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Hypoxia regulates proliferation of acute myeloid leukemia and sensitivity against chemotherapy

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ABSTRACT

Reduced oxygen partial pressure (pO_2, hypoxia) is an important component of the bone marrow microenvironment and the hematopoietic stem cell niche. It is unclear whether this applies to the leukemic stem cell as well and if differences in pO_2 between the normal hematopoetic and the leukemic stem cell niche exits. Here, we demonstrate that while there is no detectable difference in the hypoxic level of bone marrow infiltrated by acute myeloid leukemia (AML) and healthy bone marrow, physiological hypoxia of 1% O_2 itself leads to cell cycle arrest of AML blasts (both cell lines and primary AML samples) in the G0/G1 phase with upregulation of p27 and consecutive decrease of cells in the S phase. Hence, susceptibility of AML blasts toward cytarabine as S phase dependent drug is significantly decreased as shown by decreased cytotoxicity in vitro. In addition, cells exposed to hypoxia activate PI3K/Akt and increase expression of anti-apoptotic XIAP. Inhibition of PI3K can restore cytarabine sensitivity of AML blasts at hypoxic conditions. In conclusion, hypoxia mediated effects encountered in the bone marrow might contribute to chemoresistance of AML blasts.

1. Introduction

Acute myeloid leukemia (AML) is a clonal disease of a myeloid progenitor, where genetic mutations result in uncontrolled proliferation, stop in differentiation and diminished apoptosis. Mutations are usually acquired and can be organized in functionally related categories, e.g. transcription-factor fusions. The average number of coding mutations in AML is 13 [1]. While this concept validly explains the relevant biological characteristics of AML (uncontrolled proliferation, stop in differentiation and diminished apoptosis), it is difficult to explain the clinical features. While it is clear that e.g. internal tandem duplication (ITD) of the fetal liver tyrosine kinase 3 (FLT3) results in a worse prognosis [2,3], the reason for this is much less obvious and can be hardly explained by the constitutional activation of the kinase alone. The presence of the ITD does not have an impact on the achievement of complete remission but on the incidence of relapse [4], and tyrosine kinase inhibitors have been shown to only have a moderate impact [5]. Hence, it is obvious that different factors besides the mutations within the blasts have an impact on the behavior of the leukemic cells in vivo.

These factors influencing AML blasts will be commonly summed up as the microenvironment, i.e. everything that surrounds the AML. This microenvironment comprises of several components that can be either cellular or non-cellular. Cellular components can be e.g. other AML blasts, monocytes, macrophages and mesenchymal stromal cells (MSC) or osteo- and fibroblasts residing in the bone marrow. For MSC it was shown that they protect AML blasts from chemotherapy-induced apoptosis by a variety of ways, including metabolic changes and induction of anti-apoptotic proteins [6,7]. Non-cellular components are abundant and include biological (e.g. cytokines) and physico-chemical compounds like temperature, pH and O_2. The oxygen partial pressure (pO_2) has been shown to impact differentiation of normal hematological progenitors and a crucial component of the stem cell niche, which is considered to be hypoxic [8,9]. The concept of hypoxia is however far from simple, as this term is used to describe different states: everything below 21% O_2, everything below arterial blood pO_2, or any oxygen partial pressure that is below "normal". However, what is "normal" might differ between different tissues, and even if the bone marrow pO_2 is way below 21% O_2 [10], this might not be "hypoxic" for hematopoietic cells at all. Instead, these levels of hypoxia encountered in the bone

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marrow represent the proper oxygen level required for hematopoietic cells for their proper functionality or state of differentiation.

It is however especially difficult to decide which levels of hypoxia should be investigated. Hence, we decided to characterize the effects of different levels of hypoxia on proliferation and survival in AML cells. In addition, we aimed to identify responsible molecular mechanisms.

2. Materials and methods

2.1. Immunohistochemistry of bone marrow biopsies

Immunohistochemical analyses were performed on archival bone marrow trephine biopsies taken from the iliac crest after informed consent had been obtained from the patients. Diagnosis of AML was confirmed according to WHO criteria (≥20% myeloid blasts with > 3% positivity for peroxidase). Eight samples of patients with AML were used; these samples were completely anonymized for analysis, hence no further information on these patients is available. Eight normal controls consisted of patients without bone marrow involvement; again, these patients were anonymized and no further information was made available. After formalin-fixation, these specimens had been mildly decalcified overnight in acetic acid, and embedded in paraffin wax. Sections were cut at 3 μm and stained for VEGF (Dako, Copenhagen, Denmark) and HiF1α (Novus Biochemical, Littleton, USA) by the indirect immunoperoxidase-staining technique as described elsewhere [11].

2.2. Cell culture

Standard laboratory (normoxic) conditions comprised 21% O_2, 5% CO_2, and 37 °C. For experiments in a reduced oxygen environment, the hypoxic Workstation INVIVO$_2$ 400 from Ruskinn Technology (Bridgend, United Kingdom) was used. Cells were incubated in 6% or 1% O_2, 5% CO_2, and 37 °C for 48 h. Live cell numbers were evaluated after staining with trypan blue (Life Technologies, Darmstadt, Germany).

All AML cell lines used are listed in Table 1 and were obtained from the DSMZ (German collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Cell lines were maintained at a density of 2.5×10^5/ml each in RPMI 1640 containing 10% FCS (Biochrom, Berlin, Germany), 2 mM glutamine and 1% penicillin-streptomycin (all from Life Technologies, Darmstadt, Germany). Primary AML samples were collected from bone marrow aspirates from AML patients who underwent routine bone marrow aspiration or from peripheral blood. Mononuclear cells were separated by Ficoll-Hypaque (Sigma–Aldrich, St Louis, MO) density-gradient centrifugation and cultured at a density of 10^6/ml in RPMI 1640 containing 10% FCS, 2 mM glutamine and 1% penicillin-streptomycin. Experiments were performed on fresh, not frozen cells that exhibited a viability of > 90% within 24–48 h after acquisition.

2.3. WST-assay

Overall viability of cell samples was measured by the WST-1 assay. Briefly this assay is based on the cleavage of the tetrazolium salt WST-1 (Roche, Mannheim, Germany) to a (colored) formazan dye by mitochondrial dehydrogenases of viable cells. The signal can be detected spectrophotometrically in an enzyme-linked immunosorbent assay (ELISA) reader and directly correlates to the number of viable and metabolically active cells in the sample. The assay was performed in 96-well plates with 100 μl of a 2.5×10^5/ml suspension of AML blasts. After 48 h exposure to increasing concentrations of cytarabine (Sigma–Aldrich, Taufkirchen, Germany) 10 μl of WST-1 reagent were added for a further incubation period of 4 h. The ELISA reader (OptiMax, Molecular Devices, Sunnyvale, USA) was set at a wavelength of 450 nm with a reference wavelength of 690 nm. Viability of cells after drug exposure was calculated as a percentage relative to untreated controls.

Table 1
AML cell lines used for experiments. FAB: French-American-British Classification, ITD: internal tandem duplication. Apoptosis: fold change of AnnexinV positive cells (%) after 48 h of hypoxia of 1% O_2 as compared to 21% O_2.

Cell Line	FAB	Comments	Apoptosis
KG-1a	AML M6	Complex aberrant karyotype	0.4
Kasumi-1	AML M2	t(8;21)	8.6
NB-4	AML M3	t(15;17)	7.1
OCI-AML3	AML M4	NPM1A mutation	3.4
OCI-AML5	AML M4	Complex aberrant karyotype	2.6
MV-4/11	AML M5	FLT3 ITD	5.8
Molm-13	AML M5a	FLT3 ITD	18.4
HEL	AML M6	JAK2 V617F mutation	6.6
TF-1[a]	AML M6	Complex aberrant karyotype	3.1
CMK	AML M7	Complex aberrant karyotype	4.7
M-07e[a]	AML M7	Complex aberrant karyotype	1.1
Mono-Mac-6	AML M5	*t*(9;11)(p22;q23)	11.4

[a] Requires GM-CSF for in vitro growth.

2.4. Flow cytometry

Flow cytometric data were acquired using a FACSCalibur cytometer (BD Biosciences, San Jose, USA). Assessment of apoptotic cells were performed with the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, Heidelberg, Germany) after exposure to the respective drugs cytarabine and the PI3K-Inhibitor LY294002 (Cell Signaling, Danvers, USA). Analysis of Annexin-V expression was performed using BD CellQuest Pro software. For cell cycle analysis cells were fixed with 70% ice cold ethanol (Sigma–Aldrich, Taufkirchen, Germany) at the respective time points. After digest with 1 μg/ml RNAse A (Sigma–Aldrich, Taufkirchen, Germany) cells were stained with 50 μg/ml PI (Sigma–Aldrich, Taufkirchen, Germany) and DNA content was quantified by flow cytometry. For measurement of BrdU uptake, cells were exposed to BrdU (10 μM) for 4 h and staining of uptake was performed with the FITC BrdU Flow Kit (BD Biosciences, San Jose, USA) according to the manufacturer's protocol.

2.5. Western blotting

For Western blot analysis, cells were lysed in Cell lysis buffer (Cell Signaling) in either normoxia or hypoxia. Lysis buffer was supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were then separated on a 10–12% polyacrylamide gel, transferred to Hybond-P membranes (GE Healthcare, Little Chalfont, United Kingdom), probed with the appropriate antibodies (HiF1α, p27, XIAP [all from BD Pharmingen, Heidelberg, Germany], GAPDH, phospho Akt and total Akt [all from Cell Signaling, Danvers, USA]), and visualized using an enhanced chemiluminescence kit (Thermo, Rockford, USA).

2.6. Microarray analyses

Pre-treatment bone marrow samples of 533 patients at time of diagnosis were analyzed using Affymetrix U133A+B and Affymetrix U133 Plus2.0 microarrays (Affymetrix, Santa Clara, CA) as published previously [12]. The microarray data have been deposited in the Gene Expression Omnibus with the accession number GSE37642 [13]. Expression data are presented as log2 throughout the study.

2.7. Statistical analysis

Results are shown as the mean ± SEM or SD of at least 3 experiments each. Paired data were analyzed using the paired Student *t* test. For survival analyses, Cox regression and the method of Kaplan–Meier using Log Rank test with SPSS Statistics® (Version 22, IBM) were used. Microsoft Excel was used for data acquisition and storage. A P value < .05 was considered statistically significant.

3. Results

3.1. Expression of HiF1α and VEGF in normal bone marrow and AML bone marrow

The expression of Hypoxia inducible Factor 1α (HiF1α) is known to be directly influenced by the presence of hypoxia. In a first step we examined whether AML bone marrow is more hypoxic than normal bone marrow by investigating the expression of HiF1α and Vascular Endothelial Growth Factor as a downstream target of HiF1α in 8 normal bone marrows and 8 AML bone marrows. As shown in Fig. 1, there was no difference in the expression of VEGF (Fig. 1A), while there seemed to be a higher expression of HiF1α in the normal bone marrow (Fig. 1B). Interestingly, in the AML samples HiF1α seemed not to be expressed in (morphological) blasts but in more mature cells with maturated nuclei (Fig. 1C). The number of mature myeloid cells however is reduced in bone marrow infiltrated by AML blasts, which explains the reduced number of HiF1α expressing myeloid cells. Accordingly, there was no in vitro expression of Hif1α by AML at hypoxia at short (2 days) or long term culture (10 days) and in primary AML (Fig. 1D) or very short term culture (Fig. 1E).

3.2. Clinical impact of HIF1α and VEGF RNA expression

In addition, we analyzed the expression of HIF1α and VEGF in leukemic blasts from 535 AML patients that were treated within

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