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Original article

Histone modifications patterns in tissues and tumours from acute promyelocytic leukemia xenograft model in response to combined epigenetic therapy

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ABSTRACT

Xenograft models are suitable for *in vivo* study of leukemia's pathogenesis and the preclinical development of anti-leukemia agents but understanding of epigenetic regulatory mechanisms linking to adult cell functions in pathological conditions during different *in vivo* treatments is yet unknown. In this study, for the first time epigenetic chromatin modifications were characterized in tissues and tumours from murine xenograft model generated using the human acute promyelocytic leukemia (APL) NB4 cells engrafted in immunodeficient NOG mice. Xenografts were subjected to combined epigenetic treatment by histone deacetylase inhibitor Belinostat, histone methyltransferase inhibitor 3-DZNeaplanocin A and all-*trans*-retinoic acid based on *in vitro* model, where such combination inhibited NB4 cell growth and enhanced retinoic acid-induced differentiation to granulocytes. Xenotransplantation was assessed by peripheral blood cells counts, the analysis of cell surface markers (CD15, CD33, CD45) and the expression of certain genes (PML-RAR alpha, CSF3, G-CSFR, WT1). The combined treatment prolonged APL xenograft mice survival and prevented tumour formation. The analysis of the expression of histone marks such as acetylation of H4, trimethylation of H3K4, H3K9 and H3K27 in APL xenograft mice tumours and tissues demonstrated tissue-specific changes in the level of histone modifications and the APL prognostic mark, WT1 protein. In summary, the effects of epigenetic agents used in this study were positive for leukemia prevention and linked to a modulation of the chromatin epigenetic environment in adult tissues of malignant organism.

1. Introduction

Acute promyelocytic leukemia is an aggressive but a highly curable disease with the all-*trans* retinoic acid (RA) or the combination of RA and anthracycline-based chemotherapy [1–3]. However, the resistance to the differentiating effects of RA is frequently acquired during the treatment with the incidences of RA toxicity and APL differentiation syndrome [4–6]. Therefore, other novel clinical approaches are developed including combinatorial treatments by RA with epigenetic drugs. The number of studies examined treatment strategies by inhibitors of histone deacetylases (HDAC), histone methyltranferases (HMT) and DNA methyltrasferases (DNMT) [7]. The clinical trial data indicate that several HDAC inhibitors (vorinostat, valproic acid, depsipeptide, belinostat and others) have an activity in haematological malignancies and are well tolerated [8–11]. However, clinical experience with HDAC inhibitors revealed occurrence of the acute toxicities symptoms as a fatigue, anaemia, neutropenia, gastrointestinal and cytopenias [12]. The combinatorial drug treatments result in diverse effects on cancer cells but little is known about long-term toxicities and their influence on their environment and malignant organism tissues. Considering the fact that epigenetic research is one of the ways for new approaches in the prognosis, diagnosis and prevention of human diseases, we took attention on the elucidation of epigenetic regulatory mechanisms linking to adult cell functions in pathological conditions. For this purpose, we

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generated murine APL xenograft model with the application of HMT inhibitor DZNeaplanocin A (DZNep), hydroxamate-type HDAC inhibitor belinostat (Bel) and RA. It was shown in *in vitro* studies that DZNep deplete and inhibit EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2), which is overexpressed in variety of cancers including haematological malignancies [13]. EZH2 acts as a histone lysine methyltrasferase, which mediates trimethylation of lysine 27 (K27) on histone H3 to silence PRC2 target genes expression [14–16]. Belinostat is a pan-HDAC inhibitor, which promotes histone acetylation in cancer cells and inhibit tumour cell growth *in vitro* and in animal models [17,18]. Previous studies have demonstrated the action of such epigenetic drugs resulting in cell cycle arrest, apoptosis and inhibition of cell proliferation [19,20]. *In vivo* findings against AML in NOD/SCID mice indicated that combined epigenetic treatment with DZNep and HDAC inhibitor panabinostat induced AML cell apoptosis and significantly improved survival of engrafted mice [21]. Regarding the monitoring of myeloid disease progression, it should be emphasized that WT1 gene is intensely expressed in leukemias [22,23] and various types of solid tumours [24] but the expression is limited in normal adult tissues, excluding kidney podocytes [25].

In our study, by using *in vitro* and *in vivo* models, we report the rationale for choosing epigenetic drugs, Bel and DZNep, for APL treatment. For the elucidation of the possible cellular and molecular mechanisms associated with the phenomenon, we determined the changes in peripheral blood counts, the expression of certain cell surface markers' and related genes, the epigenetic chromatin environment in untreated APL xenograft mice and treated by epigenetic approach. Our observations indicate that APL xenograft mice somatic tissues are subjected to changes in specific histone modifications in response to epigenetic agents-derived treatment. The results obtained might provide new insights in our knowledge of leukemia disease biology.

2. Materials and methods

2.1. Cell culture and in vitro assays

The human acute promyelocytic leukemia cell line NB4 (from DSMZ, GmbH, Braunschweig, Germany) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were negative for mycoplasma infection using the MycoProbe™ detection kit (R&D systems Europe, Ldt.). In each experiment, logarithmically growing cells were seeded at 5×10^5 cells per ml in 5 ml of medium. NB4 cells were treated continously with 0.15 μM DZNep (Cayman Chemical, Ann Arbour, USA), 0.2 μM Bel (Selleck chemicals, Houston, USA) and 1 μM RA (Sigma, Sent Luis, USA). Cell proliferation was evaluated by the trypan blue exclusion test. Viable and dead (blue coloured) cell number was determined by counting in a haemocytometer. The degree of differentiation was assayed by the ability of cells to reduce nitro blue tetrazolium (NBT) (Sigma) to insoluble blue–black formazan after stimulation with phorbol myristate acetate (PMA) (Sigma). NBT-positive cells were counted in a haemocytometer. At least 400 cells were scored for each determination. Data was expressed as the percentage of NBT-positive cells number relative to the viable cell number.

2.2. In vivo *xenograft model*

Male/female immunodeficient NOG mice at the age of 9–12 weeks were purchased from Taconic (Ry, Denmark). Animals were housed and maintained in specific pathogen free conditions. Studies were conducted in accordance with the guidelines of "Law on the Care, Welfare and Use of Animals" of the Republic of Lithuania. License for the use of laboratory animals in this research (No. 3; 2013-11-13) was obtained from the Lithuanian Food and Veterinary Office. Xenograft model of APL was established by intravenous (i.v.) inoculation of exponentially growing NB4 cells (5×10^6/mouse), suspended in 200 μl serum-free cell cultivation medium, in immunodeficient NOG mice. Three mice per treatment group were employed. APL xenograft mice (denoted as APL) were not treated and used as a control. Xenograft mice began to receive a treatment after the appearance of first typical illness symptoms and specific cell surface markers' detection in the peripheral blood cells (PB). APL xenograft mice were treated with the combination of Bel (20 mg/kg) and DZNep (1 mg/kg); *i.p.*, 5 times/week; 5 injections and further treated orally with RA (0.45 mg/d, 5 times/week, 20 times) (denoted as APL–DBR). Xenotransplantation was assessed by monitoring the PB counts of microcapillary collected cells using Exigo EOS haematology analyser (Boule Medical AB, Stockholm, Sweden) according to the manufacturer's instructions. For assessment of human-origin PB cell surface markers, CD15, CD33 and CD45 (BD Pharmingen, California, USA), flow cytometry analysis was performed as described previously [26].

2.3. Quantitative real-time PCR (Q-PCR)

Total RNA from mice PB was extracted by TRIzol (Invitrogen), as recommended by the manufacturer, and then reverse transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Q-PCR was performed with Maxima® SYBR Green qPCR Master Mix (Thermo Scientific) on the Rotor-Gene 6000 system (Corbett Life Science). The primers (forward (F) or reverse (R), 5′-3′ orientation) for the tested genes were:

WT1 (F) GGCATCTGAGACCAGTGAGAA, (R) GAGAGTCAGACTTGAAAGCAGT;

PML-RARα (F) CAGTGTACGCCTTCTCCATCA, (R) GCTTGTAGATGCGGGGTAGA;

CSF3 (F) GCTGCTTGAGCCAACTCCATA, (R) GAACGCGGTACGACACCTC;

G-CSFR (F) CTTGTGGCCTATAACTCAGCC, (R) CCCACTCAATCACATAGCCCT;

GAPDH (F) TCCATGACAACTTTGGTATCG, (R) TGTAGCCAAATTCGTTGTCA).

The amount of mRNA was normalized to GAPDH and the relative gene expression was determined using $\Delta\Delta C_t$ method (compared to control xenograft).

2.4. Western blot analysis

Xenograft mice tumours and tissues (~50–70 mg) were frozen in liquid nitrogen and disrupted by grinding. Lysates were prepared in SDS lysis buffer homogenizing through the needle G21 on ice, than centrifuged at 20,000 × *g* for 20 min, +4 °C. Proteins were precipitated with acetone and dissolved in SDS buffer. Samples were subjected to SDS electrophoresis and transferred as described previously [26]. Immunoblotting was performed using antibodies against H4Ac (penta), H3K4me3, H3K9me3, H3K27me3 (Upstate Biotechnology, NY, USA), WT-1 (Thermo Scientific, EU) and GAPDH (Cell signalling, Danvers, USA). Immunoreactive bands were detected by enhanced chemiluminescence according to the manufacturer's instructions. Western blots were digitized using an ImageScanner™ III scanner (LabScan 6.0 software; GE Healthcare Biosciences, Germany) and image analysis was implemented in Matlab™ environment (The MathWorks, Natick MA, USA). Fold difference for each target protein was obtained by dividing normalized total density of the target protein peak by the normalized total density of the control. Normalized total densities were total densities of each peak corrected according equalizations of loading control.