Cbl-b-dependent degradation of FLIP\textsubscript{L} is involved in ATO-induced autophagy in leukemic K562 and gastric cancer cells

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Abstract

Various molecular mechanisms are involved in the efficacy of arsenic trioxide (ATO) against malignant hematologic and some solid tumors. FLICE-like inhibitory protein (FLIP) is an inhibitor of apoptosis mediated by death receptors. In this study, we identified a new link between the down-regulation of cellular FLIP\textsubscript{L} and ATO-induced autophagy. ATO induced the degradation of FLIP\textsubscript{L} in K562 and MGC803 cells, which was mediated by the ubiquitin–proteasome pathway. Moreover, the casitas B-lineage lymphoma-b (Cbl-b) was involved in this process, which interacted with FLIP\textsubscript{L} and promoted proteasomal degradation of FLIP\textsubscript{L}. Our findings lead to a better understanding of the mechanism of action of ATO, and suggest that a novel signaling pathway is required for ATO-induced autophagy in K562 and MGC803 cells.

1. Introduction

Over the last two decades, arsenic trioxide (ATO) has been used successfully for the treatment of many types of hematologic malignancies [1–4]. Recent clinical trials have also demonstrated promising results with ATO on a variety of solid tumors [5–8]. To date, various mechanisms of ATO have been investigated [9], such as those involved in the stimulation of differentiation or the induction of apoptosis. Recently, there is emerging evidence that autophagy plays an important role in ATO-induced toxicity [10–12]. However, the exact molecular mechanisms involved in ATO-induced autophagy require further investigation.

Cellular FLIP (FLICE-like inhibitory protein), which is critical for protection against death receptor-mediated cell apoptosis [13], is overexpressed in many tissues, mainly in two major isoforms, FLIP long (FLIP\textsubscript{L}) and FLIP short (FLIP\textsubscript{S}). FLIP has been reported to not only possess anti-apoptotic activity but also serve as an anti-autophagy molecule [14–15]. A previous study suggested that ATO induced apoptosis via down-regulation of FLIP in myelodysplastic syndromes and myeloid leukemia cells [16]. However, whether FLIP is involved in ATO-induced autophagy remains unclear.

The casitas B-lineage lymphoma (Cbl) family of E3 ubiquitin ligases plays a key role in the determination of ubiquitinated substrate proteins and in regulating cell function [17–20]. In tumor necrosis factor (TNF)-treated mouse macrophage cells, c-Cbl is involved in ubiquitination and degradation of FLIP\textsubscript{S} [21]. c-Cbl also degrades active Src for autophagy in focal adhesion kinase (FAK)-deficient mouse squamous carcinoma cells [22]. Our prior observations indicated that ATO could up-regulate the expression of Cbl-b, another Cbl family member, in both acute promyelocytic leukemia and gastric cancer cells [23]. However, the function of Cbl-b up-regulated by ATO is not fully understood.

In the present study, we demonstrated that degradation of FLIP\textsubscript{L} was associated with ATO-induced protective autophagy in hematologic and solid tumor cells. Most notably, the ubiquitin E3 ligase Cbl-b interacted with FLIP\textsubscript{L} and triggered proteasomal degradation of FLIP\textsubscript{L}, which facilitated ATO-induced autophagy. These observations suggest that FLIP\textsubscript{L} is a critical regulator of ATO-induced autophagy in leukemic K562 and gastric cancer cells.

Structured summary of protein interactions:

FLIP-L physically interacts with CBL-B by anti bait coimmunoprecipitation (View interaction)

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2. Materials and methods

2.1. Cell culture

Human chronic myelogenous leukemic cell line (K562), human T lymphoma cell line (Jurkat), human breast cancer cell line (MCF-7) and human gastric cancer cell line (MGC803) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37 °C under an atmosphere of 95% air and 5% CO2.

2.2. Reagents and antibodies

Arsenic trioxide (ATO) and chloroquine (CQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). PS341 (Bortezomib) was purchased from Millenium Pharmaceuticals Inc. (Cambridge, MA, USA). Anti-Cbl-b, anti-actin, anti-p62, anti-Beclin 1, anti-PARP and anti-FLIP antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-LC3 antibody was purchased from Novus Biological (Littleton, CO, USA) and anti-phosphotyrosine 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY).

2.3. Cell viability assay, flow cytometry and Western blot analysis

These experiments were performed as described previously [23].

2.4. Transmission electron microscopy

Cells were treated and collected by trypsinization, then fixed with 2.5% phosphate-buffered gluteraldehyde, postfixed in 1% phosphate-buffered osmium tetroxide. Cells were then embedded, sectioned, double stained with uranyl acetate and lead citrate, and analyzed using a JEM-1200EX transmission electron microscope (JEOL, Japan).

2.5. Immunoprecipitation assay

Cells were collected and lysed in an NP40 buffer supplemented with complete protease inhibitor cocktail (Roche). After pre-clearing with protein A/G agarose beads for 2 h at 4 °C, whole-cell lysates were used for immunoprecipitation with the indicated antibodies. Generally, 1–4 μg of the commercial antibody or immunoglobulin-G (Santa Cruz, CA, USA) was added to 1 ml of the cell lysate and incubated at 4 °C for 8–12 h. After adding protein A/G agarose beads, incubation was continued for an additional 1 h. The immunoprecipitates were then washed extensively with a lysis buffer and eluted by boiling them with an SDS loading buffer for 5 min.

2.6. Transfections of plasmid constructs

The experiment was performed as described previously [24].

2.7. Small interfering RNA transfections

Cells were seeded at a density of 3 × 10^5 cells/well in 6-well plates. After 24 h, cells were transfected with small interfering RNA (siRNA) using Lipofectamine 2000 reagent according to the manufacturer’s protocols. The FLIPs siRNA sequence used was: AAG GAA CAG CTT GCC GCT CAA. The FLIPs siRNA sequence used was: AAC ATG GAA CTG CCT CTA CTT. The Beclin 1 siRNA sequence used was: AAA GTG GTA AGA CTG TGC CAA. The Ctrl sequence used was: AAT TCT CCG AAC GTG TCA CGT. FLIPL, FLIPS, Beclin 1, Cbl-b and Ctrl siRNAs were produced from Genechem Co. (Shanghai, China). Gene silencing effect was verified by Immunoblotting.

2.8. Statistical analysis

All the presented data were confirmed in at least three independent experiments, and are expressed as the mean ± SD. Statistical comparisons were made by Student’s t test. P < 0.05 was considered statistically significant.

3. Results

3.1. ATO affects cell viability, and induces apoptosis and protective autophagy in malignant hematologic and solid tumor cell lines

K562, Jurkat, MCF-7 and MGC803 cell lines were treated with different concentrations of ATO for 24 or 48 h, and viability was evaluated. The IC50 values of ATO at 24 h in K562, Jurkat, MCF-7 and MGC803 cells were 4.59 ± 1.53 μM, 5.92 ± 1.39 μM, 49.76 ± 8.34 μM, and 59.33 ± 7.90 μM, respectively (Table 1). To investigate whether ATO is a direct activator of autophagic flux, we detected microtubule-associated protein light chain 3 (LC3) (LC3-I/LC3-II) by immunoblot analysis. As shown in Fig.1A, treatment with ATO significantly induced a time-dependent increase in the expression of both LC3-I and LC3-II in K562, Jurkat, MCF-7 and MGC803 cells accompanied by PARP cleavages. It has been reported that the sequestosome 1 (p62) protein has LC3 binding domains for serving as a selective substrate of autophagy [25]. Consistent with increased autophagic flux following treatment with ATO, there was a time-dependent decrease in the level of p62 by immunoblotting (Fig. 1A). Furthermore, ATO-induced autophagy was confirmed by transmission electron microscopy (formation of autophagosomes) in K562 and MGC803 cells (Supplementary Fig. S1). Because autophagy can result in both cell survival and death, we next determined whether ATO-induced autophagy was protective or pro-apoptotic. Treatment of K562 cells with either ATO, CQ (an inhibitor of autophagy that stops autophagy at the formation stage), or a combination of ATO and CQ for 24 h revealed that combined treatment with ATO and CQ decreased cell viability, and increased apoptotic cell death compared to ATO or CQ treatment alone. Similar results were also observed in MGC803 cells (Fig. 1B and C). To confirm the effect of autophagy inhibition by the pharmacologic agent CQ on ATO-induced apoptosis, an RNA interference approach was used to suppress the expression of Beclin 1, which is required for the initiation of the formation of autophagosomes [26]. Reduced cell viability in MGC803 cells was observed with knockdown of Beclin 1 (Fig. 1D). These data indicated that blockage of autophagy enhanced the antitumor effect of ATO in K562 and MGC803 cells.

3.2. FLIPs is involved in ATO-induced autophagy

To investigate whether FLIP expression was associated with ATO-induced autophagy, K562, Jurkat, MCF-7 and MGC803 cells were treated with ATO. Results showed that the levels of FLIPs, pro-

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC50(24h)[μM]</th>
<th>IC50(48h)[μM]</th>
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</thead>
<tbody>
<tr>
<td>K562</td>
<td>4.59 ± 1.53</td>
<td>5.92 ± 1.39</td>
</tr>
<tr>
<td>Jurkat</td>
<td>5.92 ± 1.39</td>
<td>3.68 ± 1.01</td>
</tr>
<tr>
<td>MCF-7</td>
<td>49.76 ± 8.34</td>
<td>20.48 ± 5.66</td>
</tr>
<tr>
<td>MGC803</td>
<td>59.33 ± 7.90</td>
<td>26.91 ± 4.43</td>
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tein were down-regulated in a time-dependent manner in all cell lines, but the levels of FLIP in solid tumor cells were not consistent with leukemia cells (Fig. 2A). We therefore investigated the effect of FLIPL on autophagy. To assess the effect of FLIPL on autophagy, FLIPL-targeted siRNA was transfected in MGC803 cells. Suppression of FLIPL induced significant levels of PARP cleavage and LC3-II expression (Fig. 2B), indicating activation of apoptosis and autophagy. Furthermore, specific down-regulation of FLIPL enhanced ATO-induced autophagy in MGC803 cells as indicated by the up-regulation of LC3-II (Fig. 2C). The result was further confirmed by transmission electron microscopy (Supplementary Fig. S2). However, ATO-induced autophagy in MGC803 cells remained no changes in down-regulation of FLIPS (Fig. 2D), suggest that FLIPL plays more important role in the regulation of ATO-induced autophagy.

3.3. Degradation of FLIPL protein by ATO is mediated through the ubiquitin–proteasome pathway

To explore whether FLIPL was regulated by the ubiquitin–proteasome pathway, the proteasome inhibitor PS341 was used to inhibit the function of the proteasomal pathway. In ATO-treated K562 cells, pretreatment with PS341 could effectively prevent FLIPL degradation (Fig. 3A left). A similar effect was observed in MGC803 cells (Fig. 3A right). Because phosphorylation of tyrosine residues could serve as a signal for ubiquitination [27], phosphorylation of FLIPL was examined in ATO-treated K562 and MGC803 cells. Compared to the control, the results revealed an increase in phosphorylated FLIPL at 3 h in K562 cells (Fig. 3B left) or at 1 h in MGC803 cells (Fig. 3B right). Next, ATO-induced ubiquitination of the FLIPL protein was investigated. Consistent with the fact that ubiquitination precedes proteasomal degradation, polyubiquitinat-ed FLIPL was evident within 3 h in K562 cells after ATO treatment (Fig. 3C left). A similar effect was also obtained in MGC803 cells after treatment of ATO within 1 h (Fig. 3C right). These findings suggested that FLIPL was ubiquitinated during ATO-treated K562 and MGC803 cells.

3.4. Cbl-b is involved in the ubiquitination of FLIPL

To determine whether the E3 ubiquitin ligase Cbl-b was involved in ubiquitination of FLIPL, K562 and MGC803 cells were treated with ATO, and the level of Cbl-b protein was detected. Immunoblotting showed that the expression of Cbl-b increased in a time-dependent manner (Fig. 4A). Furthermore, an interaction between Cbl-b and FLIPL was found in ATO-treated K562 and MGC803 cells (Fig. 4B). To characterize whether FLIPL was ubiquitinated by Cbl-b, previously established MGC803 cells stably transfected with the shRNA plasmid of Cbl-b [24] were used for further experiments (Fig. 4C). Knockdown of Cbl-b significantly inhibited ATO-dependent ubiquitination of FLIPL (Fig. 4D). These results indicated that Cbl-b was required for ATO-induced ubiquitination of FLIPL in K562 and MGC803 cells.

3.5. Cbl-b is involved in ATO-induced human gastric cancer cell autophagy

Next, to characterize the effect of Cbl-b on ATO-induced autophagy, Cbl-b specific shRNA was used to silence Cbl-b expression in MGC803 cells. Specific silenced Cbl-b expression induced the down-regulation of LC3-II levels and the up-regulation of FLIP protein (Fig. 5A). Moreover, shRNA-mediated knockdown of Cbl-b effectively suppressed autophagy induced by ATO or starvation,
as shown by the reduction of LC3-II levels (Fig. 5B and C). Electron microscopy studies also showed a decrease in the number of ATO-induced autophagic vacuoles in Cbl-b silencing MGC803 cells when compared to non-silencing control cells (Supplementary Fig. S3). Furthermore, the inhibition of autophagy induced by Cbl-b depletion was reverted by knockdown of FLIPₗ in Cbl-b silencing MGC803 cells (Fig. 5D). Moreover, siRNA-mediated knockdown of Cbl-b in K562 cells also suppressed ATO-induced autophagy (Fig. 5E). These observations collectively indicated that Cbl-b might enhance ATO-induced autophagy in K562 and MGC803 cells.
4. Discussion

Autophagy is a cellular catabolic degradation response to nutrient starvation or metabolic stress. Previous reports have shown that autophagy is activated by some anti-tumor drugs that induce apoptosis [28]. But, whether autophagy is a death-induced mechanism or a protective effort for cellular survival after chemotherapy is still controversial [29]. As reported recently, ATO induced not only apoptosis but also autophagy in leukemia cells [11]. It is important to understand in detail the signaling pathways involved in this process. ATO was suggested to induce autophagic cell death in acute myelogenous leukemia and malignant glioma cells [11,30]. However, in our observations, ATO was observed to induce protective autophagy and not autophagic cell death in K562 and MGC803 cells. Therefore, the
opposing effects in response to ATO may be dependent on different types of malignant cells. In our study, we showed that treatment with ATO increased both the expression of LC3-I and the expression of LC3-II in K562, Jurkat, MCF-7 and MGCC083 cells. These phenomena have also been seen in the other author’s studies on the effect of ATO [12,31–32]. However, the exact mechanism is unclear.

FLIP is a key inhibitor of death receptor signaling. Micheau et al. [33] reported that FLIP L (but not FLIP S) blocks caspase-8-mediated apoptosis. In our study, FLIP L was strongly down-regulated by ATO in a time-dependent manner in all cell lines. These findings confirmed that down-regulation of FLIP L by ATO is not a cell line specific effect. Several studies reported that down-regulation of FLIP L could induce spontaneous apoptosis [34–35]. Our results have shown for the first time that FLIP L-targeted siRNA induced not only spontaneous apoptosis but also spontaneous autophagy in MGCC083 cells. Lee et al. [15] demonstrated that in NIH3T3 cells, FLIP L suppressed autophagy by regulating the autophagy signal transduction pathway in autophagosome elongation. Our results also found that specific down-regulation of FLIP L significantly enhanced ATO-induced autophagy in MGCC083 cell lines. Collectively, these results demonstrate that FLIP L is a critical regulator of both apoptosis and autophagy in ATO-treated K562 and MGCC083 cells.

Data have emerged that post-transcriptional regulation of proteins plays an important role in regulating cell signal pathways. For example, ubiquitination of FLIP L by the ubiquitin E3 ligase Itch has been reported to control TNF-induced cell death [36]. TNF-dependent ubiquitination of FLIP L is regulated by c-Cbl in Mycobacterium tuberculosis-induced macrophage apoptosis [21]. C-Cbl is the second member of the Cbl family of proteins, and is essential for the negative regulation of T-cell activation, growth factor receptor and non-receptor-type tyrosine kinase signaling [37–39]. Here we demonstrated that ATO treatment led to up-regulation of C-Cbl and degradation of FLIP L proteins in both K562 and MGCC083 cells. Further experiments indicated that C-Cbl interacted with FLIP L in the regulation of FLIP L turnover. A recent study demonstrated that Autophagic regulation of Src was mediated by c-Cbl when the Src/FAK pathway is disrupted [22]. In our study, ATO-dependent activation of autophagy was considerably blocked by knockdown of C-Cbl, which was mediated via regulating degradation of FLIP L. This study is the first to report that C-Cbl is essential for ATO-induced autophagy in K562 and MGCC083 cells. Taken together, the present observations indicate that ATO-induced autophagy in K562 and MGCC083 cells is mediated through proteasomal degradation of FLIP L. This is the first study to show that FLIP L is involved in ATO-induced protective autophagy. Furthermore, the E3 ubiquitin ligase C-Cbl is a key participant of autophagy induced by ATO, which facilitates FLIP L degradation. These observations collectively suggest that FLIP L is a critical regulator of ATO-induced autophagy in K562 and MGCC083 cells, and that a combination of ATO and FLIP L targeted agents may have considerable therapeutic benefit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.07.067.

References