A HIGH FREQUENCY OF APOPTOSIS WAS FOUND IN CULTURES OF LYMPHOCYTES ISOLATED FROM THE VENOUS BLOOD OF CHILDREN BORN WITH A LOW BIRTH WEIGHT

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Abstract: Children born with a low birth weight (below 2500g) exhibit a slower rate of development, and a greater tendency towards morbidity and mortality, together with a deficit of weight and height. One reason could be an increase in the level of cell elimination by apoptosis. The aim of this study was to evaluate and compare the incidence of apoptotic and necrotic (dead) cells in cultures of peripheral blood lymphocytes obtained from children born with a low birth weight and from children with a normal birth weight. Peripheral blood lymphocytes were obtained by venipuncture (10 ml) and isolated using the density gradient centrifugation method. The lymphocytes were cultured for 48 h in a culture medium containing low concentrations of fetal calf serum. A comparison study was performed between low birth weight children and normal birth weight children and the susceptibility of their lymphocytes to apoptosis and to necrosis in serum-deficient feeding culture conditions. The amount of apoptotic cells and the percentage of dead cells were significantly higher in cultures of lymphocytes obtained from low birth weight children than in cultures from normal birth weight children. The two estimated parameters inversely correlated with the concentration of fetal calf serum in the culture medium. Pulsed field gel electrophoresis showed increased DNA degradation patterns in the cultures of lymphocytes obtained from low birth weight children.

Our results should be perceived as an indication that, under worse feeding conditions, the elimination of cells by apoptosis and by necrosis is significantly higher for lymphocytes of low birth weight children than for those of normal birth weight children. The enhanced elimination of lymphocytes is related to a greater susceptibility to infections, especially of the respiratory tract, as...
established in the retrospective analysis of the anamneses of the examined group of low birth weight children.

Key Words: Children Born With Low Birth Weight, Lymphocyte Cultures, Apoptosis, Necrosis

INTRODUCTION

Children born with a low birth weight (below 2500 g) need special medical consideration due to their slower rate of development and increased tendency towards morbidity and mortality in the first years of their life, and a predisposition to several diseases in adulthood [1]. Mortality and morbidity in children born with a low birth weight (LBW) is markedly higher during the first year of life than in the case of children born with a normal birth weight (NBW) [2, 3]. The incidence of psychomotor disturbances is also greater among children with LBW. In adulthood, they are more likely to develop high blood pressure, diabetes mellitus, glucose intolerance and elevated concentrations of serum cholesterol resulting in an increased risk of death due to cardiovascular disease [4-7]. Children with LBW demonstrate an increased risk of growth hormone (GH) deficiency in the latter stages of life [2, 6, 8-10]. Most infants with LBW compensate their growth deficit during the first 6-9 months of their lives. However, nearly 30% of those children show a substantial deficit of weight and height, below 5 percent (the fifth percentile) [11].

It was found that the elimination of fetus cells by apoptotic pathways was greater in children born with LBW, and probably resulted from maternal IGF1-GH axis inefficiency [6, 9, 12]. Those mechanisms of cell elimination and anabolic signals can presumably outlast the prenatal period, making the cells ‘more prone’ to apoptosis in states of malnutrition.

In developing countries, the incidence of infants born with low birth weight (below 2500g) after a normal duration of pregnancy (more than 37 weeks) is 5.8 times higher than in the rest of the world [1]. It should also be stressed that in developing countries, malnutrition of children is common, and in those countries, the pre-natal development and post-natal care of LBW children deserve particular attention.

The purpose of this study was to evaluate the incidence of peripheral blood lymphocyte apoptosis in children born with a low or normal birth weight, after 48 hours in vitro incubation with low concentrations of fetal calf serum (FCS). Also, a comparison of the patterns of DNA isolated from the peripheral blood lymphocytes of children from the two investigated groups was done by means of pulsed field electrophoreses.

METHODS

Patients

This project was approved by the Bioethical Committee of Wroclaw Medical University (appr. Nr.: KB-235/2000). The study included 10 children aged 4-11
(mean [SD], 6.95 [2.1] years) born with a low birth weight, below the tenth percentile for gestational age. The control group consisted of 10 children aged 4 - 11 (mean [SD], 6.95 [1.97] years) born with a normal birth weight. The children did not show any signs of puberty (1\textsuperscript{st} grade on Tanner’s scale).

\textbf{Lymphocyte separation and culture}

Heparinized blood (10 ml), obtained by venipuncture, was separated using the single-step continuous density-gradient centrifugation technique with Histopaque-1077 [13]. Lymphocytes were cultured in 24-well plastic dishes at a density of $5 \times 10^5$ cells/ml in a culture medium composed of Minimum Essential Eagle (MEM), 2 mM L-glutamine, 25 µg/ml gentamycin and various concentrations of FCS (concentration range: 0.0% to 10%, v/v). Cells were stimulated to mitogenesis with a lectin – PHA-M (1% v/v) – and the cultures were carried out for 48 h at 37°C in a CO\textsubscript{2} incubator.

\textbf{Detection of apoptotic cells}

The cells were stained with a mixture of fluorescent dyes, acridine orange and ethidium bromide, following the procedure described in the literature [14]. In our laboratory, this staining procedure is routinely used for the detection of apoptotic cells in lymphocyte cultures [15, 16]. The amounts of apoptotic, viable and dead cells were counted within 500 cells found randomly under a fluorescence microscope in slides prepared from cultures of lymphocytes obtained from LBW and NBW children. The amount of dead cells was estimated with a routine propidium-iodide exclusion test [17].

\textbf{Isolation of DNA from lymphocytes for Pulsed field gel electrophoresis}

Aliquots of lymphocytes (separated by single-step continuous density-gradient centrifugation) containing $2 \times 10^6$ cells were suspended in PBS (100 µl) and mixed with equal volumes of 2% low-melting point agarose at 37°C. The mixtures were then poured into a multiwell blockformer and left on ice for 30 min to solidify. Afterwards, the microslabs were transferred into eppendorff centrifuge tubes and incubated overnight at 56°C with 0.05% proteinase K in 50 mM Tris HCl, pH 7.0, containing 50 mM EDTA. The microslabs were washed twice with 50 mM Tris HCl, pH 7.0, 50 mM EDTA and polymerized into 1% agarose gel.

\textbf{Pulsed-field gel electrophoresis}

In general, the pulsed-field electrophoresis conditions were the same as previously described [16, 18]. The electrophoresis was run by means of the contour-clamped homogeneous electric field system (CHEF). The CHEF III apparatus was from BioRad, USA. The DNA size markers Mid Range I PFG Marker and Lambda Ladder PFG Marker (NEB Nucleic Acids, USA) were applied. The electrophoresis conditions were set for the separation fragments of DNA from 500 kb to 50 kb (switch time 50 seconds, run time 17 hours, angle
120°, voltage gradient 6 V/cm). Finally, the gels were stained with EtBr, inspected and photographed under a UV-lamp.

**Statistical analysis**
The relationships between the concentration of FCS in a culture medium and estimated frequency of apoptotic cells and, separately, of dead cells were estimated via the calculation of regression equations, and the significance of the estimated results was assessed with a two-way analysis of variance [19].

**RESULTS**

Children born with a low birth weight (LBW) differed significantly from children born with a normal birth weight (NBW) in their birth length, weight and body mass index (BMI). LBW children were born two weeks earlier, on average, than NBW children, although only one child from the LBW group was born after a pregnancy lasting below 37 weeks. The general characteristics of the examined groups of children are given in Tab. 1.

**Tab. 1. The general characteristics of the tested groups of children.**

<table>
<thead>
<tr>
<th>Tested group of children</th>
<th>Duration of pregnancy (weeks)</th>
<th>Birth length (cm)</th>
<th>Birth weight (g)</th>
<th>HV SDS (Standard deviation score of height)</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBW</td>
<td>mean 37.3 SD 1.15</td>
<td>mean 48 SD 2.6</td>
<td>mean 2100 SD 237</td>
<td>mean -2.35 SD 0.72</td>
<td>mean 14.98 SD 1.4</td>
</tr>
<tr>
<td>NBW</td>
<td>mean 39.3 SD 1.13</td>
<td>mean 53.9 SD 1.45</td>
<td>mean 3160 SD 388</td>
<td>mean 1.24 SD 1.29</td>
<td>mean 18.1 SD 4.71</td>
</tr>
</tbody>
</table>

p<0.05       p<0.05       p<0.001   p<10⁻⁴     p<0.05

We routinely detect apoptosis in cell cultures by means of the EtBr/AO-staining procedure, followed by the examination of cell culture smears under a fluorescent microscope. This method of cell staining permits a high quality analysis of cell morphology and the detection of apoptosis, with results that are repeatable in a series of experiments. As an example of the typical results obtained with this staining procedure, Fig. 1 shows a microphotograph taken from a microscopic image of the culture incubated with a lectin (PHA-M, 1% v/v) in the presence of 2.5% foetal calf serum (FCS) in a culture medium. The majority of the cells in Fig. 1 are yellow-to-orange-stained; they are dead cells. Two cells (top right) are early apoptotic – they display green fluorescence and contain irregularly-shaped nuclei. The membranes of these cells exhibit active blebbing, and the formation of apoptotic bodies is also evident. Another cell (top left) exhibits yellow-to-orange fluorescence and contains an irregularly-
shaped nucleus – it could be rated as a late apoptotic cell. Only one cell in Fig. 1 is viable (bottom); it has a regular, round-shaped nucleus and exhibits yellowish-to-green fluorescence.

Fig. 1. The detection of apoptosis via double-staining fluorescence (EtBr/AO) in a suspension of lymphocytes isolated from the venous blood of a LBW child, and cultured in the presence of lectin PHA-M (1% V/V) and 5% FCS in a culture medium.

A microscopic examination showed that lymphocytes obtained from LBW children more frequently exhibited apoptosis after 48 h of culture than lymphocytes isolated from NBW children. The correlation between apoptotic cell numbers and the concentration of FCS in the cell culture medium was calculated with regression equations and is given in Fig. 2. As can be seen in Fig. 2, the fraction of apoptotic cells increased in proportion to the decreasing concentration of FCS, both in the cases of LBW and of NBW blood donors, although it should be noted that the amount of apoptotic cells in LBW lymphocyte cultures was 5-12% higher than that in NBW lymphocyte cultures. Even in the optimal concentration of FCS (10%, v/v) recommended for routine in vitro cultures of lymphocytes [20], the number of apoptotic cells in LBW lymphocyte cultures was 12% higher than in NBW lymphocyte cultures. In the highest concentrations of FCS (2.5%-10%), a relative decrease in apoptotic cell frequency was seen in LBW lymphocyte cultures and in NBW lymphocyte cultures. This was probably due to an elevation in the dead cell fraction in those cultures in which necrosis prevails over apoptosis, which is an energy-consuming mode of death.
Fig. 2. Apoptotic cell frequencies in cultures of lymphocytes obtained from LBW children and from NBW children; analyses carried out in a culture medium containing various concentrations of FCS (mean, n=10).

Fig. 3. The relationship between dead cell numbers and the concentration of FCS in the culture medium, estimated in a culture of lymphocytes obtained from LBW children and NBW children (mean, n=10).
The two-way analysis of variance proved that the differences between the apoptotic cell numbers of LBW lymphocyte cultures and NBW lymphocyte cultures were significant (F = 47.46, df = 1, p = 0.001), and the effect of FCS concentration on apoptotic cell number was also significant (F = 18.47, df = 5, p = 0.0031).

The fraction of dead cells also increased in proportion to the decreased concentration of FCS in the lymphocyte culture medium, as shown in Fig. 3. This figure documents the marked differences in dead cell numbers found in cultures containing lower concentrations of FCS. In the lowest concentration of FCS, the frequency of dead cells in LBW lymphocyte cultures was 25% higher than in NBW lymphocyte cultures.

The amounts of dead cells were significantly different in both groups of children (F = 7.67, df = 1, p = 0.0394) and the impact of various concentrations of FCS on dead cell numbers was estimated as significant (F = 5.31, df = 5, p = 0.0454), as estimated with the two-way analysis of variance.

Fig. 4. CHEF electrophoresis of DNA obtained from lymphocytes from control children (lines 1-3) and from children born with a low birth weight (lines 4-7). M1, M2 – DNA size markers.

In our previous screening using the pulsed field of electrophoresis method (CHEF), including 173 children recognized as LBW patients and 30 children born with normal birth weight (NBW), we showed that DNA from the lymphocytes of LBW children exhibited DNA degradation patterns, as shown in Fig. 4. In the majority of the cultures of lymphocytes obtained from LBW children, we found a marked increase in the size of the 500 kb domain in the DNA electrophoretic profiles. This suggested non-specific DNA degradation in the lymphocyte cultures, although in some cases, in the separation slides, the 50 kb DNA fragment was observed; this is considered an apoptosis-specific electrophoretic domain [16, 17]. By contrast, the DNA separation profiles of
lymphocytes from NBW children did not contain the 50kb domain and only incidentally showed unspecific DNA degradation with the presence of the 500 kb domain.

DISCUSSION

To our knowledge, this is the first information on the higher frequency of apoptosis in lymphocytes obtained from LBW children and cultured in a serum-deficient medium. Lymphocytes are crucial cells in immunity (the immune response); therefore, it is important to enumerate the main subpopulations of lymphocytes before and after a culture in serum-deficient medium. Such data will allow us to predict the effectiveness of the immune system of LBW children and forecast their susceptibility to infections.

A retrospective analysis of our clinical documentation showed that recurrent infections of the respiratory tract were announced in anamneses, in the case of almost 60% of the LBW children studied (93 cases out of 161), whereas in the group of NBW children, only about 10% (4 cases out of 39) suffered from those infections.

The results presented in this paper are considered initial. Investigations have to be done on a larger group of LBW children. Further studies on the mechanisms of apoptosis induction in LBW children are now in progress. However, even these preliminary results seem to be important for physicians, especially for pediatricians, since they could offer a rational explanation of the worse development and greater susceptibility to infection found in low birth weight children in comparison to normal birth weight children.

REFERENCES