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Research paper

Maintenance of cellular respiration indicates drug resistance in acute myeloid leukemia

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ABSTRACT

Primary resistance to induction therapy is an unsolved clinical problem in acute myeloid leukemia (AML). Here we investigated drug resistance in AML at the level of cellular metabolism in order to identify early predictors of therapeutic response. Using extracellular flux analysis, we compared metabolic drug responses in AML cell lines sensitive or resistant to cytarabine or sorafenib after 24 h of drug treatment to a small cell lung cancer (SCLC) cell line exposed to etoposide. Only drug-resistant AML cells maintained oxidative metabolism upon drug exposure while SCLC cells displayed an overall metabolic shift towards glycolysis, i.e. a Warburg effect to escape drug toxicity. Moreover, primary AML blasts displayed very low glycolytic activity, while oxygen consumption was readily detectable, indicating an essential role of oxidative pathways in the bioenergetics of AML blasts. In line with these observations, analysis of the mitochondrial membrane potential using tetramethylrhodamine ethyl ester staining and flow cytometry allowed for clear discrimination between drug sensitive and resistant AML cell line clones and primary blasts after 24 h of treatment with cytarabine or sorafenib. Our data reveal a distinct metabolic phenotype of resistant AML cells and suggest that disrupting oxidative metabolism rather than glycolysis may enhance the cytotoxic effects of chemotherapy in AML.

1. Introduction

Chemotherapy has become a mainstay of cancer treatment during the second half of the 20th century [1]. More recently, advances in understanding the biology of cancer initiated a shift towards targeted agents as key components of therapeutic regimens [2]. However, even if the road towards precision medicine appears to be paved, personalized oncology still faces a major obstacle since individual patient outcome with a certain treatment cannot be reliably predicted [3]. This problem is of particular relevance in acute myeloid leukemia (AML) because this disease constitutes a medical emergency, and response to induction therapy represents an important prognostic parameter [4]. Currently, therapeutic response can only be assessed by cytology or flow cytometry after the first cycle of chemotherapy. On the other hand, well-established molecular biomarkers do not predict resistance to standard chemotherapy in AML [5], although AML is one of the genetically best-characterized malignancies [6,7]. Consequently, the initial treatment for virtually all AML patients, a combination of cytarabine and an anthracycline, has not been altered fundamentally throughout the last 30 years [8]. It has been suggested that risk-adapted exchange of this approach for novel therapeutic strategies might substantially improve the poor outcome in AML with overall cure rates of less than 40% [5,8]. To date, the value of genetic and genomic data as predictive biomarkers has not been fully established, given the complexity of the information and our incomplete understanding of their biological relevance [9]. This complexity might be resolved on the phenotypic level as only a few hallmarks of cancer have been identified [10,11]. Intriguingly, the fundamental observation by Otto Warburg and his colleagues, who reported already in 1924 that cancer cells maintain glycolysis and lactate production even in the presence of

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; 2-DG, 2-deoxy-D-glucose; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; MSCs, mesenchymal stromal cells; OCR, oxygen consumption rate; PB, peripheral blood; SCLC, small cell lung cancer; TMRE, tetramethylrhodamine ethyl ester

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oxygen [12], has been recognized only recently as one of these hallmarks [11]. Metabolic reprogramming has been linked not only to tumorigenesis, but also to drug resistance [13,14]. Thus, targeting cell metabolism may enhance the efficacy of other treatments [15,16]. The mechanisms of drug resistance remain largely elusive, yet are expected to be less diverse on the metabolic than on the genetic level [15,16]. Therefore, we investigated whether resistance towards antineoplastic agents in AML is associated with a characteristic metabolic phenotype. We found that only drug-resistant AML cells, but not small cell lung cancer (SCLC) cells maintained oxidative metabolism upon drug exposure, while resistant SCLC cells displayed an overall metabolic shift towards glycolysis that could not be observed in AML cell lines. We conclude that oxidative phosphorylation rather than glycolysis is the primary bioenergetic pathway in AML and that inhibition of oxidative metabolic pathways may overcome drug resistance.

2. Materials and methods

2.1. Cell lines and patient samples

Cell lines were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkultur (DSMZ) and cultured as recommended. Drug-resistant HL-60 and NCI-H82 cell lines were generated by continuous exposure to increasing doses of cytarabine (0.05 μM to 1 μM) or etoposide (0.16 μM to 34 μM). Sorafenib-resistant MV4-11 cells were derived from single cell clones and have been described previously [17]. AML samples were collected at the University Hospital Marburg after patients' informed consent and mononuclear cells were isolated by density centrifugation. Drug treatment of primary blasts was performed in 4-day mesenchymal stromal cell (MSC)-conditioned medium. MSCs were collected and cultured as described [18].

2.2. Fluorescence microscopy

For confocal imaging, cells were stained with 100 nM tetramethylrhodamine ethyl ester (TMRE, Abcam) and 50 μg/ml Hoechst 33342 (Life Technologies). Imaging was performed in 8-well μ-slides (Ibidi) using a LSM 510 Meta laser-scanning microscope (Zeiss).

2.3. Flow cytometry

For flow cytometry analyses, cells were seeded and treated in 6- or 96-well plates. Cell viability was assessed by staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich final concentration 2 μg/ml). For semiquantitative measurements of mitochondrial membrane potential, cells were stained with 50 nM TMRE provided in the TMRE-Mitochondrial Membrane Potential Assay Kit (Abcam). Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, Abcam) was used in a final concentration of 1 μM as a positive control for disruption of the mitochondrial membrane potential. DAPI-counterstaining was performed to exclude dead cells. Additionally, AML blasts were stained with antibodies for CD45 (PerCP-Cy™5.5 Mouse Anti-Human CD45, BD Biosciences) and CD34 (PE-Cy™7 Clone 8G12 (CE/IVD), BD Biosciences). Signals were acquired using a LSR II flow cytometer with FACSDiva software version 6.1 (BD Biosciences) and analyzed with FlowJo version 7.6.5 (Tree Star). Flow cytometry measurements included at least 10.000 events.

2.4. Metabolic assays

For metabolic assays, cells were seeded and treated in polyethylene −coated 96-well plates in four to six replicates. Overall metabolic activity was determined using the Cell Proliferation Kit I (MTT) (Roche). Extracellular acidification (ECAR) and oxygen consumption rates (OCR) were measured using the XF96 Extracellular Flux Analyzer (Seahorse Bioscience) along with consumables, assay medium and the XF Glycolytic Stress Test Kit supplied by the manufacturer as recommended. If not stated differently, ECAR was determined in the presence of 10 mM glucose and OCR under basal conditions. In the glycolysis stress test, final assay concentrations of 5 μM of the respiratory chain inhibitor oligomycin and 100 mM of the glycolysis inhibitor 2-deoxy-D-glucose (all from Sigma-Aldrich) were used.

2.5. Statistics

Sample means were compared pairwise using generalized least squares estimation and p-values adjusted for multiple comparisons were calculated as described [19]. Trends were assessed by Jonckheere's test. Statistical analyses were performed using the R language and environment for statistical computing [20].

3. Results

3.1. Drug sensitivity profiles of AML and SCLC cell lines

In order to uncover the metabolic features of AML cells that might be associated with their response to pharmacologic antitumor treatment, we studied two pairs of drug-sensitive and −resistant AML cell line subclones. In particular, we examined HL-60 cells sensitive or resistant to the cytostatic cytarabine and MV4-11 cells sensitive or resistant to the tyrosine kinase inhibitor sorafenib, which targets the length mutation in the FLT3 kinase (*FLT3*-ITD) present in these cells [17]. Moreover, we included etoposide-sensitive or −resistant NCI-H82 SCLC cells as a control for comparison. To confirm drug sensitivity or resistance, respectively, we treated sensitive (S) and resistant (R) cell lines with increasing doses of cytarabine, sorafenib or etoposide, and analyzed cell viability after two, three and five days via DAPI-exclusion by flow cytometry. We found that viability of cytarabine-sensitive HL-60(S) compared to −resistant HL-60(R) cells significantly decreased after 48 h, while a marked difference in survival of MV4-11(S) and MV4-11(R) cells was seen after 72 h of treatment with sorafenib. Finally, a significant reduction in viability of NCI-H82(S) compared to NCI-H82(R) cells was observed only after 120 h of treatment with low and intermediate doses of etoposide (Fig. 1, Fig. S1). These findings confirm drug resistance of the three (R) cell lines in comparison to their (S) counterparts. Subsequently, metabolic analyses after 24 h of treatment with cytarabine, sorafenib or etoposide allowed us to assess metabolic drug responses prior to the onset of cytotoxic effects induced by these agents in AML or SCLC cell lines.

3.2. Maintenance of oxygen consumption indicates drug resistance in AML cells

To further investigate whether drug resistance was associated with a distinct metabolic phenotype, we first determined the overall metabolic activity of (S) and (R) cells using an MTT assay, which measures reduction of the tetrazolium salt (4,5- dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) to a purple-colored formazan by NAD(P)H-dependent oxidoreductases. Indeed, we observed a substantial decrease of formazan-absorbance in all (S) compared to the corresponding (R) cells (Fig. 2A, Fig. S2A) after 24 h of drug treatment. In order to further dissect the metabolic pathways affected by antineoplastic treatment, we used Seahorse Bioscience XF technology to simultaneously determine extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in real-time. ECAR measurement after addition of glucose provides a measure for the rate of glycolysis, whereas OCR reflects the combined activity of several upstream oxidative pathways that supply reduced NAD(P)H or $FADH_2$ coenzymes to the electron transport chain, either directly or via the tricarboxylic acid cycle [21]. Metabolic measurements in our (S) and (R) cell lines after 24 h of drug exposure revealed that ECAR-responses differed significantly between (S) and (R) cell lines for all three pairs investigated. Specifically, all (S)

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