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Mechanisms of resistance to azacitidine in human leukemia cell lines

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The DNA methylation inhibitor azacitidine (5-azacytidine) is used against myelodysplastic syndrome and acute myeloid leukemia, but drug resistance is an ongoing, intractable problem. To investigate resistance mechanisms, we generated two azacitidine-resistant cell lines, THP-1/AR and HL60/AR, and studied genetic disparities between them and their corresponding parental lines. In cells treated with azacitidine, significant mitotic variations were noted in parental cells which were absent in resistant cells, suggesting that resistance arises from negating azacitidine-mediated activation of apoptosis signaling and reestablishing G_2/M checkpoint. Importantly, both resistant cell lines have common point mutations in the uridine-cytidine kinase 2 (*UCK2*) gene, which encodes the rate-limiting enzyme of the azacitidine activation pathway. Forced expression of mutated *UCK2* in parental THP-1 cells abrogated azacitidine-induced apoptosis, whereas overexpression of wild type *UCK2* in resistant THP-1/AR cells restored sensitivity to azacitidine, implying that *UCK2* gene mutations perturb azacitidine activation and advance azacitidine resistance. Our study provides new insights into azacitidine resistance and establishes models useful in developing effective strategies to overcome it.

In leukemia cells, the methylation states of gene regulatory regions are upregulated, thereby silencing the expression of some genes, including tumor suppressor genes and cell-differentiation related genes [1–6]. Abrogation of hypermethylation should promote re-expression of these genes, consequently inducing antitumor effects. For this purpose, two DNA methyltransferase (DNMT) inhibitors azacitidine (5-azacytidine) and decitabine (5-aza-2-deoxycytidine) were developed. Although both are nucleoside analogs of cytidine, their antitumor mechanisms differ. Decitabine incorporates into DNA, resulting in disruption of DNMT, thus suppressing DNA methylation. Azacitidine, likewise, can incorporate into DNA, suppressing methylation; however, it preferentially incorporates into RNA, developing cytotoxicity by inhibiting protein synthesis [7,8]. The antitumor effects of azacitidine are concentration dependent. Its cytotoxic activity increases with concentration, whereas its DNMT inhibition peaks at low concentrations and abates at higher concentrations [7–10]. Although their antitumor mechanisms differ, in preclinical examinations and clinical trials both reagents have been shown to possess cytotoxic properties and differentiation-inducing effects [11–15]. Accordingly, both are now approved for the treatment of myelodysplastic syndrome and acute myeloid leukemia (AML) with 20% to 30% bone marrow blasts, and their usefulness for treating AML with high bone marrow blasts is under investigation [16].

Drug resistance remains a major problem for patients treated with small molecular reagents such as azacitidine, but the underlying mechanisms are poorly understood [17]. Therefore, clarifying resistance mechanisms is central to develop effective leukemia therapies. Previous studies have shown that human equilibrative nucleoside transporters (hENTs) and concentrative nucleoside transporters (hCNTs) play an important role for transport of natural nucleosides and nucleoside analogs into cells [18–20]. Using hCNT1-expresing kidney cells, Rius et al. [21] showed that hCNT1 mediates uptake of azacitidine, thus sensitizing cells. This result suggests that decreasing levels of

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these transporters is a possible mechanism for acquiring resistance to DNMT inhibitors. However, both azacitidine and decitabine undergo a series of phosphorylation steps before incorporation into DNA or RNA. In this process, UCK2 and deoxycytidine kinase (DCK) function as key enzymes for activation of azacitidine and decitabine, respectively. Therefore, perturbation of the activation process may also result in blocking nucleoside analog activity, even with normal uptake [22]. Indeed, Qin T et al. found that the level of DCK attenuated because of a *DCK* gene mutation in decitabine-resistant leukemia cells [23]. Other scenarios postulate that DNMT inhibitors are certainly active in leukemia cells but fail to kill them, in which case resistance mechanisms probably involve continuous activation of cellular signaling pathways such as the Ras-extracellular signal-regulated kinase (ERK) pathway, or deactivation of apoptotic pathways. In fact, Cluzeau et al. [24] recently reported that increased expression of the antiapoptotic factor BCL2L10 was linked to resistance in azacitidine-resistant cell line SKM1-R [24], but this mechanism is not well elucidated. However, a recent study showed that cytidine deaminase inactivates DNMT inhibitors, decreasing their half-life [25]. Therefore, various mechanisms, either common or specific to each reagent, may be involved in acquisition of resistance to DNMT inhibitors, but most remain obscure.

In this study, we developed two azacitidine-resistant cell lines to probe the processes involved in azacitidine drug resistance. Our results demonstrate that acquisition of resistance is caused by perturbation of the azacitidine activation process because of *UCK2* gene mutations.

Methods

Reagents

Azacitidine, decitabine and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines

THP-1 and HL60 are BCR/ABL-negative human myeloid leukemia cell lines [26,27]. To determine the IC_{50} values of azacitidine, cells from the two parental lines were independently incubated in the presence of various concentrations of each reagent for 96 hours and then enumerated using a Cell Counting Kit-8 (Wako Pure Chemical Industries, Osaka, Japan) in accordance with the manufacturer's instructions. Dose response curves were prepared, and concentrations yielding 50% cellular viability were designated as IC_{50}.

To generate resistant clones, THP-1 and HL60 cells were treated with stepwise increasing concentrations of azacitidine (0.2–1.0 μmol/L) and colonized on a medium containing methylcellulose with 1.0 μmol/L azacitidine, followed by selection and cloning of surviving colonies. Clones at the highest IC_{50} value from each cell line were designated as THP-1/AR (TAR) and HL60/AR (HAR). All cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin G, and streptomycin sulfate and then split every 4 days.

Flow cytometry

For cell cycle analysis, cells were incubated with propidium iodide for 15 minutes and then analyzed by flow cytometry using a FACScan/CellFIT system (Becton Dickinson, San Jose, CA, USA). To analyze p-glycoprotein expression, cells were incubated with a phycoerythrin-labeled anti–p-glycoprotein antibody for 30 minutes. Phycoerythrin-labeled mouse IgG1 was used as a control. To examine the expression levels of surface antigens including CD11b, CD13, CD14, CD15, CD33, CD36, and HLA-DR, cells were incubated in solutions of respective antibodies conjugated with fluorescein isothiocyanate (Becton Dickinson) for 30 minutes and then analyzed by flow cytometry.

Western blot analysis

Whole cell lysates were prepared from 1×10^7 cells. Next, 30 μg of lysates was separated electrophoretically using 10% polyacrylamide gel. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously [28]. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, used as an internal control, was purchased from Chemicon International (Temecula, CA, USA). Anti–caspase-3, anti–cleaved caspase-3, anti–caspase-7, anti–cleaved caspase-7, anti–caspase-9, anti–cleaved caspase-9, anti-p44/42 (ERK1/2) mitogen-activated protein kinase (MAPK), anti–phospho p44/42 (ERK1/2) MAPK, anti–c-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and anti–phospho JNK/SAPK were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-DNA methyltransferase 1 (DNMT1), anti-DNMT3a, and anti-DNMT3b were purchased from Active Motif (Carlsbad, CA, USA). Anti-uridine-cytidine kinase 2 (UCK2) rabbit polyclonal antibody was purchased from Proteintech Group (Chicago, IL, USA). Anti-BCL2L10 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA).

DNMT activity assay

Cells were cultured in the presence or absence of azacitidine for 24 hours. Nuclear extracts were then prepared as described previously [29]. DNMT activity was determined using a DNMT activity/inhibition assay system (Active Motif) in accordance with the manufacturer's instructions.

Sequence analysis

Total RNA from cells was isolated with the acid guanidinium thiocyanate phenol chloroform method. Polymerase chain reaction (PCR) was performed using complementary DNA (cDNA) that was prepared from total RNA by Superscript II reverse transcriptase. The primers used for cDNA amplification are summarized in Supplementary Table 1 (online only, available at www.exphem.org). Direct sequence analysis was performed using primers summarized in Supplementary Table 2 (online only, available at www.exphem.org).

Real-time PCR analysis

We generated cDNA from total RNA extracted by reverse transcriptase and subjected to SYBR real-time PCR quantitation. PCR products were analyzed using an ABI PRISM 7700 system (Applied Biosystems, Foster City, CA, USA). Complimentary DNA corresponding to the *GAPDH* gene was used for the internal control of these real-time analyses. The primers used for real-time PCR are summarized in Supplementary Table 3 (online only,