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The hematopoietic tumor suppressor interferon regulatory factor 8 (IRF8) is upregulated by the antimetabolite cytarabine in leukemic cells involving the zinc finger protein ZNF224, acting as a cofactor of the Wilms' tumor gene 1 (WT1) protein

Giorgia Montano[a], Tove Ullmark[a], Helena Jernmark-Nilsson[a], Gaetano Sodaro[b], Kristina Drott[a], Paola Costanzo[b,1], Karina Vidovic[a,1], Urban Gullberg[a,*,1]

[a] Department of Hematology and Transfusion Medicine, Medical Faculty, University of Lund, Lund, Sweden
[b] Department of Molecular Medicine, and Medical Biotechnology, University of Naples Federico II, Naples, Italy

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ABSTRACT

The transcription factor interferon regulatory factor-8 (IRF8) is highly expressed in myeloid progenitors, while most myeloid leukemias show low or absent expression. Loss of IRF8 in mice leads to a myeloproliferative disorder, indicating a tumor-suppressive role of IRF8. The Wilms tumor gene 1 (WT1) protein represses the IRF8-promoter. The zinc finger protein ZNF224 can act as a transcriptional cofactor of WT1 and potentiate the cytotoxic response to the cytostatic drug cytarabine. We hypothesized that cytarabine upregulates IRF8 and that transcriptional control of IRF8 involves WT1 and ZNF224. Treatment of leukemic K562 cells with cytarabine upregulated IRF8 protein and mRNA, which was correlated to increased expression of ZNF224. Knock down of ZNF224 with shRNA suppressed both basal and cytarabine-induced IRF8 expression. While ZNF224 alone did not affect IRF8 promoter activity, ZNF224 partially reversed the suppressive effect of WT1 on the IRF8 promoter, as judged by luciferase reporter experiments. Coprecipitation revealed nuclear binding of WT1 and ZNF224, and by chromatin immunoprecipitation (ChIP) experiments it was demonstrated that WT1 recruits ZNF224 to the IRF8 promoter. We conclude that cytarabine-induced upregulation of the IRF8 in leukemic cells involves increased levels of ZNF224, which can counteract the repressive activity of WT1 on the IRF8-promoter.

1. Introduction

Interferon regulatory factor-8 (IRF8) (also called interferon consensus sequence binding protein, ICSBP) is an interferon γ-inducible transcription factor, expressed during hematopoiesis in cells of the myeloid, as well as of the B-lymphocyte lineage [1,2]. In normal hematopoiesis, IRF8 expression is high in myeloid, lymphoid and dendritic progenitors [3]. On the other hand, levels of IRF8 are low or absent in leukemic blasts from a majority of chronic myeloid leukemia (CML) or acute myeloid leukemia (AML) patients [4], often due to promoter methylation [5,6], suggesting an anti-leukemic role for IRF8. Several mechanisms downstream of IRF8 that are relevant for an anti-oncogenic function have been reported, including repression of *BCL2L1* (Bcl-X_L) [7], *MYC* [8], *BCL2* [9], *PTPN13* [10], *CTNNB1* (β-catenin) [11,12], and enhanced expression of *CASP3* [7], and *NF1* [13]. Loss of IRF8 in mice leads to deregulated myeloid differentiation with an accumulation of neutrophil-like cells, resembling human CML [14]. CML is driven by the oncogenic fusion protein BCR-ABL with constitutive tyrosine kinase activity [15]. Forced expression of IRF8 antagonizes the BCR-ABL-induced leukemic phenotype *in vitro* and *in vivo* [8,9]. Moreover, treatment of leukemic cells with the BCR-ABL-inhibitor imatinib or with retinoic acid, both clinically used in the treatment of leukemia, increases expression of IRF8 [16,17], further emphasizing the tumor suppressor function of IRF8 in myeloid malignancies.

Transcriptional control of IRF8 in leukemic cells is incompletely understood. The Wilm's tumor gene 1 (WT1) protein is a zinc-finger transcription factor normally expressed in a small subset of hematopoietic progenitor cells [18–22], suggesting a role in early hematopoiesis. WT1 is commonly overexpressed in myeloid

* Corresponding author.
E-mail addresses: giorgia.montano@med.lu.se (G. Montano), tove.ullmark@med.lu.se (T. Ullmark), helena.jernmark_nilsson@med.lu.se (H. Jernmark-Nilsson), sodaro@unina.it (G. Sodaro), kristina.drott@med.lu.se (K. Drott), paola.costanzo@unina.it (P. Costanzo), karina.vidovic@med.lu.se (K. Vidovic), urban.gullberg@med.lu.se (U. Gullberg).
[1] These authors jointly supervised this work.

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leukemias [21–26] and WT1 cooperates with the leukemia fusion protein RUNX1 (AML1-ETO) to rapidly induce leukemia in mice, demonstrating a leukemogenic role for WT1 [27]. Recently, we showed that WT1 targets the *IRF8*-promoter, resulting in transcriptional repression [16]. Moreover, expression levels of *WT1* and *IRF8* in primary acute myeloid leukemias are highly anticorrelated [16]. Thus, WT1-mediated repression of IRF8 provides one explanation for the generally low expression of IRF8 in CML and AML. Interestingly, the constitutive tyrosine kinase activity of BCR-ABL causes increased expression of WT1, indicating a BCR-ABL-WT1-IRF8 pathway in CML [16,28].

The zinc-finger protein ZNF224 was first identified as a repressor of the human aldolase A gene [29]. In contrast to the restricted expression of WT1 in adult tissues, ZNF224 is ubiquitously expressed. A functional interaction between ZNF224 and WT1 was shown as, ZNF224 functions as a coactivator of WT1 on the promoter of the vitamin D-receptor (VDR), demonstrating that ZNF224 can also activate transcription [30]. The role of ZNF224 in WT1-mediated transcriptional regulation was recently extended to a number of WT1-target genes involved in the regulation of apoptosis. On the promoter of these genes, ZNF224 acts as a co-activator of proapoptotic genes, while repressing expression of antiapoptotic WT1 target genes [31]. In this way, ZNF224 shifts the balance of antiapoptotic and proapoptotic signals in favor of the latter. Consistent with a proapoptotic role, overexpression of ZNF224 in leukemic cells potentiated the cytotoxic response to the cytostatic drug and antimetabolite cytarabine [31], while WT1 can confer resistance to this treatment [32].

Cytarabine is one of the most commonly used drugs used for treatment of AML [33]. Within the cell, the pyrimidine nucleoside analog cytarabine is activated into ara-CTP which is incorporated into DNA of proliferating cells in place of deoxycytidine triphosphate (dCTP), thus blocking DNA synthesis, resulting in proliferation arrest and cell death [34]. Cytarabine may also be incorporated into DNA repair patches of quiescent cells, leading to inhibition of DNA-repair [35,36].

The aim of this work was to elucidate whether expression of the tumor suppressor IRF8 is affected by cytarabine, and whether ZNF224 cooperates with WT1 in transcriptional regulation of the *IRF8*-promoter.

2. Material and methods

2.1. Cell culture

The human kidney cancer cell line HEK293T/17 was cultured in Dulbecco's modified Eagle's medium, (Hyclone Laboratories Inc, Utah, U.S.A.), supplemented with 10% fetal calf serum and 100 μg/ml streptomycin–penicillin mix (Bio-Whittaker Inc., MD, U.S.A.) at 37 °C in 5% CO_2. The leukemic cell line K562 (DSMZ, Braunschweig, Germany) was cultured in RPMI 1640 (Gibco Life Technologies, NY, U.S.A.) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. K562 cells were treated 1 μM cytarabine (Pfizer AB, Sollentuna, Sweden) for 72 h. Concentration of cytarabine was carefully titrated in initial experiments to generate maximal proliferation arrest.

2.2. Immunoblotting

Harvested cells for immunoblotting were resuspended in Laemmli buffer (#161-0737, Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing 0.2 M β-mercaptoethanol (Sigma–Aldrich). Proteins were separated by SDS–PAGE (12% TGX gel, #456-1043, Bio-Rad) and transferred to a Hybond ECL membrane (GE Healthcare, Uppsala, Sweden). Primary antibodies used were: rabbit anti-IRF8 antibody (MBS224027, MyBioSource.com, CA, U.S.A.), rabbit anti-ZNF224 (T3) antibody (29), rabbit anti-WT1 (C-19, Santa Cruz, CA, U.S.A.), and mouse anti-GAPDH (7-B, Santa Cruz, CA, U.S.A.). The EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel) was used for analysis of protein bands with a ChemiDoc $^{TM}XRS^{+}$ system (Bio-Rad).

2.3. shRNA-mediated knockdown of ZNF224

Pools of K562 cells expressing ZNF224 shRNA, were obtained as previously described [31]. Briefly, K562 cells were transfected with 1.5 μg of short-interfering RNA plasmid SH2351C3 (Open Biosystem, Huntsville, AL, U.S.A.) using the HiPerFect Reagent (Qiagen, Venlo, Netherlands), according to the manufacturer's protocol; transfection of a non-silencing shRNA (scrambled shRNA) (Open Biosystem) was used as negative control. Transfected cells were selected by culture in the presence of puromycin (500 μg/ml) (Promega Corporation, Wisconsin, U.S.A.) for 4 weeks. Suppression of ZNF224 protein expression in response to shRNA expression was previously characterized by immunoblotting [31].

2.4. RNA isolation, reverse transcription and quantitative PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, after which RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, U.S.A.) with random hexamer primers according to the manufacturer's instructions. Quantitative PCR (qPCR) was carried out using TaqMan probe-based chemistry (Applied Biosystems); the probe for ZNF224 (Hs00273760_m1), IRF8 (Hs00175238_m1), WT1 (Hs00240913_m1), and the endogenous controls beta-actin (Hs99999903_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were purchased as Assay-on-Demand (Applied Biosystems). The amplification reactions were all performed in triplicates in a StepOnePlus (Applied Biosystems). Data were collected and analyzed using the Applied Biosystems StepOne^{TM}Real-Time PCR Software v2.0. The relative quantification in gene expression was determined using the ΔΔCt method [37]. Efficacy of the PCR amplification of controls and test was identical; parallelism of standard curves of the control and test was confirmed.

2.5. Transient transfection and luciferase-reporter assays

HEK293T/17 cells were transiently transfected using Lipofectamine reagent (Life Technologies, CA, U.S.A.) in 12-well plates with 200 ng of a luciferase reporter plasmid containing the proximal IRF8 promoter including a WT1 response element [16], and the 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(+/−) expression plasmids at indicated combinations and concentrations. To normalize the luciferase assay a pRL-CMV plasmid (20 ng) encoding the renilla luciferase was used. Dual-Luciferase Reporter Assay System (Promega Corporation, WI, U.S.A.) was performed 48 h after the transfection, according to the manufacturer's instructions.

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed using Chromatin immunoprecipitation assay kit (Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Briefly, cross-linked chromatin was prepared from HEK293T/17 cells transfected with 3XFLAG-CMV-7.1-ZNF224 or with pcDNA3WT1(+/−), or co-transfected with 3XFLAG-CMV-7.1-ZNF224 and pcDNA3WT1(+/−). The antibodies anti-ZNF224 (G-16), anti-WT1 (C19), and anti-HA were from Santa Cruz Biotechnology, TX, U.S.A. ChIP samples were analyzed by