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CISPLATIN INDUCES THE SMALL HEAT SHOCK PROTEIN HSP25 AND THERMOTOLERANCE IN EHRLICH ASCITES TUMOR CELLS *

Steffi Oesterreich, Hildegunde Schunck, Rainer Benndorf, and Heinz Bielka

Institute of Molecular Biology, Department of Cell Physiology, Robert-Rössle-Str. 10, 1115 Berlin-Buch, FRG

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SUMMARY. Exposure of Ehrlich ascites tumor (EAT) cells to the anticancer drug cisplatin results in an elevated abundance of three isoforms of the small heat shock protein hsp25 without inducing the general stress response as commonly observed after heat shock. The most effective cisplatin concentration (2.5 μM) is also most efficient in arresting cells in S phase suggesting a relationship between hsp25 expression and cell cycle events. Exposure to cisplatin results also in an increased thermotolerance of EAT cells.

Cisplatin is a widely used drug in treatment of carcinomas (1). However, its efficiency is often reduced by innate or acquired drug resistance (1,2). Both, the mechanism of action of cisplatin and the mechanism of cisplatin resistance still remain a matter of discussion. The formation of cisplatin-DNA adducts has been considered the principal site of action of this drug (3). Cells differ in their ability to repair such lesions depending on both, the amount of DNA repair enzymes and the availability of deoxynucleotides. Concerning cisplatin resistance, three mechanisms are known so far: (I) A decreased intracellular drug accumulation, (II) an increased intracellular drug detoxification, and (III) an increased capacity for DNA repair of cisplatin-induced lesions (2,4).

Exposure of cells to cytotoxic chemicals, e.g. alcohol (5) and arsenite (6), induces the synthesis of a set of proteins known as heat shock proteins (hsp's). This reaction is accompanied by the development of transient cross-resistance to several cytotoxic effects, including heat shock. Previously it was shown that among the mammalian heat shock proteins the expression of the small hsp's (human, hamster: hsp27; mouse: hsp25) is related to the acquisition of stress resistance (7,8).

Besides stress-induced synthesis, the expression of hsp25/27 is also related to the presence of the estrogen receptor (9,10), to the growth state (11) and to the aggressive state of tumor cells (12). This concerns the abundance as well as the degree of modification (appearance of posttranslationally phosphorylated isoforms) of this protein.

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ABBREVIATIONS: heat shock protein, hsp; Ehrlich ascites tumor, EAT; Dulbecco's modified Eagle's medium, DMEM; fetal calf serum, FCS; sodium dodecylsulfate polyacrylamide gel electrophoresis, SDS-PAGE; two-dimensional polyacrylamide gel electrophoresis, 2D-PAGE.

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Here we demonstrate that cisplatin induces the synthesis of hsp25 resulting in an elevated abundance of this protein in EAT cells. In contrast to other cytotoxic chemicals, this drug induces specifically hsp25 without eliciting the general stress response. As a consequence of cisplatin-induced hsp25 synthesis, the EAT cells become thermotolerant. Furthermore arguments are provided that hsp25 induction is related to another effect of this drug, namely the arrest of the cell cycle in late S.

MATERIALS AND METHODS

Cell culture and labeling. EAT cells, maintained in DMEM supplemented with 15% heat-inactivated FCS, were used. Experiments were performed with exponentially growing cells (approximately $1x10^6$ cells per 25 cm^2 culture flask). *cis*-Dichlorodiammineplatinum [II] (cisplatin) (Sigma) was added from a stock solution (1 mM in physiological saline) to final concentrations as indicated. To obtain protein synthesis patterns, cells were labeled with [^{3}H]leucine as described in (13). For determination of total protein synthesis, 10^6 cells/ml were kept in siliconized tubes under gentle shaking at 37°C. At zero time, 5 μCi/ml [^{3}H]leucine and after 1 h, cisplatin were added. At the times indicated 50 μl cell suspension was removed, the proteins were precipitated with 10% trichloroacetic acid, and the incorporated radioactivity was measured.

Protein electrophoresis and detection. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (14) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) according to O'Farrell (15) as described in (11). Labeled protein bands were visualized by Amplify (Amersham) and the dried gels were exposed to Hyperfilm MP (Amersham). Western blot analysis was performed as described earlier (16).

Colony formation. After exposure to cisplatin or heat shock, cells were counted and 2000 cells were plated in 35 mm petri dishes in 2 ml 0.3% softagar in DMEM supplemented with 15% FCS. Colony formation was determined 5 days thereafter. Colonies larger than 50 cells were counted. The plating efficiency was approximately 70%.

Flow cytometry. Ethanol fixed single cell suspensions were stained with olivomycin and ethidium bromide. Cell cycle analysis was carried out on a fluorocytophotometer as described (17).

RESULTS

Induction of hsp25 by cisplatin

Under normal conditions, in vitro cultivated, exponentially growing EAT cells contain only traces of the small murine stress protein hsp25 (Fig. 1). A 24 h exposure of EAT cells to cisplatin results in the accumulation of this protein. Within the tested range of cisplatin concentrations (0.25...20 μM), 1-5 μM was found to be most efficient resulting in a similarly increased hsp25 abundance as obtained after heat shock (1 h: 41.0°C, 2 h: 37.0°C) (Fig. 1). Analysis of the hsp25 isoform patterns after exposure of cells for 24 h to 1 μM cisplatin (Fig. 2c) reveals an increased abundance of all three major hsp25 isoforms with an increased proportion of the phosphorylated isoforms hsp25/2 and/3 as compared to control cells (Fig. 2a) which preferentially contain the non-phosphorylated isoform hsp25/1. Thus, the cisplatin-induced hsp25 isoform pattern is similar to that induced by a single heat shock (Fig. 2b) (13).

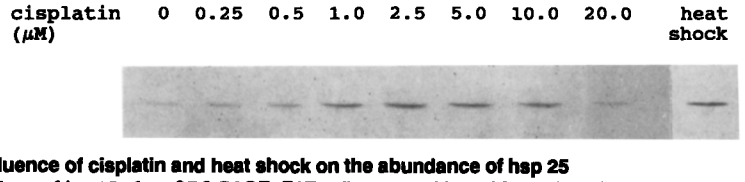

Fig. 1. Influence of cisplatin and heat shock on the abundance of hsp 25
Western blots of hsp25 after SDS-PAGE. EAT cells were cultivated for 24 h in the absence or presence of cisplatin at concentrations as indicated. The heat shock was 1 h: 41.0°C, 2 h: 37.0°C.

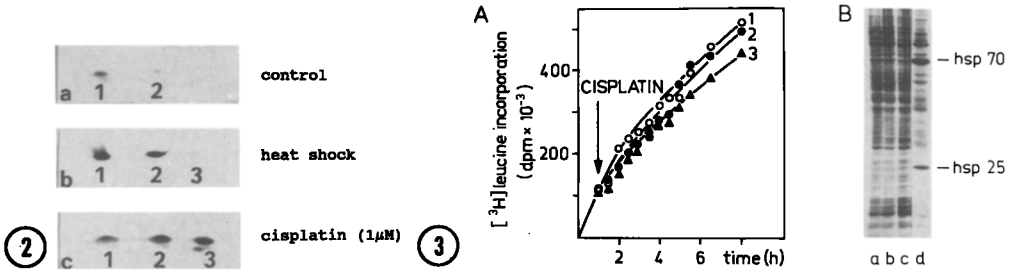

Fig. 2. Effect of heat shock and cisplatin on isoform patterns of hsp25
Western blots of hsp25 isoforms after 2D-PAGE. a, control; b, heat shock (1 h: 41.0°C, 2 h: 37.0°C); c, 24 h incubation in the presence of 1.0 μM cisplatin.

Fig. 3. Effect of cisplatin on total protein synthesis and protein synthesis pattern
A, Total protein synthesis of cells cultivated in the absence (1) or in the presence of 1.0 μM (2) or 10.0 μM (3) cisplatin.
B, Fluorogram of [^{3}H]leucine-labeled EAT proteins separated on SDS-PAGE. Cells were incubated in the absence (lanes a, control) or in the presence of 1 μM cisplatin for 3 h (lane 2) or for 24 h (lane 3) or were heat shocked (1 h: 41.0°C, 2 h: 37.0°C)(lane d). During the last hour of incubation 50 μCi/ml [^{3}H]leucine was present.

By two criteria we can exclude that cisplatin-mediated hsp25 induction is the result of a general stress response. Firstly, total cellular protein synthesis is almost unaffected by exposure of EAT cells to 1.0 μM or 10 μM cisplatin (Fig. 3A, curves 2 and 3, respectively) as compared to control cells (curve 1). Similar results for EAT cells have been described by Pinto and Lippard (3). In contrast, total protein synthesis declines immediately after heat shock treatment (18). Secondly, neither short (3 h)(Fig. 3B, lane b) nor long time (24 h)(Fig. 3B, lane c) exposure of EAT cells to 1.0 μM cisplatin results in protein synthesis pattern characteristic of the heat shock response. Compared with the control (Fig. 3B, lane a), a 3 h exposure of cells to cisplatin causes a weak (Fig. 3B, lane b) and a 24 h exposure a moderate induction of hsp25 synthesis (Fig. 3B, lane c). In any case, the synthesis of further heat shock proteins (e.g. hsp70), as observed after heat shock (Fig. 3B, lane d), is not induced by cisplatin.

Cisplatin-caused cell cycle arrest

Since cisplatin is known to interfere with the cell cycle (19), we tested its effect on the passage of EAT cells through the cell cycle at concentrations which induce hsp25 (Fig. 4). The flow cytophotometric analysis reveals that, in the range from 0.25...5.0 μM, cisplatin causes an arrest in late S resulting in an almost complete depletion of the 4C and in a reduction of the 2C cell population at 1.0 and 2.5 μM when compared with the control. Obviously, a further increase of cisplatin concentration to 10 and 20 μM additionally blocks progression through early S and G1 which is accompanied by a strong accumulation of 2C cells. Alternatively, the high cytotoxicity of this drug concentration may reduce the viability, thus preventing cells to leave their cell cycle compartment. This might explain the similarity of the profiles with the control cells. It is obvious that cisplatin concentrations which are most effective in arresting EAT cells in late S lead also to the most pronounced increase in the accumulation of hsp25.

Effect of cisplatin treatment and subsequent heat shock on cell survival

In the experiment described here we addressed the question if cisplatin-mediated hsp25 accumulation is accompanied by the acquisition of thermotolerance. A single heat shock (1 h: 42.0°C) causes a reduction in cell survival to 66% (Fig. 5A, 1b) as compared to control cells (100%)(Fig. 5A, 1a). Exposing cells to

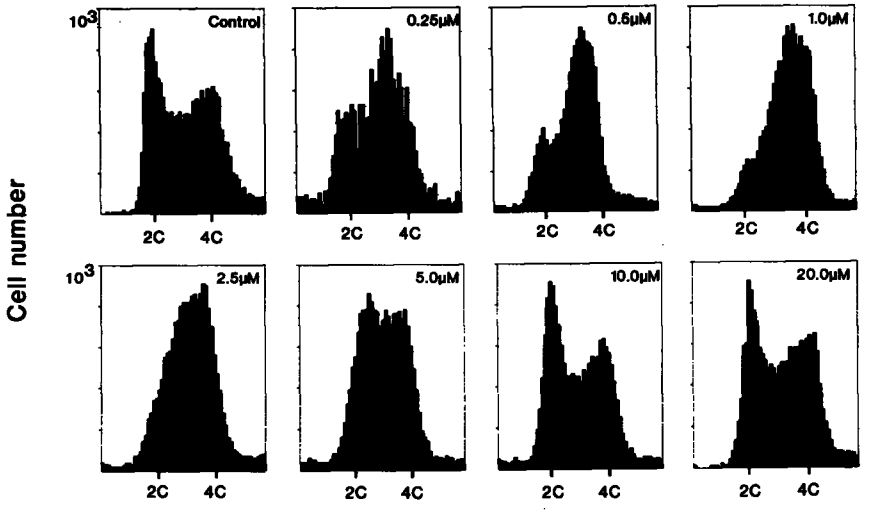

Fig. 4. Effect of cisplatin on passage of EAT cells through cell cycle
For flow cytometric analysis, EAT cells were grown for 24h in the absence (control) or in the presence of cisplatin at concentrations as indicated and were thereafter processed for flow cytometric analysis as described in (17).

1 μM cisplatin for different periods of time (0.5, 6, 12, or 24 h) yields survival rates of 74, 48, 52, and 58%, respectively (Fig. 5A, columns 2a-5a). Cells pretreated in this manner (1 μM cisplatin for 0.5, 6, 12, or 24 h) were then exposed to a subsequent heat shock (1h: 42.0°C). This results in increased thermotolerance with survival rates of 80, 78, 120, and 115%, respectively, in comparison to cells not pretreated with cisplatin. This increase in thermotolerance is obviously related to the increased abundance of hsp25 caused by pretreatmet of cells with cisplatin, since thermotolerance is most pronounced in cisplatin pretreated cells with the highest hsp25 level (Fig. 5B).

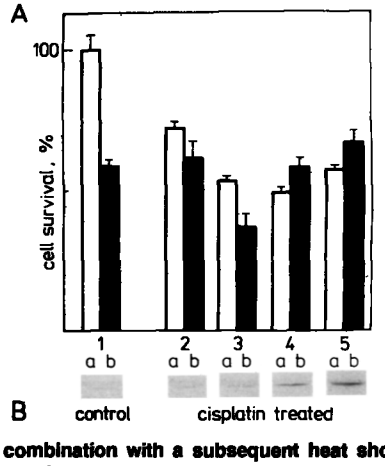

Fig. 5. Effect of cisplatin in combination with a subsequent heat shock on survival of EAT cells determined with the colony formation assay
A. Cell survival of untreated cells (1) or after exposure to 1 μM cisplatin for 0.5 h (2), 6 h (3), 12 h (4), or 24 h (5) without (a) or with a subsequent heat shock (1 h 41.0°C) (b). Only colonies larger than 50 cells were counted. Plating efficiency was approximately 70 % throughout the experiment. B. Western blots, showing the abundance of hsp25 in cisplatin treated cells used for the subsequent heat shock treatment.

DISCUSSION

This study provides evidence for a specific increase in synthesis and abundance of the small heat shock protein hsp25 after exposure of EAT cells to cisplatin. No other major changes of the protein synthesis pattern and of total protein synthesis were detected as observerd after heat shock. This excludes the possibility that hsp25 induction by cisplatin is the result of a general stress response.

The moderately increased synthesis rate of hsp25 after cisplatin treatment (Fig. 3B, lanes b,c) is obviously sufficient to accumulate similar large amounts of this protein as obtained after heat shock (Fig. 1). It is not known, whether an altered degradation of hsp25 contributes to its accumulation. Another possibility to explain the cisplatin-caused hsp25 accumulation is a cell cycle dependent expression of this protein. The cisplatin arrested late S phase cells are supposed to contain an elevated level of hsp25. This was concluded from previous findings according to which hsp25 is accumulated in in vivo-cultivated stationary EAT cells (11) which have an increased frequency of late S and G2 phase cells (20).

The hsp25 induction by cisplatin is by our knowledge a new effect of this drug which might be the basis for another mechanism of cellular resistance. Indeed it was shown by others, that the expression of the small mammalian heat shock protein, especially the occurrence of phosphorylated isoforms, is related to stress resistance (8,13,21) . This includes heat shock (8,13) and chemical stress, caused by several drugs like doxorubicin, daunorubicin, vincristin, and VP16 (recently described as atypical multidrug resistance) (21).

The cisplatin-caused increase in synthesis and abundance of the small mammalian heat shock protein seems to be of direct importance for therapeutic concepts. Cisplatin has been found to be more effective under hyperthermic conditions in in vitro systems (22, 23) and in human cancer therapy (24). However, successful growth inhibition of tumors strongly depended on the conditions of treatment, like the time interval between exposure to cisplatin and heat, which could not be explained by the authors (25). Involvement of the small mammalian stress protein sheds new light on these findings. It seems interesting to study the time kinetics of hsp25/27 induction, development of drug resistance, and therapeutic effects of chemo- and heat treatment in more detail. This line of experimentation is expected to lead to improved chemo- and hyperthermic therapy concepts, taking into account resistance of tumor cells.

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