Clinical immunology

Different sensitivity of resting and activated blood lymphocytes and B leukemic lymphoblasts on dexamethasone action

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Abstract
The aim of the study was to assess the influence of dexamethasone on these cellular parameters that turn of the cells on apoptosis process. Evaluation of intracellular pH, changes in cell cycle phases and annexin-V staining were performed. The study include peripheral blood lymphocytes isolated from 25 healthy volunteers non stimulated or stimulated with PHA or SAC to blastogenesis and 10 samples of children bone marrow taken at the day of BpC-ALL diagnosis. The cells were cultured in medium with or without dexamethasone (10^{-6} M). Analysis of pH_{i}, cell cycle and apoptosis test were performed on flow cytometer Coulter EPICS-XL.

Acidification of lymphocytes and lymphoblasts incubated with dexamethasone was seen in 10-25% percentage of cells. Rest of cells showed alkalization. Lymphocytes had lower pH_{i} than lymphoblasts, also after incubation with dexamethasone. Dexamethasone influenced phase S in cell cycle only in T lymphocytes (72 h), while it had no effect on B lymphocytes and lymphoblasts. The evaluation subG_{1} phase showed significant influence of dexamethasone on lymphocytes T and lower on lymphocytes B. Dexamethasone had also significant influence on lymphocytes T stimulated with PHA. There was no influence on lymphocytes B stimulated with SAC and lymphoblasts. After 2 and 48 h of incubation with dexamethasone there was a significant increase in percentage of lymphocytes in early apoptotic phase. Influence of dexamethasone on apoptosis of lymphoblasts was lower.

Presented results indicate that dexamethasone is not very potent inductor of B-lymphoblasts apoptosis.

Key words: cell cycle, apoptosis, pH_{i}, dexamethasone, BpC-ALL.


Introduction
Dexamethasone, one of the group of synthetic glucocorticoids possess higher anti-inflammatory than immunosuppressive properties. In human, the both activities are mediated by specific cytoplasmic receptors able to bind dexamethasone with high affinity. The occupation of the receptors results in inhibition of protein synthesis which control of inflammatory as well as immunological response [1]. Recently, it was documented that dexamethasone significantly increase surface and soluble scavenger receptors (CD163 and HCP-1) on macrophages and mononuclear cells [2]. This is probably one of mechanisms minimized in surgical patients occurrence of SIRS [3].

It was documented also that dexamethasone have antileukemic activity [4, 5] and is able to penetrate of CNS when its half-life is longer then others derivates of synthetic glucocorticoids [6]. In group of children with ALL-T close correlation was observed between in vitro susceptibility of leukemic blasts from bone marrow and peripheral blood and in vivo response on therapy including dexamethasone, measured as percentage of blast cells in bone marrow at the day 15 [7]. Significantly higher EFC (events free survival) was observed when dexamethasone was included for treatment of leukemic patients [4-6]. Because of among the various leukemias B-cell acute lymphoblastic leukemia represents the most common childhood leukemia, we decided to check whether action of dexamethasone on T and B lym-
phocytes activated in short cultures by mitogen, and malignant B lymphoblasts taken from bone marrow at the time of leukemia recognition, will be similar. The study was undertaken especially for assessment of susceptibility of these cellular parameters that turn off the cells on apoptosis process. Study include evaluation of annexin-V staining, intracellular pH and changes in cell cycle phases.

Materials and methods

Patients

The study involved 25 healthy, adult volunteers (average age 38.6 ±15.72, median 30): 15 women (average age 38.9 ±15.25, median 37) and 10 men (average age 38.1 ±17.47, median 29) and 10 children (average age 6.2 ±4.98, median 3.75): 3 girls (average age 6.5 ±4.77, median 4) and 7 boys (average age 6.1 ±5.44, median 3) on the day of diagnosis of acute B-precursor cell leukemia (BpC-ALL).

From adult volunteers 5 ml heparinized blood was collected by venipuncture from antecubital vein. In case of children, on the day of clinical diagnosis, 0.5-2.0 ml of heparinised bone marrows were obtained for routine diagnostic test (myelogram, blasts phenotyping, cytochemical examination and cytogenetic test). Remaining bone marrow cells after all these tests were used for research studies.

Cells

Peripheral blood lymphocytes and bone marrow lymphoblasts were isolated using a standard Ficoll-Histopaque®-1077 (Sigma-Aldrich Co, St. Louis, USA) gradient centrifugation. Cells concentration was adjusted to 1 × 10⁶/ml in control culture medium (RPMI1640 supplemented with 10% FCS (Fetal Calf Serum), 200 mM L-glutamine and 1 ml of 1 M Hepes). The procedures were performed in sterile condition. Viability of blood lymphocytes and bone marrow cells was 95-98% as determined by trypan blue staining.

1 × 10⁶ of isolated cells, resuspended in 100 μl of culture medium, were cultured in sterile plastic 96-flat bottom Microwells cultured plates Nunclon (Nunc A/S, Denmark) at 37°C in 95% humidity and in atmosphere containing 5% CO₂. 100 μl of control medium, medium with mitogen or medium with dexamethasone (Sigma-Aldrich Co, St. Louis, USA) was added to the appropriate cells. The final concentration of dexamethasone was 10⁻⁶ M. PHA (phytohaemagglutinin) was used for lymphocytes T stimulation (final concentration in well 20 μg/ml) and SAC (Staphylococcus aureus Cowan A) for lymphocytes B (final concentration in well 1 : 8000).

Cells isolated at day 0 and harvested at 2 and 48 hrs of culture were used for the analysis of apoptosis and intracellular pH. Analysis of cell cycle was performed with the cells cultured for 72 h.

Annexin staining

The assay was performed according to manufacturer’s specifications (Annexin-V kit, Pharmingen, BD Biosciences, Belgium). Cells (1 × 10⁵) were washed and resuspended in 100 μl medium containing Annexin-V Buffer Solution, 5 μl of Annexin-V and 10 μl Propidium Iodide. After incubation for 10 minutes at room temperature in the dark 400 μl Annexin-V Buffer Solution was added and cells were analyzed on flow cytometer within 1 h.

Cell cycle analysis

Cells were washed and resuspended in PBS (phosphate buffered saline), (8-10 × 10⁶ cells/ml). 10 μl of appropriate antibody was added (anti-CD2 FITC (fluorescein isothiocyanate) – 25 μg/ml or anti-CD19 FITC – 100 μg/ml, DAKO, Denmark) to 100 μl of cell suspension. Cells were incubated for 30 minutes at 4°C, in the dark. Cells were washed twice, 100 μl DNA Prep LPR and 2000 μl DNA Prep Stain were added (Coulter DNA Prep, Reagent Kit, BeckmanCoulter, Nyon, Switzerland). Cells were incubated for 30 minutes, at room temperature, in the dark. Cells were analyzed on flow cytometer within 2 h.

Histograms of DNA content were analyzed using MultiCycle software (Phoenix Flow System, Phoenix, USA) to determine fractions of the population in each phase of the cell cycle (subG1, G0/G1, S, G2/M).

Intracellular pH

For intracellular pH (pHi) measurement, 6 × 10⁶ purified cells were centrifuged at 1500 g for 5 minutes and resuspended in PBS. Cells were incubated for 30 minutes at 37°C in 5% CO₂ with carboxy-SNARF-1-AM (5-(and-6)-carboxy-SimiNaphthoRhodaFluor-1, acetoxymethyl ester, acetate, Molecular Probes, Oregon, USA) (final concentration 10 μM). After incubation, cells were centrifuged at 1500 g for 5 minutes then the pellet was resuspended in 1ml PBS and cells were analyzed by flow cytometry (Coulter EPICS XL). Light emission was measured at 575 nm and 635 nm and expressed as a ratio. The pH was calculated based on a standard curve. The standard curve was generated by determining the same fluorescence ratio from cells in buffers with differing pH containing the proton ionophore nigericin, thus allowing convert of the fluorescence ratio into intracellular pH.

Statistical analysis

Statistical analysis was performed using nonparametric Wilcoxon test for dependent results and nonparametric Mann Whitney U test for independent results. For assessing correlation between results Spearman test was used.

The study was approved by Independent Ethics Committee of Medical University of Warsaw.
**Results**

The intracellular pH (pHi) was assessed in normal peripheral blood lymphocytes and bone marrow B lymphoblasts (BpC-ALL) before and after 2 and 48 h of incubation in control medium and in medium supplemented with dexamethasone. The results are shown in Table 1. Bone marrow lymphoblasts had higher pHi in comparison to normal blood lymphocytes mean ± SD 7.78 ±0.19 vs 7.6 ±0.07 respectively, \( p < 0.003 \). After 2 and 48 h of incubation in control medium only about 10% of lymphocytes and 16% of lymphoblasts undergo statistically significant acidification in comparison to pHi of the cells at time 0 (\( p < 0.016 \)). On the contrary, in majority of cells (about 90% cells) pHi were alkalized. Dexamethasone added to culture medium induced slightly alkalization but the significant differences in pHi were observed only in the both of lymphocytes population after 2 hrs of incubation and in 90% of BpC-ALL (tab. 1.).

Dexamethasone decreased significantly proliferation of non stimulated and stimulated with PHA peripheral blood of CD2+ lymphocytes (Fig. 1). This decreased of phase S was seen in 24% of non-stimulated CD2+ lymphocytes and in 79% of PHA-stimulated T cells. Dexamethasone induced no significant changes in proliferation of B lymphocytes (CD19+) cultured without or with SAC. Also, no statistical difference was observed between bone marrow-derived B lymphoblasts cultured with and without dexamethasone (Fig. 1).

The evaluation of subG1 phase of cell cycle (late stage of apoptosis) in PHA-stimulated T lymphocytes (CD2+) revealed the significant increase (\( p < 0.014 \)) in subG1 phase in dexamethasone-treated cells (13.4%) in comparison to cells cultured without dexamethasone (3.1%) (Fig. 2). In the same way, dexamethasone affected CD2+ cells in the absence of PHA stimulation (1.2% vs. 7.15% cells in subG1 phase after incubation without and with dexamethasone, respectively). Dexamethasone only slightly, non statistically significantly decreased the subG1 phase in SAC-stimulated B lymphocytes (CD19+). In unstimulated B cells the significant increase in subG1 population upon dexamethasone-treatment has been observed (0.6% and 10.10% for cells cultured without and

### Table 1. Intracellular pH of cells after lymphocytes and leukemic lymphoblasts culture in medium with or without dexamethasone

<table>
<thead>
<tr>
<th>Incubation time (hrs)</th>
<th>cells</th>
<th>pHi (mean ± SD)</th>
<th>p-value for comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control medium</td>
<td>medium with DXM</td>
<td>control/ DXM</td>
</tr>
<tr>
<td>0</td>
<td>lymphocytes</td>
<td>7.6 ±0.7 (n = 20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BpC-ALL</td>
<td>7.78 ±0.19 (n = 8)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>lymphocytes</td>
<td>7.55 ±0.14 (89%)</td>
<td>7.68 ±0.11 (90%)</td>
</tr>
<tr>
<td></td>
<td>BpC-ALL</td>
<td>6.87 ±0.79 (11%)</td>
<td>7.073 ±0.30 (10%)</td>
</tr>
<tr>
<td>48</td>
<td>lymphocytes</td>
<td>7.72 ±0.15 (84%)</td>
<td>8.00 ±0.33 (90%)</td>
</tr>
<tr>
<td></td>
<td>BpC-ALL</td>
<td>7.07 ±0.30 (16%)</td>
<td>7.17 ±0.27 (10%)</td>
</tr>
<tr>
<td>48</td>
<td>lymphocytes</td>
<td>7.75 ±0.07 (90%)</td>
<td>7.80 ±0.13 (89%)</td>
</tr>
<tr>
<td></td>
<td>BpC-ALL</td>
<td>6.37 ±0.68 (10%)</td>
<td>6.83 ±0.45 (11%)</td>
</tr>
<tr>
<td></td>
<td>BpC-ALL</td>
<td>8.09 ±0.24 (80%)</td>
<td>8.12 ±0.35 (75%)</td>
</tr>
<tr>
<td></td>
<td>BpC-ALL</td>
<td>7.30 ±0.38 (20%)</td>
<td>7.46 ±0.39 (25%)</td>
</tr>
</tbody>
</table>

n – number of patients, (%): percentage of cells in which pHi was increased or decreased; 
\( p \) – probability, DXM – dexamethasone in medium, NS – non statistical significance.

BpC-ALL – acute common B lymphoblastic leukemia.
with dexamethasone, respectively). On the contrary, no effects of dexamethasone on B lymphoblasts were observed (Fig. 2).

The percentage of cells in early and late stages of apoptosis is shown in Table 2. The study was performed on population of lymphocytes without identification CD2+ and CD19+. In freshly isolated lymphocytes (time 0) about 11% of cells showed features of early stage of apoptosis while only 0.56% of cells appeared to enter the late stage of apoptosis/necrosis. After 2 and 48 h of incubation with dexamethasone the significant increase in percentage of cells in early stage of apoptosis was observed comparing to cells incubated in control medium. In the lymphocytes incubated for 2 h with dexamethasone no changes in the number of cells in late phase of apoptosis/necrosis was observed. However, the 48-hour-incubation with dexamethasone induced a significant increase of the number of cells in late apoptotic/necrotic phase (7.94% and 16.00% for cell cultured without and with dexamethasone, respectively). No significant differences in the population of cells in early phase of apoptosis was observed in B leukemic lymphoblasts incubated with dexamethasone for 2. Prolongation of time incubation to 48 h significantly increased percentage of lymphoblasts beginning apoptosis process. Significant difference was only observed in number of lymphoblasts in late apoptosis stage depend on dexamethasone presentation in culture medium fort 2 h.

However, in the population of bone marrow B leukemic lymphoblasts the number of cells in late apoptotic/necrotic phase was significantly higher than in the population of blood lymphocytes (Table 2).

![Figure 2](image_url)

**Table 2.** Percentage of cells in early and late phase of apoptosis after 2 and 48 h incubation in medium with or without dexamethasone

<table>
<thead>
<tr>
<th>Death</th>
<th>Time of incubation (hours)</th>
<th>Medium</th>
<th>Peripheral blood lymphocytes (PBL)</th>
<th>Bone Marrow Lymphoblasts (BpC ALL)</th>
<th>p-value for median comparison PBL-BpC ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>median (%) (Q1; Q3)</td>
<td>median (%) (Q1; Q3)</td>
<td></td>
</tr>
<tr>
<td>Early apoptosis</td>
<td>2</td>
<td>Control</td>
<td>11.35 (9.21; 14.70)</td>
<td>14.90 (7.11; 17.00)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dex</td>
<td>14.60 (11.80; 17.80)</td>
<td>17.10 (7.41; 21.70)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>0.046</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Control</td>
<td>15.70 (12.60; 19.60)</td>
<td>16.10 (5.48; 18.70)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dex</td>
<td>21.30 (18.10; 24.60)</td>
<td>19.40 (11.10; 23.70)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>0.002</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Late apoptosis/necrosis</td>
<td>2</td>
<td>Control</td>
<td>0.64 (0.41; 0.87)</td>
<td>2.56 (1.93; 4.28)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dex</td>
<td>0.58 (0.45; 0.77)</td>
<td>3.64 (2.06; 5.02)</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>NS</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Control</td>
<td>7.94 (6.14; 10.80)</td>
<td>26.60 (20.00; 47.50)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dex</td>
<td>16.00 (12.30; 22.90)</td>
<td>35.50 (17.60; 43.10)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>0.01</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

n – number of patients, (Q1; Q3) – 25 percentile; 75 percentile, NS – no statistical significance, p – statistical significance, control – medium without dexamethasone, Dex – medium with dexamethasone
Discussion

It is of general agreement that the best type of cellular death is apoptosis which does not generate the inflammation. Cancer cells, in contrast with normal cells have some changes in genome which enable them to avoid apoptosis. One of these is intracellular pH (pHi). Intracellular acidification is an essential event preceding endonuclease activation in apoptosis in various cell lines [8] but its role and relationship to resistance to chemotherapy is not discovered yet. It was suggested that high pHi correlates with high proliferation status of cells. Malinowska et al. [9] has found that children with lymphoblasts with acidic pHi had significantly longer EFS than those with alkaline pHi.

Earlier published results revealed significant discrepancies concerning the role of pHi in cell apoptosis indicating that acidification [8, 10, 11], alkalization [12] or even lack of changes [13] accompany the process of apoptosis. Results presented in this paper (Table 1) are in agreement with Rich et al. [8] observation that leukemic cells exhibit higher pHi values than normal PBL. In both studied populations of cells (normal peripheral blood and leukemic bone marrow) we observed very small percentage of cells with pHi below 7.0.

We were interested if dexamethasone lowered pHi of blast cells and activate apoptosis process. As it is shown in Table 1 in the both cell population (acidic and alkaline) treatment with dexamethasone slightly increased pHi was observed. Presented results documented that dexamethasone did not induce the acidification of cells as it was described by Tepel et al. [14] in study of peripheral blood lymphocytes isolated from healthy volunteers.

Percentage of leukemic lymphoblasts in S phase was also not changed by dexamethasone added to medium for three days (Table 2). Similarly to leukemic B blasts, CD19+ lymphocytes stimulated in in vitro cultures with SAC were not sensitive to dexamethasone also. At the same time dexamethasone significantly decreased number of CD2+ cells in S phase what suggests that proliferating T cells but not B cells are more dexamethasone sensitive.

Leukemic lymphoblasts in comparison with peripheral blood lymphocytes have higher pHi and higher percentage of cells in S phase. It indicates that lymphoblasts proliferate more intensively. Some authors described positive correlation between percentage of cells in S phase and their pHi [8, 9]. In our studies we did not notice such correlation (data not shown). Examining the effect of dexamethasone on cell cycle phases we observed that dexamethasone increased the percentage of cells in subG1 phase that is one of the indicators of apoptosis. This effect was more profound for non stimulated T and B blood lymphocytes. Lymphocytes stimulated with PHA were more sensitive to dexamethasone than B lymphocytes stimulated with SAC (Fig. 1). Dexamethasone did not influenced percentage of of BpC-ALL in phase subG1.

Apoptosis can be described by many parameters and examined by wide spectrum of techniques. In this study we assessed early stages of apoptosis using annexin-V binding to cell surface and late stages of apoptosis/necrosis by staining additionally with propidium iodide (PI). Results obtained in these experiments indicate that peripheral blood lymphocytes are more sensitive to dexamethasone than BpC-ALL. Moreover, dexamethasone evoke stronger early apoptosis process in normal lymphocytes than in BpC-ALL, but the characteristics changes for late apoptosis (necrosis) are expressed stronger in bone marrow lymphoblasts than peripheral blood lymphocytes.

Higler percentages of apoptotic leukemic lymphoblasts B (from cell lines) after 3 days incubation with dexamethasone were shown by Ito et al. [15]. However, authors performed only two such experiments on leukemic lymphoblasts B collected from patients. After 3 days exposure to 1000 nmol/l dexamethasone results in these two patients were quite different (17% and 83%). On the basis presented results we concluded that acidification of cytoplasm is not necessary for apoptosis that was also documented by Li and Eastman [11]. Our results demonstrated that pHi above 7.0 did not prevent this process. Also, Benson et al. [16] did not show any acidification during the apoptosis of dexamethasone-treated CEM line cells.

So far influence of dexamethasone was assessed mainly on whole lymphocyte population [14, 17] or on T lymphocytes [18, 19]. Information about influence of dexamethasone on leukemic cells mainly comes from studies conducted on cell lines, especially T-cell lines, very rarely B-cell lines. Only few authors analyze the influence of dexamethasone on blasts isolated from patients [20, 21].

The results obtained from our studies performed on the influence of dexamethasone on normal resting and activated blood lymphocytes, as well as on children’s B lymphoblasts isolated from bone marrow at the day of ALL diagnosis, allow to conclude that dexamethasone is not very potent inductor of B-lymphoblasts apoptosis.

References


