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Original Article

Induction of cytosine arabinoside-resistant human myeloid leukemia cell death through autophagy regulation by hydroxychloroquine

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ABSTRACT

We investigated the effects of the autophagy inhibitor hydroxychloroquine (HCQ) on cell death of cytosine arabinoside (Ara-C)-resistant human acute myeloid leukemia (AML) cells. Ara-C-sensitive (U937, AML-2) and Ara-C-resistant (U937/AR, AML-2/AR) human AML cell lines were used to evaluate HCQ-regulated cytotoxicity, autophagy, and apoptosis as well as effects on cell death-related signaling pathways. We found that HCQ-induced dose- and time-dependent cell death in Ara-C-resistant cells compared to Ara-C-sensitive cell lines. The extent of cell death and features of HCQ-induced autophagic markers including increase in microtubule-associated protein light chain 3 (LC3) I conversion to LC3-II, beclin-1, ATG5, as well as green fluorescent protein-LC3 positive puncta and autophagosome were remarkably greater in U937/AR cells. Also, p62/SQSTM1 was increased in response to HCQ. p62/SQSTM1 protein interacts with both LC3-II and ubiquitin protein and is degraded in autophagosomes. Therefore, a reduction of p62/SQSTM1 indicates increased autophagic degradation, whereas an increase of p62/SQSTM1 by HCQ indicates inhibited autophagic degradation. Knock down of p62/SQSTM1 using siRNA were prevented the HCQ-induced LC3-II protein level as well as significantly reduced the HCQ-induced cell death in U937/AR cells. Also, apoptotic cell death and caspase activation in U937/AR cells were increased by HCQ, provided evidence that HCQ-induced autophagy blockade. Taken together, our data show that HCQ-induced apoptotic cell death in Ara-C-resistant AML cells through autophagy regulation.
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1. Introduction

Although multidrug chemotherapy and allogeneic stem cell transplantation have improved the clinical outcomes of acute myeloid leukemia (AML) patients over the past several decades, the predominant reasons for the failure of cures in AML are relapse after obtaining complete remission and primary refractoriness to initial chemotherapy. Indeed, the relapse or refractory status, a feature of acquired chemoresistance, is associated with extremely poor prognosis [1–3].

Cytosine arabinoside (1-β-d-arabinofuranosyl-cytosine; Ara-C) is a major anticancer agent that is widely used to induce or consolidate complete remission in AML [1,4]. As it has been suggested that the development of drug resistance to Ara-C is one of the major impediments to successful AML treatment [5–8], novel therapeutic approaches must be developed to overcome Ara-C resistance in myeloid leukemia cells.

Autophagy is a homeostatic, catabolic cellular process whereby damaged or long-lived cytoplasmic proteins and organelles are sequestered in vacuoles. They are subsequently delivered to lysosomes for degradation and recycled to maintain cellular metabolism in response to adverse environmental stresses such as nutrient deprivation, metabolic stress, DNA damage, and elevation

Abbreviations: AML, acute myeloid leukemia; HCQ, hydrochloroquine; Ara-C, cytosine arabinoside; LC3, microtubule-associated protein light chain 3; PARP, poly(ADP-ribose) polymerase; FADD, Fas-associated death domain protein; CI, combination index; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; 3-MA, 3-methyl adenine; PI, propidium iodide; TEM, transmission electron microscopic; PBS, phosphate buffered saline; HRP, horseradish peroxidase; DPBS, Dulbecco's phosphate buffered saline; siRNA, small interfering RNA; SD, standard deviation.

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of reactive oxygen species [9–14]. Thus, this process provides an alternative source of energy to enable cell survival [11–14].

Recent evidence highlights the important role played by autophagy in cancer development, tumor cell survival, and death in specific microenvironments [15–17]. Although the pro-survival, or anticancer, effects of autophagy are controversial [16–18], data are accumulating indicating that autophagy is one of the critical mechanisms for developing resistance to chemotherapy and targeted therapy [12,15,16,18–20].

Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) are the autophagy inhibitors whose effectiveness in vivo and safety in clinical trials have been approved by the FDA [21–23]. Although HCQ can act through several mechanisms, autophagy inhibition by blocking autophagosome fusion and degradation [16,23] is considered to have an important function in inducing cytotoxicity in cancer cells [24–26]. Moreover, the effects of these agents on the induction of cytotoxicity or survival in chemoresistant myeloid leukemia cells, as well as their molecular mechanism(s), have not been defined. In contrast to CQ, HCQ can be safely dose-escalated in cancer patients [16].

In this study, we demonstrated that HCQ induces cell death in Ara-C-resistant AML cells via autophagy regulation.

2. Materials and methods

2.1. Cells and cultures

The human AML cell line U937 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The Ara-C-resistant U937 (U937/AR) cell line was established in our laboratory by exposing parental U937 cells to stepwise increasing concentrations of Ara-C (from 1 nM to 2 mM, usually at intervals of 10 passages) in complete RPMI-1640 media supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin in 5% CO_2 incubator at 37 °C. The resistant sublines were grown for more than 6 months. To maintain exponential growth, cells were seeded at 1×10^5 cells/ml and passaged every 3 days. Cells were cultured in serum-free RPMI-1640 media in a humidified atmosphere of 5% CO_2 incubator. In addition, we used OCI-AML-2 (AML-2) and Ara-C-resistant OCI-AML-2 (AML-2/AR) cell lines kindly provided by Dr. T.S. Kim (Korea University Graduate School, Seoul, Korea) [27]. Patients were enrolled onto protocols approved by the Institutional Review Board of Severance Hospital and provided written informed consent in accordance with the Declaration of Helsinki and all patient samples were anonymised. Upon signed informed consent, human leukemia cells were obtained from the diagnostic bone marrow (BM) aspiration samples of patients with de novo AML diagnosed at Yonsei University Severance Hospital by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. In these human leukemia cells, primary AML cells were established in our laboratory using diagnostic BM samples of AML patients with induction failure after receiving at least one induction course of intensive chemotherapy (7 + 3 with idarubicin plus cytarabine) in complete RPMI-1640 media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA) and 100 U/ml penicillin in 5% CO_2 incubator at 37 °C. The sublines were grown for more than 6 months. To maintain exponential growth, cells were seeded at 1×10^5 cells/ml and passaged every 3 days. Cells were cultured in serum-free RPMI-1640 media in a humidified atmosphere of 5% CO_2 incubator. Two patient cell lines were included in this study. The 2 patients had normal karyotype and M2 subtype according to the French-American-British classification.

2.2. Reagents

HCQ was purchased from Myung-in pharmaceuticals (Seoul, Korea) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Ara-C was from Sigma-Aldrich. Electrophoresis reagents and protein assay kits were obtained from Bio-Rad (Hercules, CA, USA). Pan-caspase inhibitor (z-VAD-fmk), specific caspase-8 inhibitor (z-IETD-fmk), and specific caspase-9 inhibitor (z-LEHD-fmk) were purchased from R & D systems (Minneapolis, MN, USA).

2.3. Apoptosis assay

The annexin V assay was performed as previously described [28]. The percentage of apoptotic cells was determined using a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). Cell death was measured using propidium iodide (PI) exclusion assays and a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA).

2.4. Transmission electron microscopy

For transmission electron microscopy (TEM) evaluation, each sample was fixed with 2% glutaraldehyde/paraformaldehyde and then post-fixed with 1% OsO_4 dissolved in 0.1 M phosphate buffered saline (PBS). After pure, fresh resin embedding and polymerization, thin sections were double stained with 7% uranyl acetate and lead citrate for contrast staining. All of the thin sections were observed by TEM (JEM-1011, JEOL, Japan) at an acceleration voltage of 80 kV.

2.5. Transfection of GFP-LC3

Plasmid green fluorescent protein (GFP)-microtubule-associated protein light chain 3 (LC3) was kindly provided by Dr. Kim (Division of Pulmonology, Yonsei University College of Medicine, Seoul, South Korea). The cell suspension of 2×10^6 leukemia cells was immediately transfected with pGFP-LC3 cDNA (5 μg) using the Nucleofector instrument (program T-20; Lonza, Cologne, Germany) in accordance with the manufacturer's instructions. Immediately after electroporation, the cells were resuspended in a complete medium and incubated at 37 °C in a humidified 5% CO_2 incubator. The cells were harvested after 24 h with HCQ and used for the subsequent experiments. U937 and U937/AR cells expressing GFP-tagged LC3 were used to demonstrate induction of autophagy.

2.6. Confocal microscopy

Cells transfected with pGFP-LC3 plus HCQ were centrifuged onto glass slides. Fluorescent images were observed and analyzed under a Zeiss LSM 700 laser-scanning confocal microscope (Göettingen, Germany). The GFP-LC3 puncta were quantified by counting the numbers in cells as described elsewhere [29]. GFP-LC3 puncta in a single cell were manually counted under a confocal microscope. For each group, 50 cells were randomly selected for estimating the average number of GFP-LC3 puncta per cell. The data presented here are from representative experiments of at least three independent repeats.

2.7. Western blot analysis

Total cell lysates were prepared and analyzed by western blot as described earlier [30]. Rabbit polyclonal antibodies against LC3, beclin-1, ATG5, and p62/SQSTM1 were obtained from Novus

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