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Induced myelomonocytic differentiation in leukemia cells is accompanied by noncanonical transcription factor expression

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ABSTRACT

Transcription factors that drive non-neoplastic myelomonocytic differentiation are well characterized but have not been systematically analyzed in the leukemic context. We investigated widely used, patient-derived myeloid leukemia cell lines with proclivity for differentiation into granulocytes by retinoic acid (RA) and/or monocytes by 1,25-dihyrdroxyvitamin D3 (D3). Using K562 (FAB M1), HL60 (FAB M2), RA-resistant HL60 sublines, NB4 (FAB M3), and U937 (FAB M5), we correlated nuclear transcription factor expression to immunophenotype, G1/G0 cell cycle arrest and functional inducible oxidative metabolism. We found that myelomonocytic transcription factors are aberrantly expressed in these cell lines. Monocytic-lineage factor EGR1 was not induced by D3 (the monocytic inducer) but instead by RA (the granulocytic inducer) in lineage bipotent myeloblastic HL60. In promyelocytic NB4 cells, EGR1 levels were increased by D3, while Gfi-1 expression (which promotes the granulocytic lineage) was upregulated during D3-induced monocytic differentiation in HL60, and by RA treatment in monocytic U937 cells. Furthermore, RARα and VDR expression were not strongly correlated to differentiation. In response to different differentiation inducers, U937 exhibited the most distinct transcription factor expression profile, while similarly mature NB4 and HL60 were better coupled. Overall, the differentiation induction agents RA and D3 elicited cell-specific responses across these common FAB M1-M5 cell lines.

1. Introduction

Differentiation induction therapy agents like all-*trans* retinoic acid (RA) and 1,25-dihyrodxyvitamin D3 (D3) show promise in many cancer cells types [1–3]. Although acute myeloid leukemias (AML) are extremely heterogeneous diseases, with over 200 known AML-related cytogenic aberrations [4], RA and D3 evoke comparable responses in human myeloid leukemia cell lines, i.e. RA induces granulocytic events while D3 induces monocytic events. Whether RA and D3 can act additively, synergistically or antagonistically is an outstanding question, since each behavior has been observed in different contexts. Although lineage-determining myeloid transcription factors are well characterized for the nonmalignant case [5–7], systematic analysis of their expression during differentiation induction therapy in leukemia is lacking. In this study we used sequentially more mature, human myeloid leukemia cell lines K562 (FAB M1), HL60 (FAB M2), NB4 (FAB M3) and U937 (FAB M5) and compared treatment-induced expression of an ensemble of well-known transcription factors that govern myelomonocytic lineage selection.

K562 is a chronic myelogenous leukemia (CML) cell line (FAB M1) that harbors the Bcr-Abl fusion protein [8,9]. K562 cells exhibit inducible erythroleukemic and megakaryocytic characteristics [10,11], but are not responsive to either RA [12,13] or D3 treatment [14], and thus serve as a negative control for RA- or D3-induced differentiation. HL60 leukemia cells are FAB M2 lineage-bipotent myeloblasts [15,16] that can differentiate along either the granulocytic lineage (using RA) or monocytic lineage (using D3). HL60 cells are t(15;17)-negative, so RA-induced therapy must act through a

Abbreviations: FAB, French–American–British [myeloid leukemia classification]; RA, retinoic acid; D3, 1,25-dihydroxyvitamin D3; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; RARα, retinoic acid receptor α; VDR, vitamin D receptor; Gfi-1, growth factor independent protein 1; EGR1, early growth response protein 1; C/EBPα, CCAAT-enhancer binding protein α; PU.1, binds PU-box, also called Spi-1; IRF-1, interferon regulatory factor 1; AhR, aryl hydrocarbon receptor; Oct4, octamer-binding transcription factor 4; CD, cluster of differentiation [marker]

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mechanism independent of PML-RARα. We previously isolated and described two sequentially emergent RA-resistant HL60 cell lines that differ in their RA-inducible CD38 expression, termed R38+ and R38− [17,18]. These lines, which do not growth arrest or exhibit other RA-induced markers when treated with RA, demonstrate that as RA resistance becomes more profound, progressive resistance to D3 also develops. NB4 is an acute promyelocytic leukemia (APL) cell line (FAB M3) that does contain the t(15;17) translocation pathognomonic for APL [19–21]. NB4 cells are highly RA-responsive, but are less responsive to D3 than wild-type HL60 cells are, and require combination treatment to achieve any degree of monocytic differentiation [22,23]. U937 monocytic leukemia cells (FAB M5), the most mature cells in this study, are highly responsive to D3-induced monocytic/macrophage differentiation. RA exerts ambiguous differentiative effects in U937, which at times have been considered either monocytic or granulocytic [24–26]. U937 cells harbor a t(10;11) translocation, a recurrent event found in AML cells and T cell acute lymphoblastic leukemia [4,27].

During non-neoplastic myelomonopoiesis, the transcription factors PU.1 (a myeloid lineage master regulator) and C/EBPα have positive effects on both granulocytic and monocytic maturation, but the ratio of PU.1 to C/EBPα determines granulocytic versus monocytic lineage selection [28]. This is due to a bistable switch described by Laslo et al. (2006) [29] that involves mutually antagonistic repressors Gfi-1 and EGR1 which lie downstream of PU.1 and C/EBPα. Gfi-1 represses monocytic differentiation and promotes granulocytic lineage selection, while EGR1 acts conversely. In addition to retinoid acid receptor α (RARα) and vitamin D receptor (VDR), other transcription factors found to be significant, specifically to RA-induced differentiation, are IRF-1, AhR and Oct4 [30,31]. Aryl hydrocarbon receptor (AhR) expression increases during myeloid differentiation of HL60 [30] as well as during monocytic differentiation of HL60 and U937 [32], and promotes Oct4 downregulation, putatively relieving stemness. IRF-1 expression is induced by RA in HL60, NB4 and U937 cells [31,33], but not K562 cells [34], and this expression appears to be Stat1-independent [35].

In this study we treated K562, wild-type and RA-resistant HL60, NB4 and U937 cells with RA, D3, or combination RA + D3 and assessed differentiation using immunophenotypic markers CD38 and CD11b (myelomonocytic markers) and CD14 (a monocytic-specific marker). Additionally we assessed G1/G0 cell cycle arrest and inducible oxidative metabolism, a functional differentiation marker of mature myelomonocytic cells. We surveyed nuclear expression of the nine aforementioned transcription factors and analyzed their coupling to cellular phenotype. Our intentions were to: (1) provide ourselves and others with a comparative index of responses by these cell lines, (2) identify departures from the norm of myelomonocytic transcription factor expression in the leukemic differentiation context, (3) determine how the transcription factor expression and phenotypic marker signatures couple to the individual treatments (RA, D3 or RA + D3) and to myeloid cell maturity, and (4) determine if combined RA + D3 treatment promotes one lineage over the other.

2. Materials and methods

2.1. Cell lines and treatments

Original HL60 patient isolates were a gift of Dr. Robert Gallagher and maintained in this laboratory. Two retinoic acid (RA)-resistant HL60 sublines (R38+ and R38−) were isolated as described previously [17]. NB4 cells were provided by Ethan Dmitrovsky (Dartmouth University). American Type Cell Culture (ATCC)-obtained U937 cells were provided by Tracy Stokol (Cornell University) and ATCC-obtained K562 cells were provided by Hening Lin (Cornell University). No human primary tissues were obtained or used in this study—all parent cell lines were established approximately 30 years ago [8,15,19,25] and are purchasable from ATCC. Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% (HL60) or 10% (K562, NB4, U937) heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% antibiotic–antimycotic (Invitrogen) and maintained in a 5% CO_2 humidified environment at 37 °C. Cells were seeded at either 0.1×10^6 cells/ml (HL60), 0.2×10^6 cells/ml (HL60, NB4, U937) or 0.3×10^6 cells/ml (K562). Cell viability consistently exceeded 95%. All-*trans* retinoic acid (RA; Sigma, St. Louis MO) was added from a 5 mM stock solution in 100% ethanol to a final concentration of 1 μM in culture. 1,25-Dihydroxyvitamin D3 (D3; Cayman Chemicals, Ann Arbor, MI) was added from a 1 mM stock solution in 100% ethanol to a final concentration of 0.5 μM in culture.

2.2. Flow cytometry

CD surface markers were detected using anti-CD38 (PE), anti-CD11b (APC) and anti-CD14 (PE) antibodies (BD Biosciences, San Jose, CA). 0.5×10^6 cells were harvested and analyzed by flow cytometry on a BD LSRII flow cytometer (BD Biosciences) as described previously [18]. For cell cycle analysis, cells were harvested and stained with propidium iodide-containing solution and analyzed as described previously [18]. For reactive oxygen species quantification, cells were treated with 0.2 μg/ml 12-o-tetrade canoylphorbol-13-acetate (TPA, Sigma, St. Louis, MO) or dimethyl sulfoxide (DMSO) carrier and stained with 5 μM 5-(and-6)-chloro methyl-2′,7′-dichlorodihydro–fluorescein diacetate acetyl ester (H_2-DCF, Molecular Probes, Eugene, OR), then analyzed by flow cytometry as described previously [18].

2.3. Western blotting

Cell nuclear lysates were isolated using the NE-PER extraction kit (Thermo Scientific, Rockford, IL). Equal amounts of protein lysate (15 μg) were resolved by SDS–PAGE and transferred onto PVDF membrane (Millipore, Billerica, MA). Blots were blocked and incubated with primary and secondary antibody as described previously [18]. Primary antibodies were specific against C/EBPα, RARα, VDR, EGR1, PU.1, Oct4 (Cell Signaling, Danvers, MA), AhR, Gfi-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and IRF-1 (BD Biosciences). Anti-Histone 3 or anti-TATA-binding protein (Cell Signaling) were used to ensure even loading.

2.4. Statistical analysis

p-Values between treatment group means were calculated using ANOVA within GraphPad software. Repeat Western blot data were quantified using ImageJ. Pearson correlation coefficient calculation and hierarchical clustering analysis (average linking method) were performed in MATLAB or Cluster 3.0.

3. Results

3.1. Induced phenotypic changes in K562, HL60, NB4 and U937 cells

Wild-type and RA-resistant HL60 maintained in our laboratory have a doubling time of approximately 20 h (Fig. 1A, B) and are grown in RPMI 1640 medium with 5% FBS. We obtained K562, NB4 and U937 cell lines which have slower doubling times and are maintained in RPMI medium with 10% FBS (Fig. 1A, B). Compared to untreated wild-type HL60, untreated NB4 and U937 are more mature, having higher basal expression levels of CD38 and