Protein expression of the glucocorticoid receptor in childhood acute lymphoblastic leukemia

Melchior Lauten, Gunnar Cario, Girma Agedom, Karl Welte, Martin Schrappe

Background and Objectives. Early treatment response is a strong predictor for treatment outcome in childhood acute lymphoblastic leukemia (ALL), treated within the protocols of the Berlin-Frankfurt-Münster (BFM) study group. In the ALL-BFM trials, early treatment response is assessed by in vivo response to glucocorticoids (prednisone response), the molecular background of which is unknown. Initial in vivo resistance to glucocorticoid (GC) treatment in childhood ALL (prednisone-poor response) is associated with a dramatically shorter event-free survival than that found in GC-sensitive patients (prednisone-good responders). The intracellular effects of glucocorticoids are mediated by the glucocorticoid receptor (GR). The protein expression of the GR has been linked to GC resistance in various diseases treated with GC. However, existing data are conflicting.

Design and Methods. We performed a case-control study for prednisone response to investigate the association of in vivo GC resistance and GR protein expression in childhood ALL. GR expression was assessed using Western blot technology.

Results. The median relative GR protein expression of all patients was 0.87. Overall, we did not find different GR protein expression in PPR and PGR patients. GR protein expression was 0.91 in PGR patients versus 0.85 in PPR ones of in vivo GC resistance and GR expression.

Interpretation and Conclusions. We conclude that the expression of GR is of minor importance for in vivo GC resistance in childhood ALL.

Key words: childhood leukemia, ALL, prednisone response, glucocorticoid resistance, glucocorticoid receptor.

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lines\textsuperscript{21,26} and in vivo studies in asthma, rheumatoid arthritis, inflammatory bowel disease and ALL\textsuperscript{12,11,25,27,28} could not clarify the importance of GR-\(\beta\) so far.

GR-\(\gamma\) RNA expression in childhood ALL has been investigated by Beger et al.\textsuperscript{29} Using an in vitro stimulation assay she showed longer GR-\(\gamma\) RNA up-regulation in prednisone-poor responders than in prednisone-good responders after in vitro stimulation with dexamethasone.\textsuperscript{29}

In a different study investigating GR-\(P\) expression levels in hematologic malignancies it was suggested that GR-\(P\) increases the activity of GR-\(\alpha\). The authors concluded that, by increasing GR-\(\alpha\) activity, GR-\(P\) could be involved in GC resistance during GC treatment.\textsuperscript{21} The function of GR-\(A\) has not yet been subject of investigations.

Comparative in vivo studies investigating the protein expression of GR isoforms and their association with GC resistance in childhood ALL have not yet been published. Unfortunately, studies on GR isoform protein expression are generally hampered by technical problems (see discussion). Therefore, the current study investigated the protein expression of the sum of all GR isoforms using an isoform unspecific antibody.

**Design and Methods**

**Patients and study design**

Our study was designed as a case-control study for prednisone response to assess the association of glucocorticoid resistance and glucocorticoid receptor protein expression in childhood ALL. Cases were patients with poor in vivo prednisone response (n=20), controls were patients with good in vivo prednisone response (n=20). The study utilized spare peripheral blood and bone marrow samples as well as clinical data from patients enrolled in the ALL-BFM 90 and ALL-BFM 95 trials. Criteria used to match the patients in the two groups were initial white blood cell count (WBC; <10,000, 10,000-<50,000 and \(\geq\)50,000 leukocytes/\(\mu\)L), sex, immunophenotype (T-ALL, non T-ALL) and age at diagnosis (<1 year, 1-9 years and \(\geq\)10 years) (Table 1). All patients who did not die from progressive disease or relapsed ALL had a follow-up of at least five years.

Fifteen matched patient pairs of the current study were also investigated for heat shock protein 90 (Hsp90) expression in a different study, which was recently published.\textsuperscript{30} The Hsp90 data of these patients will be referred to in the discussion.

**Cell samples**

The spare bone marrow and peripheral blood samples analyzed in this study were taken from ALL patients at diagnosis before administration of glucocorticoids. Peripheral blood samples had to have a minimum of 70% leukemic blasts prior to gradient centrifugation. Mononuclear cells were isolated using gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Freiburg, Germany). After isolation, cells were frozen in Roswell Park Memorial Institute medium (RPMI-1640) containing 10% dimethyl sulfoxide (DMSO).

**Western blotting**

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with two gels at a time. Forty micrograms of total cell lysate protein were separated on 10% SDS gels for 90 minutes at 180V (Owl Scientific Inc., Wobum, USA). Every gel was run with 40 \(\mu\)g protein from HeLa S3 cells to normalize protein quantity. The gels were transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, BioRad Laboratories, Rich-

**Table 1. Distribution of the clinicopathological features in patients with childhood ALL, divided into two groups according to in vivo response to prednisone.**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Prednisone Poor response n=20 (100%)</th>
<th>Prednisone Good response n=20 (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>14 (70.0)</td>
<td>14 (70.0)</td>
</tr>
<tr>
<td>female</td>
<td>6 (30.0)</td>
<td>6 (30.0)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 year</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-9 years</td>
<td>15 (75.0)</td>
<td>15 (75.0)</td>
</tr>
<tr>
<td>(\geq)10 years</td>
<td>5 (25.0)</td>
<td>5 (25.0)</td>
</tr>
<tr>
<td>Initial WBC count ((\mu)L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10,000</td>
<td></td>
<td></td>
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<tr>
<td>10,000-&lt;50,000</td>
<td>4 (20.0)</td>
<td>4 (20.0)</td>
</tr>
<tr>
<td>(\geq)50,000</td>
<td>16 (80.0)</td>
<td>16 (80.0)</td>
</tr>
<tr>
<td>Immunophenotype</td>
<td></td>
<td></td>
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<tr>
<td>non T-ALL</td>
<td>10 (50.0)</td>
<td>10 (50.0)</td>
</tr>
<tr>
<td>T-ALL</td>
<td>10 (50.0)</td>
<td>10 (50.0)</td>
</tr>
</tbody>
</table>
mond, USA) and blotted in parallel at 20V for 2 hours. After blotting, gels (Comassie) and membranes (Ponceau S) were stained to document blotting failure. The membranes were incubated with a polyclonal isoform unspecific GR antibody (sc-1003, Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C overnight. After washing (three times with PBS-T), membranes were incubated with a horse-radish peroxidase labeled goat-anti-rabbit secondary antibody (sc-2030, Santa Cruz Biotechnology) at 4°C overnight. Subsequently, the membranes were incubated with a polyclonal CRKL antibody (sc-9005, Santa Cruz Biotechnology) at 4°C overnight. Washing and incubation with the secondary antibody was performed as described above. Every gel was run with HeLa S3 cells as positive controls and with pre-stained markers (Wide range color marker, Sigma, Deisenhofen, Germany).

Finally, the bands were visualized on a X-ray film (Cronex 5, AGFA, Mortsel, Belgium) using the electro-chemo-luminescence method according to the manufacturer’s instructions. Bands of the GR protein appeared at approximately 90kD, bands of the CRKL protein at approximately 40kD. An example of the Western blots is shown in Figure 1. After scanning of the X-ray film, image analysis was performed on a Macintosh iMAC computer using the public domain National Institutes of Health (NIH) Image program (available on the Internet at http://rsb.info.nih.gov/nih-image).

Statistical analysis

Frequencies were calculated for descriptive purposes. GR protein expression was calculated normalized to CRKL and relative to the GR expression in HeLa S3 cells. The correlation between median GR protein expression and prednisone response was calculated by logistic regression analysis and a χ² test. Computations were performed using SAS software (SAS-PC Version 6.04, SAS Institute Inc., Cary, NC, USA).

Results

We performed a case-control study for in vivo prednisone response on 20 paired patients’ samples to assess the association of GC resistance and GR protein expression in childhood ALL. The clinicopathologic features of all patients investigated in our study are shown in Table 1. The median relative GR protein expression of all patients was 0.87. Overall, we did not find different GR protein expression in PPR and PGR patients (Table 2). GR protein expression was 0.91 in PGR patients versus 0.85 in PPR ones. In addition, subgroup analysis for risk factors known to have an impact on event-free survival in childhood ALL (sex, age at diagnosis, initial WBC count, immunophenotype) revealed that even within these subgroups GR protein expression did not differ significantly between the PPR and PGR groups (Table 3).

Discussion

In 1983 Pui et al. investigated GR-binding sites in blasts of patients with relapsed ALL and found no association with clinical characteristics, initial response to treatment, or duration of remission. Since then, expression of GR and its isoforms has been subject of various investigations in patients with a broad spectrum of diseases that are treated with glucocorticoids. In some of these studies, GR-β and GR-P were suggested to inhibit GR-dependent activation of transcription and, therefore, contribute to GC resistance. In one study examining leukemic blasts from 13 ALL patients and peripheral blood mononuclear cells from 9 healthy volunteers, the ALL samples showed
reduced GR-α protein expression with no concomitant decrease in GR-β compared to the findings in healthy controls. In addition, GR-α expression was lower in mononuclear leukocytes of T-cell lineage than in leukocytes from the B-cell precursor immunophenotype. The authors considered that the reason for the reduced sensitivity to glucocorticoids in T-ALL might be different expression of the GR isoforms. GR isoform RNA expression may be up-regulated for longer in PPR samples than in PGR ones after in vitro stimulation with dexamethasone. The investigation of protein expression of GR isoforms is hampered by biological as well as technical problems. For example, Haarman and colleagues found that GR-β protein is not detectable in primary ALL cells, whereas it is detectable in CEM-C7 cells. In addition, Beger et al. described that sequences from GR-α, GR-β, and GR-γ can form the carboxy-terminal end of GR-γ and that GR-γ RNA expression may be up-regulated for longer in PPR samples than in PGR ones after in vitro stimulation with dexamethasone. Beger’s study reveals a fundamental problem concerning the interpretation of studies examining differential protein regulation of GR isoforms. GR isoform specific antibodies are commercially available only for GR-α, GR-β, and GR-γ. However, since we are now aware that GR-γ exists with the carboxy-terminal ends of various other GR isoforms, and since the structure of GR-γ may differ from each of these isoforms by only 1 amino acid within the DNA binding domain (arginine), we must conclude that the GR-α and GR-β specific antibodies currently used are highly likely to recognize not only the respective specific GR isoform, but also GR-γ. Thus, potential effects of GR-α or GR-β on GC resistance could have been mimicked by a GR-γ effect. We think that, currently, it is not possible to study GR isoform protein expression using an isoform specific antibody approach.

Table 3. Median GR expression in subgroups of patients with childhood ALL, further divided according to in vivo response to prednisone.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of patients</th>
<th>Median GR expression ((P_{25}-P_{75}))</th>
<th>Poor PDN* response</th>
<th>Good PDN* response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
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<tr>
<td>20-0.67</td>
<td>28</td>
<td>(0.55-1.21)</td>
<td>0.98</td>
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<tr>
<td>10-1.28</td>
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<td></td>
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<tr>
<td><strong>Female</strong></td>
<td>12</td>
<td>1.20</td>
<td>0.70</td>
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<tr>
<td>(0.88-1.48)</td>
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<td>(0.50-1.60)</td>
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<td></td>
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<tr>
<td><strong>Age</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-9 years</td>
<td>30</td>
<td>0.86</td>
<td>0.88</td>
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<tr>
<td>(0.59-1.35)</td>
<td></td>
<td>(0.53-1.36)</td>
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<tr>
<td>≥ 10 years</td>
<td>10</td>
<td>0.83</td>
<td>1.13</td>
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<tr>
<td>(0.64-1.52)</td>
<td></td>
<td>(0.70-1.46)</td>
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<tr>
<td><strong>Initial WBC count (µlL)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>10,000-49,999</td>
<td>8</td>
<td>0.86</td>
<td>1.06</td>
<td></td>
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<tr>
<td>(0.73-1.38)</td>
<td></td>
<td>(0.55-1.45)</td>
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<tr>
<td>≥ 50,000</td>
<td>32</td>
<td>0.78</td>
<td>0.91</td>
<td></td>
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<tr>
<td>(0.58-1.32)</td>
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<td>(0.55-1.31)</td>
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<tr>
<td><strong>Immunophenotype</strong></td>
<td></td>
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</tr>
<tr>
<td>Non T-ALL</td>
<td>20</td>
<td>1.03</td>
<td>1.19</td>
<td></td>
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<tr>
<td>(0.80-1.48)</td>
<td></td>
<td>(0.78-1.56)</td>
<td></td>
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<tr>
<td>T-ALL</td>
<td>20</td>
<td>0.67</td>
<td>0.74</td>
<td></td>
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<tr>
<td>(0.43-1.22)</td>
<td></td>
<td>(0.53-1.05)</td>
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PDN: prednisone, *PDN*: relative to the normalized expression of the target protein in HeLa S3 cells. All differences of GR expression between the two responder groups were statistically not significant.

The formation of a GR heterodimer (e.g. GR-α GR-β) seems to prevent induction of transactivation or transrepression of GC responsive genes. Furthermore, competitive binding of GR-α and GR-β or GR-P to co-activators or co-repressors has been suggested to play a role in GC resistance. The investigation of protein expression of GR isoforms is hampered by biological as well as technical problems. For example, Haarman and colleagues found that GR-β protein is not detectable in primary ALL cells, whereas it is detectable in CEM-C7 cells. In addition, Beger et al. described that sequences from GR-α, GR-β, and GR-γ can form the carboxy-terminal end of GR-γ and that GR-γ RNA expression may be up-regulated for longer in PPR samples than in PGR ones after in vitro stimulation with dexamethasone. Beger’s study reveals a fundamental problem concerning the interpretation of studies examining differential protein regulation of GR isoforms. GR isoform specific antibodies are commercially available only for GR-α, GR-β, and GR-γ. However, since we are now aware that GR-γ exists with the carboxy-terminal ends of various other GR isoforms, and since the structure of GR-γ may differ from each of these isoforms by only 1 amino acid within the DNA binding domain (arginine), we must conclude that the GR-α and GR-β specific antibodies currently used are highly likely to recognize not only the respective specific GR isoform, but also GR-γ. Thus, potential effects of GR-α or GR-β on GC resistance could have been mimicked by a GR-γ effect. We think that, currently, it is not possible to study GR isoform protein expression using an isoform specific antibody approach.

The protein data of this study support the RNA data published by Tissing et al., who investigated GR-α, GR-β and GR-P RNA expression as well as the expression of the sum of all GR isoforms (GR-total) in a case-control study of prednisone response in childhood ALL at diagnosis. In that study there was no differential expression of any GR isoform RNA. Twelve of the patients of the current study (6 matched pairs) were also investigated in the study by Tissing et al. This now allows us to hypothesize that a defect of GR mRNA translation as a cause of GC resistance in childhood ALL can be excluded by the current study to a certain extent. However, our study does not definitively exclude that GR expression could influence outcome of childhood ALL, since the initial co-existence of small cell fractions with distinct GR expression patterns cannot be ruled out. The analysis of resistant blasts after 1 week of GC treatment could be a next step to approach this issue.

In a recently published study we showed that differential expression of hsp90, the main protein of the GR activating heterodimer, does not con-
Glucocorticoid receptor protein expression in childhood ALL

tribute to GC resistance in childhood ALL.30 Fifteen matched pairs of the current study were also investigated in the hsp90 study. Therefore, we calculated hsp90/GR ratios for each of the 15 responder pairs to look for an imbalance between hsp90 and GR expression in each of the patients. GC resistance in patients with normal GR expression could occur, for instance, because of low hsp90 expression and thus low GR activating capacity. However, hsp90/GR ratios were not significantly different in sensitive and resistant patients (data not shown).

To summarize, using Western blot technology we did not find a correlation between GR protein expression and GC resistance in childhood ALL. In addition, there was no difference in the ratio of hsp90 to GR expression in GC sensitive and resistant ALL patients. Further studies might focus on elucidating activation defects of the GC-GR complex in detail. In particular, in vivo stimulation studies focusing on the dynamics of GR expression under GC treatment would be helpful to clarify, once and for all, the role of GR expression in GC resistance in childhood ALL.

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Pre-publication Report & Outcomes of Peer Review

Contributions
GC was involved in planning the study and writing and reviewing the manuscript. GA performed the protein expression experiments. KW and MS took part in planning the project. ML was responsible for designing the case-control study, for the statistical analysis and for writing the report.

We gratefully acknowledge the excellent co-operation of all participants of trials ALL-BFM 90 and ALL-BFM 95 as well that of all doctors and nurses at the participating medical centers.

Disclosures
Conflict of interest: none
Redundant publications: parts of the results were presented at the 5th International Symposium on Leukaemia and Lymphoma, Amsterdam, Netherlands (oral presentation, abstract #028). The abstract of the presentation was published in Leukemia (Leukemia 2003;17:665).

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Manuscript processing
This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received June 17, 2003; accepted October 3, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic
Early treatment response is a strong predictor for treatment outcome in childhood acute lymphoblastic leukemia (ALL), treated within the protocols of the Berlin-Frankfurt-Münster (BFM) study group. In the ALL-BFM trials, early treatment response is assessed by in vivo response to glucocorticoids (prednisone response), the molecular background of which is unknown.

What this study adds
This paper reports negative results, suggesting that the expression of glucocorticoid receptor does not influence prednisone response in childhood acute lymphoblastic leukemia.