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Transcriptional up-regulation of SOD1 by CEBPD: A potential target for cisplatin resistant human urothelial carcinoma cells

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Abstract

Bladder cancer is the fourth most common type of cancer in men (ninth in women) in the United States. Cisplatin is an effective agent against the most common subtype, urothelial carcinoma. However, the development of chemotherapy resistance is a severe clinical problem for the successful treatment of this and other cancers. A better understanding of the cellular and molecular events in response to cisplatin treatment and the development of resistance are critical to improve the therapeutic options for patients. Here, we report that expression of the CCAAT/enhancer binding protein delta (CEBPD, C/EBP δ , NF-IL6 β) is induced by cisplatin in the human bladder urothelial carcinoma NTUB1 cell line and is specifically elevated in a cisplatin resistant subline. Expression of CEBPD reduced cisplatin-induced reactive oxygen species (ROS) and apoptosis in NTUB1 cells by inducing the expression of Cu/Zn-superoxide dismutase (SOD1) via direct promoter transactivation. Several reports have implicated CEBPD as a tumor suppressor gene. This study reveals a novel role for CEBPD in conferring drug resistance, suggesting that it can also be pro-oncogenic. Furthermore, our data suggest that SOD inhibitors, which are already used

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Disclosure of potential conflicts of interest

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as anti-angiogenic agents, may be suitable for combinatorial chemotherapy to prevent or treat cisplatin resistance in bladder and possibly other cancers.

Keywords

C/EBP transcription factor; Cisplatin drug resistance; Superoxide dismutase; Urothelial carcinoma; SOD inhibitor

1. Introduction

Urothelial carcinoma (UC) is the most common type of bladder cancers, accounting for more than 90% of all bladder cancers [1]. The treatment strategy for UC is mainly surgery followed by chemotherapy or radiotherapy [2]. Chemotherapeutic agents can be effective in the treatment of advanced bladder cancer [3]. Cis-platinum (II)-diammine dichloride (cisplatin) is a platinum-based DNA damaging molecule widely used to treat bladder tumors [4]. Cisplatin induces DNA lesions that stall DNA replication, leading to cell cycle arrest, and ultimately induces cell death via apoptosis [5,6]. However, the clinical efficacy of cisplatin is limited by its toxic effects on normal cells and by the development of drug resistance within tumor cells. Cisplatin resistance can be achieved by different mechanisms, including reduced intracellular drug accumulation, increased detoxification of drug by thiol-containing molecules, increased DNA damage repair, and altered cell signaling pathways and apoptosis mediators [7,8].

Factors contributing to cisplatin cytotoxicity include reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals, and hydrogen peroxide, which target DNA, proteins and lipids to generate byproducts of the normal metabolism of oxygen [9–11]. ROS also serve as signaling molecules in physiological and pathological pathways. Tumor cells escape the ROS mediated cytotoxicity in part by up-regulating ROS scavengers such as thioredoxin and superoxide dismutases [12]. Many transcription factors are induced by cisplatin and may contribute to the development of drug resistance [13]. However, none of these or their targets have so far provided clinically proven drug targets. A better understanding of the cellular and molecular events in response to cisplatin treatment and the development of resistance are of critical importance to improve the therapeutic options for patients.

Tumor hypoxia is one of the tumor-specific conditions that promote the development of drug resistance [14]. The transcription factor CCAAT/enhancer binding protein delta (CEBPD, C/EBP δ , NF-IL6 β) is one of the genes that are induced by hypoxia [15–17]. CEBPD belongs to the C/EBP gene family, whose members have unique properties in regulating cell-type-specific growth and differentiation [18–20]. According to current knowledge, CEBPA functions purely as an anti-proliferation agent [21,22]. In contrast, CEBPB can be both a growth inhibitor and growth promoter depending on context [23]. CEBPD was originally identified as an acute phase response gene [24]. However, the functions of CEBPD specifically in response to stress stimuli have still not been fully addressed. In addition, CEBPD is involved in a wide range of cell-type-specific processes including differentiation and proliferation [25,26]. Several findings point collectively to a tumor suppressor function of C/EBP δ . C/EBP δ -deficiency causes genomic instability in mouse embryo fibroblasts [27] and delays apoptosis during mammary gland involution [28]. C/EBP δ expression is down-regulated in a significant proportion of acute myelomonocytic leukemias, and cancers of the cervix, liver, and breast [29–33] while forced expression of CEBPD inhibits the growth of tumor cell lines in vitro [31].

In this study, the role of CEBPD in cisplatin resistance was investigated in the human urothelial carcinoma cell line NTUB1. We identified a novel potentially pro-oncogenic activity of CEBPD whose expression was sufficient to reduce cisplatin cytotoxicity through activation of SOD1, which reduces cisplatin-induced reactive oxygen species.

2. Materials and methods

2.1. Reagents and antibodies

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), triethylenetetramine dihydrochloride (TETA), arsenic trioxide, 4'-6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), RNase A, 2',7'-dichlorofluorescein diacetate (DCF-DA), DMSO and G418 were purchased from Sigma Chemical Inc. (St. Louis, MO, USA). CEBPD (M-17, SC-636), CEBPD (5.61, SC-135734), CEBPB (C-19, SC-150) and PRX (SC-23969) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SOD1 (65151A), β -actin (AC-15) and α -tubulin (Ab-1, CO06) antibodies were purchased from BD Biosciences (San Jose, CA, USA), Novus Biologicals (Littleton, CO, USA) and Oncogene Res. Prod. (Merck & Co., Taiwan), respectively. Goat anti-rabbit and anti-mouse IgG conjugated with peroxidase antibodies were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Enhanced chemiluminescence (ECL) was from Amersham Life Sciences Inc. (Amersham, England). Cisplatin was from Pharmacia & Upjohn (Milan, Italy). Paclitaxel was from Bristol-Myers Squibb (Princeton, NJ, USA). Gemcitabine was from Fegersheim (France).

2.2. DNA constructs

The expression vector for human CEBPD in pcDNA3.1 (+) was as described previously [34]. Site-specific mutations of CEBPD were generated with the Quickchange™ mutagenesis kit (Stratagene, Co.) according to the manufacture's protocol and using the following primers. R198A mutation: 5'-CGAGTACCGG-CAGCGGGCCGAGCGCAACAACATC-3' and 5'-GATGTTGTTGCGCTGGCCCCGCTGCCGGTACTCG-3'. The deletion of the carboxy-terminal amino acids 197–296 of CEBPD (Δ DBD) was constructed by PCR amplification by 5'-primer CAAGCTAGCATGAGCGCCGCTCTTC and 3'-primer AAGGATCCAATCTGCCGGTACTCGGGGC, and cloned into shuttle vector 5-Flag-SK⁺. The flag CEBPD- Δ DBD cDNA cassette was then subcloned into pcDNA3 vector. CEBPD shRNA (CEBPD-pSi) plasmid was as described previously [35]. The shRNA target sequence for SOD1 (NM_000454) is TCCCTTGATGTAGTCTGAGG and cloned in pLKO.1-puro vector (clone ID: TRCN0000009869; obtained from the National RNAi Core Facility, at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica in Taiwan).

2.3. Cell culture and establishment of stable clones

NTUB1, an immortalized human urothelial carcinoma cell line, was established from a high-grade bladder cancer [36]. Cisplatin (NTUB1/P(14)), gemcitabine (NTUB1/G(1.5)), arsenic trioxide (NTUB1/As(0.5)) and paclitaxel (NTUB1/T(0.017)) resistant sub-lines were maintained in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 100 unit/ml penicillinG, 100 μ g/ml streptomycin and 2 mM L-glutamine (Invitrogen, CA, USA) at 37 °C, 5% CO₂, as indicated with addition of 14 μ M cisplatin, 1.5 μ M gemcitabine, 0.5 μ M arsenic trioxide, and 0.017 μ M paclitaxel, respectively [37,38]. pcDNA3 (V), full-length-CEBPD (CEBPD; D), CEBPD-R198A (R198A) and a deletion mutant lacking the C-terminal amino acids 197–296 (Δ DBD) were stably transfected into the NTUB1 cells by the Lipofectamine 2000 method as specified by the manufacturer (Invitrogen, CA, USA) followed by drug selection with 800 μ g/ml G418. Resistant colonies

were selected and expanded. The clones were then maintained in culture supplemented with 200 µg/ml G418.

2.4. Cytotoxicity analysis by MTT assay

Cellular cytotoxicity of cisplatin was assessed with a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay as previously described [37]. Briefly, the cells were plated at a density of 2×10^3 cells/well in 96-well plates and incubated at 37 °C overnight before drug exposure. Cells were cultured in the presence of graded concentrations (0.1, 0.3, 1, 3, 10, and 30 µM) of cisplatin alone or combined with various doses of triethylenetetramine dihydrochloride (TETA) at 37 °C for 72 h. 50 µl of MTT (2 mg/ml in PBS) was added to each well and allowed to react for 4 h. Media were removed by centrifugation at $1000 \times g$ for 10 min and 150 µl DMSO were added to each well. The proportions of surviving cells were determined by absorbance spectrophotometry at 540 nm using a microplate reader, MRX-2 (Dynex, Chantilly, VA, USA). The cell viability was expressed as the ratio to the untreated control. The IC₅₀ values of each group were calculated by the median-effect analysis [39] and presented as mean $\pm$ standard deviation (SD).

2.5. RNA isolation and real-time quantitative RT-PCR (reverse transcription-polymerase chain reaction; Q-RT-PCR)

Total RNAs from indicated treatments were isolated using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacture's recommendations. Two micrograms total RNA was treated with DNase I (Promega, Madison, WI, USA) and reverse transcribed by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) following the manufacture's instruction. LightCycler Fast Start DNA Master SYBR Green I (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) were used for real-time Q-PCR following the manufacture's instruction. Cyclophilin A gene was served as normalization control. The data were analyzed using the comparative C_t method. A ratio of specific mRNA/cyclophilin amplification was then calculated, to correct for any differences in efficiency. All samples were independently analyzed at least three times in duplicate. Primers used:

CEBPD-UTR for: 5'-GGACATAGGAGCDCAAAGAA-3'

CEBPD-UTR rev: 5'-GCTTCTCTCGCAGTTTAGTGG-3'

Cyclophilin A for: 5'-GTCAACCCACCGTGTCTT-3'

Cyclophilin A rev: 5'-CTGCTGTCTTTGGGACCTTGT-3'

hSOD1 for: 5'-CTGAAGGCCTGCATGGATTC-3'

hSOD1 rev: 5'-CCAAGTCTCCAACATGCCTCTC-3'

2.6. Western blot analysis

Cells were harvested by trypsinization and resuspended with suitable amount of PBS adjusted with the cell numbers. The cells were mixed with equal volume of 2× sample buffer and boiled for 5 min twice to denature the proteins. Cell extracts were separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in TBST buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20). The membranes were incubated with specific antibodies at 4 °C overnight. The membranes were washed with TBST buffer and incubated with secondary antibody at room temperature for

another 1 h. Signals were detected by chemiluminescence ECL reagent after TBST wash and visualized on Fuji SuperRX film.

2.7. Nuclear staining

Cells were seeded onto serum-coated coverslips. After treating with indicated dose of cisplatin for 24 h, the cells were fixed with 2% formaldehyde in PBS and stained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) for 1 h. The coverslips were then mounted with 80% glycerol and photographed by microscopy.

2.8. Flow cytometry analysis

DNA content was determined following propidium iodide (PI) staining of cells as previously described [27]. Briefly, 8×10^5 cells were plated and treated with 0, 10 or 20 μ M cisplatin for 24 h. Cells were harvested by trypsinization, washed with PBS, and fixed by ice-cold methanol. After overnight incubation, the cells were washed with PBS and incubated with 50 μ g/ml PI and 50 μ g/ml RNase A in PBS at room temperature for 30 min. The proportions of cells in each cell cycle phases were analyzed using FACScan flow cytometer and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

2.9. Quantitative analysis of intracellular reactive oxygen species (ROS)

Production of ROS was analyzed by flow cytometry as described previously [40]. Briefly, 3×10^5 cells were seeded in 6-well plates. The following day, cells were treated with cisplatin as indicated doses. One hour prior to harvest, 10 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA) was added to the medium. The cells were collected by trypsinization and washed with PBS. The green fluorescence of intracellular DCF (2',7'-dichlorofluorescein) was analyzed immediately by FACScan flow cytometer (Becton Dickinson). The ROS production efficiency (M1 ratio) was calculated as "[counts of treated sample in M1 – counts of control in M1]/counts of control in M1 $\times 100$ ".

2.10. Luciferase reporter assay

6×10^4 NTUB1 cells were seeded in 3.5-cm dishes. 0.1 μ g of SOD1-luciferase reporter construct, p1499-sod1 [41], were co-transfected with vector pcDNA3 (V) or CEBPD expressing plasmids (CEBPD, R198A and Δ DBD) by Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The cells were harvested 24 h post-transfection and analyzed for luciferase activity (Luciferase assay system, Promega, Madison, WI, USA) by luminometer. Total protein was quantified by Bradford reagent (Bio-Rad, USA) and used for normalization.

2.11. Chromatin immunoprecipitation (ChIP) assays

The ChIP assay was carried out as described previously [42]. Briefly, NTUB1 cells were treated with 0 or 20 μ M cisplatin for 24 h and fixed with 1% formaldehyde for 15 min. The cross-linked chromatin was then prepared and sonicated to an average size of 500 bps. The DNA fragments were immunoprecipitated with CEBPD (SC-636) or control rabbit IgG antibodies at 4 °C overnight. After reversal of the cross-linking, the immunoprecipitated chromatin was amplified by primers related to the specific regions of the SOD1 genomic locus: SOD1-797(S): 5'-TCCAGCCTGGGTGACAGAGCGAGAC-3' and SOD1-531(AS): 5'-GTTTGGCAAGGCCACAACCCTACAG-3'. The amplified DNA products were resolved by agarose gel electrophoresis, stained with ethidium bromide and photographed.

2.12. Statistical analysis

All data were presented as mean $\pm$ SD and compared with Student's *t*-test.

3. Results

3.1. Cisplatin induces CEBPD expression

We had previously generated sublines of the human urothelial carcinoma cell line NTUB1 which exhibit resistance to specific chemotherapeutic drugs such as cisplatin (NTUB1/P(14)), gemcitabine (NTUB1/G(1.5)), arsenic trioxide (NTUB1/As(0.5)), and paclitaxel (NTUB1/T(0.017)) [37,38]. Expression analysis revealed that CEBPD was specifically expressed in the cisplatin resistant subline but not the parental line or the other sublines. Similar results were observed in a nasopharyngeal carcinoma cisplatin resistant subline (unpublished data). In contrast, the related protein CEBPB was expressed in all of these cell lines and was only slightly increased in the cisplatin resistant subline (Fig. 1A). In all cell lines, expression of CEBPA was barely detectable at either protein or RNA level (data not shown). To explore whether CEBPD expression is induced by the acute cisplatin response, NTUB1 cells were treated with various concentrations of cisplatin for 12 or 24 h. CEBPD protein levels gradually increased upon cisplatin exposure in a concentration-dependent manner and reached maximal levels after 12 h at 20 μ M (Fig. 1B). Similarly, the expression of *CEBPD* mRNA was up-regulated 3–20-fold by cisplatin in a time- and concentration-dependent manner (Fig. 1C). In contrast, CEBPB protein did not change upon cisplatin treatment (Fig. 1B).

3.2. CEBPD reduces cisplatin sensitivity in NTUB1 cells

To determine the role of CEBPD in the cisplatin response, a CEBPD expression construct was transfected into NTUB1 cells for transient expression. Cells were treated with graded concentrations of cisplatin for 72 h. The sensitivity of cells was determined by MTT cell viability assay (data not shown) and the IC_{50} values of tested cells were calculated accordingly (Fig. 1D). Over-expression of CEBPD increased the IC_{50} to 3.31 μ M as compared to 2.14 μ M for vector transfected cells and 1.75 μ M for parental cells. Thus, forced expression of CEBPD promoted NTUB1 cell survival in the presence of cisplatin (Fig. 1D; $P < 0.001$). To further investigate the contribution of CEBPD to cisplatin resistance, WT CEBPD and a CEBPD variant that lacks amino acids 197–269 (Δ DBD) were transfected into NTUB1 cells. Clones were selected and the level of CEBPD was determined by Western analysis (Fig. 1E and data not shown). Although CEBPD was shown to possess growth arresting or apoptosis inducing properties in many cell types [28,30,31], we could easily establish CEBPD over-expressing clones of NTUB1 cells. This observation shows that CEBPD does not arrest NTUB1 cell growth. Vector control clone (N-V), WT CEBPD (N-D#1) and the deletion of amino acids 197–269 CEBPD variant (N- Δ DBD) expressing clones were used for subsequent experiments. The sensitivity of these cells to cisplatin was determined by MTT cell viability assay. As shown in Fig. 1E, WT CEBPD clone (N-D#1) showed significant higher IC_{50} to cisplatin compared to vector (N-V) and DBD deletion mutant (N- Δ DBD). These data demonstrate that forced expression of CEBPD promoted NTUB1 cell survival in the presence of cisplatin in NTUB1 cells. Furthermore, the DNA-binding activity of CEBPD is necessary for cisplatin resistance in NTUB1 cells, indicating that a target gene of CEBPD mediates resistance.

3.3. The DNA-binding domain of CEBPD is essential to confer cisplatin resistance in NTUB1 cells

DNA damage by cisplatin induces cell cycle arrest primarily during G1 and/or S phases followed by programmed cell death [6]. We assessed apoptosis in response to cisplatin by nuclear morphology as well as flow cytometry of cellular DNA content, considering cells with sub-G1 DNA content as apoptotic. Compared to control cells, the N-D#1 cells exhibited significantly less cells with apoptotic nuclear condensation (Fig. 2A). Flow cytometry showed that 24 h of exposure to 10 μ M cisplatin led to an increase of cells in S-

phase in all three cell lines, indicative of S-phase arrest (Fig. 2B). The higher dose of 20 μ M cisplatin triggered an apoptotic response in the control cells N-V ($21.32 \pm 4.49\%$) and N- Δ DBD ($18.15 \pm 7.19\%$). This effect was completely abolished in CEBPD expressing N-D#1 ($5.89 \pm 1.01\%$) cells ($P < 0.05$), which instead exhibited S-phase arrest. These results suggest that over-expression of intact CEBPD but not of a DNA-binding mutant of CEBPD enhance cisplatin resistance by lowering the effective dose of the drug.

3.4. CEBPD represses cisplatin-induced ROS production

The cytotoxicity of cisplatin is mediated to a large part by ROS [9]. As expected and confirmed with others, the cellular ROS levels were increased in dose- and time-dependent manner by cisplatin in NTUB1 cells (data not shown). In addition, over-expression of WT CEBPD (N-D#1) significantly reduced basal level of ROS. However, this effect was not observed with the DNA-binding deletion clone, N- Δ DBD (Fig. 2C). To explore the role of ROS in CEBPD-associated cisplatin resistance, we chose 20 μ M of cisplatin to determine if ROS levels were modulated by CEBPD expression at 24 h. The peak intensity of untreated control cells (N-V) was set at 100. The percentage of cells with values higher than 100 is assigned as M1 value as indicated in Fig. 2C. The ROS production efficiency was further transformed into the M1 ratio (see Section 2). As shown in Fig. 2D, cisplatin significantly induced ROS production efficiency (M1 ratio) in parental NTUB1 cells but not in N-D#1 cells ($P < 0.001$). Since complete deletion of the DBD also removes the nuclear localization signal, we also tested an R198A point mutation (N-R198A), which does not interfere with nuclear localization but destroys the DNA binding activity [43]. In contrast to WT CEBPD (N-D#1), the IC_{50} of the N-R198A stable clone to cisplatin was significantly reduced and comparable to N-V and N- Δ DBD cells (data not shown). As shown in Fig. 2D, the M1 ratio of N-R198A clone was similar to N-V control cells ($P < 0.005$ between N-D#1 and N-R198A). Taken together, these results demonstrate that expression of wild-type CEBPD efficiently reduced the induction of ROS by cisplatin.

3.5. SOD1 is a target gene of CEBPD in the cisplatin response

Antioxidant enzymes serve as ROS scavengers and reduce intracellular ROS levels. To evaluate whether CEBPD affects cisplatin sensitivity by this pathway, proteins were examined by Western analysis. Interestingly, Cu/Zn-superoxide dismutase (SOD1) was highly expressed in N-D#1 but not in N- Δ DBD and N-V control cells, while another antioxidant enzyme, peroxiredoxin (PRX), was equally expressed in all three cell lines (Fig. 3A). In addition, SOD1 was induced by cisplatin along with CEBPD in clone N-V. However, in CEBPD expressing N-D#1 cells, cisplatin did not further induce the already elevated level of SOD1 (Fig. 3B). Consistent with these data, we found SOD1 expressed in the NTUB1/P14 cisplatin resistant subline and RNAi-mediated knockdown of endogenous CEBPD expression significantly reduced SOD1 levels in these cells (Fig. 3C). Interestingly, silencing of SOD1 expression also reduced *CEBPD* mRNA expression. We speculate that this may be due to a feedback mechanism. Superoxide dismutases are metalloenzymes and convert superoxide radicals into hydrogen peroxide. Hydrogen peroxide is then converted to water and oxygen by catalase [44]. Thus, our results suggest that expression of wild-type CEBPD reduces the level of ROS through SOD1 induction, which then results in reduced cisplatin cytotoxicity.

To examine whether CEBPD directly regulates SOD1 at the transcriptional level, the 1500 bp region upstream of transcription start site of the human SOD1 gene was analyzed. Fig. 4A shows the location of five potential C/EBP binding sites upstream of the transcription start site, which were identified by the "TFSEARCH" program [45]. Next, we co-transfected NTUB1 cells with a luciferase reporter construct containing this region of the SOD1 promoter [41] and increasing amounts of CEBPD expression plasmid. As shown in Fig. 4B,

the SOD1 promoter activity was induced by full-length wild-type CEBPD in a dose-dependent manner, while the R198A mutation significantly impaired transactivation activity (Fig. 4C). To demonstrate direct binding of endogenous CEBPD to the SOD1 promoter, we performed chromatin immunoprecipitation (ChIP) with NTUB1 cells. In agreement with the induction of CEBPD expression by cisplatin in these cells (Fig. 1B), CEBPD binding was detected specifically in cisplatin treated cells (Fig. 4D). Consistent with this result, *SOD1* mRNA levels increased significantly in NTUB1 cells treated with 20 μ M cisplatin ($P < 0.005$, Fig. 4E). In addition, *SOD1* mRNA levels were elevated in NTUB1/P(14) cells (Fig. 4E) concurrent with the high expression of CEBPD in these cells (Fig. 1A). These results demonstrate that CEBPD directly regulates SOD1 gene expression at the transcriptional level.

3.6. SOD1 inhibition potentiates cisplatin cytotoxicity

To further explore the potential clinical utility of SOD1 as a drug target, we used the copper chelator triethylenetetramine (TETA), which potently inhibits the activity of SOD1 [46]. The intracellular ROS levels were significantly elevated in both cisplatin and TETA treated cells and further induced by combined treatment suggesting that blockage of SOD1 activity potentiates cisplatin-induced ROS production in NTUB1 cells (Fig. 5A). Most importantly, the cisplatin cytotoxicity was further enhanced by TETA in both NTUB1 and cisplatin resistant subline NTUB1/P(14) cells (Fig. 5B and C, $P < 0.005$).

4. Discussion

In this report, we have shown that the transcription factor CEBPD is a cisplatin response gene, which reduces cisplatin cytotoxicity in a human urothelial carcinoma cell line while up-regulating SOD1 and reducing levels of free radicals. The model in Fig. 6 illustrates how this mechanism can lead to cisplatin resistance. To our knowledge, present study is the first to establish a direct link between CEBPD and platinum-based drug response as well as resistance in tumor cells. CEBPD is down-regulated in several types of cancer (see Section 1) while it is up-regulated in high-grade glioma [47] and mesothelioma [48]. Many studies reported that CEBPD arrests the growth of different tumor cell lines in vitro (see Section 1), our data show that CEBPD does not affect the growth of a bladder carcinoma cell line. Furthermore, our results suggest that CEBPD may promote the survival of tumor cells specifically in response to chemotherapeutics. Therefore, this study provides novel evidence that CEBPD may not only be a tumor suppressor but also act as a tumor promoter. Growing evidences suggested that many important regulators exhibit both oncogenic and tumor suppressor functions in different cell/tissue context. CEBPB, another member of C/EBPs, has been suggested exhibiting oncogenic and anti-oncogenic function in skin and fibroblasts [23].

Tumor cell resistance to chemotherapeutic drugs and acquired resistances are among the most serious problems for effective cancer patient treatment today. The cellular mechanisms of resistance to platinum-based drugs are multi-factorial and most of them are due to the induction of specific proteins [7]. Knowledge to predict clinical response to chemotherapy is emerging but far from fully established [49]. Studies have revealed the role of several transcription factors in anti-cancer drug resistance. However, their relevant target genes are either unknown or have not revealed useful molecular targets to combat resistance [13]. It has been shown that the ETS-1 transcription factor, which is associated with cisplatin resistance in several tumor cell types, induces CEBPD expression [50]. However, this is the first study to identify CEBPD as a cisplatin-induced gene and as a critical factor in the cellular defense against cisplatin toxicity. Thus, mechanisms leading to persistent CEBPD expression may provide selective advantage to the tumor cell. However, the underlying mechanism by which cisplatin-induced CEBPD warrants further investigation.

Cisplatin not only damages DNA directly but also triggers production of ROS, which is a critical step in apoptotic cell death and tumorigenesis [12,51]. Several lines of evidence suggest that cisplatin-induced cell death is associated with altered cellular redox state and DNA damage responses [8]. Byun et al. demonstrated that reducing cellular glutathione and glutathione-S-transferase (GST) enzyme activity by buthionine sulfoximine (BSO) reduced cisplatin resistance suggesting that cellular ROS levels plays an important role in mediating cisplatin cytotoxicity [52]. In the present study, we have obtained compelling evidence that CEBPD reduces ROS levels and concomitantly cisplatin cytotoxicity. Use of CEBPD mutants established that CEBPD-mediated gene expression is critical for its contribution to cisplatin resistance in NTUB1 cells, and subsequent analyses identified SOD1 as one relevant target gene.

Antioxidant enzymes are promising targets for cancer therapy [53,54]. Cytoplasmic enzyme Cu/Zn-superoxide dismutase (SOD1) is an important cellular defense molecule against damages caused by superoxide radicals. SOD1 plays a critical role in response to diverse stimuli, including stresses, pro-inflammatory cytokines, growth factors and in the aging process [55,56]. Heavy metals and environmental hormones are known to activate SOD1 gene expression [57–59] and a study in budding yeast demonstrated that SOD1 plays a role in the DNA damage response [60]. Over-expression of SOD1 was found in the radio-resistant and cisplatin cross-resistant human glioblastoma cell line U251 [61] and targeted over-expression of SOD1 in an animal model reduced cisplatin toxicity [62]. Proteomic approaches demonstrated that SOD1 is highly expressed in cisplatin resistant human ovarian cancer cell lines, and that SOD1 inhibition could enhance cisplatin sensitivity in vitro [46]. Interestingly, specific copper chelators that inhibit SOD1 activity are well tolerated and have already been used in clinical trials in cancer patients due to their anti-angiogenic activity [63,64]. Furthermore, it has been shown that Cu/Zn chelators dramatically reduce cisplatin toxicity in animal models [65]. Our data suggest that SOD1 can contribute to cisplatin resistance in bladder carcinoma cells. Collectively, these reports call for an assessment of CEBPD and SOD1 expression in tumors as a potential means to predict cisplatin resistance.

In conclusion, our study provides further insight into the molecular mechanism of cisplatin resistance in urothelial carcinoma by showing CEBPD-mediated SOD1 induction. Furthermore, this study also suggests that SOD1 inhibitors should be explored for use in combinatorial therapies to prevent or combat cisplatin resistance. Understanding pathways that lead to persistent expression of CEBPD and consequently SOD1 in cisplatin resistant tumors warrants further investigation and offer better rational treatment strategies.

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Abbreviations

CEBPD	CCAAT/enhancer binding protein delta
ROS	reactive oxygen species
SOD1	Cu/Zn-superoxide dismutase
TETA	triethylenetetramine dihydrochloride

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Fig 1. CEBPD protein is expressed specifically in a cisplatin resistant subline and over-expressing CEBPD enhances cisplatin resistance. (A) Western blot analysis of CEBPD and CEBPB protein expression in NTUB1 and drug resistant sublines (NTUB1/P(14), NTUB1/G(1.5), NTUB1/As(1.5) and NTUB1/T(0.017)). Expression of α -tubulin was used as a loading control. (B) NTUB1 cells were treated with 0, 10, 20, 30 or 40 μ M of cisplatin and the proteins were harvested at 12 or 24 h as indicated. Western blot analysis was performed with antibodies for CEBPD, α -tubulin, CEBPB and β -actin. (C) Q-RT-PCR analysis of *CEBPD* mRNA expression from the same treatment as (B). *CEBPD* mRNA expression normalized to cyclophilin A expression is shown as fold over untreated samples at 12 h. Data represent means $\pm$ standard deviation (SD) of five independent samples, each analyzed in duplicate (*** P < 0.005 and * P < 0.05). (D) IC_{50} values of transiently transfected cells were performed by MTT assay as described in Section 2 (*** P < 0.005). Data represent means $\pm$ standard deviation (SD) of three independent experiments. (E) A representative MTT assay of established CEBPD expressing clones and the IC_{50} values of each group were calculated by the median-effect analysis and presented as means $\pm$ SD with *** P < 0.005 compared to N-V and N-ΔDBD.

Fig 2.

The DNA-binding activity of CEBPD is essential for the inhibition of cisplatin-induced apoptosis. (A) Representative images of N-V, N-D#1 and N-ΔDBD clones, seeded onto glass coverslips and treated with 0, 10, or 20 μM of cisplatin for 24 h. The nuclei were stained with DAPI and photographed by microscopy. The arrows point to apoptotic cells. (B) The indicated cells were treated with 0, 10, or 20 μM of cisplatin for 24 h and the cell cycle profile of each sample was determined by flow cytometry. The means ± SD of 2–4 independent experiments is shown with * $P < 0.05$ comparing N-D#1 and N-V at 20 μM cisplatin. (C) A representative flow cytometry of ROS in untreated N-V, N-D#1 and N-ΔDBD cells. The M1 region is indicated. (D) The N-V, N-D#1 and N-R198A cells were treated with 0 or 20 μM of cisplatin for 24 h. The ROS levels were determined and transformed to M1 ratios as described in Section 2. The data represent the percentage of ROS production efficiency compared to the N-V untreated samples (means ± SD) of four independent experiments with *** $P < 0.005$.

Fig 3.

CEBPD expression is positively correlated with SOD1 protein levels. (A) Western blot analysis of SOD1 and PRX protein expression in NTUB1 stable clones. α -tubulin was used as a control for protein loading. (B) N-V and N-D#1 cells were treated with 20 μ M cisplatin for 24 h followed by Western analysis of CEBPD and SOD1 expression. β -Actin was used as a control for protein loading. (C) Cisplatin resistant subline NTUB1/P14 was transiently transfected with shRNA expression constructs directed against GFP (GFPsi) as negative control, CEBPD (CEBPDsi), or SOD1 (SODsi). CEBPD and SOD1 expression were assessed 24 h later compared to β -actin as control.

Fig 4.

SOD1 is a target gene of CEBPD. (A) Scheme of the human SOD1 promoter from positions +74 to -1499 bp. The potential C/EBP binding sites were predicted by the program “TFSEARCH” [45]. Five predicted C/EBP binding sites (filled ovals) are shown at positions -1321 to -1311, -723 to -713, -707 to -697, -694 to -684 and -248 to -241 bp. The primers (arrows) and PCR product (bar) used for ChIP (panel D) are indicated. (B) Luciferase reporter activity from 0.1 μg of SOD1 luciferase reporter plasmid transiently co-transfected with 0.1, 0.2 or 0.3 μg of CEBPD expression plasmid in NTUB1 cells. The results were normalized by total protein concentration and are represented relative to vector control (means ± SD from three independent experiments performed in duplicate each) with ****P* < 0.005. (C) Luciferase reporter activity from 0.1 μg of SOD1 luciferase reporter plasmid transiently co-transfected with 0.3 μg of vector (V), WT CEBPD (WT), or R198A mutant (R198A) CEBPD expression plasmids in NTUB1 cells. Shown are the means ± SD from at least three independent experiments performed in duplicate, relative to vector transfected cells with **P* < 0.05 and ***P* < 0.01. (D) NTUB1 cells were treated with 0 or 20 μM cisplatin for 24 h. The sonicated chromatin was subjected to ChIP analysis by CEBPD antibody or control IgG. The amplified DNA products were resolved by agarose gel electrophoresis, stained with ethidium bromide and photographed. The SOD1 promoter-specific PCR product is indicated by the arrow. (E) SOD1 mRNA expression was examined by Q-RT-PCR in NTUB1 cells treated with 20 μM cisplatin for 24 h and NTUB1/P(14) cells compared to untreated NTUB1 (Results are shown as fold of change to NTUB1 cells in mean ± SD, from three independent experiments performed in duplicate each; ****P* < 0.005).

Fig 5. SOD1 inhibition potentiates cisplatin cytotoxicity. (A) Representative flow cytometry of ROS levels in NTUB1 cells treated for 24 h with 20 μ M cisplatin and/or 20 mM TETA. (B) NTUB1 and (C) NTUB1/P(14) cells were subjected to cytotoxicity analysis with combinations of cisplatin and TETA (0.5, 2 and 5 mM) by MTT assay as described in Section 2. The percentage of cell survival relative to untreated cells is presented. *** P < 0.005 and * P < 0.05 between cisplatin alone and combined samples, respectively.

Fig 6.

Model for CEBPD-mediated cisplatin resistance. Cisplatin causes DNA damage as well as reactive oxygen species (ROS), which trigger cell cycle arrest or/and apoptosis. Cisplatin induces CEBPD by an as of yet unidentified mechanism which directly activates superoxide dismutase (SOD1) gene expression. Superoxide anion ($O_2^{\bullet-}$) is dismutated by SOD1 and converted to H_2O_2 which can be further neutralized to water and oxygen by catalase. The reduced ROS levels in NTUB1 cells cause the cisplatin resistant phenotype. The inhibition of SOD1 by triethylenetetramine (TETA) potentiates the cisplatin cytotoxicity and abrogates cisplatin resistance.