PLZF overexpression in T-ALL cell line CEM-C7H2-2C8 downregulates glucocorticoid receptor (GR) and its target genes

Muhammad WASIM1*, Muhammad MANSHA2, Muhammad TAYYAB1, Ali Raza AWAN1, Sehrish FIRyal1, Christine MANTINGER3, Tahir YAQUB1
1Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan
2Department of Zoology, Division of Science and Technology, University of Education, Lahore, Pakistan
3Tyrolean Cancer Research Institute, Medical University of Innsbruck, Austria

Abstract: Glucocorticoids (GCs) induce cell cycle arrest and apoptosis in lymphoid cells and constitute a central component in the treatment of lymphoid malignancies. The molecular basis of this clinically important phenomenon remains, however, poorly understood. Using whole genome expression profiling we have previously identified glucocorticoid response genes in children with acute lymphoblastic leukemia (ALL). The promyelocytic leukemia zinc finger (PLZF) appeared as one of the most promising candidate genes, which has been implicated in the pathogenesis of several leukemia types. We have already established that transgenic PLZF reduced the sensitivity to GC-induced apoptosis in the CEM-C7H2-2C8 leukemic cell line and knockdown of PLZF resulted in a small but significant increase in cell death in this cell line. The present study was proposed to find a plausible molecular explanation for this protective effect of PLZF against GC-induced cell death. It was found that doxycycline-regulated PLZF overexpression in the CCRF-CEM T-ALL cell line downregulates the GC-induced GR expression and its target genes, which resulted in reduced apoptosis induced by GC.

Key words: PLZF, GR, glucocorticoid-response gene, glucocorticoid-induced apoptosis, leukemia cell line

1. Introduction
Glucocorticoids (GCs) are key elements in the treatment of childhood acute lymphoblastic leukemia (ALL) and are components of treatment protocols for other lymphoid malignancies as well. GCs induce G1 cell cycle arrest and massive apoptosis in immature lymphoid cells and have a significant antileukemic effect in the majority of children with ALL (Schrappe et al., 2000). GC-induced apoptosis is a phenomenon of considerable biological and clinical significance. A long list of GC-regulated genes has been reported in lymphoid lineages and leukemic cell lines (Schmidt et al., 2004) along with related clinical samples (Schmidt et al., 2006; Tissing et al., 2007). The most promising GC-regulated gene identified among them was the promyelocytic leukemia zinc finger (PLZF) (Schmidt et al., 2006). Pathogenesis of different types of leukemia has been reported to be enhanced by impaired PLZF function, e.g., myeloid leukemia arising from a fusion protein product of AML-1/ETO. Reports have proven that in vitro transrepression activity of PLZF is inhibited by the AML-1/ETO fusion protein product with the ETO zinc finger domain. This is potentially done by exclusion of PLZF from the nucleus to prohibit its binding with response elements on regulatory areas of target genes (Melnick et al., 2000).

Most effects of GC are conveyed through the GC receptor (GR) (Cidlowski et al., 1996). The GR is a ligand-activated transcription factor of the large nuclear receptor super family that resides in the cytoplasm and, upon ligand binding, translocates to the nucleus (Laudet and Gronemeyer, 2002). In the nucleus, it dimerizes and binds to so-called GC response elements (GREs), thereby altering the expression of a plethora of target genes. Transrepression of gene expression occurs either by direct binding to negative GREs or, without direct DNA binding, by protein–protein interactions with other DNA-binding transcription factors, sequestration of transcription inducers, or competition for coactivators (Geley et al., 1997).

In one of our previous studies, we extended our analysis of GC regulation of PLZF to several leukemic cell lines and performed functional analyses on the role of PLZF in the antileukemic effects of GC. We found that transgenic PLZF alone had no effect on cell proliferation and survival,
but reduced sensitivity to apoptosis induced by the GC analogue dexamethasone in the CCRF-CEM model for T-ALL (Wasim et al., 2010). In this study, we aimed to propose a molecular explanation for this protective effect of PLZF on GC-induced apoptosis in the above-mentioned cell line. Here we found that doxycycline (dox)-induced overexpression of PLZF downregulates the GR and its target genes, which might be the likely explanation.

2. Materials and methods

2.1. Cell lines and tissue culture

The T-ALL cell line CEM-C7H2-2C8 (Schmidt et al., 2004), a CEM-C7H2 derivative with constitutive expression of the tetracycline-regulated reverse transactivator (rtTA; Schmidt et al., 2006), and the HEK293T (ATCC) cell line used in this study were tested for mycoplasma infection and their authenticity was verified by DNA fingerprinting (Parson et al., 2005). CEM-C7H2-2C8 was maintained in RPMI 1640 medium, and HEK293T (packaging cell line) cells were maintained in DMEM. FCS 10%, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin were added to these culture media as described earlier (Wasim et al., 2010, 2012). Cells were cultured at 37 °C in 5% CO2 and saturated humidity and were diluted 1:2 daily or every other day to maintain mid log-phase cultures (2.5–6 × 10^5 cells/mL). β-Dexamethasone (10^−4 M) was dissolved in 100% ethanol. The final ethanol concentration in the dexamethasone-treated and control cultures was maintained at 0.1%. All above reagents were purchased from Sigma (Vienna, Austria).

2.2. Immunoblotting

Our immunoblotting procedure was published earlier in detail (Wasim et al., 2010, 2012). In brief, cell pellets (5 × 10^6 cells) were washed by PBS, lysed in 100 µL of RIPA buffer, clarified by centrifugation, quantified by Bradford assay, and mixed with 40 µL of 4X SSB buffer containing 5% β-mercaptoethanol. Total lysate was size-fractioned by 12.5% SDS-PAGE, blotted onto nitrocellulose membranes, and incubated overnight with primary antibody for PLZF (Prestige Antibodies, Sigma, HPA00149), GR (E20 from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and α-tubulin (DM1A, Calbiochem, Nottingham, UK) as a protein loading control. The membranes were washed and then incubated with the antiserum or anti-rabbit horseradish-peroxidase conjugated secondary antibody for 45 min at room temperature. After another washing step immune complexes were visualized using an enhanced chemiluminescence reagent, ECL Plus (Amersham Pharmacia Biotech, Amersham, UK) and exposure to AGFA Curix X-ray films.

2.3. Real-Time RT-PCR

For PLZF, GR, SLA, and PFKFB2 mRNA quantification by real-time RT-PCR, 20 ng of transcribed cDNA for each gene was amplified in TaqMan Universal MasterMix (Applied Biosystems) in 96-well plates using real-time RT-PCR kits for PLZF (Hs00232313_m1), GR (Hs00230813_m1), SLA (Hs00277129_m1), and PFKFB2 (Hs01015408_m1), as well as TBP (Hs00427620_m1) as the normalization control. Two-step thermo protocol was followed, which included 94.5 °C for 5 min in the first step, and then 40 cycles of 94 °C for 15 s alternating with 60 °C for 1 min, and fluorescence signal intensities were read during the 60 °C temperature step (iQ5 Multicolor Real-Time PCR Detection System; Bio-Rad Laboratories, Vienna, Austria). Data from 3 technically replicated measurements were averaged and normalized to the internal TBP control as described earlier (Wasim et al., 2010, 2012). Log2-fold change values (M-values) were calculated for 3 biological replicates by comparing normalized real-time RT-PCR data from GC-treated samples against data from the corresponding control samples. M-values were averaged for the 3 biological replicates and P-values were calculated (Student’s t-test) to test against the null hypothesis of no differential expression (mean M = 0).

3. Results and discussion

We have already reported (Wasim et al., 2010) the generation, characterization, and functional analysis of 2 stably transduced derivatives of the CCRF-CEM T-ALL cell line with conditional expression of PLZF, termed CEM-C7H2-2C8-ZBTB16#19 and #58. Results showed that transgenic expression of PLZF alone, even at levels that clearly exceeded those induced by GC, had no detectable effect on survival or cell cycle progression. However, it significantly reduced the extent of apoptosis induced by 10^−7 M and, even more so, 10^−8 M dexamethasone in this T-ALL in vitro model. This finding was surprising and needed a plausible explanation. Hence, this study was carried out to explore a logical molecular answer. Therefore, we hypothesized a model (Figure 1) that explains that GCs transactivate a plethora of genes (proapoptotic), including PLZF, through GR which ultimately induces apoptosis. Being a bona fide transcription repressor, PLZF transrepresses GR and hence its target genes involved in the induction of the intrinsic pathway of apoptosis, eventually resulting in reduction of sensitivity to GC-induced apoptosis.

Since GC-induced overexpression of PLZF resulted in a protective effect (Wasim et al., 2010), we undertook to investigate whether PLZF overexpression downregulates GR or not. Real-time RT-PCR confirmed our hypothesis. We have already established (Wasim et al., 2010) that PLZF overexpression is regulated by dox. Here we showed that there is more than 23-fold (ΔΔCT (log2 M value) 5.54) increase in PLZF level after dox induction (Figure 2A). This dox-regulated overexpression of PLZF significantly
downregulated the GC-induced GR expression at mRNA (Figure 2B) as well as protein (Figure 3) level. Although apparently inactive as a transcription factor when tested alone, PLZF significantly interfered with the transcriptional response to GC in this CCRF-CEM T-ALL cell line. Since both gene inductions and repressions were attenuated in the presence of PLZF, and since regulations of many transcription units were affected, a general mechanism of action seems likely, such as sequestration of the activated GR by PLZF (Wasim et al., 2010). A previous report suggested that PLZF might bind to and inhibit DNA binding of GR (Martin et al., 2003) to its response elements, resulting in attenuated expression of its target genes. Our immunoblot results (Figure 3) tend to suggest same conclusion. Here the GR band is less prominent due to PLZF overexpression and slightly translocated towards PLZF, which might be due to protein–protein interaction between GR and PLZF.

Since GC-regulated expression of GR is under the inhibitory effect of dox-induced PLZF expression, its target genes, SLA and PFKFB2, are also downregulated in same manner (Figures 4A and 4B). It has already been shown that regulation of GC-response genes is attenuated under the effect of PLZF expression. One of these attenuated genes was BCL2L11/Bim (Wasim et al., 2010), the induction of which was previously shown to play a crucial role in GC-induced apoptosis in this model system (Ploner et al., 2008).

In conclusion, we report that dox-regulated PLZF overexpression in the CCRF-CEM T-ALL cell line downregulates the GC-induced GR expression and its target genes, which results in reduced apoptosis induced by GC. Furthermore, this conclusion suggests the studying of the possible interaction between PLZF and GR.

Acknowledgments
The authors are thankful to Prof Dr Reinhard Kofler, Johannes Rainer, Christian Ploner, Anita Kofler, and Arno
Helmberg for unlimited support and fruitful discussions during the experimental work at DMP, Medical University of Innsbruck and Tyrolean Cancer Research Institute, Innsbruck, Austria. This work was financially supported by the Higher Education Commission of Pakistan and the Cancer Aid Society, Tyrol, Austria.

References


