-18th week was 6.5% and fetal cells constituted 5.1% of the apoptotic cell population. In the 26th-28th week it was 10.1%/7.5% and in the 37th-38th week 12.5%/9.9%, respectively. In samples obtained 30 min, 12h and 24h postpartum, the mean apoptosis was 25.1%, 12.5 and 6.1%, respectively and fetal cells constituted 14.8%, 2.1% and 0.16% of the apoptotic cells. Forty-eight h after delivery, apoptosis was 2.3% and no fetal cells were detected. Accurate estimation of the proportion of fetal cells undergoing apoptosis may facilitate the optimization of protocols for non-invasive prenatal diagnosis of chromosomal abnormalities.*

Apoptosis, or programmed cell death, is a mechanism used for the removal of all types of unwanted cells. It results in the rapid recognition and degradation of the nuclear DNA and the fragmentation of the cell into "bite-size" pieces for efficient disposal by phagocytes (1,2).

We have previously reported an increased apoptosis rate in maternal peripheral blood during pregnancy, partly accounting for the presence of cell-free fetal DNA in the plasma of pregnant women (3). Fetal apoptotic cells were present in the circulation of pregnant women, since TUNEL-positive, Y-positive cells were identified in pregnancies with male fetuses. Nuclear fragmentation, however, allowed successful FISH application in a limited number of cells, probably because the majority were in late apoptosis and the nuclei were destroyed. For this reason it was not possible to calculate the proportion of fetal cells among the mononuclear apoptotic cell population.

Annexin V has a high affinity for phosphatidylserine (PS), normally absent from the outer leaflet of the plasma membrane. During the early phase of apoptosis, when DNA fragmentation has not yet occurred and cells are still intact, PS is translocated to the outer layer of the membrane (4,5) allowing for the identification of early apoptotic cells bound to Annexin V.

The aim of the present study was to identify, by FISH, the proportion of fetal cells undergoing apoptosis in maternal peripheral blood at different weeks of pregnancy as well as postpartum. The apoptotic cell population was positively selected by MACS, based on the ability to bind Annexin V-conjugated microbeads (6,7).

Materials and Methods

Materials. Apoptosis was determined in 207 peripheral blood samples obtained after informed consent: 112 came from pregnant women, 65 in the 16th-18th week of gestation, 23 in the 26th-28th week and 24 in the 37th-38th week of pregnancy. Samples were obtained before any invasive procedure. Fetal sex was subsequently confirmed by karyotypic analysis of amniotic fluid cells.

Sixty samples were collected from 15 women after delivery, at 30 min, 12 h, 24 h and 48 h postpartum. These women were free of any medical disease or antenatal complications and gave birth vaginally to phenotypically normal babies. Thirty-five samples, used as controls, came from healthy nonpregnant women, 25-40 years of age, who were not under hormonal contraception.

Isolation of the PS exposing cells. From each case, 3ml peripheral blood were collected in EDTA. All samples were processed within 1-2 h after collection. Mononuclear cells were isolated by centrifugation on a 1.077mg/ml density gradient (Histopaque 1077, Sigma, USA), washed twice in PBS and passed through 30-μm nylon mesh filter to remove clumps. Cells were enumerated using a Coulter Counter, suspended in 1X Binding Buffer and incubated for 10 min with Annexin V-labelled microbeads (20μl of Annexin V microbeads per 10^7 total cells) (Miltenyi, Biotech, Germany).

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Key Words: Annexin V, apoptosis, maternal peripheral blood, non-invasive prenatal diagnosis.

Subsequent immunofluorescence staining was performed by incubating cells for 10 min with 100μl of FITC-conjugated Annexin V (Serotec, USA) diluted 1:50 in Tris- EDTA pH 7.4. They were next washed twice with 1X Binding Buffer and separated using mini-MACS separation columns, according to the manufacturer's instructions.

From each case 3 slides were prepared using a microcentrifuge (Cytospin 2, Shandon Southern Products Ltd, Runcorn,UK) and examined under a fluorescence microscope (Zeiss Axioskop, Germany) with an FITC filter. The apoptosis rate was expressed as the percentage of Annexin V-positive-stained apoptotic cells to the total number of mononuclear cells counted.

FISH. Dual color FISH was performed using directly labelled X (DXZ1, Oncor, Q Biogene, France) - and Y- (DYZ1, Oncor) chromosome specific probes. Slides were hybridized following the manufacturer's protocol, examined under a fluorescence microscope (Zeiss Axioskop, Germany) and photographed with a CCD camera. Cells showing an XX and an XY hybridization pattern were recorded and the proportion of fetal cells was calculated. Only intact cells showing intense cytoplasmic fluorescence, with no overlaps from adjacent cells, were analyzed.

Validation of FISH analysis on MACS separated cells. The sensitivity of the technique in isolating and detecting fetal cells undergoing apoptosis in maternal peripheral blood was determined in artificial cell mixtures designed to mimic feto-maternal hemorrhage.

Three separate experiments were performed with two replicates of artificial mixtures containing 0, 1, 5, 10, 15, 20 and 25 male cells of the CCRF-SB human B lymphoblastoid cell line (ECACC, Salisbury, UK) under apoptosis mixed into 3ml of adult female whole peripheral blood. Annexin V-positive-stained cells were separated and processed according to the FISH protocol described above. Statistical analysis was performed in order to compare the number of male cells under apoptosis detected by FISH and the estimated number of male cells spiked into the constructed mixtures.

Statistical analysis. Statistical analysis was performed using the SPSS statistical analysis software. The first phase of analysis included descriptive statistics and some graphical displays in order to understand the distributional properties of the study variables.

The second phase of analysis consisted of some independent *t*-tests that were used in order to examine the differences in the mean number of male apoptotic cells among the total number of mononuclear apoptotic cells detected. Analysis of variance (ANOVA) was used to test for differences in the mean numbers of male cells detected, while least squares means were compared to assess significance between pairs of apoptotic cells. Although the independent samples *t*-test has been shown to be robust in the presence of skewed distributions, we also investigated non-parametric tests. In all cases the results of the parametric and non-parametric tests were consistent. Thus, the results of the parametric tests are presented here as more powerful.

Results

In artificial mixtures, a correlation coefficient of 0.99 was observed for the number of male apoptotic cells detected by FISH and the estimated number of cells added into the constructed mixture.

The mean apoptosis rate in the control group was 2.28 ±0.32% (range 0.8-2.3%).

Annexin V-positive-stained mononuclear cells were identified in all samples obtained from pregnant women.

In samples from the 16th-18th week of pregnancy, the mean apoptosis rate was 6.4±0.19% (range 3.7-7.9%) (Figure 1a). As gestation progressed the apoptotic cell population increased. The mean apoptosis rate in the 26th-28th week of pregnancy was 10.1±0.65% (range 8.2-13.9%, $p<0.05$ *vs.* pregnancies in the 16th-18th weeks) and in the 37th-38th week it was 12.5±0.62% (range 9.2-14.7%, $p<0.05$ *vs.* pregnancies in the 26th-28th).

In samples collected shortly after delivery, the mean apoptosis rate was 25.1±1.54% (range 16.8-28.5%, $p<0.05$ *vs.* pregnancies in the 37th-38th weeks of gestation) (Figure 1b). At 12 and 24 h postpartum, the mean apoptosis rate was 12.5±0.62% (range 10.9-14.1%, $p<0.06$ *vs.* samples at 30 min postpartum) and 6.1±0.48% (range 4.8-7.1%, $p<0.05$ *vs.* samples at 12 h postpartum), respectively. In samples obtained 48 h after delivery it was 2.3±0.20% (range 1.3-3.0%, $p<0.05$ *vs.* samples at 24 h postpartum).

FISH. Annexin V-positive cells showing an XY hybridization pattern were not detected in any control samples.

Although the majority of Annexin V-positive cells were XX, cells showing an XY hybridization pattern were clearly seen in 30 of the 35 samples taken from women carrying male fetuses in the 16th-18th week of gestation. The mean proportion was 5.1±0.69% (range 3.6-12%) (Figure 1a).

Male apoptotic cells were identified by FISH in all cases with male fetuses tested at 26-28 (n=12) and 37-38 (n=12) weeks of gestation and the mean proportion was 7.5±0.40 (range 7.6-9.6%; $p+$ 0.16 *vs.* pregnancies in the 16-18 weeks) and 9.9%±0.8 (range 9.9-13%; $p<0.17$ *vs.* pregnancies in the 26-28 weeks), respectively.

The proportion of fetal cells among the apoptotic cell population 30 min after delivery was 14.8±3.24% (range 12.5-25.5%; $p<0.05$ *vs.* pregnancies in the 37th-38th weeks of gestation) (Figure 1b) and 12 h postpartum the proportion was 2.1± 1.15% (range 0.8-4.1%; $p<0.05$ *vs.* samples at 30 min postpartum). Male fetal apoptotic cells were detected only in 3 out of 8 samples collected 24 h after delivery from women that delivered males, at frequencies 0.10%, 0.15% and 0.25% (mean 0.16%). Fetal cells under apoptosis were not detected in any sample collected 48 h after delivery.

Discussion

Conflicting reports exist in the literature concerning the presence of fetal cells undergoing apoptosis in maternal peripheral blood during pregnancy. Sekizawa *et al.* (8) identified fetal apoptotic nucleated red blood cells (NRBCs) in post termination samples, van Wijk *et al.* (9) reported the presence of TUNEL-positive apoptotic cells of fetal origin in the maternal plasma after discontinuous Percoll gradient and Poon *et al.* (10) successfully used fetal cells from the plasma

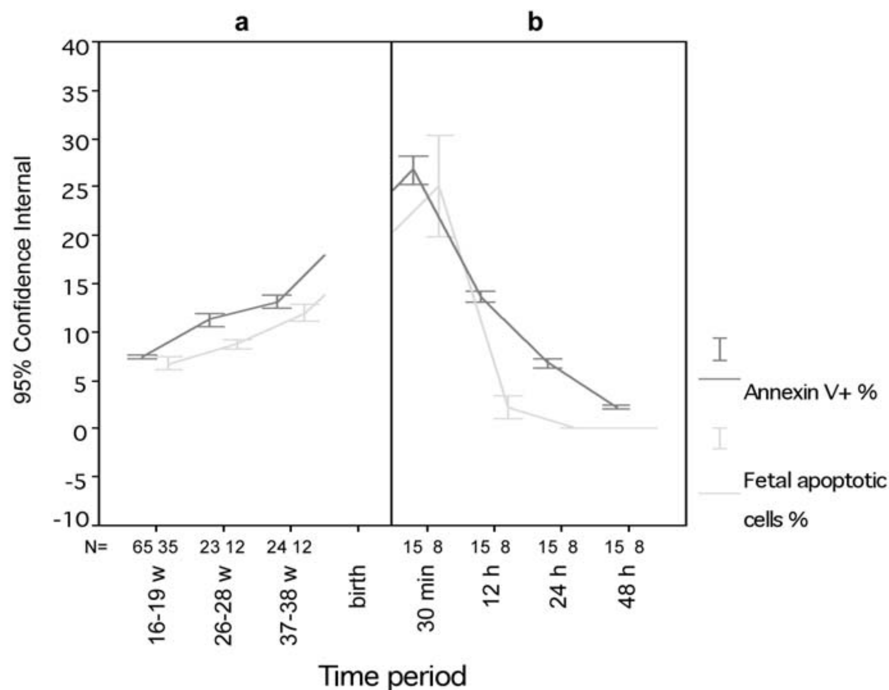

Figure 1. *Mean apoptosis rate and mean proportin of male apoptotic cells in samples obtained at different times during pregnancy (a) and in samples sequentially collected postpartum (b). Time is plotted on the x-axis, y-axis shows 95% interval confidence of Annexin V-positive cells and fetal apoptotic cells in maternal blood.*

for prenatal diagnosis. On the contrary, Bischof *et al.* (11) failed to detect any fetal, presumably apoptotic, cells in the plasma of pregnant women, possibly due to technical reasons (12). In these studies, however, the proportion of all fetal cells in the apoptotic cell population could not be determined, since Sekisawa *et al.* (8) studied only fetal NRBCs present in maternal peripheral blood, while van Wijk's (9) and Poon's (10) studies probably refer to cells in late apoptosis with decreased density, which rise to the top of the gradient. As already mentioned, in our previous study DNA fragmentation of the nucleus allowed successful FISH application in only a limited number of apoptotic cells (3).

In the present study, binding of Annexin V to PS was used in order to isolate and determine the apoptotic cell population, including all types of mononuclear cells of fetal origin entering the maternal circulation. It is known (5) that PS also becomes accessible in necrotic cells due to the disruption of membrane integrity but, since the number of necrotic cells in peripheral blood is normally negligible, the Annexin V-positive population was considered mainly as apoptotic. The apoptosis rate was determined on the basis of Annexin V binding only, since a 0.96 correlation was found with values previously obtained using ethidium bromide staining (3).

The sensitivity of the technique to recover apoptotic cells was first tested in artificial mixtures and minimal manipulations of the specimens were performed in order to avoid cell degradation due to the fragility of apoptotic cells.

According to the results obtained by FISH, fetal cells undergoing apoptosis in the 2nd trimester of pregnancy constitute approximately 5.1% of the isolated total apoptotic cell population. This proportion increased with gestational age and reached 11.9% in late pregnancy. Our estimate of the percentage of fetal apoptotic cells at 16-18 weeks of pregnancy is lower than the one obtained from 20 preliminary samples tested using FISH on Annexin V immunocytochemically-stained cells (13) and corresponds approximately to 13 fetal cells/ml of maternal peripheral blood. This number is higher than the one originally reported by Bianchi *et al.* (14) using PCR (1.2 cells/ml). Krabchi's (15) estimate of the number of all mononuclear fetal cells entering maternal circulation using FISH was also lower (4 cells/ml), possibly due to the destruction of fetal apoptotic cells during the preparation. This figure is nevertheless lower than the 25.4 genome equivalents/ml of cell-free fetal DNA in maternal plasma as quantitated by Lo *et al.* (16).

In samples obtained shortly after labor, we noted an increase in the proportion of fetal cells undergoing apoptosis, probably because of a delivery-associated increased feto-maternal transfusion and exposure of fetal cells to the maternal immune system. As already mentioned, all women gave birth vaginally and not by caesarian section. This is in agreement with Lo's observation that the amount of cell-free fetal DNA decreases rapidly after labor (17). In samples collected 12 hours postpartum, fetal apoptotic cells constituted 2.1% of the total

apoptotic cell population, while at 24 hours they were detected at very low frequencies in only 3 out of 8 cases tested. At 48 hours postpartum, we were not able to detect any apoptotic cells of fetal origin, although the methodology used was validated and was sensitive enough to detect even one target cell.

It is possible that more than one mechanism may be involved in the clearance of fetal cells from the maternal circulation, since it is reported to take place over a period of 2-3 months (18, 19) and fetal progenitor cells have been shown to persist in some women even decades after delivery (20). Since apoptosis is a rapid reaction that is completed within 2-3 hours (21), the persistence of some fetal cells is possibly due to apoptosis-resistant lymphoid or myeloid progenitors or to a defective regulation of apoptosis in some individuals, leading to fetal cell microchimerism associated with autoimmune diseases (22-24).

To our knowledge, this is the first time that the percentage of the fetal apoptotic cell population in maternal peripheral blood at various weeks of gestation has been determined. However, more research is required to determine the cellular origin of the examined fetal cells. Accurate estimation of this proportion is crucial for the optimization of the procedures related to the isolation of intact fetal cells for non-invasive prenatal diagnosis of chromosomal abnormalities.

Acknowledgements

The first two authors contributed equally to this work and both should be considered as primary authors. All the authors gratefully acknowledge the generous contribution of SPSS Hellas for the statistical analysis and Mrs. G. Bitziou for sample collection.

References

1 Alles A, Alley K, Barret JC, Buttyan R, Columbiano A, Cope FO, Copelan EA, Duke RC, Farel PB, Gershenson LE *et al*: Apoptosis: a general comment. FASEB J *5*: 2127-2128, 1991.

2 Bursch W, Oberhammer F and Schulte-Herman R: Cell death by apoptosis and its protective role against disease. Th Pharmac Sci *13*: 45-251, 1992.

3 Kolialexi A, Tsangaris GTh, Antsaklis A, Tzortzatou F, Amentas C, Koratzis A and Mavrou A: Apoptosis in maternal peripheral blood during pregnancy. Fetal Diagn Ther *16*: 32-37, 2001.

4 Koopman G, Reutelingperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST and Van Oers MHJ: Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood *84*: 1415-1420, 1994.

5 Martin SJ, Reutlingsperger CPM, McGahon AJ, Rader JA, van Schie RCAA, LaFace DM and Gree DR: Early redistridution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating, stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med *174 (21)*: 5191-5200, 1995.

6 Kuypers FA, Lewis RA, Hum M, Schott MA, Discher D, Emst JD and Lubin BH: Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled Annexin V. Blood *87*: 1179-1187, 1996.

7 Rickers A, Brocckstedt E, Mapara MY, Omo A, Dorken B and Bommert K: Inhibition of CPP32 blocks surface IgM-mediated apoptosis and D4-GD1 cleavage in human BL60 Burkitt lymphoma cells. Eur J Immunol *28*: 296-304, 1998.

8 Sekisawa A, Samura O, Zhen D, Falco V, Farina A and Bianchi DW: Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. Prenat Diagn *20*: 886-89, 2000.

9 Wijk IJ, Hoon AC, Jurhawan R, Tjoa ML, Griffioen S, Mulders MAM *et al*: Detection of apoptotic fetal cells in plasma of pregnant women. Clin Chem *46*: 729-731, 2000.

10 Poon LLM, Leung TN, Lau TK and Lo YMD: Prenatal detection of fetal Down's syndrome from maternal plasma. Lancet *356*: 1819-1820, 2000.

11 Bischoff FZ, Hahn S, Johnson KL, Simpson JL, Bianchi DW, Lewis DE, Weber WD, Klinger K, Elias S, Jackson LG, Evans MI, Holzgreve W and de la Cruz F: Intact fetal cells in maternal plasma: are they really there? Lancet *361*: 139-140, 2003.

12 Lo YMD and Poon LLM: The ins and outs of fetal DNA in maternal plasma. Lancet *361*: 193, 2003.

13 Kolialexi A, Tsangaris GTh, Mavrou A, Antsaklis A, Tzortzatou F, Touliatou V and Metaxotou C: Use of Annexin V antibody to identify apoptotic cells during pregnancy. Proc NY Acad Sci *945*: 145-150, 2001.

14 Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW and Shuber AP: PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. Am J Hum Genet *61*: 822-829, 1997.

15 Krabchi K, Gross-Louis F, Yun J, Bronsard M, Massé J, Forest JC and Drouin R: Quantification of all fetal nucleated cells in maternal blood between 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. Clin Genet *60*: 145-150, 2001.

16 Lo YMD, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PMK, Wainscoat JS, Johmson PJ, Chang AMZ and Hjelm NMl: Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet *62*: 768-775, 1998.

17 Lo YMD, Zhang J, Leung TN, Lau TK, Chang AMZ and Hjelm M: Rapid clearance of fetal DNA from maternal plasma. Am J Hum Genet *64*: 218-24, 1999.

18 Hamada H, Arimani T, Hamaguchi H and Kubo T: Fetal nucleated cells in maternal peripheral blood after delivery. Am J Obstet Gynecol *170*: 1188-1193, 1994.

19 Thomas MR, Tutchek B, Frost A, Robeck CH, Yasdani N, Craft I and Williamson R: The time of appearance and disappearence of fetal DNA from maternal circulation. Prenat Diagn *15*: 641-646, 1995.

20 Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S and DeMaria MA: Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci *93*: 705-708, 1996.

21 Coles HSR, Burne IF and Raft MC: Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development *118*: 777-784, 1993.

22 Mountz JD, Wu J, Cheng J and Zhou T: Autoimmune disease. A problem of defective apoptosis. Arthritis Rheum *37*: 1415-1420, 1994.

23 Bianchi DW: Fetomaternal cell trafficking: a new cause of disease. Am J Med Genet *91*: 22-28, 2000.

24 Bianchi DW: Fetal cells in the mother: from genetic diagnosis to diseases associated with fetal cell microchimerism. Eur J Obstet Gynecol Reprod Biol *92*: 103-108, 2000.

Received February 25, 2004
Accepted May 10, 2004