Oncology and Translational Medicine
DOI 10.1007/s10330-019-0355-5

February 2020, Vol. 6, No. 1, P16–P20

ORIGINAL ARTICLE

Cisplatin selects for CD133+ cells in lung cancer cells

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Abstract

Objective Platinum-based chemotherapy is the first-line treatment for non–small cell lung cancer, but the chemoresistance of tumor cells continues to be a considerable challenge in the management of NSCLCs, leading to recurrence of most patients. CD133 (prominin-1) is a five-transmembrane glycoprotein, and recent evidence suggests that CD133+ cells are the cause of drug resistance and tumor recurrence. In this study, the correlation between cisplatin and CD133+ cells was investigated systematically.

Methods Four lung cancer cell lines, including A549, H460, 801D and H1299, were treated with different concentrations of cisplatin. Cell viability was determined by MTT assay. Sphere-forming assay was performed to detect the capability of sphere-forming. CD133+ cells was detected by BD FACScaliber flow cytometer.

Results The results showed that cisplatin could increase the number of CD133+ cells in both time- and dose-dependent manner. The enrichment would weaken but the proportion of CD133+ cells was still higher than the basic level as incubation time extended after cisplatin was withdrawn. Compared with adherent culture, the proportion of CD133+ cells was higher when the cells were maintained suspension culture. The proportion of CD133+ cells significantly increased when cisplatin was provided in suspension culture.

Conclusion These results revealed that cisplatin induces the enrichment of CD133+ cells and CD133 is a new therapeutic target. Our data partially explained drug resistance to second-line chemotherapy in cisplatin-treated patients with NSCLCs.

Received: 6 May 2019
Revised: 25 May 2019
Accepted: 10 June 2019

Key words: CD133; Cisplatin; lung cancer cells

Lung cancer is the leading cause of cancer deaths worldwide because of its high incidence and mortality. Non-small cell lung cancer (NSCLC) has an estimated 10% 5-year survival rate [1]. Platinum-based chemotherapy is the standard first-line therapeutic approach to treat patients with NSCLCs. Although the overall median survival of patients who received platinum-based therapy has reached 9 to 12 months [2], the chemoresistance of tumor cells continues to be a considerable challenge in the management of NSCLCs. Tumor cells often show initial sensitivity to chemotherapy drugs, but resistance is acquired during treatment, leading to tumor recurrence and further tumor progression.

One emerging hypothesis that explains how cancer cells can withstand therapeutic assaults, acquire resistance, and establish distant metastasis, is the cancer stem cell (CSC) hypothesis. CSCs may be inherently resistant to the cytotoxic effect of chemotherapy because of their low proliferation rate and resistance mechanisms, including the expression of multidrug transporters of the ATP-binding cassette (ABC) superfamily. However, there is no effective treatment strategy to override these transporters for clinical therapy.

CD133 (prominin-1), a five-transmembrane glycoprotein, was initially described as a marker specific to CD34+ human hematopoietic progenitor cells [3–4], the normal stem cells of the neural [5–6], epithelial [7–8], and endothelial lineages [9], and their tumor counterparts [10–14]. A recent study showed that the expression of CD133 is associated with levels of resistance-related proteins in patients with NSCLCs [15]. Furthermore, a combination of CD133 and ABCG2 can be used as an independent predictor of postoperative recurrence for patients with stage I NSCLCs [16]. Although the drug action of cisplatin has been widely explored [17], the correlation between cisplatin and CD133+ cells has not been systematically investigated. Here, we provide evidence showing that cisplatin treatment significantly increased the ratio of CD133+

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cells. The proportion of CD133+ cells would increase as treatment time and dose increased. The enrichment would disappear after cisplatin was withdrawn and incubation time was extended but the proportion of CD133+ cells was still higher than normal levels. The proportions of CD133+ cells were significantly increased when cisplatin treatment and suspension culture coexisted. However, the high proportions only lasted a short time in culture and the high proportions disappeared as culture time extended. Cisplatin induces the enrichment of CD133+ cells. Based on our results, CD133 can be considered a new therapeutic target, suggesting that a new therapeutic strategy may be necessary to prevent the production of CSCs, whereas platinum-based chemotherapy is the standard of care for the management of NSCLCs.

Materials and methods

Cell lines and cisplatin

The human NSCLC cell lines, A549, H460, and H1299 were purchased from Peking Union Medical College Cell Center. The 801D cells were kindly provided by the People's Liberation Army General Hospital. A549, H460, 801D, and H1299 cell lines were maintained in RPMI-1640 supplemented with 10% FBS (Gibco Life Technologies, USA). Cell lines were maintained in a humidified incubator containing 5% CO_2 at 37°C. Cisplatin was purchased from Qilu Pharmaceutical Factory (China) and dissolved in saline to a final concentration of 0.3 mg/mL.

Detection of CD133+ cells

After cisplatin treatment for 1 or 24 h, cells were washed with PBS. For some experiments, single cells were dissociated from tumor spheres and analyzed by this method. One million trypsinized cells were incubated with an anti-CD133 antibody or isotype control IgG (Miltenyi Biotec Inc., Miltenyi Biotec, Germany) for 10 min. The cells were washed and resuspended in a suitable amount of buffer (PBS) before analysis using a BD FACScaliber flow cytometer (BD Biosciences, USA).

Cell proliferation assay

Tumor cells (3,000/well) were seeded in flat-bottom 96-well plates (NUNC, Denmark). Cell proliferation was evaluated by a 3-(4, 5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega) assay, which was performed at a fixed time every day for 5 consecutive days. MTS (20 μL) was added to each well, followed by incubation for 3 h at 37°C. The absorbance was recorded at 490 nm using an EL-800 universal microplate reader (Bio-Tek Instruments, Winooski, VT, USA). This experiment was repeated in triplicate.

Sphere-forming assay

Cells were expanded to spheres in a 10-cm ultra-low adhesion culture dish (Corning, USA) containing DMEM/F-12 with N2 supplement (Invitrogen, USA), 20 ng/mL EGF, and 20 ng/mL basic fibroblast growth factor (FGF; PeproTech, USA), referred to stem cell medium, for 2 weeks. The tumor sphere formation efficiency was calculated as the ratio of sphere number to the plated cell number.

Statistical analysis

Data are presented as the mean ± SE. To analyze the results of the experiments in triplicate, quantitative variables were compared using one way analysis of variance. $P \leq 0.05$ was considered statistically significant.

Results

Cisplatin treatment elevates the ratio of CD133+ cell

To determine whether cisplatin can elevate the ratio of CSCs in lung cancer, we first tested the cytotoxic effect of cisplatin in NSCLC cell lines. Four lung cancer cell lines, A549, H460, 801D, and H1299 were treated with different concentrations of cisplatin for 1 and 24 h, and cell viability was determined by MTT assay. With the increase of drug concentration, the cytotoxicity of cisplatin to the cell is stronger (Table 1). With the increase of drug concentration, the percentages of CD133+ cells were remarkably increased in all four cell lines when the treatment time was same (Table 2). With the increase of the drug concentration, the percentages of CD133+ cells were remarkably increased when the treatment time was different and cisplatin had the same killing effect. For example, the CD133 positive ratio of

Table 1 The cisplatin concentration of IC_{50} and IC_{80} at different treatment times

Cells	24 h (μg/mL)		1 h (μg/mL)	
	IC_{50}	IC_{80}	IC_{50}	IC_{80}
A549	3.5 ± 0.3	5.0 ± 0.2	64.0 ± 3.6	128.0 ± 6.2
H460	1.5 ± 0.2	4.0 ± 0.1	40.0 ± 2.5	80.0 ± 3.2
H1299	3.0 ± 0.1	6.0 ± 0.3	64.0 ± 3.2	128.0 ± 5.5
801D	1.0 ± 0.1	3.0 ± 0.1	20.0 ± 2.5	35.0 ± 4.2

Table 2 the ratios of CD133+ cells after cisplatin treatment

Cells	0 h (%)	24 h (μg/mL)		1 h (μg/mL)	
		IC_{50}	IC_{80}	IC_{50}	IC_{80}
A549	0.01	0.17±0.02	0.24±0.03	0.48±0.06	0.76±0.10
H460	0.03	0.07±0.02	0.10±0.01	0.16±0.06	0.19±0.02
H1299	0.02	0.11±0.02	0.19±0.02	0.20±0.04	0.19±0.05
801D	0.02	0.07±0.01	0.09±0.03	0.13±0.03	0.59±0.06

Table 3 The ratios of CD133+ cells at high dose in different treatment time

Cells	Treatment concentration (μg/mL)	Action time (h)				
		0	12	24	48	72
H1299	128.00	0.02	0.02±0.00	0.07±0.02	0.07±0.02	0.15±0.05
801D	35.00	0.02	0.03±0.01	0.05±0.00	0.09±0.02	0.59±0.02
A549	128.00	0.01	0.04±0.01	0.06±0.01	0.50±0.10	0.76±0.06

Table 4 The ratios of CD133+ cells at low dose in different treatment time

Cells	Treatment concentration (μg/mL)	Action time (h)				
		0	12	24	48	72
H1299	3.00	0.02	0.05 ±0.01	0.11±0.03	0.14±0.02	0.15±0.01
801D	1.00	0.02	0.04±0.00	0.07±0.01	0.09±0.02	0.14±0.02
A549	3.50	0.01	0.04±0.00	0.17±0.03	0.21±0.03	0.23±0.03

IC_{50} to 24 h of drug treatment was lower than that of drug treatment for 1 h. The CD133 positive ratio of IC_{80} to 24 h of drug treatment was lower than that of drug treatment 1 h of drug treatment. These suggested that high doses of cisplatin acting on cells for a short amount of time increased enrichment of CD133+ cells compared to low doses of cisplatin acting on cells for a long period of time. In other words, drug concentration plays a more important role in enriching CD133+ cells than length of treatment.

The ratio of CD133+ cell depends on treatment time and dose of cisplatin

The above results suggested cisplatin treatment elevated the ratios of CD133+ cells in lung cancer cells. We also explore whether the change in ratio is related to the drug concentration and treatment time. In Table 1 and 2, the data showed that with the increase of drug concentration, the ratios of CD133+ cells increased in four lung cancer cell lines. Table 3 showed the conditions at a high dose and Table 4 showed the conditions at a low dose, which suggests that with the increase of treatment time, the ratios of CD133+ cells increased. In summary, the ratios of CD133+ cell depended on treatment times and dose levels of cisplatin.

The proportion of CD133+ cell decreased, but was higher than the basal level with extended culture time after the withdrawal of cisplatin

The above results suggested that the ratio of CD133+ cells depended on treatment times and doses of cisplatin. The data suggest that drug concentration plays a more important role in enriching CD133+ cells. We used high dose cisplatin to treat cells for 1 h (Table 3). Cells were cultured for one month after removal of cisplatin and the proportion of CD133+ cells were examined. The data (Table 5) show the ratios of CD133+ cells decreased but were still higher than the basal level, which was observed when the culture time was extended after the withdrawal of cisplatin.

Table 5 The ratios of CD133+ cells after the removal of cisplatin

Cells	Before cisplatin treatment	Cisplatin treatment for 1 h	One month after removing cisplatin
H1299	0.02	0.19 ± 0.05	0.05 ± 0.01
801D	0.02	0.59 ± 0.06	0.04 ± 0.01
A549	0.01	0.76 ± 0.10	0.06 ± 0.02

Table 6 The cisplatin concentration of IC_{50} and IC_{80} when culture time became long after the removal of cisplatin

Cells	1 h (μg/mL)		Culturing for 1 month after removing cisplatin	
	IC_{50}	IC_{80}	IC_{50} (μg/mL)	IC_{80} (μg/mL)
A549	64.0 ± 3.6	128.0 ± 6.2	85.0 ± 4.5	135.0 ± 5.3
H1299	64.0 ± 3.2	128.0 ± 5.5	80.0 ± 4.9	140.0 ± 6.2
801D	20.0 ± 2.5	35.0 ± 4.2	30.0 ± 3.5	60.0 ± 5.6

Cells tolerated cisplatin when culture was extended after the removal of cisplatin

After being treated with cisplatin for 1 h, the cells were cultured for one month. Then we examined the response of cells to cisplatin. The results showed that the corresponding concentrations of IC_{50} and IC_{80} were higher than the previous concentrations in lung cancer cells (Table 6). These results suggested that the cells could tolerate cisplatin even if they were cultured for a long time after the removal of cisplatin.

The ratio of CD133+ cell increased in sphere-forming assay when cells were treated by cisplatin

Some studies showed that CD133+ cells are highly tumorigenic, and are endowed with stem-like features; importantly, they are unaffected by cisplatin treatment [18]. Tumor-initiating cells (TICs) have been reported to grow in serum-free conditions to form spheres in several solid tumors; the sphere formation assay is widely used to detect TICs [19–20]. For this reason, we performed the sphere-forming assay. We used a high concentration of cisplatin to treat cells for 1 h and then removed it. Then cells were suspended in culture. When cells were cultured for 2 weeks, the ratios of CD133+ cells were higher than those of cells grown using adherent culture. The ratios of CD133+ cells after cisplatin treatment were higher than cells with no cisplatin treatment (Table 7). When the culture time was prolonged to 4 weeks, CD133+ ratios in cells treated with cisplatin reduced, but were higher than those in cells not treated with cisplatin (Table 8).

Table 7 The ratios of CD133+ cells in sphere-forming assay when cisplatin was treated

Cells	Without cisplatin treatment (%)	After cisplatin treatment (%)
A549	0.17 ± 0.02	2.16 ± 0.23
H1299	0.12 ± 0.03	3.10 ± 0.12
801D	4.37 ± 0.10	21.50 ± 3.60
H460	0.54 ± 0.12	7.79 ± 1.20

Table 8 The ratios of CD133+ cells in sphere-forming assay after the withdrawal of cisplatin

Cells	Without cisplatin treatment (%)	After cisplatin treatment (%)
A549	0.10 ± 0.02	1.12 ± 0.23
H1299	0.09 ± 0.01	0.19 ± 0.05
801D	0.14 ± 0.03	0.22 ± 0.06
H460	0.10 ± 0.02	1.61 ± 0.20

Discussion

Platinum-based combination chemotherapy is a standard first-line treatment for advanced NSCLCs. Despite the efficacy of first-line chemotherapy, tumor recurrence is common in most cases [4]. Here, we provide direct evidence that cisplatin enriches CD133+ cells, which exhibit stem-like properties. Our data partially explained drug resistance to second-line chemotherapy in cisplatin-treated patients with NSCLCs. The proportion of CD133+ cells was reduced but was still higher than the normal levels as incubation time was extended. Our results showed that even a small rise of treatment concentration could increase the tolerance of cells to cisplatin. We explored whether this tolerance is due to cisplatin selecting resistant cells rather than selecting CD133+ cells. Studies showed that the treatment of H460 and H661 cells with cisplatin could enrich CD133+ cells. This cisplatin-induced enrichment of CD133+ cells was mediated through Notch signaling, as evidenced by increased levels of cleaved Notch1[21]. CD133+ tumor cells represent a group of cells that are not sensitive to radiotherapy and chemotherapy. These drug resistant cells are the source of tumor recurrence after radiotherapy and chemotherapy [18, 22].

In previous studies with gastric TICs, cells were cultured on non-adherent substrata to form floating spheres [10], and a substratum was used to induce their differentiation into non-tumorigenic cells. This induction effect could not have resulted from the elimination of CD133- cells—which are more sensitive to cisplatin—and preservation of the existing CD133+ cells before treatment because a limited number of cells were killed by a low dose of cisplatin—around the IC20—for each cell line during treatment. However, a high-dose (> IC_{50}) of paclitaxel, which induced significant cell death, showed no effect on the CD133+ cell number in the H460 and H661 cell lines. These results indicated that cisplatin might induce dedifferentiation of NSCLCs. Cells were treated with cisplatin and suspended in culture in this study. When cells were cultured for 2 weeks, the ratios of CD133+ cells were higher than those of a cells cultured under adherent conditions. The ratios of CD133+ cells after cisplatin treatment were higher than those in cells not treated with cisplatin. When the culture time was prolonged to 4 weeks, the CD133+ ratios of cells treated with cisplatin reduced, but were still higher than those of cells not treated with cisplatin.

In conclusion, we provided a direct evidence that cisplatin could enrich CD133+ cells. Compared with CD133- cells, the capability of sphere-forming increased in CD133+ cells. These results revealed that cisplatin induces the enrichment of CD133+ cells, and that CD133 could be a new, promising therapeutic target.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-019-0355-5
Cite this article as: Li JM, Jiang M, Zhao XT, *et al*. Cisplatin selects for CD133+ cells in lung cancer cells. Oncol Transl Med, 2020, 6: 16–20.