Neutrophil apoptosis in neonates with intrauterine growth restriction

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ABSTRACT: Background. Intrauterine growth restriction (IUGR) is associated with caloric deprivation, hypoxia and increased corticosteroid levels, which may affect neutrophil apoptosis.

Objectives. In this study, we aimed to assess neutrophil apoptosis in neonates with IUGR not associated with maternal hypertension or congenital infection.

Patients and methods. Eighteen neonates with IUGR born to normotensive mothers, without evidence of congenital or perinatal infection, and 18 gestational age matched, appropriate for gestational age (AGA) neonates were studied within the first 6 hours after birth and on day 5 of life. Neutrophil apoptosis was assessed utilizing three flow cytometric assays including the Annexin V/propidium iodide (PI) assay, analysis of DNA content after staining with PI (sub-G1 peak) and assessment of Fas (CD95) receptor expression on neutrophils using a whole blood technique. Plasma levels of soluble Fas ligand (sFasL) and IL-15 were also measured.

Results. The percentages of neutrophils being Annexin V+PI− or in the sub-G1 peak (analysis of cell cycle) or Fas+ as well as plasma sFas-L concentrations did not differ between groups either on day 1 or on day 5. Plasma IL-15 concentrations were undetectable (less than 20pg/ml) in all neonates on both days 1 and 5 of life. Comparisons between days 1 and 5 showed that certain apoptotic markers tended to increase on day 5, especially in the IUGR group.

Conclusion. Neutrophil apoptosis at birth is not affected by IUGR in the absence of maternal hypertension or infection.

Key Words: Annexin V, Apoptosis, Fas, sFasL, IUGR, Propidium iodide.

INTRODUCTION

Neutrophils are very important host defense cells in neonates, in which innate immunity has not been fully developed. Adequate neutrophil number and function are necessary for efficient killing of the pathogens. Changes in the neutrophil number may be due to alterations in either production or apoptosis. For instance, neutropenia often found in infants of hypertensive or preeclamptic mothers, regardless of the intrauterine growth status, has been attributed to decreased neutrophil production1,2, as a result of a placental inhibitor found in the circulation2. Interestingly, data regarding the neutrophil count in neonates with isolated IUGR born to normotensive mothers are sparse3. Animal studies by Ernst et al showed a significant decrease in mature neutrophils in the bone marrow of guinea pig fetuses in which IUGR was induced by uterine artery ligation4.

The other important regulator of neutrophil number and function is apoptosis. The latter, a process by which the cell programs its own death, serves several purposes and is distinguished from necrosis by its unique cytological, biochemical and molecular characteristics5. Besides its other biological roles, apoptosis interferes with various neutrophil functions, and increased apoptosis has been associated with functional impairment of the neutrophils6,7.

It is well recognized that IUGR fetuses are exposed to chronic hypoxia and a metabolic as well as endocrine response characterized by increased corti-
sol levels. Both hypoxia and corticosteroids are factors that have been demonstrated to decrease neutrophil apoptosis. Apart from that, there is evidence that malnutrition, the hallmark of IUGR, influences immunity, and IUGR neonates have been found to have impaired bactericidal and fungicidal activity of neutrophils. Nevertheless, the effect of malnutrition on neutrophil apoptosis is unknown.

On the basis of these data, we made the hypothesis that the intrauterine combination of hypoxia, increased endogenous corticosteroid levels and caloric deprivation might affect neutrophil apoptosis in the IUGR neonate, independently of maternal hypertension or infection. To this end, we utilized three flow cytometric assays including the Annexin V/propidium iodide (PI) assay, analysis of cell cycle after staining with PI and assessment of Fas receptor expression on neutrophils using a whole blood technique. In addition, we measured plasma levels of the sFasL and plasma levels of IL-15, a known inhibitor of neutrophil apoptosis. Blood sampling was performed within the first 6 hours of life representing the effect of intrauterine factors and on the 5th day thereafter reflecting the development of the apoptotic process postnatally.

PATIENT AND METHODS

Eighteen neonates with IUGR and 18 healthy appropriate for gestational age (AGA) controls matched for gestational age were included in this study. IUGR was defined as BW<10th percentile for gestational age. Neonates born to hypertensive mothers and those with major congenital abnormalities or clinical evidence of intrauterine infections were excluded. All neonates initially enrolled in the study underwent a TORCH investigation and those having laboratory evidence of infection were excluded. The study was approved by the ethical committee of our institution and an informed consent was obtained from all parents. The scientists who performed the measurements were unaware of the infants' assignment groups.

Assessment of neutrophil apoptosis was performed within the first 6 hrs after birth and at the 5th day of life. Peripheral venous blood samples were collected in test tubes containing heparin as anticoagulant. Three-hundred micro liters (µl) of the samples with an equal quantity of nutrient medium (RPMI + 10% fetal calf serum supplemented with 1mM Glutamine) were incubated in a 5% CO2 atmosphere for 12 hours, prior to assessment of the apoptotic neutrophils. Of the remaining whole blood, plasma was separated with centrifugation at 300 g for 10 min and stored at -80°C up to the sFasL measurement.

Assessment of neutrophil apoptosis

Cells were processed on a Coulter EPICS-XL flow cytometer (Coulter Electronics, Hialeah, FL, USA). Analyses performed in this study included: a) the apoptosis assay by Annexin V/PI binding, b) analysis of cell cycle (DNA content analysis) and c) determination of Fas (CD95) receptor expression on neutrophils. All measurements were performed on unstimulated peripheral blood cells.

Annexin V/PI assay

We utilized a whole blood technique with the use Annexin V conjugated with the fluorochrome FITC (Coulter Immunotech) and PI (Coulter Immunotech) for flow cytometric analysis of cells undergoing apoptosis according to manufacturer’s instructions. Annexin V is a 35 kD phospholipid binding protein that has a high affinity for phosphatidylserine, which is translocated to the outer leaflet of cell membrane during apoptosis. PI is a nuclear dye for which living and apoptotic cells are impermeable and was used to distinguish apoptotic from necrotic cells. After red cell lysis, samples were washed with ice-cold culture PBS (GIBCO, Invitrogen Ltd, Inchinnan Business Park, United Kingdom). After centrifugation for 5 min at 500 g at 4°C cells were suspended in Ca2+ enriched binding buffer (Kit component - Apoptosis Detection kit - Coulter Immunotech, Maiami,FL,USA). Then, cells were stained with 10 µl of anti-CD15 PE-Cy5 MoAb (Coulter Immunotech) for 15 min, 40 µl FITC–Annexin V and 10 µl of PI. Following 2 washes in PBS cells were suspended in 150 µl binding buffer (GIBCO, Invitrogen Ltd, Inchinnan Business Park, United Kingdom) and analyzed immediately by flow cytometry. Annexin V fluorescence emission was detected in the FL-1 (green fluorescence) channel and PI was detected in FL-2 channel (orange fluorescence). Gate A was set on the neutrophil region in the for-
ward scatter/side scatter plot to exclude lymphocytes, monocytes and debris. CD15 positive cells (neutrophils) were selected in gate B from gate A.

Finally, apoptosis in neutrophil population was measured by plotting the events from gates A and B in a (dual) biparametric histogram using FL-1 channel (Annexin V) and FL-2 channel (PI). The biparametric representation showed three distinct populations: 1) the viable cells (Annexin V−/PI−), 2) the apoptotic cells (Annexin V+/PI−) and 3) the late apoptotic and necrotic cells (Annexin V+/PI+) (Figure 1).

Analysis of the cell cycle
DNA-Prep kit (Coulter Immunotech) was used in the preparation of samples for the quantitative measurement of cellular DNA content. The measurement is based upon the ability of PI to bind stoichiometrically to double stranded DNA, under appropriate staining conditions. PI staining is a dye-exclusion assay that discriminates between cells with intact membranes (PI negative) and permeabilized membranes (PI positive). After staining with PI cells, which have lost DNA due to DNA fragmentation during the apoptosis process, will take up less stain and will appear to the left of the G1 peak (apoptotic peak, Figure 2). M-CYCLE Software was used for the calculation of sub-G1 peak.

Figure 1. Flow cytometric analysis of 12h incubated blood samples labelled with CD15 PE-Cy5, PI and Annexin V-FITC. (a) Gate A was set on the neutrophils in the FSC/SSC plot. (b) Gate B represents CD15+ cells from gate A. (c) Annexin staining in neutrophils: F3 quadrant viable cells, F2 quadrant necrotic cells, F4 quadrant apoptotic cells.

Figure 2. Detection of apoptotic cells (sub-G1 peak) by flow cytometry based on cellular DNA content analysis.
<table>
<thead>
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<th></th>
<th>AGA GROUP</th>
<th>IUGR GROUP</th>
<th>p</th>
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<tr>
<td>(n=18)</td>
<td>(n=18)</td>
<td></td>
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<td>Gestational age (wks, X±SD, range)</td>
<td>34.7±3.9</td>
<td>34.8±3.7</td>
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<td>Birth weight (g, X±SD, range)</td>
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<td>Head circumference (cm, X±SD)</td>
<td>32±3.5</td>
<td>29±2.5</td>
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<td>Length (cm, X±SD)</td>
<td>47±5.5</td>
<td>41±4.0</td>
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<td>Sex (Male/Female)</td>
<td>10/8</td>
<td>8/10</td>
<td>N.S.</td>
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<tr>
<td>Prenatal steroid administration (n, %)</td>
<td>5 (28)</td>
<td>4 (22)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cesarean section (n, %)</td>
<td>9 (50)</td>
<td>13 (72)</td>
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<td>Apgar score 1 min (X±SD)</td>
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<td>Blood glucose on admission (g/dL, X±SD)</td>
<td>54.1±11</td>
<td>31.3±16</td>
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<td>Respiratory distress (n, %)</td>
<td>7 (30)</td>
<td>10 (55.6)</td>
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<td>4 (22.2)</td>
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<tr>
<td>IPPV on day 5 (n, %)</td>
<td>2 (11)</td>
<td>1 (5.6)*</td>
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<td>Oxygen on day 1 (n, %)</td>
<td>5 (27.8)</td>
<td>6 (33.3)</td>
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<tr>
<td>Oxygen on day 5 (n, %)</td>
<td>0 (0)</td>
<td>1 (5.9)</td>
<td>N.S.</td>
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<tr>
<td>IVH of grade III-IV (n, %)</td>
<td>0 (0)</td>
<td>1 (5.5)</td>
<td>N.S.</td>
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</table>

IPPV: intermittent positive pressure ventilation, IVH; intraventricular hemorrhage.
* 1 died on day 2.

Table 1. Perinatal and neonatal characteristics.

Detection of Fas receptor expression on neutrophils

Briefly, to determine surface Fas expression, 100 μl of whole blood were stained with 10 μl anti-CD95 FITC MoAb (Coulter Immunotech), 10 μl of anti CD15 PE MoAb (Coulter Immunotech) and 10 μl of anti-CD45 PE-Cy5 MoAb (Coulter Immunotech) for 10 min at room temperature. After erythrocyte lysis and cell fixation samples were analyzed by flow cytometry. Neutrophil population was selected using specific forward and side scatter characteristics. The percentage of CD15+ CD95+ positive cells were extracted from dual plot histograms gated on CD45+ positive cells.

Measurement of plasma concentrations of soluble Fas Ligand and IL-15

The quantitative determination of sFasL and IL-15 concentrations in plasma samples was performed with enzyme—linked immunoassay with commercially available kits (Quantikine R&D Systems, Abingdon, United Kingdom) and Anogen (Ontario, Canada) for FasL and IL-15, respectively. The minimum detectable levels were 1.01–8.05 pg/ml and 20 pg/ml for FasL and IL-15, respectively.

Statistical analysis

Epidemiological characteristics are expressed as mean and standard deviation or proportions whereas values of the apoptotic markers are presented in the figures as medians, 25% and 75% quartiles and ranges (box plots). Comparisons of values between the IUGR and the AGA group, as well as between the 1st and 5th days of life were made using the Mann-Whitney U test, as values did not follow the normal distribution. Categorical differences between the two groups were compared with the Fisher's exact test. The Spearman correlation coefficient was used for correlation between the proportions of the Annexin V+/PI− neutrophils and those in the sub-G1 peak. The level of 0.05 was set as the level of significance. Statistical analysis was performed by using the SPSS
12.0 for windows software (LEAD Technologies, Inc) and the INSTAT.

RESULTS

Perinatal and neonatal characteristics
The two groups had comparable gestational age, whereas the birth weight was significantly lower in the IUGR group (Table 1). The incidence of prenat al steroid administration and cesarean section did not differ significantly between the two groups. The mean Apgar scores at 1 and 5 min were significantly lower in the IUGR group as were the glucose levels after birth (Table 1). The two groups did not differ significantly in either the incidence of respiratory distress and respiratory support with mechanical ventilation on admission or the proportion of neonates needing mechanical ventilation or oxygen on day 5 (Table 1).

Comparisons of neutrophil apoptosis between the IUGR and AGA neonates
The IUGR neonates tended to have lower neutrophil counts as compared with the AGA ones on both days 1 and 5, although the difference was not significant (Figure 3). No neonate developed neutropenia (<1500/mm³). The percentage of Annexin V⁺/PI⁻ neutrophils, as well as those being in the sub-G₁ peak was similar in the two groups on day 1. On day 5, there was a trend toward a higher proportion of Annexin V⁺/PI⁻ or sub-G₁ neutrophils in the IUGR group (Figure 3). There was a significant correlation between the percentage of Annexin V⁺/PI⁻ neutrophils and those being in the sub-G₁ peak (Spearman correlation coefficient = 0.615, p < 0.001). The percentage of neutrophils expressing the Fas receptor did not differ significantly between IUGR and AGA neonates either on day 1 or 5 (Figure 3). Plasma concentrations of sFasL were similar in the two groups at birth whereas tended to be higher in the IUGR group on day 5 (Figure 3). Levels of IL-15 were undetectable (less than 20 pg/ml) in all neonates on both days 1 and 5.

Comparison of neutrophil apoptosis between days 1 and 5 within each group
The absolute neutrophil counts did not change significantly in any group between days 1 and 5. In the AGA group, the percentage of Annexin V⁺/PI⁻ or Fas⁺ neutrophils and the plasma levels of sFasL remained unchanged whereas the percentage of neutrophils in the sub-G₁ peak tending to increase on day 5. In the IUGR group, all the above parameters increased between days 1 and 5 although not significantly (Figure 3).

DISCUSSION
This study is the first to examine the effect of isolated IUGR on neutrophil apoptosis. The rationale to exclude IUGR neonates born to mothers with hypertension and chorioamnionitis as well as those with perinatal or congenital infections was based on the knowledge that these conditions are strongly associated with changes in maternal blood and amniotic fluid levels of various cytokines[15-19] which could exert a biological effect on neonatal neutrophil apoptosis[20].

Chronic hypoxia and fetal distress have been associated with increased levels of TNFα, IL-1, IL-6 and IL-8, which could mediate the effect of hypoxia on apoptosis[21-24]. IL-8 has been proved to inhibit neutrophil apoptosis, prolonging their survival, whereas the effect of IL-6 is variable[25-27]. The acute intrapartum distress in growth restricted neonates, as indicated by the lower Apgar score in the IUGR neonates, could represent an additional factor that might have an effect on neutrophil apoptosis, possibly mediated by the elevated IL-1, IL-6, IL-8 and TNFα[21-23]. Vaginal delivery, that has also been associated with elevated levels of certain cytokines in neonatal serum, is unlikely to have influenced our results since comparable proportion in each group was delivered vaginally[28,29]. Increased corticosteroid level is an additional factor that could influence apoptosis, as several studies showed that they inhibit neutrophil apoptosis[8,20]. Malnutrition has been reported to induce macrophage apoptosis in an animal model[1]. Yet, there are no published data regarding its effect on human neutrophil apoptosis.

Contrary to our hypothesis, the percentage of apoptotic neutrophils within the first 6 hours of life did not differ between the AGA and IUGR neonates, even though they were assessed by using different techniques. Therefore, it is possible that chronic
Figure 3. Values of the apoptotic parameters assessed in the AGA and IUGR neonates on days 1 and 5 of life. Data are expressed as box plots, with the boxes representing the 25th and 75th percentiles, the horizontal lines in the boxes indicating medians, and the whiskers (vertical lines) representing the range. No difference between the groups or between days 1 and 5 within each group was significant.
stress in vivo may be a less potent stimulus as compared to hypoxia in vitro\textsuperscript{10}, thus minimizing any changes in neutrophil apoptosis. Changes of neutrophil apoptosis during the first few days of life when a decrease in the neutrophil counts is normally observed have not so far been investigated. In our study, assessment of neutrophil apoptosis on day 5 of life showed a tendency to increase during the first postpartum days in the IUGR but not in the AGA neonates.

Apoptosis is a complex process involving several pathways requiring different laboratory techniques for assessment. The use of DNA-binding dyes and fluochrome-labeled Annexin V is among the most popular in flow cytometric assays. Annexin V, an impermeable plasma protein which specifically binds to the surface phosphatidylserine, is translocated to the outer leaflet of the cell membrane during apoptosis\textsuperscript{33}. An additional staining with propidium iodide is used to distinguish apoptotic from necrotic cells\textsuperscript{32}. Another approach that has been widely used to detect cell apoptosis is analysis of cell cycle using flow cytometry. Apoptotic cells can be detected as hypodiploid cells in flow cytometric histograms where they appear as a peak to the left of the G\textsubscript{1} peak (sub-G\textsubscript{1} peak)\textsuperscript{32}. In our study, the percentage of Annexin V\textsuperscript{+}/PI\textsuperscript{+} neutrophils was significantly correlated with those being in the sub-G\textsubscript{1} peak. Measurement of Fas receptor expression on neutrophils represents an additional means for assessing apoptosis. Neutrophils are susceptible to Fas induced apoptosis, which is mediated by interaction of Fas receptor and its ligand FasL\textsuperscript{33,34}. The physiological role of the FasL shedding has not been fully characterized. In some studies, increased serum FasL levels have been associated with increased neutrophil apoptosis and chronic neutropenia\textsuperscript{35}, whereas others suggest that sFasL rather suppresses apoptosis\textsuperscript{34,36}. Data regarding the Fas expression on neutrophils and the plasma levels of sFasL in IUGR neonates are limited\textsuperscript{37}.

In the present study different techniques were used in order to obtain a more reliable apoptosis quantification. Results of all the techniques used support the notion that isolated IUGR does not significantly affect neutrophil apoptosis, justifying the rare appearance of neutropenia in these neonates. Presumably, intrauterine malnutrition, hypoxia and increased corticosteroids levels do not significantly affect neutrophil apoptosis, which ultimately remains unaffected, as a consequence of counterbalancing apoptotic/anti-apoptotic factors.

In conclusion, results of the present study suggest that IUGR does not affect neutrophil apoptosis at birth, in the absence of maternal hypertension and infection. The neutrophil apoptosis in neutropenic IUGR neonates and in those born to hypertensive or pre-eclamptic mothers requires further investigation.
Απότυπωση των ουδετερόφιλων σε νεογνά με ενδομήτρια καθυστέρηση στην ανάπτυξη

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ΠΕΡΙΛΗΨΗ: Εισαγωγή. Η ενδομήτρια καθυστέρηση της ανάπτυξης (EMKA) συνδέεται με θερμιδική ανεπάρκεια, υποξία και ανυψημένα επίπεδα χορηγοκιτοστεροιδών. Σχετικές μελέτες έδειξαν ότι άλλες προςέγγισες αυτού μπορούν να επηρεάσουν την απότυπωση των ουδετεροφίλων.

Σκοπός. Σκοπός της μελέτης αυτής ήταν η εκτίμηση της απότυπωσης των ουδετεροφίλων σε νεογνά με EMKA, η οποία δε συνδέεται με υπερτίτωση της μητέρας.

Ασθενείς και μέθοδοι. Σε 18 νεογνά με EMKA που γεννήθηκαν από μητέρες χωρίς υπερτίτωση, χωρίς ενδείξεις ενδομήτριας ή περιφερειακής λοιμώξεις, και 18 έγκυοι για την ηλικία της χώρης νεογνών, μελετήσαμε την απότυπωση των ουδετεροφίλων στις πρώτες 6 ημέρες από τη γέννηση και την 5η ημέρα ζωής. Το ποσοστό των αποτελεσμάτων ουδετεροφίλων σε νεογνά εκτιμήθηκε με 3 μεθόδους κυτταρομετρίας ροής: α) χρώση fluorescein isothiocyanate (FITC)-annexin-V μαζί με propidium iodide (PI), β) συνάλλαξη της περικυτικότητας σε DNA μετά από χρώση με PI (sub-G1 peak) και γ) μέτρηση της εκπαραγωγής έξοχος του μορίου Fas (CD95). Επίσης, μετρήθηκαν τα επίπεδα του διαλυμού υποοξέα Fas και της iντελεχενίνης-15 (IL-15) στο πλάσμα.

Αποτελέσματα. Το ποσοστό των ουδετεροφίλων που ήταν FITC-annexin-V+/PI- ή στην sub-G1 peak και εκείνων που ήταν Fas+ καθώς και τα επίπεδα του διαλυμού υποοξέα Fas στο πλάσμα δεν διέφεραν σημαντικά μεταξύ των δυο ομάδων νεογνών оύτε την 1η ούτε την 5η ημέρα ζωής. Τα επίπεδα της IL-15 δεν ήταν ανιχνευτές (κάτω από 200pg/ml) σε κανένα νεογνό ούτε την 1η ούτε την 5η ημέρα. Σύγκριση των τιμών μεταξύ της 1ης και 5ης ημέρας σε κάθε ομάδα νεογνών έδειξε μια τάση για αύξηση ορισμένων δεικτών απότυπωσης, που ήταν πιο εκτιμημένο στα νεογνά με EMKA.

Συμπεράσματα. Η απότυπωση των ουδετεροφίλων στη γέννηση δεν επηρεάζεται σημαντικά από την EMKA σε απουσία μητρικής υπερτίτωσης και ενδομήτριας ή περιφερειακής λοιμώξεις.

Αδειες: Κλειδιά: Annexin V, Απότυπωση, Fas, sFasL, Ιδιοκύτταρο προσιτοί.

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