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Comparison of the Cellular Glutathione Levels in Response to Cisplatin in Different Cell Lines

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Abstract: In this study, we have evaluated the effects of cisplatin on the cellular total GSH level in different tumor and normal cell lines. Five different cell lines of human hepatic carcinoma (HePG2), human lung adenocarcinoma (A549), human ovarian carcinoma (SKOV3), dog kidney (LLCPK1), Chinese hamster ovary (CHO) and Human gingival fibroblast (GHF1) cell lines were exposed to their respected IC_{50} concentrations of cisplatin for two hours. cisplatin cytotoxicity was measured using clonogenic assay and the total cellular GSH level was analyzed using a photometrical assay. The results showed that cisplatin had different degrees of cytotoxicities on different cell lines as shown by IC_{50} values; o.87 for HepG2, 3.27 for A549, 0.99 for SKOV3, 5.50 for LLCPK1, 5.50 for CHO and 1.60 for GHF1 cell lines and GSH level alterations after exposure to cisplatin were also different for different cell lines that 85.33 for HepG2, 637.00 for A549, 2691.00 for SKOV3, 1388.30 for LLCPK1, 412.60 for CHO and 783.24 for GHF1 cell lines. This study showed that the cellular GSH level increased in LLCPK1, A549, SKOV3 and GHF1 cell lines, but decreased in CHO and HepG2 cell lines versus to the matched controls. The highest significant variation of GSH in cancer cell line was belonging to SKOV3 and in normal cell lines for LLCPK1, after treated with cisplatin. It is concluded that the total GSH variation after exposure to cisplatin is different for different cell lines. We were not able to correlate between the level of resistance to cisplatin (based on the IC_{50} levels) and GSH level or variations in this study. It might indicate the GSH neither the unique, nor the most important mechanism of resistance to cisplatin in these cell lines, in spite of many publications in its favor.

Key words: Cisplatin, Glutathione, HepG2, A549, SKOV3, LLCPK1, CHO, GHF1

Introduction

The discovery and development of platinum compounds has been one of the greatest achievements of cancer chemotherapy in the past three decades. Cisplatin was accidentally discovered by Rosenberg in 1968 as an antibiotic and very rapidly as a very powerful antiproliferative agent against tumor cells. It was rapidly introduced in clinical use, particularly for the treatment of germ cell tumors of testis and ovary with cisplatin (Meister, 1991).

Cisplatin (cis-dichlorodiammineplatinum-II) gained a widespread use against various malignant tumors in different experimental animals (Jeanne and Amy, 2002; Paolicchi and Sotiropuolou, 2003) and in a variety of human malignancies (Hanigan *et al*., 2001). Many of the biological properties and

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effects of cisplatin have been well documented (Meister, 1991; Rooseboom *et al*., 2002) with numerous reports indicating that the cellular DNA could be the primary target in its anticancer activity (Masahiko and Akinori, 2000; Takehiko and Kato, 2001). However, the therapeutic efficiacy of cisplatin is limited due to the development of drug resistance (Brian and Rick, 2002) and major side effect, nephrotoxicity (Kazuhiko and Masanori, 2003). An increased carcinogenic risk with the development of secondary malignancies in animals/patients treated with cisplatin has also been reported (Fokkema *et al*., 1996; Kociba *et al*.,1970). In an attempt to overcome these impediments, the development of many new cisplatin analogues or the use of cisplatin in combination with other agents have been tried with different degrees of success (Prasad and Giri, 1994; Pinto and Lippard, 1985).

Cisplatin is known a source to generate oxygen free radicals. Though the role of free radicals in cisplatin induced nephrotoxicity remains a matter of debate, free radical scavengers have been shown to ameliorate the toxicities of cytotoxic agents (Zamble and Lippard,1995; Timmer-Bosscha and Mulder, 1992; Krakoff, 1979).

Thiol (sulfhydryl) groups, such as those on glutathione (GSH2) and metallothionein (MT), defend the cell against cisplatin (Kempf and Ivankovic, 1986; Treskes and Vander Vijgh, 1993; Masuda and Takahama, 1994; Masahiko and Shimada, 2000). Since the thiolate anion has a high affinity for Pt^{-2}, Pt ions entering the cell may preferentially bind to sulfur atoms rather than the bases of DNA. Although it is easy to over view this protective mechanism in first-time cisplatin receiving patients, continued exposure to the drug ultimately produces resistance due to increased sulfhydryl levels (e.g., GSH and MT). Plant and animal cells eliminate a broad range of hydrophobic toxins from the cytosol to the extra-cellular space after their conjugation with GSH. This transport is mediated by a novel class of organic anion transporters belonging to the family of ATP-binding cassette (ABC) carriers, the GS-X pumps. GS-X pump activity was localized in canalicular and basolateral rat hepatocyte plasma membranes, heart sarcolemma vesicles, human erythrocytes and tumor cells (Brian and Schnellmann, 2002; Kazuhiko and Tokumoto, 2003; Fokkema *et al*., 2002).

Glutathione (L-y-glutamyl-L-cysteinyl-glycine; GSH) is claimed to be the key component of a ubiquitous antioxidant system that defends the cell against the toxic effects of cisplatin.As much as GS-X pumps extrude OS-conjugates of anticancer electrophiles, such as cisplatin and other unmodified anti tumor drugs such as vinblastine and methotrexate, they prompt a threat to cancer therapeutic approaches by causing resistance because the drugs do not accumulate in tumor cells as expected. Also, GS-X pump activity is over-expressed in cisplatin-resistant human leukemia, thus causing acquired resistance to a number of electrophilic agents (Kazuhiko and Tokumoto, 2003; Fokkema *et al*., 2002).

Whether GSH is the principle parameter in the cisplatin cytotoxicity is the main question of this study. If it is so, the intracellular amount of initial GSH, or its consumption after exposure to cisplatin should present the degree of cellular resistance. Variations in cellular GSH content and consumption after the exposure to cisplatin should then represent the degree of sensitivity and resistant to this drug in different cell lines.In this manuscript, we are presenting the cellular toxicity of cisplatin in different cell lines, as well as the cellular GSH levels before and after the exposure to cisplatin.

Materials and Methods

Chemicals

All chemicals (cisplatin, media serum, antibiotics, FBS, OPT, EDTA buffer, buffer IV, tris buffer, TCA%10, DMSO, HEPES, Trepsin) were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisen- hofen, Germany). Standard GSH solutions were prepared freshly for each experiment in 6% (v/v) met phosphoric acid and diluted in phos-phate-EDTA buffer (pH 7.4).

Cell Lines and Clonogenic Assay

All procedures were done in Central Laboratory of Shaheed Beheshti University of Tehran -Iran at September 2004. Human hepatoma cell (HepG2) was purchased from the Pasteur institute in Tehran (Iran). Human lung carcinoma (A549), Human Ovary carcinoma (SKOV3), Human renal normal cell (LLCPK1), Chinese hamster ovary (CHO) and Human gingival fibroblast (GHF1) were as generous gift from Dr. Rakesh Goel, Ottawa Regional Cancer Center and Ottawa, Canada. All cells were grown in DMEM/ F12 media (Gibco BRL, USA) supplied with 10% fetal bovine serum and penicillin (100IU/mL)/streptomycin (100IU/mL), Cells were maintained in a humidified 37°C incubator with 5% CO_2 for three passages before the start of experiments. Cells were exposed to the different concentrations of cisplatin in a range of 0.5 to 10 μM for 1 h. Clonogenic assay was carried out after the cell exposure to cisplatin based on Kazuhiko and Masanori (2003).

Gsh Assay

GSH analysis was performed spectrophotometrically according to the enzymatic method. In brief, different cell lines (HepG2, A549, SKOV3, LLCPK1, CHO and GHF1) were grown to 80% confluences as was described above, in 25 mm plates. Experiment and control cells were harvested and collected in 1 mL of PBS, Commented and subsequently stored at -20°C. Determination of GSH was performed by a modification of the method of Cohn and Lyle. To 0.5 mL of the 100,000 g supernatant, 4.5 mL of the phosphate-EDTA buffer, pH 8.0 was added. The final assay mixture (2.0 mL) contained 100 μL of the diluted cell lines supernatant, 1.8 mL of phosphate- EDTA buffer and 100 μL of the OPT solution. (Fluorescence material which compound in the GSH). Containing 100 μg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to quartz cuvette. Fluorescence at 420 nm was determined with the excitation at 350 nm. The total amount of glutathione in the samples was determined as GSH (μmol) Per million cells using a standard curve obtained by plotting the known amounts of GSH (100; 80; 50; 40; 25 and 12.5 μm), incubated under the same experimental conditions, versus the rate of change of absorbance at 420 nm (r =0.989).

Statistical Analysis

The results are expressed as mean±SEM Differences between means were elaborated by one way analysis of variance or the Kraskal-Wallis method for multiple comparisons. Differences at $p<0.05$ were considered to be significant. IC_{50} was calculated using Graph pad prism software.

Results

Table 1 represents the IC_{50} amounts of cisplatin in different under-investigation cell lines (HepG2, A549, SKOV3, LLCPK1, CHO and GHF1). The IC_{50}s of cisplatin in these cell lines are HepG2: 0.87±0.07, A549: 3.27±0.35, SKOV3: 0.99±0.08, LLCPK1: 5.5±0.35, CHO: 5.5±0.21 and GHF1: 1.60±0.21.

As shown in Table 1, the rank order of IC_{50}s A549 > SKVO3> HepG2 for cancer cell lines and CHO> LLCPKl>GHF1 in normal cells. There is a statistically significant difference ($p<0.05$) between the IC_{50} of cisplatin among normal and cancer cell lines. Among normal cell lines (between LLCPK1 and CHO with GHF1 and then cancer cell lines between HePG2 and SKOV3 with A549, a significant relation was seen ($p<0.05$).

Table 1: The IC_{50} amounts of cisplatin in different under-investigation cell lines

	Cell lines		Cis $\bar{x}$±SEM
IC_{50} (g mL^{-1})	Normal cell lines	HEPG2	0.87±0.07
		A549	3.27±0.35
		SKOV3	0.99±0.08
		LLCPK1	5.50±0.35
		CHO	5.50±0.21
		HGF1	1.60±0.21

Table 2: Comparison GSH Levels in the cancer and normal cell line before and after the exposure to cisplatin

Cancer cell line						Normal cell line					
HePG2		A549		SKOV3		LLCPK1		CHO		HGF1	
control	+ cis	control	+ cis	control	+ cis	control	+ cis	control	+ cis	control	+ cis
$\bar{x}$±SEM	$\bar{x}$SEM	$\bar{x}$±SEM	$\bar{x}$SEM	$\bar{x}$±SEM	$\bar{x}$SEM	$\bar{x}$±SEM	$\bar{x}$SEM	$\bar{x}$±SEM	$\bar{x}$SEM	$\bar{x}$±SEM	$\bar{x}$SEM
177±19	85±8	353±53	637±81	957±108	2691±416	1035±81	10381±261	480±25	412±32	635±23	783±30

Table 2 represents the concentrations of GSH in these cell lines in nmol cells, before and after the 1 h exposure to the IC_{50} of cisplatin for each cell line. Based on this figure, the rank order of total GSH concentration for under investigation cell lines is as LLCPKl>GHF1>CHO in normal cell lines and SKOV3> A549>HepG2 in tomur cell lines. The rank order of cellular GSH level among these cell lines is the same before and after the exposure to cisplatin.

Discussion

Cell lines from many different tumors may develop resistance to cisplatin. In most cases, the level of resistance is less than 50-fold, although there are reports of up to 1000-fold resistance Nevertheless, even a small increase in resistance of a tumor to cisplatin can be clinically important (Meister, 1991).

Biochemical studies have not succeeded in identifying conclusively the basis of resistance in any type of cell selected with cisplatin, but they have defined several mechanisms that can contribute to resistance. The effectiveness of cell killing is a function of how much drug gets into the cell, how much of this actually reacts with DNA, how tolerant the cell is of lesions in its DNA and how effectively it removes these adducts from DNA (Jeanne and Amy, 2002; Paolicchi and Sotiropuolou, 2003).

Glutathione (GSH) is a tripeptide thiol, y-glutamylcysteinylglycine. Concentrations of 0.5-10 mM make it the most abundant thiol in the cell. Glutathione is synthesized in a two-step pathway involving the ATP-dependent enzymes glutamylcysteine synthetase and glutathione synthetase. The first step is rate-limiting and inhibited by glutathione itself and by buthionine sulfoximine (BSO). As a potent nucleophile, GSH reacts with alkylating agents and with cisplatin. The reaction of GSH and cisplatin in a 2:1 molar ratio forms a GSH-platinum complex that is then eliminated from the cell by an ATP-dependent glutathione S-conjugate export pump GSH may protect cells by intercepting reactive platinum complexes before they can react with DNA. GSH also protects cells by supporting DNA repair, possibly by stabilizing repair enzymes such as DNA polymerase. Increased glutathione levels have been found in some cisplatin resistant cells but not in all cell lines. On the other hand, continued exposure to the drug in patients receiving cisplatin, ultimately produces resistance due to increased sulfhydryl levels, e.g., GSH and MT (Masahiko and Akinori, 2000; Takehiko and Kato, 2001).

It has been suggested that the capacity of the cell to synthesize GSH in response to stress may be more important than the steady state GSH level. We have therefore examined the hypothesizes that different cell lines have variable potency in adapting a higher level of GSH level as a protective mechanism in long-term or short-term exposure to platinum compounds, which might reflect in their level of sensitivity to cisplatin (Brian and Schnellmann, 2002; Kazuhiko and Tokumoto, 2003; Fokkema *et al*., 2002).

To investigate on this postulation, we have investigated the effects of cisplatin on the cellular total GSH level of three tumor (HePG2, A549, SKOV3) and normal (LLCPK1, CHO, GHF1) cell lines. Present results showed that different cells have different levels of GSH. A comparison of normal GSH level with the IC_{50} of cisplatin for each cell line does not reveal any kind of correlation, to conclude that intracellular amount of GSH might be the critical index for the cell line resistance to this drug. As an example, although LLCPK1 cell line has the highest IC_{50} value and also GSH level among these cell lines (1035.7 nM/106 cells), but the CHO cell line with the same concentration of IC_{50} contain a median level of GSH (480.1 nM/106 cells) within this group of cells. Our results were therefore failed to show any correlations between the level of intracellular GSH and resistance to cisplatin (Kazuhiko and Tokumoto, 2003; Fokkema *et al*., 2002).

To examine the importance of GSH increase in response to express, cells were exposed to cisplatin. IC_{50} concentrations in the same exposure time were selected for the cisplatin to be exposed for each cell line to produce the same level of potency for the drug and stress on the cell line. As is shown in this study, compare to the matched controls, the cellular GSH level following exposure to cisplatin were increased in LLCPK1, A549, SKOV3 and GHF1 cell lines, but decreased in CHO and HepG2 cell lines. The cellular total level of GSH in various cell lines after exposure to the corresponding IC_{50} concentrations of cisplatin for each cell line was hence different and not as a good indicator of sensitivity to this drug (Fokkema *et al*., 2002; Brian and Schnellmann, 2002; Kazuhiko and Tokumoto, 2003).

On the other hand, intracellular GSH level was evaluated in some but decreased in other cell lines after exposure to cisplatin. We have therefore tried to find any correlation between the decrease and/or increase in GSH and cellular resistant to cisplatin, but was not successful to find any pattern. As examples, SKOV3 (IC_{50} = 0.99) and HepG2 (IC_{50} = 0.87) have shown almost the same level of sensitivity, however, the GSH level was increased dramatically(about 2.5 times), but decreased significantly (about 2 times) in these cell lines, respectively. LLCPK1 and CHO cell lines with the same level of IC_{50} showed the same pattern, as GSH level increased in LLCPK1, but decreased in CHO cell line after exposure to the same toxic level of cisplatin for the same duration of time (Prasad and Giri, 1994; Pinto and Lippard, 1985).

There was also not significant difference between normal and tumor cells in their pattern of GSH level or variation for cisplatin. The highest significant GSH variation in cancer cell line was belong to SKOV3 and between normal cell lines to LLCPK1 after treated with cisplatin (Kempf and Ivankovic, 1986; Treskes and Vander Vijgh, 1993; Masuda and Takahama, 1994; Masahiko and Shimada, 2000).

Answer to questions of cellular recognition of stress after exposure to cisplatin, level and speed of cellular reaction to express GSH related genes and the two-step process of GSH production in different cells and specially possible differences in the cellular uptake and hence the actual exposure of cellular GSH molecule exposure to cisplatin, are remained to be answered in further more detail studies. However, in spite of many publications on the GSH critical role, the fact of our study was not able to show any kind of correlation between GSH level and/or variations with cisplatin sensitivity,

suggests a more important role for other cellular thiol pools (e.g., MT and thiol groups on the chromatin) and resistance mechanisms (e.g., cellular entrance and DNA) repair in cisplatin clinical response (Treskes and Vander Vijgh, 1993; Masuda and Takahama, 1994).

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