Distribution of fetal erythroblasts enriched from maternal blood in multifetal pregnancies

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BACKGROUND: The aim of this study was to establish the frequency of fetal cells in the maternal blood of multifetal pregnancies and compare this figure with singleton pregnancies. METHODS: We obtained maternal blood from 31 pregnancies with 2–6 fetuses at 11–16 weeks gestation and from 50 normal singleton controls (11–14 weeks gestation). Fetal erythroblasts were isolated from maternal blood using triple density gradient separation and anti-CD71 magnetic cell-sorting techniques. The enriched erythroblasts were stained with Kleihauer–Giemsa and with fluorescent antibodies for the zeta (ζ), epsilon (ε) and gamma (γ) globin chains. The percentage of fetal cells positive for each stain was calculated. Fluorescence in-situ hybridization (FISH) for X and Y chromosomes was also performed. RESULTS: The percentage of erythroblasts enriched from maternal blood that stained positive for ζ, ε and γ globin chains and with Kleihauer–Giemsa was significantly higher in the multifetal compared with singleton pregnancies. The median enriched percentage of positively stained erythroblasts was about three times higher in the twin than in singleton pregnancies (P < 0.0001), nearly twice as high in the triplet than in twin pregnancies (P < 0.01) and five times higher in the triplet than singleton pregnancies (P < 0.001). FISH for Y chromosome confirmed the increase in fetal cell proportion in the multifetal pregnancies. CONCLUSIONS: These findings suggest that there is an increase in the physiological feto-maternal cell trafficking in multifetal pregnancies compared with singleton pregnancies, which is likely to be due to the increased placental surface area and vasculature.

Key words: embryonic (ζ,ε) and fetal (γ) globins/fetal cells/maternal blood/multifetal pregnancy

Introduction

In normal singleton pregnancy fetal blood cells escape into the maternal circulation and the frequency of these fetal cells has been estimated to be one fetal cell in 103–108 maternal cells (Gänsshirt-Ahlert et al., 1995; (Al-Mufti et al., 1999a). The proportion of fetal cells in maternal blood is increased in pregnancies with fetal chromosomal abnormalities (Bianchi et al., 1997; (Al-Mufti et al., 1999b) and in those complicated by pre-eclampsia, fetal growth restriction and fetal anaemia (Holzgreve et al., 1998; (Al-Mufti et al., 2000a,b, 2001a).

In multifetal pregnancies, there is an increase in the level of maternal serum alpha-fetoprotein (AFP), which is proportional to the number of fetuses. This may be due to associated increase in feto-maternal haemorrhage (Abbas et al., 1994) or twinning by itself. The aim of this study is to investigate further feto-maternal cell trafficking in multifetal pregnancies.

Materials and methods

Subjects

We examined 31 multifetal pregnancies at 11–16 weeks gestation, including 21 twins (6 monochorionic and 15 dichorionic), 7 triplets, and one each of quadruplets, pentuplets and sextuplets. The women were referred to our centre for first trimester fetal assessment or for embryo reduction. Screening for chromosomal defects by maternal age and fetal nuchal translucency (NT) measurement was carried out, and in all cases studied the NT risk was low. Gestational age was calculated from the date of the last menstrual period and confirmed by the ultrasound examination. None of the fetuses had any obvious anatomical defects or sonographic markers of chromosomal abnormalities. Maternal blood was obtained prior to any intrauterine intervention. Maternal blood was also obtained from 50 normal singleton pregnancies at 11–14 weeks gestation as controls. Patients gave written informed consent to participate in the study. Details of pregnancy and outcome were obtained from the patients’ notes. All mothers studied were healthy, normotensive and with no medical or other pregnancy complications. Outcome showed pregnancies were uneventful and resulted in healthy live infants with no obvious defects.

Isolation of fetal cells and staining

Maternal venous blood (20 ml) samples were collected into Lithium heparinized vacutainers (Beckton Dickinson, Franklin Lakes, NJ, USA), stored at 4°C, and processed within 24 h of collection. Triple density gradient centrifugation was carried out as previously described and the middle layer containing the erythroblasts was separated and isolated ((Al-Mufti et al., 1999b, 2000a,b, 2001a,b). Cells were incubated with magnetically-labelled CD71 antibody to the transferrin receptor antigen (Miltenyi Biotech, Bergisch Gladbach, Germany) for
Figure 1. Percentage of fetal erythroblasts enriched from maternal blood positive for the Kleihauer-Giemsa stain (a), γ (b), ε (c) and ζ-globin chains (d) in the multifetal and singleton pregnancies. In twin pregnancies closed circles = dichorionic; open circles = monochorionic.
15 min at 4°C. Magnetic cell sorting was then performed to enrich these erythroblasts as previously described (Al-Mufti et al., 1999b, 2000a,b, 2001a,b). In each case, four aliquots were obtained from the positively selected fractions, cells were cytocentrifuged at 14.3 g for 10 min (Shandon, Frankfurt, Germany) and were cytopun onto four slides. The fetal cells were detected and quantitated using two methods; firstly by morphology staining by the Kleihauer–Betke method (GTI, North Patrick Boulevard, Brookfield, Wisconsin, USA) and counterstaining with methylene blue (Gurr-Giemsa, BDH Merck Ltd, Poole, UK), and secondly by immunocytochemistry using monoclonal fluorescein isothiocyanate (FITC) conjugate fluorescent antibody against zeta (ζ), epsilon (ε) and gamma (γ) haemoglobin chains. Cells were fixed and permeabilized, as previously described (Al-Mufti et al., 2000a,b,c, 2001a,b) using a commercial ‘Fix and Perm’ reagents (Caltag Burlingame, CA, USA). Slides were then incubated with monoclonal FITC conjugate fluorescent antibody for the ζ, ε and γ chains respectively ((Al-Mufti et al., 2000a,b,c, 2001a,b). After antibody incubation, the slides were washed in phosphate buffered saline solution and mounted with 4,6-diamidino-2-phenylindole (DAPI). The slides were examined under fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Gottingen, Germany), using DAPI/FITC/TRITC (tetramethyl rhodamine isothiocyanate) triple band pass filter set. Image capture and processing was by a Microsoft computerized system (Vysis Inc., Downers Grove, Illinois, USA). Nucleated cells that showed specific staining above the DAPI background stain were counted as positive. At least 100 nucleated cells were counted.

The remaining cells in the positive fractions were centrifuged, treated with KCl and fixed with methanol/glacial acetic acid. Cells were transferred to glass slides and fluorescence in-situ hybridization (FISH) was carried out using a dual chromosome-specific DNA probes kit (Vysis Inc.) to detect chromosomes X and Y as previously described (Al-Mufti et al., 1999b, 2000a,b,c, 2001a,b). The number of nucleated cells and Y-signal positive cells were calculated. At least 100 nucleated cells were examined on each slide and the percentage of cells with one signal for the Y chromosome probe, and one, two and three signals for the X chromosome probe were calculated. Only intact cells that were not overlapping were chosen for the analysis. The slides were examined under fluorescence microscope (Zeiss Axioskop microscope; Carl Zeiss), using DAPI/FITC/TRITC triple band pass filter set. Image capture and processing was by a Microsoft computerized system (Vysis Inc.). Enrichment of fetal cells and analysis were carried out without knowledge of the clinical details of the patients.

Statistical analysis

For morphology and immunocytochemistry, the number and percentage of fetal cells was calculated out of the total nucleated cells for each of the globin chains and for the Kleihauer–Giemsa stain. On FISH analysis, the number and percentage of cells positive for Y- and X-signals were calculated. Comparison was made between the proportion of cells positive for each staining method in the multifetal and singleton pregnancies using Mann–Whitney U-test. Spearman correlation coefficient analysis was used to determine the significance of the association between the different methods of staining.

Results

The median maternal age in the 31 multifetal pregnancies was 34 years (range 19–41) and in the 50 singleton control pregnancies was 35 years (range 17–44). The median gestation at the time of blood sampling was 12 weeks (range 11–16) for the multifetal pregnancies and 12 weeks (range 11–14) for the singleton pregnancies.

In the multifetal pregnancies, positive erythroblasts were detected in all cases using the Kleihauer–Giemsa, γ and ε-chains stains and in 16 of the 31 (52%) cases with the ζ-chain stain, while in the singleton pregnancies positive erythroblasts were identified in 49 of the 50 (98%) cases with the Kleihauer–Giemsa, γ and ε-chains and in 10 (20%) cases with the ζ-chain. The proportion of fetal erythroblasts was significantly higher in the multifetal than in the singleton pregnancies, being three times higher in the twin than in singleton pregnancies (Kleihauer–Giemsa, γ and ε-chains, P < 0.0001; ζ-chain, P < 0.0004; Figure 1), twice higher in the triplet than in twin pregnancies (Kleihauer–Giemsa, γ, ε and ζ-chains, P < 0.01; γ-chain, P < 0.02; ε-chain, P = 0.45; ζ-chain, P = 0.48; Figure 1), and five times higher in the triplet than in singleton pregnancies (Kleihauer–Giemsa and γ-chain, P < 0.0001; ε-chain, P < 0.001; ζ-chain, P < 0.02; Figure 1). The median percentages of positive-erythroblasts for Kleihauer–Giemsa, γ, ε and ζ-chains for the singleton pregnancies were 2 (range 1–5), 2 (range 0.5–4), 1 (range 0.3–4) and 0.5 (range 0.3–1) respectively, for twin pregnancies were 6 (range 3–9), 4 (range 2–8), 3 (range 1–7) and 1 (range 0.4–2) respectively and for triplet pregnancies were 10 (range 5–11), 7 (range 4–9), 5 (range 2–9) and 2 (range 0.2–3) respectively. There was no significant difference in the percentage of positive erythroblasts between the monochorionic and dichorionic twin pregnancies (Kleihauer–Giemsa, monochorionic = 4.7, dichorionic = 5.9; γ-chain, monochorionic = 4, dichorionic = 4.5; ε-chain, monochorionic = 3.4; ζ-chain, monochorionic = 0.7, dichorionic = 1.1%).

There was a significant correlation between the Kleihauer–Giemsa stain and each of the γ-chain (r = 0.88, P < 0.001), ε-chain (r = 0.8, P < 0.001) and ζ-chain (r = 0.56, P < 0.001). A significant correlation was also found between the ζ and ε chains (r = 0.66, P < 0.001), ζ and γ chains (r = 0.43, P < 0.001), and ε and γ chains (r = 0.65, P < 0.001).

Using FISH for Y chromosome, the proportion of fetal erythroblasts positive for Y-signals was significantly higher in the multifetal than in the singleton pregnancies. Of the 50 singleton pregnancies, 28 were carrying male fetuses. In these 28 males, 11 cases had positive signals for Y chromosome with a median percentage of 3 (range 1–4). In the 31 multifetal pregnancies there were 28 pregnancies with twins and triplets. Of these 28 pregnancies 14 were carrying male fetuses. In the six monochorionic twins, two were with male fetuses. In the dichorionic twins, seven were with male fetuses, of which four cases had both fetuses being male and three had one male and one female fetus. In the six twin pregnancies with both fetuses being male (two of monochorionic and four of dichorionic), the median percentage of cells positive for Y-signals was 8 (range 3–9). This was significantly higher than the singleton pregnancies (P < 0.001). In the three cases of dichorionic twins with one male and one female fetus, the median percentage of positive cells for Y-signals was 4 (range 1–4), which is similar to that in singletons. In triplet pregnancies, five of the seven cases studied were carrying male fetuses. Of these five male pregnancies, two cases had all three fetuses being male and
three cases had one female and two male fetuses. In the two cases with all three fetuses being male, the percentages of positive cells for Y-signals were 12 and 11 respectively. This was significantly higher than singleton (P < 0.02) and twin pregnancies (P < 0.05). In the three triplet pregnancies with one female and two male fetuses, the median percentage of positive cells for Y-signals was 8 (range 3–10), which is similar to the values in twin pregnancies with both fetuses of male gender.

There was a significant correlation between Y-signal FISH and the Kleihauer–Giemsa (r = 0.85, P < 0.001), γ-chain (r = 0.75, P < 0.001), ε-chain (r = 0.7, P < 0.001) and ζ-chain (r = 0.5, P < 0.003).

Discussion
Our data demonstrated that in multifetal pregnancy, compared with singleton pregnancy, there is a higher number of fetal erythroblasts in the maternal circulation. We found the higher the number of fetuses, the greater the number of erythroblasts, with the percentages of erythroblasts being three times higher in the twins and five times higher in the triplets compared with singletons. For quadruplet, pentuplet and sextuplet pregnancies, one case of each was studied and therefore it is not possible to make the same conclusion as for the twins and triplets. The only previous study in multifetal pregnancies (Thomas et al., 1995) reported the presence of fetal cells in maternal blood in one triplet pregnancy at 5 weeks and 5 days gestation. The study did not comment on the quantity of fetal DNA detected or the proportion of the enriched fetal cells in the triplet compared to singleton pregnancies.

We previously reported the use of embryonic and fetal haemoglobins as fetal cell markers to detect the presence of fetal erythroblasts enriched from maternal blood in the first trimester of pregnancy and confirmed these findings by the presence of Y-signals on FISH in the male cases (Al-Mufti et al., 2001b). We demonstrated the variation in the normal frequency of the embryonic and fetal haemoglobin chains in these normal singleton pregnancies (Al-Mufti et al., 2001b). We have also shown that the embryonic haemoglobin chains were absent in the non-pregnant females and male adults, providing strong evidence of the specificity of the staining and utility of this technique in identifying fetal cells by immunocytochemistry techniques (Al-Mufti et al., 2000c, 2001b). In this study we demonstrated the distribution of embryonic and fetal haemoglobins in multifetal pregnancies and found an increase in cells positive for the embryonic and fetal haemoglobin chains and an increase in the percentage of cells positive for Y-signals from pregnancies with male fetuses. The data of the current study and previous study therefore exclude the possibility of the enriched erythroblasts that positively stained for embryonic haemoglobins are maternal in origin. In addition, all the pregnancies included in this study were normal and therefore the increase in fetal cell number cannot be attributed to an abnormal pathology such as chromosomal defects (Bianchi et al., 1997; (Al-Mufti et al., 1999b) or utero-placental insufficiency (Holzgreve et al., 1998; (Al-Mufti et al., 2000a;b) that result in an increase in the proportion of fetal cells in maternal blood.

In the twin pregnancies, the type of placental chorionicity might hypothetically have an effect on transfer of fetal cells across the placenta into the maternal circulation. However, our data showed that chorionicity did not appear to influence the number of fetal cells enriched from maternal blood, with the proportion of enriched erythroblasts being similar in monochorionic and dichorionic twins.

The increase in fetal cells enriched from maternal blood in multifetal pregnancies is most likely to be simply related to the number of fetuses, as well as the increased placental mass, resulting in a larger feto-maternal cell trafficking.

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References

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