Original Paper

Role of Beclin-1-Mediated Autophagy in the Survival of Pediatric Leukemia Cells

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Key Words

Pediatric leukemia (PL) • Oxidative stress • Autophagy • Apoptosis • Beclin-1 • miR-93

Abstract

Background/Aims: Acute and chronic leukemia are severe malignant cancers worldwide, and can occur in pediatric patients. Since bone marrow cell transplantation is seriously limited by the availability of the immune-paired donor sources, the therapy for pediatric leukemia (PL) remains challenging. Autophagy is essential for the regulation of cell survival in the harsh environment. However, the role of autophagy in the survival of PL cells under the oxidative stress, e.g. chemotherapy, remain ill-defined. In the current study, we addressed these questions. **Methods:** We analyzed the effects of oxidative stress on the cell viability of PL cells *in vitro*, using a CCK-8 assay. We analyzed the effects of oxidative stress on the apoptosis and autophagy of PL cells. We analyzed the levels of Beclin-1 and microRNA-93 (miR-93) in PL cells. Prediction of binding between miR-93 and 3'-UTR of Beclin-1 mRNA was performed by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay. The relationship between levels of miR-93 and patients' survival was analyzed in PL patients. **Results:** We found that oxidative stress dose-dependently increased autophagy in PL cells. While low-level oxidative stress did not increase apoptosis, high-level oxidative stress increased apoptosis, seemingly from failure of autophagy-mediated cell survival. High-level oxidative stress appeared to suppress the protein levels of an autophagy protein Beclin-1 in PL cells, possibly through induction of miR-93, which inhibited the translation of Beclin-1 mRNA via 3'-UTR binding. **Conclusion:** Beclin-1-mediated autophagy plays a key role in the survival of PL cells against oxidative stress. Induction of miR-93 may increase the sensitivity of PL cells to oxidative stress during chemotherapy to improve therapeutic outcome.

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Introduction

Leukemia is a severe malignant cancer worldwide [1]. Although leukemia is mainly detected in adults, some are indeed detected during pediatric period as pediatric leukemia (PL) [1]. The etiology and pathology of leukemia in children remain elusive [2-5]. However,

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some evidence have shown that leukemia in children with Down syndrome (DS) possesses a GATA1 mutation that cooperates with trisomy 21, followed by the acquisition of additional somatic mutations in epigenetic and kinase signaling genes [6-8]. This represents a transient over-proliferation of myeloid cells at the early years of life, followed by either spontaneous resolution or development into leukemia in a portion of patients [6-8]. However, there are other types of leukemia with unknown causes. Moreover, current therapies with bone marrow cell transplantation are seriously limited by the availability of immune-paired donor sources [9, 10]. Hence, development of efficient therapy independent of bone marrow transplantation, e.g. a specific chemotherapy, may substantially improve the therapeutic outcomes.

The resistance of leukemia to chemotherapy may result from enhanced anti-apoptotic potentials of the cancer cells in a particular setting [11-13]. Cellular apoptosis is regulated by apoptosis activating proteins, e.g. Bid, Bak, Bad, and apoptosis suppressors, e.g. Bcl-2 [14-20]. Autophagy is a catabolic biological event characterized by the degradation of the cellular compartments and their recycling in order to improve cell survival upon harsh living environment [21-25]. Among all autophagy-associated proteins, autophagy-associated protein 6 (ATG6, or Beclin-1) is a key regulator [26]. Recent studies have demonstrated a critical role of autophagy in the tumor initiation, growth and metastases, and especially in the mechanisms underlying chemo-resistance of tumor cells during chemotherapy [27-30]. Nevertheless, autophagy has not been extensively studied for its association with chemo-resistance of PL cells.

MicroRNAs (miRNAs) are a class of non-coding small RNAs that regulate the protein translation of target mRNA at the 3'-untranslated region (3'-UTR) [31, 32]. There are accumulating evidence showing that miRNAs play very important roles in tumor [33-35]. Among all miRNAs, the involvement of miR-93 as either a tumor enhancer [36, 37], or a tumor suppressor [38-40] in various cancers has been reported. However, the role of miR-93 in the chemo-resistance of PL cells against oxidative stress has not been studied.

In the current study, we aimed to analyze the association between apoptotic cell death and autophagic cell survival of PL cells in response to oxidative stress, which is the major mechanism for killing cancer cells during chemotherapy.

Materials and Methods

Experimental protocol approval

All experimental protocols were approved by the Research Bureau of the Second Hospital of Hebei Medical University. The experiments were carried out in accordance with the approved guidelines based on the Declaration of Helsinki.

Patient tissue specimens

Forty PL specimens in this study were histologically and clinically diagnosed at the Second Hospital of Hebei Medical University from 2010 to 2014. For the use of these clinical materials for research purposes, informed consent was obtained from all subjects, and approval from the Institutional Research Ethics Committee were obtained. The methods were carried out in accordance with the approved guidelines.

Cell lines and reagents

Kasumi-4 and 8E5 were two PL cell lines purchased from ATCC (ATCC, Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified chamber with 5% CO₂ at 37°C. The Kasumi-4 cell line was established from the peripheral blood of an chronic myeloblastic leukemia patient of 6 year-old female Japanese individual. The 8E5 cell line was derived from acute lymphoblastic leukemia cells obtained from the pleural effusion of a 4 year-old female Caucasian patient. These two lines have been widely used for studying PL. Hydrogen peroxide (H₂O₂, Sigma-Aldrich, St. Louis, MO, USA) was applied to the cultured PL cells at 0, 0.5, 2 and 5 mmol/l, respectively. 3-Methyladenine (3-MA, Sigma-Aldrich) was prepared and used fresh at a concentration of 5 mmol/l.

Cell transfection

MiRNAs mimics (miR-93) and miRNAs antisense oligonucleotides (as-miR-93) were obtained from Origene (Beijing, China). For a control of these plasmids for modifying miR-93 levels, a plasmid carrying a null sequence (null) was used. These constructs were generated and cloned into the TOPO plasmid (Invitrogen, Carlsbad, CA, USA). The plasmids were transfected into cells at a concentration of 50nmol/l using Lipofectamine-2000 (Invitrogen), receiving a 95% transfection efficiency. The cells were analyzed after 24 hours, according to the manufacturer's instruction.

MicroRNA target prediction and 3'-UTR luciferase-reporter assay

MiRNAs targets were predicted with the algorithms TargetSan (<https://www.targetscan.org>) [41]. Luciferase-reporters were successfully constructed using molecular cloning technology. The Beclin-1 3'-UTR reporter plasmid (Beclin-1 3'-UTR) and Beclin-1 3'-UTR reporter plasmid with a mutant at the miR-93 binding site (Beclin-1 3'-UTR mut) were purchased from Creative Biogene (Shirley, NY, USA). PL cells were co-transfected with Beclin-1 3'-UTR/Beclin-1 3'-UTR mut and miR-93/as-miR-93/null by Lipofectamine 2000 (5×10^4 cells per well). Cells were collected 24 hours after transfection for assay using the dual-luciferase reporter assay (Promega, Beijing, China), according to the manufacturer's instructions.

Cell counting kit-8 (CCK-8) assay

The CCK-8 detection kit (Sigma-Aldrich) was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well microplate at a density of 5000/ml. After 24 h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20 ml/well) was added and the plate was incubated at 37°C for 2 h. The viable cells were counted by absorbance measurements with a microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

Apoptosis assay by flow cytometry

For analysis of cell proliferation, the dissociated tissue cells or cultured cells were re-suspended at a density of 10^6 cells/ml in PBS. After double staining with FITC-Annexin V and propidium iodide (PI) from a FITC Annexin V Apoptosis Detection Kit I (Becton-Dickinson Biosciences, San Jose, CA, USA), cells were analyzed using FACSscan flow cytometer (Becton-Dickinson Biosciences) equipped with Cell Quest software (Becton-Dickinson Biosciences) for determination of Annexin V+ PI- apoptotic cells.

Western blot

Protein was extracted from the cultured cells with RIPA lysis buffer (Sigma-Aldrich) on ice. The supernatants were collected after centrifugation at $12000 \times g$ at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and the proteins were separated on SDS-polyacrylamide gels, and then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish hydrogen peroxide-conjugated second antibody, enhanced chemiluminescent system was applied to visualize the protein antigen. Primary antibodies were rabbit anti-Beclin-1 and anti- α -tubulin (Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). α -tubulin was used as a protein loading control. The protein levels were first normalized to α -tubulin, and then normalized to the experimental controls.

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from cultured cells using miRNeasy kit (Qiagen), for cDNA synthesis. Quantitative real-time PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green NSCLCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with $2^{-\Delta\Delta Ct}$ method for quantification. Values of genes were first normalized against α -tubulin, and then compared to experimental controls.

Statistical analysis

All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups (GraphPad Prism, GraphPad Software, Inc. La Jolla,

CA, USA). Kaplan-Meier curves were used to analyze the patient survival by miR-93 levels. All values are depicted as mean $\pm$ standard deviation and are considered significant for $p < 0.05$.

Results

High hydrogen peroxide increases PL cell apoptosis

We uses hydrogen peroxide as an oxidase stress to mimic the effect of chemotherapy on PL cells. Thus, we examined the effects of hydrogen peroxide on PL cell apoptosis using 2 PL cell lines, 8E5 and Kasumi-4. We gave hydrogen peroxide at different doses (0, 0.5, 2 and 5mmol/l, respectively) to the PL cells. We found that hydrogen peroxide did not appear to increase 8E5 cell apoptosis at low doses (0.5 and 2 mmol/l), but significantly increased 8E5 cell apoptosis at high dose (5 mmol/l), shown by quantification (Fig. 1A), and by representative flow charts (Fig. 1B). Similarly, we found that hydrogen peroxide did not appear to increase Kasumi-4 cell apoptosis at low doses (0.5 and 2 mmol/l), but significantly increased Kasumi-4 cell apoptosis at high dose (5 mmol/l), shown by quantification (Fig. 1C), and by representative flow charts (Fig. 1D).

Suppression of autophagy increases hydrogen peroxide-induced PL cell death at low dose

We next examined the effects of hydrogen peroxide on PL cell survival to see whether PL cell autophagy may be responsible for the little increases in cell apoptosis by low-dose hydrogen peroxide. For this purpose, we examined the effects of hydrogen peroxide on PL cell death also when autophagy is inhibited. We gave hydrogen peroxide at different doses (0, 0.5, 2 and 5mmol/l, respectively) to PL cells, with or without an autophagy inhibitor

Fig. 1. High hydrogen peroxide increases PL cell apoptosis. The effects of hydrogen peroxide on apoptosis in 2 PL cell lines, 8E5 and Kasumi-4. We gave hydrogen peroxide at different doses (0, 0.5, 2 and 5mmol/l, respectively) to the PL cells. (A-B) 8E5 cell apoptosis, shown by quantification (A), and by representative flow charts (B). (C-D) Kasumi-4 cell apoptosis, shown by quantification (C), and by representative flow charts (D). * $p < 0.05$. NS: non-significant. N=5.

Fig. 2. Suppression of autophagy increases hydrogen peroxide-induced PL cell death at low dose. We gave hydrogen peroxide at different doses (0, 0.5, 2 and 5 mmol/l, respectively) to PL cells, with or without an autophagy inhibitor 3-MA (5 mmol/l). (A) 8E5 cell survival in an CCK-8 assay. (B) Kasumi-4 cell survival in an CCK-8 assay. *p < 0.05. NS: non-significant. N = 5.

Fig. 3. Hydrogen peroxide increases Beclin-1 to activate PL autophagy. We gave hydrogen peroxide at different doses (0, 0.5, 2 and 5 mmol/l, respectively) to PL cells, with or without an autophagy inhibitor 3-MA (5 mmol/l). (A) Beclin-1 protein in 8E5 cells by Western blot. (B) Beclin-1 mRNA in 8E5 cells by RT-qPCR. (C) Beclin-1 protein in Kasumi-4 cells by Western blot. (D) Beclin-1 mRNA in Kasumi-4 cells by RT-qPCR. *p < 0.05. NS: non-significant. N = 5.

3-MA (5 mmol/l). We found that high-dose hydrogen peroxide decreased 8E5 cell survival in an CCK-8 assay, while low-dose hydrogen peroxide did not significantly decrease 8E5 cell survival (Fig. 2A), consistent with apoptosis data. However, when autophagy was suppressed by 3-MA, low-dose hydrogen peroxide already significantly decreases 8E5 cell survival (Fig. 2A). Similarly, we found that high-dose hydrogen peroxide decreased Kasumi-4 cell survival, while low-dose hydrogen peroxide did not significantly decrease Kasumi-4 cell survival (Fig. 2B), consistent with apoptosis data. However, when autophagy was suppressed by 3-MA, low-dose hydrogen peroxide already significantly decreases Kasumi-4 cell survival (Fig. 2B). Together, these data suggest that hydrogen peroxide not only increases apoptotic death of PL cells, but also increases PL cell autophagy to contradict hydrogen peroxide-induced apoptotic cell death at low dose.

Fig. 4. Hydrogen peroxide modulates miR-93, which targets Beclin-1 to regulate PL cell autophagy. (A) Among all predicted binding miRNAs, we found that miR-93 targeted the 135-141 base pairs of 3'-UTR of Beclin-1 mRNA. (B-C) Moreover, miR-93 levels were found to be dose-dependently inhibited by hydrogen peroxide in 8E5 cells (B), and in Kasumi-4 cells (C). * $p < 0.05$. N=5.

Hydrogen peroxide increases Beclin-1 to activate PL autophagy

We next examined the effects of hydrogen peroxide on Beclin-1, a key autophagy-associated protein, in PL cells. We gave hydrogen peroxide at different doses (0, 0.5, 2 and 5 mmol/l, respectively) to PL cells, with or without an autophagy inhibitor 3-MA (5 mmol/l). We found that hydrogen peroxide dose-dependently upregulated Beclin-1 in 8E5 cells, which was significantly suppressed by 3-MA (Fig. 3A). However, the Beclin-1 mRNA was not affected by hydrogen peroxide (Fig. 3B). Similarly, we found that hydrogen peroxide dose-dependently upregulated Beclin-1 in Kasumi-4 cells, which was significantly suppressed by 3-MA (Fig. 3C). However, the Beclin-1 mRNA was not affected by hydrogen peroxide (Fig. 3D). These data suggest that hydrogen peroxide dose-dependently increases Beclin-1 levels and cell autophagy in PL cells, which may contribute to its chemo-resistance. Moreover, the regulation of Beclin-1 by oxidative stress may be at post-transcriptional level.

Hydrogen peroxide modulates miR-93, which targets Beclin-1 to regulate PL cell autophagy

Since miRNAs are critical regulators for protein translation, we performed bioinformatics studies to screen miRNAs that may target 3'-UTR of Beclin-1 mRNA, and were regulated by hydrogen peroxide treatment. Among all predicted binding miRNAs, we found that miR-93 targeted the 135-141 base pairs of 3'-UTR of Beclin-1 mRNA (Fig. 4A). Moreover, miR-93 levels were found to be dose-dependently inhibited by hydrogen peroxide in 8E5 cells (Fig. 4B), and in Kasumi-4 cells (Fig. 4C). Together, these data suggest that miR-93 may be a Beclin-1-targeting miRNA that is suppressed by hydrogen peroxide treatment in PL cells.

MiR-93 inhibits Beclin-1 protein translation in PL cells

In order to verify that the binding of miR-93 to the 3'-UTR of Beclin-1 mRNA is functional, we either overexpressed miR-93, or inhibited miR-93 (by antisense of miR-93; as-miR-93) in both PL cell lines. The PL cells were also transfected with a plasmid carrying a null sequence as a control (null). The overexpression or inhibition of miR-93 in PL cells was confirmed by RT-qPCR (Fig. 5A). MiR-93-modified PL cells were then transfected with 1 μ g plasmids carrying luciferase reporter for 3'-UTR of Beclin-1 mRNA. Moreover, null-transfected PL cells were also transfected with 1 μ g plasmids carrying luciferase reporter for 3'-UTR of Beclin-1 mRNA with one mutate at the miR-93 binding site (mut). The luciferase activities were quantified in miR-93-modified PL cells, suggesting that miR-93 specifically targets 3'-UTR of Beclin-1 mRNA to inhibit its translation (Fig. 5B). Thus, our data suggest that the binding of miR-93 to the 3'-UTR of Beclin-1 mRNA in PL cells is functional.

MiR-93 levels predict the outcome of chemotherapy of PL patients

Next, we investigated whether the levels of miR-93 in peripheral leukemia cells after chemo-therapy may predict overall survival of PL patients. The 40 PL patients (aged 2-10 year-old) who received same chemotherapy were selected in this study. One month after treatment, levels of miR-93 in peripheral leukemia cells were taken for examination of miR-93 levels. Then, the patients were followed-up for 5 years. The median value of all 40

Fig. 5. MiR-93 inhibits Beclin-1 protein translation in PL cells. (A) In order to verify that the binding of miR-93 to the 3'-UTR of Beclin-1 mRNA is functional, we either overexpressed miR-93, or inhibited miR-93 (by antisense of miR-93; as-miR-93) in both PL cell lines. The PL cells were also transfected with a plasmid carrying a null sequence as a control (null). The overexpression or inhibition of miR-93 in PL cells was confirmed by RT-qPCR. (B) MiR-93-modified PL cells were then transfected with 1 μ g plasmids carrying luciferase reporter for 3'-UTR of Beclin-1 mRNA. Moreover, null-transfected PL cells were also transfected with 1 μ g plasmids carrying luciferase reporter for mutated 3'-UTR of Beclin-1 mRNA at the miR-93 binding site (mut). The luciferase activities were quantified in miR-93-modified PL cells. * p <0.05. NS: non-significant. N=5.

Fig. 6. MiR-93 levels predict the outcome of chemotherapy of PL patients. The 40 PL patients (aged 2-10 year-old) who received same chemotherapy were selected in this study. One month after treatment, levels of miR-93 in peripheral leukemia cells were taken for examination of miR-93 levels. Then, the patients were followed-up for 5 years. The median value of all 40 cases was chosen as the cutoff point for separating miR-93-high cases (n=20) from miR-93-low cases (n=20). Kaplan-Meier curves were performed, showing that miR-93-low PL patients had a significantly poorer survival, compared to miR-93-high PL patients. ** p <0.01. N=40.

Fig. 7. Schematic of the model. Chemotherapy may decrease miR-93 levels in PL cells, which subsequently upregulates Beclin-1 to increase PL cell autophagy to antagonize hydrogen peroxide-induced cell death. The levels of depression of miR-93 may decide the activation of autophagic cell survival of PL cells against chemo-therapy and the outcome of the chemo-treatment.

cases was chosen as the cutoff point for separating miR-93-high cases (n=20) from miR-93-low cases (n=20). Kaplan-Meier curves were performed, showing that miR-93-low PL patients had a significantly poorer survival, compared to miR-93-high PL patients (Fig. 6). Together, the findings in the current study suggest that chemotherapy may decrease miR-93 levels in PL cells, which subsequently upregulates Beclin-1 to increase PL cell autophagy to antagonize hydrogen peroxide-induced cell death. The levels of depression of miR-93 may decide the activation of autophagic cell survival of PL cells against chemo-therapy and the outcome of the chemo-treatment (Fig. 7).

Discussion

Chemotherapy induces mitotic arrest at concentrations typically used in human tumor cell (including PL cells) culture. On the other hand, the compromised effects of chemotherapy on cancer mitotic arrest and cell death may be resulting from the molecular responses of

cancer cells to oxidative stress, in which enhanced survival is reached by activation of cell autophagy.

To identify cellular and molecular markers of resistance or sensitivity to chemotherapy-associated oxidative stress, great efforts have been performed by different researchers. Although a number of candidates have been identified, including proteasome subunits, cyclin G1, and solute carrier genes [42-46], so far a validated biomarker to be used in clinic for predicting the outcome of chemotherapy has not been achieved.

In the current study, we analyzed the effects of hydrogen peroxide (as an oxidase stress induced in chemotherapy) on the cell viability and autophagy of PL cells. We found that hydrogen peroxide decreased PL cell viability only at high dose. Similarly, hydrogen peroxide only induced significant cell apoptosis at high dose. Interestingly, inhibition of cell autophagy significantly increased cell death at low dose treatment of hydrogen peroxide, suggesting that the effects of low-dose hydrogen peroxide on PL cell death might be contradicted by enhanced autophagy-associated cell survival.

Here, we found a regulatory axis miR-93/Beclin-1 in PL cells in response to oxidase stress. We have examined all Beclin-1-targeting miRNAs and only the levels of miR-93 were altered in our experimental settings. Moreover, besides the data shown here, we also found that miR-93 levels were unaltered by peroxidase at the presence of 3-MA. Furthermore, the miR-93-modified cells still regulated Beclin-1 in response to hydrogen peroxide, which seemingly resulted from the incompletely inhibition of miR-93 by as-miR-93. The loss of Beclin-1 regulation by hydrogen peroxide may be seen in miR-93-Knockout cells.

In line with this view, it seemed that hydrogen peroxide induce both apoptotic cell death and autophagic cell survival in PL cells, and autophagy appears to be a negative feedback of PL cells to antagonize the toxicity of hydrogen peroxide-induced oxidative stress, which generated a harsh environment for PL cells. In order to survive, PL cells decreased miR-93 levels to induce upregulation of Beclin-1 to augment autophagic cell survival. The levels of miR-93 depression and Beclin-1 upregulation may decide the levels of PL cells against chemotherapy. Here, we used 2 different PL lines (8E5 and Kasumi-4) and achieved same result, which potentially excluded a possibility of cell-line dependence of the current findings.

Together, our data shed light on a previously non-appreciated signaling regulatory pathway that decides the chemotherapeutic outcome against PL. Future experiments may be applied to further dissect the details of the pathway, e.g. other involved key proteins, other involved miRNAs, and the clinic relevance. Our study also suggests that strategies that increase miR-93 levels or inhibit cell autophagy may improve the outcome of PL chemotherapy.

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Disclosure Statement

The authors have declared that no competing interests exist.

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