RESEARCH ARTICLE

Short Low Concentration Cisplatin Treatment Leads to an Epithelial Mesenchymal Transition-like Response in DU145 Prostate Cancer Cells

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Abstract

Background: Prostate cancer is one of the main causes of cancer death, and drug resistance is the leading reason for therapy failure. However, how this occurs is largely unknown. We therrfore aimed to study the response of DU145 cells to cisplatin. Materials and Methods: Du145 prostate cancer cells were treated with a low dose of cisplatin for 24 h and cell viability and number were determined by MTT assay and trypan blue exclusion assay, respectively. The real time polymerase chain reaction (PCR) was used to assess responses to cisplatin treatment. Results: After 24h 2 μg/ml treatment did not result in significant reduction in cell viability or number. However, it led to enhanced cancer cell invasiveness. E-cadherin mRNA was reduced, and vimentin, Snail, Slug, metalloproteinase 9 (MMP9) mRNA expression increased significantly, a feature of epithelial-mesenchymal transition (EMT). Conclusions: Short time low concentration cisplatin treatment leads to elevated invasiveness of DU145 cancer cells and this is possibly due to EMT.

Keywords: Epithelial-mesenchymal transition - prostate cancer - cisplatin - cancer invasiveness

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Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death(Jemal et al., 2011). Its incidence is higher in developed countries, in America it is the most common cancer and is the second leading cause of cancer death in men (Siegel et al., 2014). In Asia, its incidence is relatively low, but shows a increasing trend (Jung et al., 2013; Du et al., 2014). Although multiple approaches has been used to treat this cancer, 10-25% prostate cancer patients still die of , mainly cancer metastasis (D'Amico et al., 2002).

Cisplatin is a commonly used chemotherapeutic drug for varied types of cancers including prostate cancer. It kills cancer cells by covalently binding to DNA and forming DNA adducts, which activates various signal-transduction pathways, such as DNA-damage recognition and repair, cell-cycle arrest, and programmed cell death/ apoptosis (Siddik, 2003; Kelland, 2007).

It has been reported that the residual ovarian cells that survived 3 or 5 days of cisplatin treatment display EMT and stem cell properties including increased invasiveness(Latifi et al., 2011; Baribeau et al., 2014), And the MAPK/ERK signaling pathway activation was reported to be one of the possible mechanisms underlying cisplatin's this effect (Baribeau et al., 2014). Similar to this study, Han and colleagues (Han et al., 2013; Han et al., 2014) showed that Gastric Cancer Cell that survived 48 h Doxorubicin treatment showed EMT, and this phenotype is maintained by those cancer cells, indicating an epigenetic mechanism may be responsible for the EMT.

Inspired by those studies, we wondered if short time (24h) low concentration (2 μg/ml) of cisplatin treatment leads to more malignant phenotype *in vitro* in DU145 cell line modal.

Materials and Methods

Cell lines and reagents

DU145 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), dimethylformamide, sodium dodecyl sulfate (SDS) and cisplatin were purchased from Sigma (Shanghai, China).

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was based on the method by Hansen et al. (1989). Cell viability assays were carried out using

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the MTT dye conversion assay in 96-well plates. DU145 cells were planted into 96-well plates with a density of 4000 cells/well. After overnight, cells were treated with cisplatin-containing medium. 24 h later, MTT (25 μl of 5 mg/ml MTT in sterile PBS) was added to the medium and incubate for 2h at 37°C. The reaction was stopped, and the cells were lysed by the addition of 100 μl lysis buffer consisting of 20% SDS in a water/dimethylformamide (1:1) solution (pH 4.7). Cell lysates were placed at 37°C overnight to allow cell lysis and dye solubilization. The absorbance OD was read at 570 nm and 630 nm as reference using a Thermo MULTISKAN MK3 microplate reader. The absorbance of each well was calculated as OD_{570}-OD_{630}. MTT assays were carried out on three independent experiments, each performed in triplicate.

Typan blue cell number counting

Cells were planted in 24-well plates at a density of 1×10^5 cells/well. And after overnight, medium was changed into cisplatin-containing medium. 24h later, cells were collected by using trpsin-EDTA solution, and live cells are counted by the TC10 (Bio-Rad) cell counter using trypan blue exclusion test (Strober, 2001). Every group has 3 replicates and the assay was repeated 3 times.

Scratch assay

This assay was done using Ibidi Culture-Insert (Ibidi, Germany, No.80209). This approach provides two cell culture reservoirs with a separation wall of 500 μm thick. The culture-Inserts were placed in the individual wells of 24-well plate, and 100 μl of DMEM containing 3×10^4 cells was added into each insert. After overnight, the inserts were removed and scratch was created. The cells were washed by pre-warmed PBS and each well was filled with 1ml DMEM containing 2 μg/ml cisplatin, or DMEM alone as control. Then pictures were then taken as 0 h, and 4 or 24 h later, pictures were taken again. The area of the wounds were determined by image J (http://rsb.info.nih.gov) using the freehead selection mode. The migration of cells toward the wounds was expressed as percentage of wound closure:

% of wound closure = $[(at=_{0h}-at=_{\Delta h})/at=_{0h}]\times100\%$, where at=0h is the area of wound measured immediately after scratching, and $at=_{\Delta h}$ is the area of wound measured 4 or 24h after scratching (Yue et al., 2010).

RNA Isolation and cDNA Synthesis

Total RNA was prepared using Trizol reagent (Invitrogen, USA) according to the instructions. First-strand cDNAs were synthesized from 1μg DNaseI-treated total RNA using a TIANGEN FastQuant RT Kit (with gDNase) (TIANGEN, China) according to the manufacturer's instructions.

Real-Time PCR

The expression of a set of gene transcripts was determined in triplicate with real-time polymerase chain reaction using SYBR Green PCR Master Mix(CWBIO, China). The primers for the set of mRNAs were summarized in Table 1. Each PCR reaction was carried out in a final volume of 20 μl containing 10 μl of 2×SYBR Green Master Mix, 0.8 μl of each 10 μM forward and reverse primers and 8.2 μl DEPC treated water and 1μl cDNA. PCR amplification was performed in 40 cycles using the following sequence: 95ºC for 10 min, 95ºC for 15 sec, 60ºC for 1 min . Data were normalized to GAPDH expression as housekeeping gene. LinRegPCR software (Ruijter et al., 2009) was used for quantification of expression, and mRNA expression fold change was calculated.

Statistical analysis

The absorbance value of MTT assay and the expression of the set of mRNA were expressed as mean±SD. ANOVA with Bonferroni post test was used to determine the difference among 3 or more groups while students' T-test was used to determine the difference between 2 groups.

Results

2 μg/ml cisplatin treatment for 24h does not lead to significantly reduced cell viability/ number.

DU145 cells were treated with 0 (as a control), 2, 10 μg/ml cisplatin for 1, 2 and 3 days. And MTT assay was used to assess the viability of each group. As shown in Fig 1A, 2 μg/ml cisplatin treatment for 24h does not significantly decrease the viability compared with the control group, while all other treatment sharply reduced the viability of DU145 in a time- and dose-dependent manner. To confirm the effect of low concentration of cisplatin treatment on the cells, cells were planted in 24-well plate and treated with 2 μg/ml cisplatin for 24h and counted using TC10 (Bio-Rad) cell counter. It revealed no obvious difference between 2 μg/ml cisplatin treatment and control group (Figure 1B).

Cell invasiveness was enhanced by low concentration of cisplatin treatment.

It has been reported that cells surviving cisplatin treatment displayed increased migratory potential (Latifi et al., 2011). And we wanted to see whether low dose of cisplatin, which did not kill cells at 24h time point, could leads to elevated invasiveness. Cell scratch assay showed that after 24h of 2 μg/ml cisplatin treatment, the migratory ability was elevated. Actually, at as early as 4h after cisplatin treatment, the difference between treatment

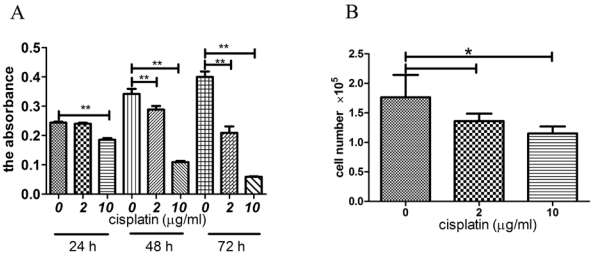

Figure 1. Low Concentration of Cisplatin Treatment for 24h does not Reduce the Viability/Number Significantly. (A) Cell viability was assessed by MTT assay after treatment of 0, 2 and 10 μg/ml cisplatin for 24, 48 and 72h; **(B)** cells were treated with 0, 2 and 10μg/ml cisplatin for 24h and cell number was determined by cell number counting. *p<0.05. **p<0.01

Table 1. The Primers for the Set of mRNAs

Gene	Forward primer	Reverse primer
E-cadherin (Entrez Gene ID 999, approved symbol CDH1)	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG
Vimentin (Entrez Gene ID 7431, approved symbol VIM)	AGTCCACTGAGTACCGGAGA	CATTTCACGCATCTGGCGTT
Snail (Entrez Gene ID 6615, approved symbol SNAI1)	TCGGAAGCCTAACTACAGCG	AGATGAGCATTGGCAGCGA
Slug (Entrez Gene ID 6591, approved symbol SNAI2)	CGAACTGGACACACATACAGTG	CTGAGGATCTCTGGTTGTGGT
OCT-4 (Entrez Gene ID 5460, approved symbol POU5F1)	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA
Nanog (Entrez Gene ID 79923, approved symbol NANOG)	CAGAAGGCCTCAGCACCTACCT ACCCCAGCC	TCTCTGCAGTCCTGCATGCAG TTCCAGCCAAA
MMP2 (Entrez Gene ID 4313, approved symbol MMP2)	TACAGGATCATTGGCTACACACC	GGTCACATCGCTCCAGACT
MMP9 (Entrez Gene ID 4318, approved symbol MMP9)	AGACCTGGGCAGATTCCAAAC	CGGCAAGTCTTCCGAGTAGT
GAPDH (Entrez Gene ID 2597, approved symbol GAPDH)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

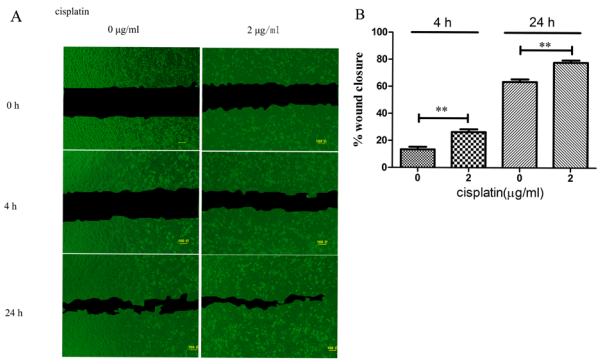

Figure 2. The Migratory Abilities of DU145 Cells in the Presence and Absence of Cisplatin Was Determined by Scratch Assay. (A) The phase contrast images of the assay. Bar: 100 μm; **(B)** The graphs represent percentage of wound closure from three independent experiments. $^{**}p<0.01$

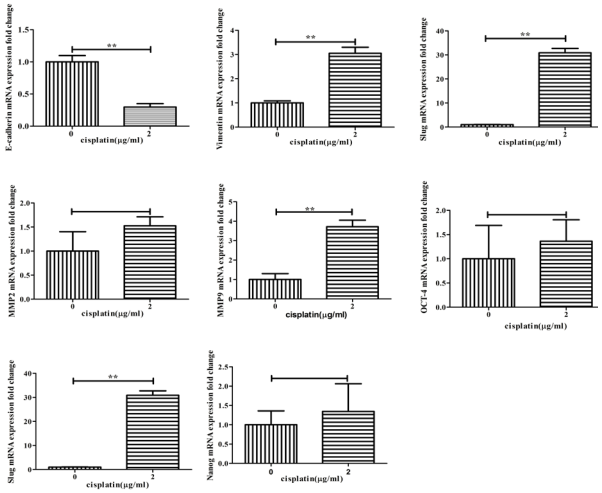

Figure 3. the EMT Markers mRNA Expression Change in Response to Cisplatin Treatment for 24h. $^{**}p<0.01$

group and control group is clear (Figure 2).

EMT markers mRNA expression change in response to cisplatin.

It is now increasingly accepted that EMT confers invasive ability on cancer cells (Gavert and Ben-Ze'ev, 2008; Kalluri and Weinberg, 2009). So we determine whether EMT occurred in the process of cisplatin-induced enhanced migration. The expression of EMT markers including E-cadherin, vimentin, Slug, Snail, Twist-1, OCT-4, metalloproteinase 2 (MMP2), and MMP9 were assessed by real time PCR. As Fig 3 showed, in response to treatment, the expression of E-cadherin mRNA reduced, while that of mRNA of vimentin, Snail, Slug, MMP9 increased significantly. Although MMP2, Nanog, OCT-4 mRNA expression increased, the differences do not reach significance. Furthermore, no obvious change in morphology was observed in the cells treated with 2 μg/ml cisplatin for 24h (Figure 4).

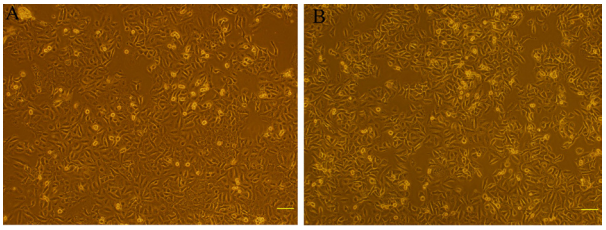

Figure 4. DU145 Cell Morphology Treated with 0 (A) and 2μg/ml Cisplatin; (B) for 24h Respectively. Scale bar, 100μm

Discussion

Platinum complexes are clinically used as adjuvant therapy of cancers aiming to induce tumor cell death and combination-chemotherapy with cisplatin is a cornerstone for the treatment of multiple cancers (Florea and Busselberg, 2011). However, cancer relapses after initial tumor regression often occur, with cancer cells with more malignant phenotype such as resistant to the initial drugs and enhanced invasiveness. However, the detailed mechanisms underlying this phenomenon are not completed understood.

We hypotheses cancer cells' response to cisplatin may provide insights about the process of cisplatin-induced invasive phenotype. So we examined the response of Du145 cells to low concentration of cisplatin in 24h.

Our results showed that 24h low concentration of cisplatin treatment did not reduce the viability/number of

DU145 cells obviously. However, it significantly increased the migratory ability of the cells as assessed by scratch assay. And similar to the increasingly accepted notion that EMT confer cancer cells invasive ability (Gavert and Ben-Ze'ev, 2008; Kalluri and Weinberg, 2009), our data showed some of the EMT markers' mRNA expression changed in the EMT-like way, such as E-cadherin, vimentin, Snail, Slug, and MMP2, while that of OCT-4, MMP9, and Nanog, did not increase that much. This observation was similar to the study of Lang et al (Lang et al., 2012) revealing that in response to heat shock for 30 min or less, cancer cells displayed increased migration with partial EMT.

In conclusion, our study showed that in response to cisplatin, DU145 cells displayed elevated migratory ability and an EMT-like phenotype. Disrupting this response may increase cisplatin's cancer-killing efficiency.

References

Baribeau S, Chaudhry P, Parent S, et al (2014). Resveratrol inhibits cisplatin-induced epithelial-to-mesenchymal transition in ovarian cancer cell lines. *PLoS One*, **9**, 86987.

D'Amico AV, Cote K, Loffredo M, et al (2002). Determinants of prostate cancer-specific survival after radiation therapy for patients with clinically localized prostate cancer. *J Clin Oncol*, **20**, 4567-73.

Du LB, Li HZ, Wang XH, et al (2014). Analysis of cancer incidence in Zhejiang cancer registry in China during 2000 to 2009. *Asian Pac J Cancer Prev*, **15**, 5839-43.

Florea AM, Büsselberg D (2011). Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. *Cancers (Basel)*, **3**, 1351-71.

Gavert N, Ben-Ze'ev A (2008). Epithelial-mesenchymal transition and the invasive potential of tumors. *Trends Molecular Medicine*, **14**, 199-209.

Han R-F, Ji X, Dong X-G, et al (2014). An epigenetic mechanism underlying doxorubicin induced EMT in the human BGC-823 gastric cancer cell. *Asian Pac J Cancer Prev*, **15**, 4271-4.

Han R, Xiong J, Xiao R, et al (2013). Activation of β-catenin signaling is critical for doxorubicin-induced epithelial-mesenchymal transition in BGC-823 gastric cancer cell line. *Tumor Biol*, **34**, 277-84.

Hansen MB, Nielsen SE, Berg K (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunological Methods*, **119**, 203-10.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA: Cancer J Clin*, **61**, 69-90.

Jung K-W, Won Y-J, Kong H-J, et al (2013). Cancer statistics in Korea: incidence, mortality, survival and prevalence in 2010. *Cancer Res Treatment*, **45**, 1-14.

Kalluri R, Weinberg RA (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest*, **119**, 1420-8.

Kelland L (2007). The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer*, **7**, 573-84.

Lang BJ, Nguyen L, Nguyen HC, et al (2012). Heat stress induces epithelial plasticity and cell migration independent of heat shock factor 1. *Cell Stress Chaperones*, **17**, 765-78.

Latifi A, Abubaker K, Castrechini N, et al (2011). Cisplatin treatment of primary and metastatic epithelial ovarian carcinomas generates residual cells with mesenchymal stem cell-like profile. *J Cell Biochem*, **112**, 2850-64.

Ruijter J, Ramakers C, Hoogaars W, et al (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res*, **37**, 45

Siddik ZH (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*, **22**, 7265-79.

Siegel R, Ma J, Zou Z, et al (2014). Cancer statistics, 2014. *CA Cancer J Clin*, **64**, 9-29.

Strober W (2001). Trypan blue exclusion test of cell viability. *Current protoc immunol*, Appendix 3:Appendix 3B.

Yue PY, Leung EP, Mak NK, et al (2010). A simplified method for quantifying cell migration/wound healing in 96-well plates. *J Biomol Screen*, **15**, 427-33.