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Cisplatin treatment leads to changes in nuclear protein and microRNA expression

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ABSTRACT

Using a proteomic approach, we have previously shown that exposure to different concentrations of cisplatin during a 12-h period can lead to changes in nuclear protein expression and alternative splicing in HeLa cells. To further shed light on the DNA damage response (DDR) induced by cisplatin, we examined the nuclear proteome profiles of HeLa cells treated with 5 μM cisplatin for different times (2, 12, and 24 h). Two-dimensional electrophoresis (2-DE) identified 98 differentially expressed proteins in cisplatin-treated cells as compared to control cells. Among them, 54 spots (55%) were down-regulated and 44 spots (45%) were up-regulated. 51 spots were subjected to Matrix-assisted-laser-desorption-ionization Time-of-flight/time-of-flight Mass spectrometry (MALDI-TOF/TOF MS) identification, and 40 spots were identified. Among these, 22 proteins were located in nucleus. These proteins were involved in stress response, cell cycle and division, apoptosis, mRNA processing, transport, splicing and microRNA (miRNA) maturation. The changed expression of Annexin A1 and Lamin B1 were confirmed by Western blot. The role of Annexin A1 in the response to cisplatin-induced DNA damage was further analyzed, and it was shown that after Annexin A1 knockdown, cisplatin-induced DNA damage was significantly increased. In addition, the changed expression of several miRNAs was also observed by quantitative real-time PCR (qRT-PCR). Taken together, these data indicate that cisplatin-induced DDR is a complex process, and that those proteins identified by proteomics can lead to new directions for a better understanding of this process.

1. Introduction

As one of most effective anticancer drugs for the last 30 years, cisplatin (cis-diammine-dichloro-platinum, CDDP) has been widely used in the treatment of many types of solid tumors, including ovary, testes, bladder, cervical, head and neck and small-cell lung cancers [1,2]. Cisplatin probably enters the cell both by passive diffusion and through the actions of a number of influx transporters [3], followed by aquation and hydrolysis to form reactive platinum species. Although aquated cisplatin is able to interact with a variety of cellular components including DNA, RNA and protein, DNA is generally recognized as the primary target, and these interactions eventually lead to the formation of interstrand and/or intrastrand crosslinks [4]. Damaged DNA can then be detected by the DNA-damage response (DDR) system, after which diverse signaling pathways are activated. As a result, cells can repair the damage and go through the cell cycle checkpoints. Alternatively, if the damage is too severe, the lesions cannot be repaired, and can either trigger cell death by apoptosis or cause the cell to go into cellular senescence [5,6]. However, even 40 years after the discovery of the anticancer activity of cisplatin, it remains a challenging task to fully describe the detailed mechanism of its cytotoxic action. In addition, the presence or acquisition of drug resistance to cisplatin during cancer treatment is becoming a major obstacle for its chemotherapeutic efficacy. Therefore, a better understanding of the cellular responses to cisplatin would not only improve strategies for cancer therapy, but would also benefit the design of novel platinum-based anticancer agents.

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Recently, proteomic analysis has become a powerful approach for the study of protein interactions and cellular changes on a global scale, which has the potential to reveal previously unknown and unanticipated associations. Several studies have applied such approaches, focusing on cisplatin's mode of action, as well as the acquisition of resistance. For example, Le Moguen et al. found that decreased amino-acid and nucleotide synthesis may be associated with cell cycle blockade, enhanced glycolysis and proliferation, possibly related to the acquisition of resistance to cisplatin [7]; while D'Aguanno et al. showed that the Nrf2 (nuclear factor-erythroid 2-related factor 2) pathway may represent a potential target to counteract cisplatin resistance [8]. In addition, it has been shown that cisplatin treatment-induced NF-kappaB down-regulation is mediated by TRAF2, and triggers the membrane death receptor/mitochondria-mediated apoptotic pathway [9]. All these findings may offer new insights into the mode of action and resistance to cisplatin. However, most of these studies have emphasized changes to the whole cell proteome, and few proteomic studies regarding cisplatin's mode of action have been conducted for nuclear proteins. One limitation of the whole-cell proteomic method is its relatively poor performance in detecting 'low-abundance' proteins. In addition, given that the main target of cisplatin is DNA [4] and that the DDR mainly occurs in the nucleus, a nuclear protein analysis for cisplatin is preferred.

For these reasons, we previously used an organelle proteomic approach to analyze the nuclear protein expression profiles in HeLa cells treated with different concentrations of cisplatin (0.1, 1, 5 and 10 μM) for 12 h. We found that the expression of many nuclear proteins was affected by cisplatin treatment, and that these proteins were involved in various cellular processes such as stress response, cell cycle progression and mitosis [10]. In particular, many of the identified proteins were associated with pre-mRNA splicing, indicating that alternative splicing might occur during cisplatin treatment. Indeed, alternative splicing was observed for the *Fas* gene [10]. Using the same method, we also examined the nuclear proteome response to benzo(a)pyrene, an indirect-acting DNA damaging agent. Similar to the cisplatin study, many proteins associated with pre-mRNA splicing were identified, and alternative splicing was observed for the *CD44* gene [11]. Together, these data pointed out the involvement of alternative splicing in cisplatin-induced DDR, which was initially revealed by the proteomic method.

In the present study, to further expand our knowledge regarding cisplatin-induced DDR, the time-response of HeLa cell to cisplatin, e.g., exposed to 5 μM cisplatin for 2, 12, and 24 h, was examined using the nuclear proteomic approach. As reported here, there were a total of 98 spots with different expression levels after cisplatin exposure, and 22 proteins were identified by mass spectrometry. The functions of some of the identified proteins were further explored for their possible roles in cisplatin-induced DDR.

2. Materials and methods

2.1. Cell culture and treatments

Human cervical adenocarcinoma HeLa cells were grown at 37 °C under a 5% CO_2 atmosphere in Eagle's Minimum Essential Medium (Gibco, CA) supplemented with bicarbonate and 10% newborn calf serum (PAA, Austria). HeLa cells were treated with 5 μM cisplatin (Sigma, St. Louis, USA) prepared freshly in 0.9% NaCl for 2, 12, and 24 h unless otherwise indicated.

2.2. Cytotoxicity analysis

The cytotoxic effect of cisplatin on HeLa cells was examined by MTT and Trypan blue assays as described previously [12].

Briefly, 5×10^3 cells in 100 μl medium per well were seeded into a 96-well plate, and allowed to incubate overnight before treatment. For the MTT assay, 20 μl MTT (5 mg/ml in PBS) was added into each well and incubated another 3 h. Then the solution was discarded and 150 μl DMSO was added to each well. The plate was shaken to thoroughly mix the formazan into the solvent. Absorbance was measured at 490 nm using a microplate reader (Tecan infinite M200, Switzerland). Relative survival was represented as the absorbance of the treated group/absorbance of the control group. For the Trypan blue assay, cells were treated with trypsin, removed from the plate and centrifuged for 5 min at 250 g. The pellet was suspended in PBS. Equal volume of 0.4% Trypan blue and the cell suspension were mixed and 10 μl of the mixture was applied to a hemacytometer. The stained (nonviable) and unstained (viable) cells were counted under a microscope, and viability was represented as the number of viable cells/number of total cells × 100. Relative viability was represented as viability of treated group/viability of control group.

2.3. Sample preparation

Nuclear proteins were prepared using the Nuclear Extraction Kit (Chemicon, Millipore, MA, USA) following the manufacturer's instructions. The proteins were concentrated using an Amicon Ultra centrifugal filter unit with a 3 kDa molecular cut-off (Millipore). Then, concentrated proteins were mixed with sample buffer (7 M Urea, 2 M Thiourea, 0.1% CHAPS, 65 mM DTT) and centrifuged at 14,000 × *g* for 20 min three times in order to remove salt. Protein concentration was determined by the Bradford Assay [13].

2.4. 2-Dimentional electrophoresis (2DE)

Isoelectric focusing (IEF), SDS-PAGE, staining and analysis were conducted as previously described [10]. Briefly, 200 μg nuclear extracts were loaded onto 17 cm, pH 5-8 linear immobilized pH gradient strips (Bio-Rad, CA, USA) for IEF. After 12 h of rehydration, the strips were transferred to the IEF cell. The parameters were set as follows: 250 V for 30 min, slow; 1000 V for 2 h, rapid; 10,000 V for 5 h, linear; 10,000 V until 60,000 Vh, rapid. After IEF was completed, the strips were equilibrated and the second dimension was performed by vertical 12% SDS-PAGE. Gels were stained using silver staining and scanned with a Bio-Rad GS-800 scanner. Images were analyzed by PDQuest software Version 7.4.0 (Bio-Rad). A statistical analysis was performed using the Student's *t*-test. Spots with significant differences (two-fold change, $P < 0.05$) were selected for MS identification. Three gels were set for each sample and three biological replicates were used for each time point.

2.5. MALDI-TOF/TOF MS identification

Selected spots were excised from the stained gels. Gel pieces containing the peptides were covered by 50 μl of a 1:1 ratio of 30 mM $K_3Fe(CN)_6$ and 100 mM NaS_2O_3 and vortexed until the color disappeared, then washed twice with 200 μl distilled water for 5 min. The dried gel was washed with 50% and 100% acetonitrile (ACN), and incubated in 25 mM NH_4HCO_3 for 5 min at 37 °C. After draining, 50% ACN and 100% ACN were added alternately until gel turned opaque white and dried at 40 °C for 5 min. Enzymatic digestion was performed as previously described [14]. Briefly, sequencing-grade trypsin was prepared in 25 mM NH_4HCO_3 at a concentration of 12.5 ng/μl to rehydrate the dried gel. The digestion was conducted overnight at 37 °C. Peptides were extracted three times with 10 μl of 50% ACN containing 0.1% TFA (trifluoroacetic acid, GE HealthCare) for 5 min at 37 °C. The extracts were then vacuum concentrated to 5 μl. The peptide solution was mixed with the same volume of matrix solution consisting of saturated CHCA (β-cyano-4-hydroxycinnamic acid) in 50% CAN/0.1% TFA. The mixture was then loaded onto a MALDI-TOF-TOF mass spectrometer (ABI4700 System, USA). A standard peptide mixture (des-Arg1-Bradykinin, Mr 904.468; Angiotensin I, Mr 1296.685 Da; Glu1 Fihrinopeptide B, Mr 1570.677 Da; ACTH (1–17), Mr 2093.087 Da; ACTH (18–39), Mr 2465.199 Da; ACTH (7–38), Mr 3657.929 Da) was used as an external standard calibration. The following settings were used: detected mass range of 700–3200 Da (optimal resolution for the quality of 1500 Da); 50 Hz laser frequency; 200 Hz repetition rate; 355 nm UV wavelength, and 20,000 V accelerated voltage. Peptide mass fingerprint data were matched to the NCBInr database using the MASCOT software search engine (http://www.matrixscience.com). Protein hit with score >62 was considered significant in the database search.

2.6. Small interfering RNA (siRNA) transfection

Knockdown of *ANXA1* expression was achieved following transfection of ANXA1 siRNA. The sense strand of the siRNA was ACUCCAGCGCAAUUUGAUGTT (nucleotides 414-432) of the human *ANXA1* mRNA sequence, and was synthesized by Genepharma Corporation (Shanghai, China). A nonspecific control with nucleotides UUCUCCGAACGUGUCACGUTT was used as a negative control (referred to as NC). The siRNA was delivered by lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The final concentration of siRNA was 50 nM and the medium was changed after 6 h incubation. After another 12 h incubation, the effect of silencing was checked and the cells were subjected to further treatment.

2.7. Apoptosis analysis

The Annexin V-FITC/PI kit (Multiscience, Hangzhou, China) was used to analyze apoptosis. Briefly, cells were washed with phosphate-buffered saline (PBS) and