ACTIVATION OF NF-κB IN LEUKEMIC CELLS IN RESPONSE TO INITIAL PREDNISONE THERAPY IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKAEMIA: RELATION TO OTHER PROGNOSTIC FACTORS

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Nuclear factor κB (NF-κB) is a transcription regulator of proliferation and cell death. Increased activation of NF-κB may be responsible for treatment failure in children with acute lymphoblastic leukaemia (ALL). This study aimed to assess changes in NF-κB activation in peripheral blood mononuclear cells prior to and after 6 and 12 h of prednisone administration in relation to age, initial WBC count at diagnosis and early treatment response in childhood ALL. The study comprised 55 children with de novo ALL. Cells were stained with mouse anti-NF-κB (p65) antibody followed by goat anti-mouse antibody conjugated with FITC and measured by laser scanning cytometer. The nuclear/cytoplasmic (N/C) ratio of NF-κB reflecting activation of NF-κB was decreased 12 h after treatment in the standard risk group patients, whereas it remained statistically unchanged in the non-standard risk group patients. Changes in the N/C ratio of NF-κB were not associated with age and early treatment response; however, in children with an initial WBC count higher than 20,000/µl at diagnosis, this ratio was increased after 6 and 12 h from prednisone administration. The association of higher activation of NF-κB with an elevated initial WBC count suggests that activation of NF-κB may be responsible for treatment failure in children with ALL.

Key words: childhood ALL, NF-κB, prognostic factors.

Introduction

Acute lymphoblastic leukaemia (ALL) is one of the most common malignancies in children [1]. The prognostic factors in ALL are still a matter of debate; the most commonly accepted include age, certain chromosomal abnormalities (e.g. t(9;22), t(4;11)), peripheral white blood cell (WBC) count at diagnosis and early response to treatment including a response to preliminary therapy with glucocorticoids (GCs) [2-4]. Nuclear factor-κB (NF-κB) is a transcriptional factor that targets genes that promote inflammation, proliferation of tumour cells, angiogenesis, tumour invasion, metastases, and cell survival [5-7]. NF-κB is a family (Rel/NF-κB family) comprising RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) which form homo- and heterodimers. These proteins are retained in the cytoplasm in an inactive state, bound to IκB protein. The NF-κB activation occurs through classical and alternative pathways by activation of Iκ kinases (IKK), degrada-
tion of IκB protein, cytoplasmic release of sequestered NF-κB protein, translocation of NF-κB from cytoplasm to the nucleus and activation of the target genes (Fig. 1). Activation of the classical NF-κB pathway releases active p50/p65 and p50/C-Rel dimers, while the alternative pathway leads to nuclear accumulation of the p52/RelB complex. The rapid translocation of NF-κB from the cytoplasm to the nucleus is considered to be a marker of its activation. In the classical pathway, one of the target genes activated by NF-κB is that which encodes IκB. Newly-synthesized IκB can enter the nucleus, remove NF-κB from DNA, and export the complex back to the cytoplasm to restore the original latent state. Thus, the activation of the NF-κB pathway is generally a transient process, lasting from 30-60 min in most cells [5, 8, 9].

Constitutive or aberrant activation of NF-κB is frequently encountered in solid tumours and hematologic malignancies such as acute myelogenous leukaemia (AML) and ALL [10-12]. In these clinical conditions, constitutive activation of NF-κB results in increased proliferation and decreased apoptosis and, therefore, is usually associated with a phenotype resistant to therapy and poor prognosis [5].

GCs used for initial treatment of ALL impair activity of transcriptional factors including NF-κB which may result in increased apoptosis and response to treatment [13].

Studies of NF-κB expression in ALL have been mostly limited to analysis of cell lines [14-17]. To the best of our knowledge, only one has focused on the significance of NF-κB expression as a prognostic factor in childhood ALL [18]. Therefore, the purpose of this study was to assess changes in NF-κB activation in peripheral blood mononuclear cells collected prior to and after 6 and 12 h of prednisone administration as well as the relation between activation of NF-κB and prognostic factors defining risk groups according to Berlin-Frankfurt-Münster (BFM) Group criteria such as age, WBC count at diagnosis and early treatment response in childhood ALL.

Material and methods

Patients

The study comprised 55 children (21 girls, 34 boys) aged 6-192 months (mean 78.1 months; median 72 months) with de novo ALL diagnosed between June 2002 and November 2005. The diagnosis was based on morphologic examination of bone marrow smears stained with MGG and confirmed by results of flow cytometry immunophenotyping of bone marrow cells. The characteristics of patients are given in Table I.

All children were treated according to one of the consecutive BFM Group protocols: BFM ALL 90 or
ALL-IC 2002 Protocols. The initial phase of therapy was the same for all patients and consisted of oral administration of prednisone 60 mg/m²/day in three divided doses plus one age-related dose of intrathecal methotrexate on the first day of treatment (6 mg < 1 y., 8 mg 1-2 y., 10 mg 2-3 y., 12 mg > 3 y.). During the first day of treatment, three consecutive doses of prednisone were given at 6 hours’ intervals. Based on two criteria: prednisone response (as defined by the BFM Group) and bone marrow day 15 leukemic cells rate, patients were classified as good early treatment responders (i.e., patients with peripheral blood leukemic cell count of not more than 1 × 10³/µl on day 8 and with no more than 5% of leukemic cells in their bone marrow on day 15) and poor early treatment responders (i.e., patients with peripheral blood leukemic cell count exceeding 1 × 10³/µl on day 8 and/or with more than 5% of leukemic cells in their bone marrow on day 15). Children aged less than 6 years with an initial WBC count of less than 20 000/µl, with no t(9;22) and/or t(4;11) translocation and with good early treatment response were classified as standard risk patients, whereas the remaining children as non-standard (intermediate or high) risk patients.

The study was approved by the Ethics Committee of the Pomeranian Medical University.

Preparation of peripheral blood cells for analysis

Peripheral blood was sampled prior to prednisone administration and 6 and 12 h after the first dose of prednisone (prior to 2nd and 3rd dose of oral prednisone) into polystyrene tubes containing 50 µl of Heparin (Polfa Warszawa, Poland). 2-5 ml of peripheral blood was mixed with equal volume of PBS (phosphate buffered saline), pH 7.4 without Ca and Mg. The mixture was then laid onto ficoll (Gradisol, Polfa Kutno, Poland) at a ratio of 2 volumes of diluted blood to 1 volume of ficoll followed by centrifugation at 800 g for 30 minutes at room temperature (Eppendorf Centrifuge 5403, Germany). Mononuclear cells containing layer was collected and rinsed twice in PBS each time at 200 g for 5 min (Eppendorf Centrifuge 5403, Germany). Cell pellet was resuspended in an appropriate volume of PBS to obtain final cell concentration of 5 × 10⁶/ml. Aliquots of 200 µl were centrifuged at 300 g for 5 min (Shandon CytoSpin 3, USA). CytoSpin preparations were fixed in cold (0-4°C) 1% buffered paraformaldehyde for 15 min followed by cold (-20°C) 70% ethanol for up to 24 hours. Slides were subsequently air dried, then stored at -20°C.

Immunocytochemistry

For immunocytochemical detection of NF-κB, slides were rinsed in PBS twice, each time for 5 min, then 100 µl of 1 : 50 diluted mouse monoclonal anti-NF-κB antibody (p65) (Chemicon, Temecula, CA) was added and the slides were incubated for 1 h at room temperature. Then, the slides were rinsed in PBS for 5 min and 100 µl of 1 : 40 diluted polyclonal goat anti-mouse antibody conjugated with FITC (DakoCytomation, # F 0479) was added. The slides were incubated for 1 hour at room temperature, rinsed once again in PBS and then incubated for 30 min at room temperature with 200 µl of propidium iodide (PI, Sigma Aldrich) in concentration of 10 µl/ml in the presence of ribonuclease A (Ribonuclease A type I-AF from bovine pancreas, Sigma Aldrich) in concentration of 100 µl/ml. During all incubations cells were deposited in a humid chamber and protected from light. Slides prepared from the same blood sample and stained according to the described procedure but with PBS instead of the primary antibody served as negative controls.

Fluorescence measurements

Slides were mounted with coverslips and NF-κB – associated FITC green fluorescence (emission maximum 520 nm) and DNA-associated PI red fluorescence (emission maximum 580-nm) excited by 488-nm argon laser were measured simultaneously by laser scanning cytometry (LSC, CompuCyte, USA). DNA-associated PI red fluorescence was used as a contouring parameter (threshold contour). Values of integrated NF-κB – associated FITC green fluorescence were measured separately over the cell nucleus and over the rim of cytoplasm, and recorded as FCS

<table>
<thead>
<tr>
<th>Table I. Clinical characteristics of patients</th>
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<td>PARAMETER</td>
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<td>Age:</td>
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<td>&lt;72 months</td>
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<td>WBC at diagnosis:</td>
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<td>&lt;20 000 cells/µl</td>
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<td>≥20 000 cells/µl</td>
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<td>Early response to treatment:</td>
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3.0 files by WinCyte 3.4 software. The integrated fluorescence is the sum of fluorescence of all the pixels within the integrated fluorescence contour.

Principles of evaluating NF-κB – associated FITC green fluorescence are presented in Fig. 2. Based on two parameters DNA-associated PI red fluorescence maximal pixel and cell area cells were selected for further analysis. To measure integrated NF-κB – associated FITC green fluorescence (Green Fluorescence Integral) over the nucleus, the integration contour was set at +0 pixels from the threshold contour, whereas peripheral inner and outer contours were introduced at +2 and +4 pixels from the threshold contour in order to measure green fluorescence intensity (Green Fluorescence Priph Int) over a rim of cytoplasm. Background fluorescence was subtracted automatically by the software. In each slide, at least 5,000 cells were measured. Since activation of NF-κB comprised rapid translocation of NF-κB from cytoplasm to nucleus, the N/C ratio of NF-κB associated fluorescence was automatically calculated by the software by dividing the values of relative units of nuclear and cytoplasmic fluorescence.

Statistical analysis

Values of NF-κB were expressed as means ± SD of NF-κB – associated FITC green fluorescence. Shapiro-Wilk test was used to evaluate data distribution, whereas U Manna-Whitney test, analysis of variance as well as Student’s t-test were used to analyze the differences between the means of analyzed parameters. P values equal or less than 0.05 was considered statistically significant.

Results

There were no statistically significant differences between mean values of NF-κB bound green FITC fluorescence measured in both standard and non-standard risk patients prior to and 6 and 12 h from treatment.

Fig. 2. Principles of measurements of NF-κB expression
ACTIVATION OF NF-κB IN ALL in response to initial prednisone therapy

prednisone administration. However, in the standard risk patients, mean N/C ratio of NF-κB bound green FITC fluorescence after 12 hours from prednisone administration was significantly lower as compared with the respective value prior to therapy (7.09 vs. 6.53; \( p = 0.01 \); Fig. 3). In the group of non-standard risk patients, the respective ratios did not differ (6.68 vs. 6.9; NS; data not shown).

There were no statistically significant differences between mean values of NF-κB as well as mean N/C ratios of NF-κB bound green FITC fluorescence prior to and 6 and 12 hours from prednisone administration with respect to age (children younger than 6 years vs. older children) and early treatment response (good vs. poor).

In the group of children with an initial WBC count of less than 20 000/μl as well as in the group of remaining patients mean values of NF-κB measured prior to and 6 and 12 hours after prednisone administration remained unchanged. However, in the group of children with an initial WBC count of less than 20 000/μl, a drop of mean N/C ratio of NF-κB was seen after 6 and 12 hours from prednisone administration, whereas this ratio measured at the same time points was significantly higher in the

Changes in NF-κB-bound green fluorescence intensity measured separately over nucleus (white bars) and over cytoplasm (light gray bars) and the N/C ratio of NF-κB fluorescence (dark gray bars) in peripheral blood mononuclear cells before treatment, 6 and 12 h after treatment (mean ± SD).

**Fig. 3.** Changes in NF-κB expression in the standard risk group

Changes in NF-κB-bound green fluorescence intensity measured separately over nucleus and over cytoplasm and the N/C ratio of NF-κB fluorescence in peripheral blood mononuclear cells before treatment, 6 and 12 h after treatment in relation to the WBC count (mean ± SD).

**Fig. 4.** Changes in NF-κB expression in relation to the WBC count at diagnosis
group of children with a WBC count higher than 20 000/µl (6.44 vs. 7.29, p = 0.01 and 6.21 vs. 7.15, p = 0.008, respectively; Fig. 4).

Discussion

In normal cells, activation of NF-κB is tightly regulated and transient. This activation results in its rapid translocation from the cytoplasm to the nucleus where it upregulates the transcription of its target genes [5]. Activators of NF-κB include proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), epidermal growth factor, T- and B-cell mitogens, bacteria and lipopolysaccharides, viruses, viral proteins, double-stranded RNA, physical and chemical stresses, cellular stresses such as ionizing radiation and chemotherapeutic agents [5, 20].

Genes triggered by NF-κB activation include genes promoting cell survival such as Bcl-2, Bcl-xL, IAP 1/2, survivin, XIAP and cell proliferation (cyclin D, c-Myc, GADD 45, p21) [5, 6]. In cancer cells, various types of molecular alterations impair regulation of NF-κB activation that results in the persistent presence of NF-κB in the nucleus. This constitutive activation and subsequent deregulated expression of NF-κB controlled genes includes genes promoting proliferation and survival of tumour cells. While many NF-κB stimuli have been identified, those responsible for constitutive activation of NF-κB in most cell types are poorly understood [10-12].

NF-κB activation can be easily determined with the use of LSC by computing the ratio of NF-κB associated fluorescence measured over the area of cell nucleus to the intensity of the NF-κB associated fluorescence measured over the cytoplasm [20]. In our study, the unchanged N/C ratio of NF-κB observed after 6 and 12 hours from prednisone administration in the group of non-standard risk patients is likely to reflect the constitutive activation of NF-κB in the population of malignant cells. This is consistent with NF-κB constitutive activation in many human cell lines as well as tumour samples from patients with hematologic malignancies (ALL, AML, NHL, HL) and carcinomas (e.g. of the breast, prostate, lung and colon) [5]. Cells that express constitutively activated NF-κB are resistant to various chemotherapeutic agents and radiation treatment [5]. The precise mechanism of such resistance is not clear. It has been suggested that in human cutaneous T-cell lymphoma cells, it results from the resistance to apoptosis [21]. If GGs are unable to inhibit activation of NF-κB, constitutive activation of NF-κB promotes tumour cell proliferation and increased survival [13].

Possible mechanisms of constitutive NF-κB activation are diverse and include aberrant IKK activity and a shorter IκB half-life (as seen in B cell lymphoma), IκB mutation (as seen in Hodgkin lymphoma), IL-1β production (as seen in AML), TNF-α production (as seen in cutaneous T-cell lymphoma and Burkitt lymphoma), autocrine or paracrine activation of NF-κB resulting from overexpression of ligands and receptors of epidermal growth factor and HER-2/neu (as seen in breast carcinoma), TNF-α, IL-1, hepatocyte growth factor (as seen in prostatic carcinoma), and integrins [5].

In contrary to non-standard risk patients, in the group of the standard risk patients, the N/C ratio of NF-κB was significantly lower 12 h after prednisone administration. This observation may suggest decreased activation of NF-κB which reflects cells susceptibility to GCs used in the initial treatment of ALL. GCs inhibit NF-κB activation by following mechanisms:

- induction of the synthesis of the NF-κB inhibitor IκB which subsequently binds to and sequesters NF-κB in the cytosol, thus preventing it from translocating to the nucleus [13, 22-24],
- impaired NF-κB activation and/or function either by blocking its access to its DNA (a priori) site [24, 25] or by forming a complex with NF-κB which loses DNA capacity [26, 27], and/or by competition with NF-κB for nuclear co-activators [28].

The activation of NF-κB as expressed by the N/C ratio of NF-κB was not associated with age and early treatment response but with the WBC count at diagnosis. The N/C ratio of NF-κB was higher in children with an initial WBC count higher than 20 000/µl which is a priori a negative prognostic factor in childhood ALL. It has been suggested that the elevated WBC count at diagnosis and increased blast percentages in the bone marrow of patients with AML is related to cyclin D1 expression which is regulated by NF-κB and overexpressed in many human cancers [6].

It seems that persistent activation of NF-κB in ALL which we found after 6 and 12 h of prednisone treatment is responsible for increased proliferation and decreased apoptosis and, therefore, a worse prognosis. We conclude that changes in activation of NF-κB may confer the risk of treatment failure on a subgroup of children with ALL and an elevated WBC count at diagnosis. Our preliminary observations would require confirmation by the analysis of NF-κB activation in relation to treatment results in a long follow-up time.

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