Involvement of mitochondrial dynamics in the antineoplastic activity of cisplatin in murine leukemia L1210 cells

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Abstract. Leukemia is a type of hematopoietic stem cell malignant cloned disease with high mortality. Cisplatin-based chemotherapy is one of the most common treatments for leukemia. Similar to other chemotherapeutic agents, cisplatin resistance has become a serious issue in cancer therapy. In the present study, we investigated the role of mitochondrial dynamics in the antineoplastic activity of cisplatin in murine leukemia L1210 cells. Firstly, the L1210 cell line resistant to cisplatin (L1210/DDP) was established. Compared to its parental cell line, the IC₅₀ value of cisplatin in the L1210/DDP cells was increased 10-fold. Mitofusins (Mfn1 and Mfn2), mitochondrial outer membrane fusion proteins, were markedly upregulated in the L1210/DDP cells, whereas the expression of fission protein Drp1 and inner membrane fusion protein OPA1 were not significantly altered. In addition, mitofusins were also upregulated in the parental L1210 cells subjected to cisplatin stress. To investigate the role of mitochondrial dynamics in the antineoplastic activity of cisplatin, the effect of mitochondrial division inhibitor (Mdivi)-1 on cisplatin-induced cell death, caspase-3 cleavage and ROS production was examined in L1210 cells. We found that 5 µM of Mdivi-1 efficiently attenuated cisplatin-induced cell death, caspase activation and intracellular ROS increase in L1210 cells. Our data indicated that mitochondrial dynamics play an important role in the antineoplastic activity of cisplatin, and mitofusin-mediated mitochondrial fusion may be involved in the process of cisplatin resistance in leukemia cells. Therefore, the present study revealed that mitochondrial dynamics may be a potential target used to improve the antineoplastic activity of cisplatin in leukemia in the future.

Introduction

Leukemia is also known as blood cancer and has a high mortality rate. It is pathologically characterized by the massive proliferation of abnormal hematopoietic stem cells (1,2). Although the precise pathogenesis of leukemia remains to be elucidated, various genetic mutations occurring in response to factors including genetic factors, immune deficiency, toxic agents and the leukosis virus in the process of leukocyte differentiation have been widely accepted as the main causes of leukemia (3). Genetic mutations may lead to the unlimited proliferation of leukemia cells in bone marrow and other hematopoietic tissue (4). As a result, the development of mature blood cells is disrupted. Due to the high mortality rate, leukemia is considered as a malignant cancer which seriously threatens human health. It has also been reported that the incidence rate of leukemia is relatively high in the elderly population and children in China (5). At present, the main treatment methods for leukemia are combined chemotherapy and marrow transplantation. However, the high expense, donor deficiency and immunological rejection are important issues in marrow transplantation (6-8). Thus, combined chemotherapy is the most important treatment for leukemia. However, among conventional chemotherapeutic agents, cisplatin is also widely used in combined chemotherapy for leukemia (9). Unfortunately, evidence has shown that with treatment more and more cancer cells become resistant to cisplatin (10). Therefore, it is urgent to further investigate the precise mechanism of cisplatin resistance.

Platinum-based drugs are widely used in the treatment of cancer such as leukemia, lymphomas, melanoma, head-neck cancer, bladder cancer and gynecological tumors (11). Cisplatin is one of the first platinum-based drugs discovered in the 1960s (12). Cisplatin interacts with DNA double strands by forming interstrand and intrastrand adducts, thereby inducing apoptosis in cancer cells through the interference with DNA
Mitochondria are also highly dynamic organelles and move through the cell with frequent fission and fusion events (19). Various highly conserved dynamin-related GTPases are identified as the mediators of mitochondrial dynamics. Dynamin-related protein 1 (Drp1) is involved in the process of mitochondrial fission, while mitofusin 1/2 (Mfn1/2) and OPA1 are required for mitochondrial outer or inner membrane fusion in mammalian cells, respectively (20). In addition, recent studies have suggested the involvement of mitochondrial dynamics in the acquired cisplatin resistance or sensitivity in several cancer cell lines (21,22). It has been reported that OPA1-mediated mitochondrial fusion is potentially responsible for cisplatin-induced resistance in neuroblastoma B50 rat cells (21). By contrast, Drp1-dependent mitochondrial fission was found to regulate piceatannol-induced cisplatin sensitivity in ovarian cancer (22). Therefore, it is of interest to investigate the role of mitochondrial dynamics in the antineoplastic activity of cisplatin in leukemia cells.

In the present study, we established the L1210/DDP cell line, and found that the IC$_{50}$ value of cisplatin in the L1210/DDP cells was increased 10-fold. In addition, mitochondrial outer membrane fusion proteins, Mfn1 and Mfn2 were upregulated in L1210/DDP cells. In addition, mitofusins were also upregulated in the parental L1210 cells subjected to cisplatin stress. The Drp1 inhibitor, Mdivi-1, efficiently attenuated cisplatin-induced cell death, caspase activation and intracellular ROS increase in L1210 cells. Our data indicate that mitofusins and Drp1-mediated mitochondrial dynamics may be involved in the antineoplastic activity of cisplatin in L1210 cells, and suggest that mitochondria may be potential targets used to improve the clinical outcomes of leukemia in the future.

Materials and methods

Cell culture. Leukemia cell line L1210 was obtained from the China Center for Type Culture Collection (CCTCC; Wuhan, China). The L1210/DDP cell line was generated according to the dose-escalation strategy as previously described (23,24). In brief, the parental L1210 cells were treated with cisplatin at an IC$_{50}$ concentration, and the surviving cells were cloned in soft agar. A clone was selected and cultured in medium supplemented with a first dose of 0.4 mg/l of cisplatin (Sigma-Aldrich, St. Louis, MO, USA). The dose of cisplatin in the medium for the L1210 cells was increased 50% at every time-point of subculture. The surviving cells followed by six subcultures were cloned again in soft agar. A clone was selected as the cisplatin-resistant cell line for culture in the medium. The cells were grown in suspension in medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; TransGen Biotech, Beijing, China) and 1% penicillin/streptomycin (P/S) (Solarbio, Beijing, China), and maintained in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with an atmosphere containing 5% CO$_2$.

Cell viability assay. Cell viability was determined by trypan blue exclusion assay as previously described (25). In brief, L1210 and L1210/DDP cells were firstly seeded at 5x10$^5$ in 24-well plates and maintained in suspension culture. At 72 h after incubation with DDP or Mdivi-1 (Sigma-Aldrich) + DDP, samples were centrifuged at 1,000 x g for 5 min at 25°C. The cells were stained with 0.04% trypan blue (Sigma-Aldrich) after being washed with phosphate-buffered saline (PBS). The number of dead cells (blue) and viable cells (uncolored) were counted using a hemacytometer. The ratio of the number of dead cells/all counted cells represented the percentage of cell death. The IC$_{50}$ value of cisplatin to L1210 or L1210/DDP was determined in the same way.

Western blot analysis. L1210 and L1210/DDP cells were harvested and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio) according to the manufacturer's instructions. Whole cell lysates were mixed with an equal volume of 2X loading buffer (25% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.01% bromophenol blue and 1 M Tris-HCl), sonicated, boiled for 5 min and stored at -20°C prior to use. The cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBST) buffer for 1 h at room temperature, and then, immunoblotted for 2 h at room temperature with the following primary antibodies: rabbit anti-Drp1, Mfn2 and caspase-3 (1:1,000; Cell Signaling, Boston, MA, USA), rabbit anti-Mfn1 and OPA-1 (1:1,000; Abcam, Cambridge, UK), and rabbit anti-GAPDH (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After three washes with TBST, the membranes were further incubated with an HRP-conjugated goat anti-rabbit secondary antibody (1:2,000; TransGen Biotech) for 2 h at room temperature. A chemiluminescence assay was carried out with Amersham ECL Prime Western Blotting Detection reagents (CWBIO, Beijing, China), and the immunoblotting signal was detected using Molecular Imager ChemiDoc™ XRS+ system (Bio-Rad, Hercules, CA, USA).

Annexin V-FITC/PI apoptosis assay. Subsequent to the indicated treatments, the cells were harvested from each group for the apoptosis assay using Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI)
Table I. Cisplatin cytotoxicity in the L1210 cells.

<table>
<thead>
<tr>
<th>Cisplatin concentration in the L1210 cells (mg/l)</th>
<th>Percentage of cell death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.43</td>
</tr>
<tr>
<td>0.025</td>
<td>23.77</td>
</tr>
<tr>
<td>0.05</td>
<td>27.46</td>
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<tr>
<td>0.1</td>
<td>28.18</td>
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<tr>
<td>0.2</td>
<td>30.70</td>
</tr>
<tr>
<td>0.4</td>
<td>39.53</td>
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<tr>
<td>0.8</td>
<td>49.28</td>
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<tr>
<td>1.2</td>
<td>53.23</td>
</tr>
<tr>
<td>1.6</td>
<td>65.30</td>
</tr>
<tr>
<td>2</td>
<td>73.25</td>
</tr>
<tr>
<td>3.2</td>
<td>86.78</td>
</tr>
<tr>
<td>6.4</td>
<td>90.57</td>
</tr>
</tbody>
</table>

L1210 cells were exposed to different concentrations of cisplatin. At 72 h after cisplatin treatment, the cell viability was examined by trypan blue exclusion assay, and the percentage of cell death was calculated.

Table II. Cisplatin cytotoxicity in the L1210/DDP cells.

<table>
<thead>
<tr>
<th>Cisplatin concentration in the L1210/DDP cells (mg/l)</th>
<th>Percentage of cell death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>0.25</td>
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<tr>
<td>0.5</td>
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<tr>
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<td>32</td>
<td>60.74</td>
</tr>
<tr>
<td>64</td>
<td>93.85</td>
</tr>
</tbody>
</table>

L1210/DDP cells were exposed to different concentrations of cisplatin. At 72 h after cisplatin treatment, the cell viability was examined by trypan blue exclusion assay, and the percentage of cell death was calculated.

Detection of the intracellular ROS level. To examine the role of Drp1-dependent mitochondrial fission in intracellular ROS production, cells were pretreated with 5 µM Mdivi-1 for 2 h prior to CDDP treatment. At 72 h after cisplatin treatment, the cells were incubated with 10 µM of the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) for 30 min at 37°C in the dark. After incubation, the cells were washed twice with PBS and harvested. The fluorescence intensity was assessed using flow cytometry with the excitation source at 488 nm and the emission wavelength at 525 nm, thus detecting the intracellular ROS. Data analysis was carried out using inCyte software (both from Guava, Millipore Corp.).

Statistical analysis. The quantitative data are displayed as the mean ± SD. Data were analyzed using either Student's t-test to compare two conditions or ANOVA followed by planned comparisons of multiple conditions, and p<0.05 was considered to indicate a statistically significant result.

Results

Establishment of the L1210/DDP cell line and confirmation of its cisplatin resistance. In order to investigate the
mechanism of cisplatin resistance, we successfully generated the L1210/DDP cell line according to a dose-escalation strategy (23,24). The parental cells were grown in suspension in RPMI-1640 medium supplemented with 10% FBS and 1% P/S, whereas the L1210/DDP cells were maintained in medium containing 4 mg/l of cisplatin to retain their resistance. The cisplatin resistance of the L1210/DDP cells was determined by trypan blue exclusion assay. As shown in Fig. 1A and Table I, the parental L1210 cells were sensitive to 0.8-6.4 mg/l of cisplatin. In contrast, the L1210/DDP cells were sensitive to 4-64 mg/l of cisplatin (Fig. 1B and Table II). In addition, the dose-response curves of the two cell lines to cisplatin were generated (Fig. 1A and B), and the IC_{50} values for cisplatin were also calculated as 0.795 and 8.131 mg/l in the L1210 and L1210/DDP cell lines, respectively. Compared to the parental cell line, the IC_{50} value for cisplatin in the L1210/DDP cells was increased 10-fold.

Expression of mitochondrial dynamic-related proteins in L1210 and L1210/DDP cells. It has been reported that mitochondrial dynamics are involved in acquired cisplatin resistance (7). To investigate the possible role of mitochondrial dynamics in the cisplatin resistance of leukemia cells, the expression level of mitochondrial dynamic-related proteins was examined in the two cell lines using western blot assay. Notably, we found that both Mfn1 and Mfn2 were upregulated in L1210/DDP cells. However, there was no significant difference in the expression level of Drp1 and OPA1 in the two cell lines (Fig. 2). Since both Mfn1 and Mfn2 are important components required for mitochondrial outer membrane fusion, the results revealed the possibility that mitofusin-mediated mitochondrial fusion may contribute to the mechanism of cisplatin resistance in leukemia cells.

Effect of cisplatin stress on mitochondrial dynamic-related protein expression in the parental L1210 cells. Although mitofusins were upregulated in the L1210/DDP cells, direct evidence that mitofusin-mediated mitochondrial fusion leads to the development of cisplatin resistance in leukemia cells remains unclear. Therefore, to further confirm the involvement of mitofusins in the development of cisplatin resistance in leukemia cells, cisplatin stress at 0.4 mg/l (the concentration below the IC_{50} value) was introduced to the parental L1210 cells. The expression of mitochondrial dynamic-related proteins in cells was examined at 0, 4, 6 and 8 days after treatment with 0.4 mg/l of cisplatin. As shown in Fig. 3, it was found that both Mfn1 and Mfn2 were obviously upregulated during the period of cisplatin stress, whereas the expression of OPA1 was not significantly altered. In contrast, Drp1 was downregulated at 8 days after cisplatin stress. Although there were a few differences in the expression patterns of mitochondrial dynamic-related proteins, the results revealed that the shift of mitochondrial dynamics to fusion may contribute to the development of cisplatin resistance in L1210 cells.

Drp1 inhibitor Mdivi-1 efficiently attenuates cisplatin-induced cell death in L1210 cells. The aforementioned data revealed that mitochondrial fusion may contribute to the antineoplastic activity of cisplatin in leukemia cells. To further investigate the role of mitochondrial dynamics in the sensitivity of leukemia cells to cisplatin, we used different concentrations of Mdivi-1, a Drp1 inhibitor, to decrease Drp1-dependent mitochondrial
fission, and examined the effect of Mdivi-1 on cisplatin-induced cell death in L1210 cells. In the trypan blue exclusion assay, L1210 cells were divided into five groups: control, DDP, 2.5 µM Mdivi-1 + DDP, 5 µM Mdivi-1 + DDP and 10 µM Mdivi-1 + DDP. The representative image in each group showed the cell death (in blue) after cisplatin exposure. (B) Effect of Mdivi-1 on cisplatin cytotoxicity in L1210 cells. The ratio of the number of dead cells/all counted cells represents the percentage of cell death in each group. Data are presented as the mean ± SD of at least three independent experiments; *p<0.01.

Effects of Mdivi-1 on cisplatin-induced ROS production and caspase activation in L1210 cells. It has been reported that cisplatin induces cancer cell death through the promotion of intracellular ROS production and caspase activation (26,27). The aforementioned data revealed that mitofusins were upregulated in L1210/DDP cells, and that the Drp1 inhibitor, Mdivi-1 attenuated cisplatin-induced L1210 cell death. It was necessary to further investigate the mechanism of mitochondrial dynamics in cisplatin-induced cell death in L1210 cells. Thus, the effect of Mdivi-1 on cisplatin-induced ROS production and caspase activation was examined in L1210 cells. Consistent with previous studies (26,27), 4 mg/l of cisplatin significantly increased the intracellular ROS level in L1210 cells. Pretreatment with 5 µM of Mdivi-1 significantly attenuated cisplatin-induced intracellular ROS production in L1210 cells, although it did not completely block the increment of ROS (Fig. 6). Moreover, the results of western blotting revealed that 4 mg/l of cisplatin markedly stimulated the cleavage of caspase-3. Pretreatment with 5 µM of Mdivi-1 attenuated the cleavage of caspase-3 in L1210 cells (Fig. 7). These data demonstrated that mitochondrial dynamics may be involved in cisplatin-induced L1210 cell death through the regulation of intracellular ROS production and the caspase pathway.

Discussion
Leukemia is the most common hematopoietic malignancy. The development and progression of leukemia is a multifactorial and multi-step process that involves genetic and
epigenetic changes (28). Leukemia is also one of the most common malignant tumors, particularly in children. At present, combined chemotherapy is the major treatment method for leukemia, and cisplatin is one of the most common agents used for combined chemotherapy in clinical treatment. Cisplatin was also the first platinum-based drug to obtain approval of the US Food and Drug Administration (29). The antineoplastic effects of cisplatin have been linked to its ability to crosslink with DNA, then interfere with DNA repair mechanisms, cause DNA damage and eventually lead to apoptosis in cancer cells (14,24). In contrast, mitochondria are also important targets of cisplatin. Cisplatin can induce the decline of ATP enzyme activity and activate the endogenous apoptosis pathway (26). Cisplatin is currently applied for the clinical management of patients suffering from leukemia, testicular, ovarian, head and neck, colorectal and lung cancers (11,30-32). However, the resistance of cancer cells to cisplatin has become a serious problem, and greatly limits its therapeutic effect in clinical treatment.

In the present study, the role of mitochondrial dynamics in cisplatin resistance of leukemia L1210 cells was investigated. Firstly, we established the L1210/DDP cell line using the dose-escalation strategy. The tolerance of L1210/DDP cells to cisplatin was increased 10-fold, compared to the parental cells (Fig. 1). Notably, mitofusins Mfn1 and Mfn2 were obviously upregulated in L1210/DDP cells (Fig. 2). Mfn1 and Mfn2 are vital components required for mitochondrial outer membrane fusion (33). In addition, it has been reported that mitofusins are involved in apoptosis by interacting with Bak and Bax (34-36). To examine the role of mitofusins in the development of cisplatin in L1210 cells, the expression of mitochondrial dynamic-related proteins was detected after cisplatin stress. Similarly, Mfn1 and Mfn2 were upregulated in L1210 cells subjected to cisplatin stress (Fig. 3). The results revealed the upregulation of mitofusins not as an effect, but as a cause in the development of cisplatin resistance. To clarify the role of mitofusin-mediated mitochondrial fusion in cisplatin resistance, it is necessary to knockdown mitofusins in the L1210/DDP cells or overexpress mitofusins in parental L1210 cells, and examine the antineoplastic effects of cisplatin in these cells. Unfortunately, we failed to transfect any siRNAs or plasmids into L1210 cells, and failed to infect L1210 cells with a lentivirus or adenovirus (data not shown). Thus, it is impossible to knockdown or overexpress mitofusins in L1210 cells using gene manipulation technology.

The balance between mitochondrial fission and fusion is important to maintain mitochondrial morphology. Thus, we disrupted the dynamic balance using chemical agents, to further investigate the mechanism of mitochondrial dynamics in the development of cisplatin resistance. It has been well documented that Mdivi-1 is a potent Drp1 inhibitor, and is most widely used (37,38). Obvious mitochondrial fusion in cells can be observed when Drp1-dependent mitochondrial fission was inhibited by Mdivi-1 (39,40). In the present study, 5 µM of Mdivi-1 efficiently attenuated cisplatin-induced cell death, intracellular ROS production and caspase activation
in L1210 cells. These results demonstrated that mitochondrial dynamics play an important role in the antineoplastic activity of cisplatin in leukemia cells. However, there are some deficiencies that warrant improvement in the present study. First, it is necessary to observe and compare mitochondrial morphology in L1210 and L1210/DDP cells, since mitofusins are upregulated in L1210/DDP cells. The two common methods used to label transfected mitochondria are Mito-DSRed and MitoTracker staining (41,42). As L1210 cells are difficult to be transfected, we stained cells with MitoTracker, and found that mitochondria were clustered around the perinuclear region in the two cell lines. The two cell lines cultured in suspension appear as small spheres. As a result, it was difficult to distinguish the morphology of clustered mitochondria in the two cell lines (data not shown). Therefore, it may be interesting to further investigate the role of mitochondrial dynamics in the development of cisplatin resistance in other adherent cancer cells with high transfection efficiency.

In conclusion, our data revealed that mitofusins Mfn1 and Mfn2 were upregulated in leukemia L1210 cells resistant to cisplatin. In addition, cisplatin stress increased the expression of mitofusins in parental L1210 cells. Inhibition of Drp1-dependent mitochondrial fission by Mdivi-1 efficiently attenuated cisplatin-induced cell death, intracellular ROS production and the activation of the caspase pathway in L1210 cells. These results demonstrate that mitochondrial dynamics play an important role in the antineoplastic effects of cisplatin in leukemia cells. Thus, targeting mitochondrial dynamics may provide a novel strategy in order to improve the chemotherapeutic effect of cisplatin in the future.

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References