Reduction of *cis*-Dichlorodiammineplatinum-Induced Cell Inactivation by Methionine*

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Abstract

The effect of *cis*-dichlorodiammineplatinum(II) (cisplatin) in combination with methionine was investigated on human NHIK 3025 cells cultivated *in vitro*. Simultaneous treatment with cisplatin and high dose methionine was less lethal by means of colony-forming ability than treatment with cisplatin alone. This reduction in cell inactivation was seen only when methionine was present during the exposure with cisplatin and was strongly dependent upon the concentration of methionine. Cells pretreated with methionine before treatment with cisplatin were found to be more sensitive than cells treated with cisplatin alone.

The protective effect of methionine was also compared with benzaldehyde since we have previously found that this drug may inhibit the uptake of cisplatin through the cell membrane. The data suggest that methionine and benzeldehyde protect against cisplatin cytotoxicity independently of each other and by entirely different mechanisms.

Introduction

We have previously reported that the cytotoxicity of *cis*-dichlorodiammineplatinum(II) (cisplatin) against human NHIK 3025 cells is reduced in the presence of benzaldehyde as well as certain other aromatic aldehydes [1, 2], and that this protection is due to aldehyde-induced reduction of the uptake of cisplatin through the cell membrane [2, 3] These findings support the idea that the uptake of cisplatin into cells is due to a transport mechanism which is either active, and energy requiring, or passive, but rate-limited. The influence of the plasma membrane as a barrier against the cytotoxicity of cisplatin has also recently been demonstrated in our laboratory by using the method of electropermeabilization [4]. Furthermore there is a strong correlation between variations in sensitivity to cisplatin and uptake into mammalian cells *in vitro* [5]. According to Byfield and Calabro-Jones it is possible that the uptake of cisplatin into the cells may follow an amino acid transport mechanism [6–8]. This could also explain the inhibition of methionine uptake in the presence of cisplatin, as has been reported by other workers [9, 10]. If cisplatin and methionine are taken up by the same mechanism one might expect high dose methionine to protect cells against cisplatin.

In the present paper we have studied the cell-inactivating effect of cisplatin in combination with high dose methionine in human NHIK 3025 cells. The results show that methionine induces a strong protective effect against cisplatin. As benzaldehyde has been shown to protect against cisplatin by reducing its uptake into the cells, we found it interesting to compare the protective effects of methionine and benzaldehyde. The results indicate that the mechanism of protection is different for the two compounds although both reduce the amount of cell-associated Pt as measured by atomic absorption spectroscopy. These results are discussed in light of their relevance to the mechanism of cellular uptake of cisplatin.

Experimental

Cells and Cell Culturing Techniques

Cells of the established cell line NHIK 3025, derived from human uterine cervix carcinoma *in situ* [11, 12] were used. The cells were routinely grown as a monolayer, at 37 °C in medium E2a [13] containing 30% serum (*i.e.* 20% human serum prepared in the laboratory and 10% horse serum (Gibco)). In order to maintain cells in continuous exponential growth, the cell cultures were trypsinized (0.25% trypsin, Difco 1:250) and recultured three times a week [14]. Cells were routinely recultured the day before use in experiments.

Cell Survival

The inactivating effect of cisplatin was measured as loss of colony-forming ability of cells treated with cisplatin either alone or in combination with benz-

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aldehyde or methionine. The cells were trypsinized and seeded into 6 cm Falcon plastic Petri dishes with 5 ml medium. The cell number was adjusted to give 150 colonies per dish after drug treatment. Two hours after seeding the cells had attached to the dishes, and the medium was replaced by medium containing the drug(s). After treatment the medium was replaced by fresh medium, and the cells were incubated for a total of 12–14 days with a medium change on day 6. The cells were then fixed in ethanol and stained with methylene blue. Only colonies containing more than 40 cells were counted.

Atomic Absorption Spectroscopy

The cells were trypsinized and resuspended in fresh medium. The drugs were added to the cells which were kept in suspension for a 2 h drug treatment period. After centrifuging the cells were washed once in fresh medium before they were dissolved in 100 µl 16 N HNO_3. The day after, 100 µl H_2O was added to each sample, and the amount of cellular-bound platinum was measured using a Varian Spectra A-30 atomic absorption spectrometer fitted with a GTA-96 graphite tube atomizer. Instrument control and data acquisition were by a Varian DS-15 data station using Varian atomic absorption software. The atomic absorption signal was measured in 50 µl aliquots with a platinum lamp at 256.9 nm. Automatic background correction with a modulated deuterium lamp was utilized. The amount of Pt was calculated from a calibration curve run immediately before the samples. Each point was represented by three parallels from which the mean value and standard error (S.E.) were calculated.

Drugs

Benzaldehyde was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Berks., U.K.) and was vacuum-distilled and stored under N_2. *cis*-Dichlorodiammineplatinum(II) (cisplatin) was from Farmitalia Carlo Erba (Barcelona, Spain). It was first dissolved in glycozole [15] as a stock solution with a concentration of 1000 µM before it was further diluted in the growth medium and added to the cells.

Results

In order to study the effect of methionine in combination with cisplatin, both compounds were dissolved in growth medium and added simultaneously to the cells by a change of medium. In Fig. 1 cell survival is shown after 2 h treatment with 15 µM cisplatin and with different concentrations of methionine. While there was a strong inactivating effect of cisplatin alone, cell survival increased with increasing concentrations of methionine up to at least 10 mM which was the highest concentration tested.

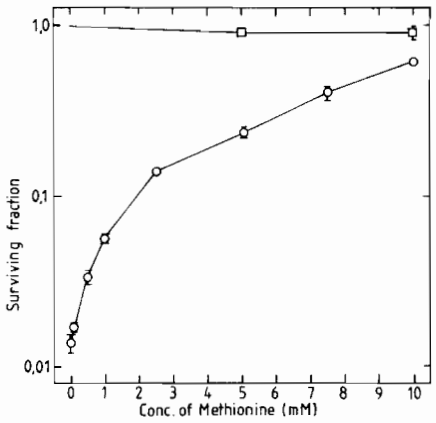

Fig. 1. Survival of NHIK 3025 cells after treatment with different concentrations of methionine either without (□) or with (○) 15 µM cisplatin for a 2 h drug treatment. Standard errors of the mean (S.E.) are indicated when they exceed the size of the symbols.

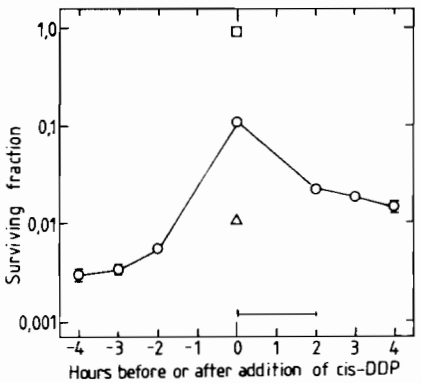

Fig. 2. Survival of NHIK 3025 cells treated with 15 µM cisplatin (*cis*-DDP) for 2 h as a function of scheduling of 2 h pulses of 2.5 mM methionine (○). Cell survival is also shown for cells treated for 2 h with 2.5 mM methionine (□) or 15 µM cisplatin (△) alone. The horizontal bar represents the treatment period for cisplatin and data points are plotted from the time at which drug incubation began. S.E. are indicated when they exceed the size of the symbols.

The data indicate that the cytotoxicity of cisplatin may be almost completely inhibited by methionine. For cells treated with up to 10 mM methionine alone there was no significant effect on cell survival (Fig. 1, upper curve).

The effect on cell survival was also studied when methionine was present either before or after treatment with cisplatin. Figure 2 shows an experiment where the cells were treated for 2 h with 15 µM cisplatin and in addition with a 2 h pulse of 2.5 mM methionine starting either before, simultaneously with, or after the cisplatin pulse. The horizontal line marking from 0 to 2 h is drawn to visualize the cisplatin pulse. Cell survival after this treatment alone was about 1%. The abscissa represents the time when methionine was added. In line with the data of Fig. 1,

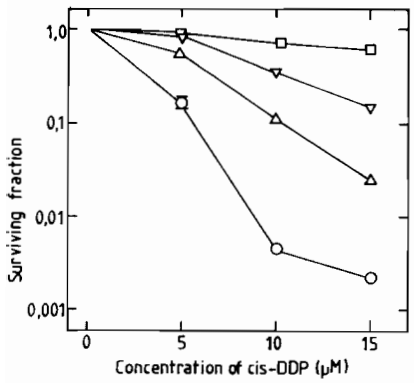

Fig. 3. Survival of NHIK 3025 cells after treatment for 2 h with cisplatin (*cis*-DDP) as a function of drug concentration, either with cisplatin alone (○) or with 2.5 mM benzaldehyde (△); with 2.5 mM methionine (▽); or with both 2.5 mM benzaldehyde and 2.5 mM methionine (□). S.E. are indicated when they exceed the size of the symbols.

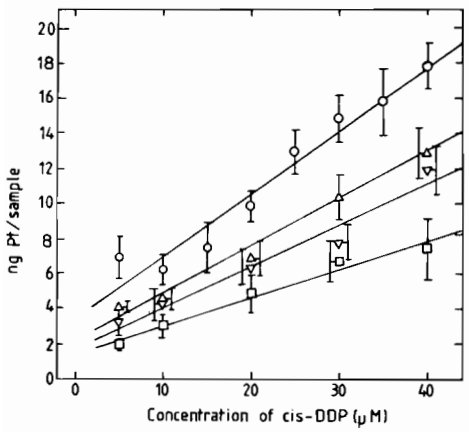

Fig. 4. The amount of cell-associated Pt in NHIK 3025 cells after treatment for 2 h with cisplatin (*cis*-DDP) as a function of drug concentration. The cells were treated either with cis platin alone (○) or with 2.5 mM benzaldehyde (△); with 2.5 mM methionine (▽); or with both 2.5 mM benzaldehyde and 2.5 mM methionine (□). S.E. are indicated by vertical bars.

cell survival was 10 times higher when methionine and cisplatin were present simultaneously than with cisplatin alone. However, when the cells received methionine after treatment with cisplatin, a small or not significant protection was found. In contrast to this, cells pretreated with methionine were found to be somewhat more sensitive to cisplatin than cells treated with cisplatin only.

To compare the protection against cisplatin induced by methionine and by benzaldehyde, cells were treated with different concentrations of cisplatin either in combination with 2.5 mM methionine or with 2.5 mM benzaldehyde or with both methionine and benzaldehyde simultaneously (Fig. 3). In comparison to cells treated with cisplatin alone, there was a strong protection when cisplatin was given in combination with methionine or benzaldehyde. An even stronger protection was, however, found when the cells were treated with both methionine and benzaldehyde in combination.

To measure the amount of cell-associated Pt after these four variants of treatment, atomic absorption measurements were performed (Fig. 4). The data show that while cell-associated Pt increases with increasing concentration of cisplatin, it is reduced by a certain factor by the presence of methionine as well as by benzaldehyde. Furthermore, when benzaldehyde and methionine are present simultaneously, cell-associated Pt is reduced more than when each of the protective compounds is present separately. In Fig. 4 the data are fitted by straight lines by the method of least squares. As indicated, the slope of these lines is lower for cells treated with either benzaldehyde or methionine in combination with cisplatin than for cells treated with cisplatin alone. Within the experimental deviation, the fall in the slope for cells treated simultaneously with both methionine and benzaldehyde together with cisplatin represents a direct addition of the protection induced by each protective compound separately (p = 78%).

Discussion

From Fig. 1 it can be seen that the cell survival increases with increasing concentrations of methionine for all methionine concentrations tested (up to 10 mM). Within this dose range there is no indication of any maximum degree of protection and the data show that the cytotoxicity of cisplatin may be nearly completely inhibited by the simultaneous presence of methionine. This is different from the protection induced by benzaldehyde, which was shown to reach a maximum at about 2 mM benzaldehyde resulting in a 10 times increase in cell survival compared to cisplatin alone [1] (Fig. 3). Thus, the present data suggest that benzeldehyde and methionine may protect the cells against cisplatin by different mechanisms.

The sequencing experiment (Fig. 2) resulted in three main observations. First, if the pulse of methionine was given after cisplatin, no significant protection was found. This suggests that methionine is not active in reversing DNA crosslink formation by cisplatin, as previously found for thiourea [16], and the data also accord with the findings that methionine is not active in reversing protein crosslinks by cisplatin [17]. Secondly, the protective effect of methionine was seen only when treatment with cisplatin and methionine was given simultaneously. Thirdly, if the pulse of methionine was given before cisplatin, the cellular sensitivity to cisplatin was increased. Thus, the effect of methionine on cisplatin cytotoxicity is dual, depending on the sequencing of

the treatment pulses of the two compounds. While the protection induced by methionine during simultaneous treatment is possibly explained by a reduced uptake of cisplatin in the presence of methionine, as observed by the atomic absorption measurements (Fig. 4), the enhanced cisplatin cytotoxicity seen after methionine pretreatment is more difficult to explain. A possible explanation emerges from the observation that methionine reacts with cisplatin to form complexes having different cytotoxicity as compared with cisplatin [18]. Thus, if the intracellular concentration of methionine is elevated at the start of cisplatin treatment as a result of the methionine pretreatment, complexes which are more toxic than cisplatin might be formed intracellularly during cisplatin treatment. While this offers an explanation of the enhanced cisplatin sensitivity after methionine pretreatment it also may explain, at least partly, the cisplatin protection induced by methionine under simultaneous treatment. In this case the complexes between the two compounds are formed extracellularly and have to penetrate through the plasma membrane to exert cellular damage. The plasma membrane may have little permeability to the complex, or the complex could be rapidly inactivated in the extracellular surroundings. Thus, the reduced cisplatin uptake observed during simultaneous treatment with cisplatin and methionine could have two reasons. On the one hand, methionine competes with cisplatin for a transport mechanism. On the other hand, many cisplatin molecules could be bound to methionine extracellularly to form complexes having little ability to enter cells, although they could be toxic once they are inside the cells. However, we also recognize the possibility that cells pretreated with methionine may have changed sensitivity to cisplatin as a result of an altered metabolism.

The data of Figs. 3 and 4 indicate that for both methionine and benzaldehyde protection against cisplatin, as measured by increased cell survival, seems to be a secondary effect due to cellular uptake of cisplatin. Furthermore, the reduced uptake induced by methionine and benzaldehyde, respectively, are additive, suggesting that the two compounds protect the cells against cisplatin by different mechanisms. It was previously found that transport of cisplatin into the cells is influenced by benzaldehyde binding to the plasma membrane [2, 3]. Suggesting that cisplatin is taken up by two different mechanisms, one influenced by benzaldehyde and the other influenced by methionine, one would expect a maximum degree of protection also by methionine in contrast to the results seen in Fig. 1. If an uptake mechanism for cisplatin is influenced by methionine, this could therefore not be the whole explanation for the protective effect of methionine. A more possible explanation in accordance with the data may, however, be that complexes formed between cisplatin and methionine in the extracellular environment may be impermeable to the cells.

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