Resistance to Cisplatin in NSCLC is Related to Autophagy Induced by PI3K/AKT/mTOR Signaling Pathway Inhibition

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Abstract

Background: Cisplatin is among the most widely used compound in chemotherapy for the treatment of many cancer types, including non-small cell lung cancer. Many patients with NSCLC relapse after cisplatin treatment because of resistance to this compound but the molecular mechanisms underlying cisplatin-resistance is still unknown.

Materials and Methods: A549 cell lines were used and their molecular response to cisplatin treatment and in combinatory treatment with 3MA, LY294002 or TUDCA was explored through transmission electron microscopy, Western blotting and RT-qPCR.

Results: Cisplatin induced endoplasmic reticulum stress and autophagy but inhibited PI3K/AKT/mTOR signaling pathway.

Conclusion: These results show that cisplatin induces ERS response but inhibits the PI3K/AKT/mTOR signaling pathway inducing by the way autophagy that finally reduces the signal cascade of cell death.

Keywords: Non-small cell lung cancer; Cisplatin; Autophagy; Cisplatin resistance; PI3K/AKT/mTOR pathway

Introduction

Non-Small Cells Lung Cancer (NSCLC) is a fast growing and highly metastatic carcinoma arising in the lung’s epithelia. It is the most prevalent malignant disease affecting human lungs and counts for more than 12.4% of total new cancer cases each year in the world [1,2]. This cancer shows a particular insensibility to chemotherapy and radiotherapy rendering surgery the most effective approach in its treatment so that chemicals are used mostly as either neo-adjuvant or adjuvant chemotherapeutic agent [3,4]. Among the compounds used in NSCLC treatment regimen is cisplatin (cis-diaminedichloroplatinum II) which is also used in the treatment of many other cancer types because of its high anti-proliferative activity [5].

The cytotoxic effect of cisplatin is believed to result from the chemical’s ability to bind DNA and induce DNA damages that could lead to cellular death or to induce special cell death signals such as mitochondrial or Endoplasmic Reticulum Stress (ERS) responses or autophagy [6-8]. Despite its efficacy, the use of cisplatin is hindered by the occurrence of resistance that in turn leads to cancer relapse and reductions in the patient’s survival rate [9]. Thus far, for patients with advanced NSCLC and unsuitable for surgery, cisplatin is recommended to be used in combinatory therapy regimen with other compounds that enhance cisplatin cytotoxicity or restore cancer cells’ sensitivity to cisplatin [10,11].

Molecular mechanisms underlying this resistance are not well understood but it has been shown elsewhere that autophagy, in certain circumstances, can also promote cell proliferation by degrading damaged organelles and dysfunctional macromolecules that could otherwise induce cellular death [12,13]. In the special context of NSCLC, it revealed that autophagy could play an important role in the cancer resistance to cisplatin treatment in vitro [14].

The hypothesis in this study was that resistance to cisplatin in NSCLC arises from the interactions...
between this compound and cellular metabolic factors involved in autophagy. The main objective was then to study the link between exposition to cisplatin and autophagy occurrence and to identify the molecular elements involved in this resistance.

**Materials and Methods**

**Cell cultures and drug treatment**

The NSCLC cell line A549 was used in this study. Cells were cultured in Roswell Park Memorial Institute 1640/Keaigh’s Modification of Ham’s F-12 Medium (RPMI 1640/F-12K) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), penicillin (100 U/ml), streptomycin (100 U/ml), and streptomycin (100 U/ml). Cell cultures were incubated at 37°C in a 5% CO₂ incubator with humidified atmosphere.

The effect of cisplatin on cells was measured by seeding cells in logarithmic growth phase in 96 well plates (5000 cells/well). The culture medium was added to plates with cisplatin at varying concentration (0.50 µg/ml) for a final volume of 200 µl and incubated in 37°C incubator for 24 or 48 h. Further experiments with PI3K inhibitors 3-methyladenine (3-MA), LY294002 and Tauroursodeoxycholic Acid (TUDCA) were also performed in combination with cisplatin. These treatment cultures were realized in triplicate for each concentration of cisplatin and incubated at 37°C in a 5% CO₂ incubator with humidified atmosphere.

**Cell viability assays**

Cell viability was analyzed using the MTT assay. A549 cells were collected with trypsin, suspended in a final concentration of 10⁵ cells/ml and cultures in 96 well plates (200 µl/well). At the end of culture incubations, 20 µl MTT (5 mg/ml; Bi-yun Tian Biology Company) was added to wells and kept for 4 h in the dark before adding 150 μl DMSO to dissolve formazan crystals. Absorbance at 570 nm was measured using a plate reader spectrophotometer. The relative cell viability (%) was expressed as a percentage relative to that seen in control cells at all time points studied. All experiments were performed in triplicate.

**Transmission electron microscope**

A549 cells were trypsinized, washed with PBS and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH=7.2) overnight at 4°C. Next day, the cells were washed three times with 0.1 M phosphate buffer before fixation in 1% aqueous osmium. The preparation was then dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90% and 100%) and embedded in araldite. The ultrathin sections were prepared with a microtome and mounted on copper grids. The samples were stained with 2% aqueous uranyl acetate and lead citrate. The transmission electron microscope was used to observe dyed samples.

**Western blot assay**

After cisplatin (15 µg/ml) treatment with or without other compounds, cells were trypsinized and washed twice with cold PBS and suspended in 120 µl of RIPA buffer. Cell lysates were sonicated for 5 sec on ice and then static at 4°C for 45 min. After centrifuging at 3000 g for 15 min, protein concentrations were measured using the BCA protein assay (Skylight Bioengineering Co., Ltd.). Samples of extracted proteins were boiled for 10 min at 100°C, and equivalent amounts of proteins (30 µg to 90 µg) were separated on 12% SDS-polyacrylamide gel electrophoresis (Shanghai Yu Sen Biotech Co., Ltd.). Protein bands were then transferred onto membranes and blocked with 5% (w/v) skimmed milk for one hour at room temperature. Membranes were then treated overnight at 4°C with specific primary antibodies (anti-GRP78, LC3, Beclin 1, p-mTOR, p-AKT, P70, p-P70, PERK and IRE1). Transfer membranes were washed three times with TBST before incubation with horseradish peroxidase conjugated secondary antibodies (1:2,000, Sigma) for 1 h at room temperature. Membranes were washed three times with TBST before taking photographs using a chemiluminescence imaging system.

**Flow cytometry**

After treatment, cells were harvested and washed twice with PBS. Cell death was determined using propidium iodide (PI, 1 µg/ml) and Annexin V-FITC (1 µg/ml; Invitrogen). After 15 min incubation at 37°C, cells were detected using a FACScan flow cytometer (BD Biosciences FACSCalibur).

**Real-time PCR**

After appropriate treatment and culture, cells were harvested and total RNA extraction was performed using Trizol RNA extraction Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s recommendations. The extracted RNA was purified using ethanol and stored in RNase-free water. This RNA was used to synthesize cDNAs using PrimeScript™ RT reagent Kit (TAKARA) according to instructions in the manual. Briefly, purified RNA (5 µl of 200 ng/µl solution) was mixed in 0.2 ml RNase free tubes with 4 μl 5X PrimeScirp buffer; 1 µl PrimeScirp RT Enzyme; 1 µL Oligo dt Primer (50 µM); 1 µl Random 6 mers (100 µM); 8 µl RNase free water. The mixture was kept 15 min at 37°C, 5 s at 85°C and 60 min at 4°C.

The obtained cDNA was then subjected to real-time PCR using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TAKARA). For each targeted gene, the reaction mixture was composed of 10 µl SYBR premix ex Taq; 0.4 µl 50 ROX Reference Dye; 0.4 µl Primer F (10 µM); 0.4 µl Primer R (10 µM); 2 µl cDNA; 6.8 µl distilled deionised Water. Primers sequence and the size of amplified fragment for each gene are presented in Table 1. Reaction tubes are placed in the LightCycler® 96

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**Table 1: Primers used in this study for real time PCR experiments.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’----3’)</th>
<th>Reverse primer (5’----3’)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP78</td>
<td>GAAGCCCGGTCTCCGAAAAGGT</td>
<td>GAATTTGAGTGCGGGCACCACCA</td>
<td>89</td>
</tr>
<tr>
<td>PERK</td>
<td>GGAAAGCGAGAGCGGAGTTTATT</td>
<td>ACTAGTGGCATTATGGCAGCTTC</td>
<td>111</td>
</tr>
<tr>
<td>IRE1</td>
<td>AGAAAGCGAGCGACCTTTGTC</td>
<td>GTTTGCGTGTCAGCATGGGA</td>
<td>111</td>
</tr>
<tr>
<td>mTOR</td>
<td>TCCTAGAGATGATGAGTCAGGAG</td>
<td>CACCTCCACCTCTATAGGCC</td>
<td>141</td>
</tr>
<tr>
<td>P70s6k</td>
<td>AGAACCTTCGTGCTGAAAGGT</td>
<td>CGACAGGTTGTTCAGGTGAA</td>
<td>104</td>
</tr>
<tr>
<td>AKT</td>
<td>TCCTCTCAAGAATGGTGCCA</td>
<td>GTGCGTTGATGAGCTGTTG</td>
<td>181</td>
</tr>
<tr>
<td>p70</td>
<td>TCATCGGCCCTTTGTGTAAGGA</td>
<td>ACATGCACCTCGGTTTTGGAT</td>
<td>97</td>
</tr>
</tbody>
</table>
(Roche) for quantitative amplification. The amplification reaction was performed with an initial denaturation at 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec. Relative levels of RNA expression were assessed using the 2-ΔΔCt method with GAPDH as an internal control.

**Cell migration assay**

Cells (5.10⁴ cells/ml) in the logarithmic growth phase were seeded in the upper Transwell chamber with serum-free medium. The lower chamber was filled with 600 µl of medium supplemented with 20% serum. After 48 h of incubation at 37°C, remaining cells in the upper chamber were mechanically removed, and those that migrated onto the lower side were fixed, dyed, photographed and counted. The experiment was repeated three times. In each experiment more than five fields of view were counted.

**Statistical analysis**

For statistical analysis, all data obtained were analyzed using Prism 6 for Windows software (GraphPad Software, San Diego, USA). Statistically significant differences between studied groups were evaluated using the unpaired Student’s t-test and Fisher’s exact test. P values less than 0.05 were considered statistically significant.

**Results**

**Cisplatin inhibited A549 cell proliferation**

The effect of cisplatin on A549 cells viability was evaluated by the MTT assay after treatment with varying concentration of the drug for increasing incubation times. As shown in Figure 1A, the cells survival rate decreased in a dose dependent manner in the three incubation time experiments. In order to evaluate the level of apoptosis in cells treated with cisplatin, flow cytometry was made after staining. Results from flow cytometry examinations are presented in Figure 1B and showed that the level of apoptotic cells increases with drug dose. In cells treated with 30 and 50 µg/ml, the level of necrosis was particularly high. The level of apoptosis was barely observable after 12 h treatment and no significant difference was observed between treatments times 24 h and 48 h. Based on these results, all further experiments were chosen to be performed with 15 µg/ml of cisplatin and an incubation period of 24 h.

**Treatment with cisplatin induced autophagy**

A549 cells were treated with cisplatin (15 µg/ml) added with rapamycin, 3MA, LY294002 or TUDCA and examined with transmission electron microscopy in order to detect autophagic structures (Figure 2). A549 untreated cells did not show any sign of autophagy whereas cisplatin alone induced an observable level of autophagy. Addition of rapamycin significantly increased the level of cisplatin-induced autophagy whereas 3MA and LY294002 inversed the autophagic effect of cisplatin. Inhibition of apoptosis by TUDCA reduced the formation of autophagosomes induced by cisplatin.

Cisplatin and rapamycin decreased the level of p-mTOR and upregulated the expression of LC3 and Beclin 1

Western blot experiments were used to estimate les expression level of proteins GRP78, LC3, Beclin1, p-mTOR, p-AKT, P70, p-P70, PERK and IRE1 after treatments of A549 cells. Results of these experiments are shown in Figure 3. As expected, rapamycin treatment reduced the level of p-mTOR in comparison to control cells and cisplatin treated cells. Meanwhile, the expression level of autophagy related proteins LC3 and Beclin 1 were increased by rapamycin addition to treatment. Rapamycin also inhibited the expression of GFP78 that contributes to apoptosis escape confirming the decrease in apoptosis rate observed by flow cytometry. Treatment with PI3K inhibitors, 3-MA and LY294002 significantly increased the expression level of autophagy-related proteins confirming the results observed by TEM. RT-qPCR experiments were made to evaluate the level of respective mRNAs production. As shown in Figure 5, the expression profile of mRNAs was comparable to the protein expression profile observed with western blot experiments.
Cisplatin and rapamycin increased the rate of cell migration

Transwell experiments were used to determine the effect of different chemicals on A549 cells migration. In comparison to control cells, cisplatin treatment for 24 h significantly decreased the migration rate (Figure 5). Addition of rapamycin to cisplatin treatment increased cells migration but did not completely restore the migration rate observed for control cells. Addition of TUDCA to cisplatin slightly inversed the decrease in cell migration rate induced by cisplatin. Addition of 3-MA and LY294002 markedly enhanced the cisplatin-induced decrease in the migration rate.

Discussion

The cytotoxic effect of cisplatin has been exploited in the fight against cancer for many decades and since its first approval by the American Food and Drug Administration for cancer treatment, it has been effectively used for the treatment of many cancer types including lung cancers [15]. Several studies investigated the molecular mechanism behind its cytotoxicity and cisplatin seems to interfere with a number of cellular signaling pathways perturbing the normal behavior of cells. In fact, cisplatin was shown to bind to genomic DNA leading to DNA damages that induce apoptosis cascade of reactions [16]. Cisplatin also induces other damages that end up to cell death through mitochondrial stress or endoplasmic reticulum stress [8,17]. The first part of the present study effectively showed the cytotoxic effect of cisplatin even at low concentrations.

Electron microscopic observations confirmed the fact that cisplatin induces the formation of autophagosomes as shown in several previous studies [6]. In order to further study the induction of autophagy, the effect of various compounds on the cisplatin-induced autophagy was explored. Rapamycin, significantly enhanced the effect of cisplatin on autophagosome induction showing the involvement of mTOR in the cisplatin-induced autophagy. Additional experiments with mTOR upstream elements, PI3K inhibitors LY294002 and 3-MA inversed the autophagic action of cisplatin. These results suggest that cisplatin-induced autophagy is regulated through the cellular PI3K/AKT/mTOR signaling pathway which is central to many metabolic processes including endoplasmic reticulum stress response, apoptosis and cell proliferation [18,19]. It was previously shown that ERS usually occurs in cancer cells and their survival is due to processes modulated by the PI3K/AKT/mTOR signaling pathway such as the Unfolded Protein Response (UPR) and autophagy that restore homeostasis [20,21]. It makes sense to consider that cisplatin induced in NSCLC cells an ERS which was followed by autophagy related to the PI3K/AKT/mTOR signaling elements as shown elsewhere [14,22]. It is important to mention that autophagy being induced by a cell stress state could result in rescuing the cell from death.

Based on the suspicion that cisplatin interacts with the PI3K/AKT/mTOR signaling pathway inducing autophagy that in turn protects cancer cells from cisplatin-induced cytotoxicity, western blotting experiments and RT-qPCR experiment were made to study the expression pattern of the signaling pathway proteins as well as the proteins involved in autophagy. Results showed that cisplatin significantly increases the expression level of autophagy related proteins (LC3 and Beclin1) and ERS markers (PERK and IRE1). It therefore decreases the expression of antiapoptotic protein P70, and...
Figure 4: Level of mRNA expression (A-G). GFP78 (A), mTOR (B), AKT (C), P70 (D), P70S6K (E), PERK (F) and IRE1 (G) mRNA expression levels were estimated by RT-qPCR in A549 cells (control) or cells treated with cisplatin alone or in combination with rapamycin, 3-MA, LY294002 or TUDCA. One-way ANOVA was used as statistical analysis approach. *P<0.05, **P<0.01; ***P<0.001; ****P<0.0001 and ns = no significance when compared to cisplatin treatment group. #P<0.05, ##P<0.0001 and ns ctrl = no significance when compared to untreated control cells.

the activated PI3K downstream element p-AKT. It also decreases the level of phosphorylated mTOR. Rapamycin enhanced the effect of cisplatin in comparison to normal cells whereas 3-MA and LY294002 inverted it. Frankly speaking, there were no animal experiments, but it was still an inspiring scientific research for the future.

Altogether, these results showed that cisplatin induces ERS response but concomitantly inhibits the PI3K/AKT/mTOR signaling pathway. This inhibitory effect induces autophagic reactions that finally reduce the signal cascade for apoptosis.

Acknowledgement

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References

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