Correlation between HSP90 Induction Kinetics in Murine Leukemia Cells and the Amount of Cisplatin over a Wide Range of Cytostatic Concentrations

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The induction of HSP90 in murine erythroleukemia cells, clone F4 N, by *cis*platin (DDP) was examined using indirect immunofluorescence and avidin-biotin technique, and compared with *cis*platin cytotoxicity. A reverse dependence of HSP90 induction time was found on a wide range of *cis*platin concentrations (0.5–10 μM), which proved to be cytostatic up to 48 h of continuous treatment. Thus, the observed induction pattern of HSP90 in F4 N cells strictly correlated with their high tolerance toward DDP. This indicates that HSP90 might be responsible, at least in part, for *cis*platin resistance of F4 N cells.

Introduction

HSP90 (90-kDa heat shock protein) is one of the most abundant cytosolic proteins in eucaryotes (up to 1% of soluble protein even in absence of stress). It is involved in conformational stress regulation of key signaling molecules as steroid receptors and kinases (Buchner, 1999). In addition to this role, HSP90 is thought to contribute to protein homeostasis under physiological and stress condition. Heat induced chaperone activity (Yonehara *et al*., 1996), as well as participation of HSP90 in cooperative action with HSP70 (70 kDa HSP) in protein renaturation (Schumacher *et al*., 1996, Pearl and Prodromou, 2000) has been shown.

Cisplatin (*cis*-diamminedichloroplatinum, DDP) is a potent antitumor drug worldwide used in chemotherapy of human cancers. Its cytotoxicity is believed to be due to the formation of intra- and inter-strand DNA crosslinks (Sherman and Lippard, 1987). DNA damage induced by *cis*platin may be a trigger for induction of HSPs. In fact, DDP has been shown to induce the accumulation of a variety of stress proteins, including HSP25, HSP60 and HSP70 (Wu and Welsh, 1996, Matsumoto *et al*., 1996). The induction of HSPs, their profile and timing after treatment with platinum complexes has been suggested to be indicative of the cytotoxicity of these complexes (Fujita *et al*., 1995). There is accumulating evidence that HSP27 and HSP70 are associated with drug resistance in human tumor cells (Ciocca *et al*., 1992, Vargas-Roig *et al*., 1998, Abe *et al*., 1999). Moreover, HSP70 was suggested as a marker of cellular resistance toward chemotherapeutics (Roigas *et al*., 1998).

The data about the effect of *cis*platin on the induction of HSP90 are limited. Elevated synthesis of HSP90 has been reported in rat kidney upon *in vivo* treatment with DDP (Satoh *et al*., 1994). The role of HSP90 in the increased resistance to cancer chemotherapy is not fully understood.

Here, we examined the effect of *cis*platin on the induction of HSP90 in murine leukemia cells. We present evidence that there is a reverse dependence of the time-course of HSP90 induction on the amount of DDP over a wide range of concentrations, proved to exert cytostatic activity.

Results and Discussion

The induction of HSP90 in murine F4 N cells by *cis*platin was studied using a monoclonal anti-human HSP90 antibody. This antibody is specific for HSP90 as can be seen in Fig. 1. To test whether this antibody recognizes also murine HSP90, F4 N cells were heated for 1 h at 42 °C and allowed thereafter to recover at 37 °C for 2–9 hours. At different recovery times cells were fixed, treated with the monoclonal anti-human HSP90 antibody, followed by FITC-conjugated antimouse IgG, to visualize the induced HSP90 by indirect immunofluorescence. The antibody recognized the

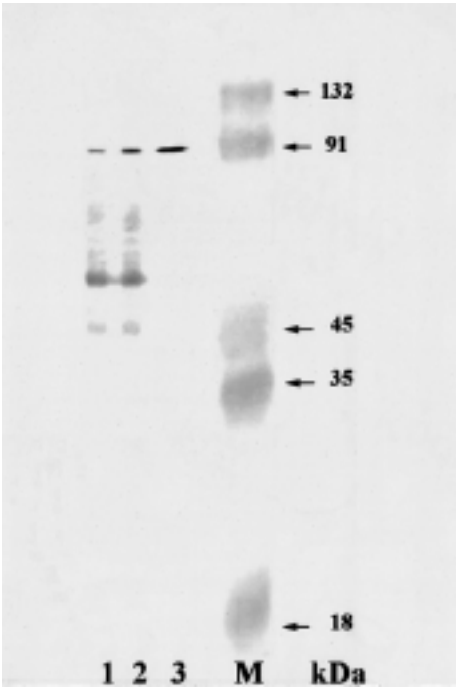

Fig. 1. 12% SDS-PAGE of liver cell lysate followed by Western transfer onto PVDF membrane and immunostaining with 3F12 monoclonal antibody (lane 3), goat anti-human HSP90a polyclonal antibody (StressGen Biotechnologies Corp., Victoria BC, Canada) (lane 1), goat anti-human HSP90b polyclonal antibody (StressGen Biotechnologies Corp., Victoria BC, Canada) (lane 2), prestained molecular weight markers (Bio-Rad, München, Germany) (M).

increased levels of murine HSP90 in the heat-shocked cells in comparison with the background HSP90 levels of unstressed cells (controls). Elevated levels of HSP90 were observed after 5 and 7 h of recovery, reaching a maximum after 9 hours. Upon 24 h recovery time, the HSP90 level returned to that of controls (results not shown). These data are in accordance with the results of Samali and Cotter (1996) on heat induction of HSP90 in human monoblastoid cells.

Further, the induction of HSP90 in F4 N cells by *cis*platin was examined. The cells were incubated for 6–48 h with *cis*platin in concentration range of 0.05–20 μM. At defined times the cells were fixed and analyzed for HSP90 level by indirect immunofluorescence, or using avidin-biotin technique. Both of the methods gave identical staining pattern. As early as 6 h treatment with 20 μM DDP resulted in a strong increase of HSP90 cellular level (Fig. 2).

For quantitative evaluation of 90 kDa staining, the mean optical density of treated cells (15–20 cells) was determined using absorption-transmission photo mode program at dual-wave length measurement (690 and 500 nm), and expressed as a percent of control cell density (Table I). Upon 12 hr of continuous drug treatment of F4 N cells, the highest 90 kDa protein staining was observed

Table I. Induction of HSP90 in F4 N cells by cisplatin. HSP90 induction was followed by indirect immunofluorescence and avidin-biotin technique using a monoclonal HSP90 antibody. The mean optical density of treated cells (15–20) was determined on the film by a dual-wave length measurement (690 nm and 500 nm) using absorption-transmission photo mode program, and expressed as a percent of control cell density.

Cisplatin	Mean optical density (% of control)			
[μM]	Time of treatment (h)			
	12	18	36	48*
0.5	–	–	310	334
1	–	150	473	261
5	128	236	403	118
10	143	189	250	–

* Determined by avidin-biotin technique.
Values are representative of 3 independent experiments.

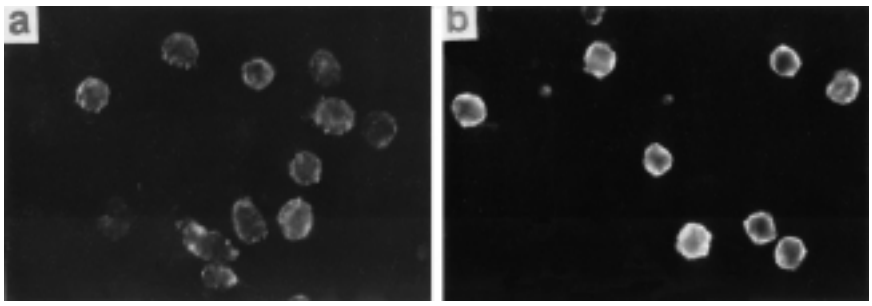

Fig. 2. Induction of HSP90 in F4 N cells by cisplatin as analyzed by indirect immunofluorescence. The cells were treated for 6 hours with 20 μM cisplatin (b); untreated cells (a).

with 10 μM *cis*platin. After prolonged drug exposure, the pattern of HSP90 induction and/or accumulation changed: upon 18 h incubation, the maximum of HSP90 staining was reached with 5 μM *cis*platin; after 36 h, a most intense fluorescence was registered with 1 μM *cis*platin, and after 48 h of drug exposure, the cells exhibited a most intense 90 kDa staining with 0.5 μM *cis*platin as detected by the highly sensitive avidin-biotin technique. Interesting correlation could be observed when putting together this induction kinetics and the cytotoxicity of DDP at the above concentrations. The cytotoxicity of *cis*platin was assessed by a cell growth assay. The growth-inhibitory effect of 18, 24 and 48 h incubation of F4 N cells with DDP are presented in Table II. In the range of 0.5–10 μM, and up to 48 h of continuous treatment *cis*platin produced only a cytostatic effect. Thus, over a wide range of cytostatic DDP concentrations, there is a reverse dependence of HSP90 induction time on *cis*platin amount, as schematically presented in Fig. 3. Cisplatin concentrations that did not significantly influence cell growth (0.05 μM) did not induce HSP90 (results not shown).

There are limited data on the role of HSP90 in DDP resistance of tumor cells. No correlation has been reported between expression of HSP90, and the extent of intrinsic DDP sensitivity of some human tumor cell lines (Hettinga *et al.*, 1996). In the present study, however, the observed induction pattern of HSP90 upon DDP treatment of F4 N cells strictly correlated with their high tolerance toward this agent. This indicates that HSP90 might be responsible, at least partly, for *cis*platin resistance of these cells.

Table II. Cytotoxicity of cisplatin in F4 N cells.

Concentration [μM]	Cell number (x 10^6/ ml) 18 h	24 h	48 h
10	0.33 ± 0.05	0.26 ± 0.04	0.25 ± 0.03
5	0.36 ± 0.04	0.36 ± 0.03	0.30 ± 0.03
1	0.36 ± 0.02	0.37 ± 0.04	0.34 ± 0.04
0.5	0.37 ± 0.02	0.52 ± 0.07	0.54 ± 0.06
0.2	–	0.48 ± 0.06	0.83 ± 0.07
0.05	–	0.49 ± 0.05	1.20 ± 0.11
Control	0.31 ± 0.03	0.57 ± 0.06	1.77 ± 0.24

Values are means ± SD of triplicate determinations in 3 independent experiments.

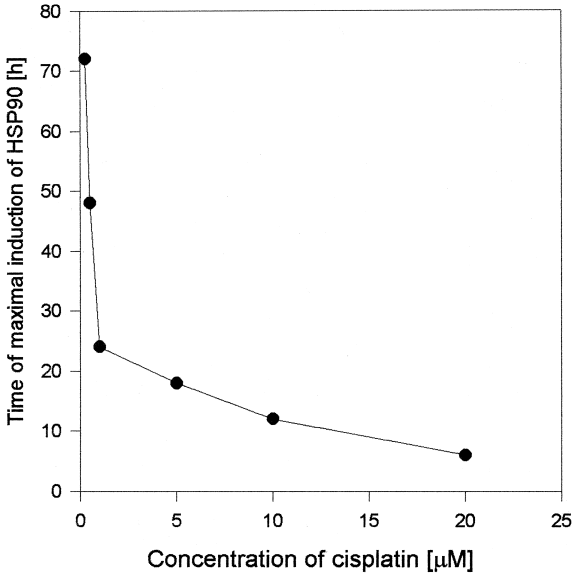

Fig. 3. Dependence of the time of maximal induction of HSP90 in F4 N cells on DDP concentration.

Experimental

Murine erythroleukemia cells, clone F4 N, were cultured in minimum essential medium supplemented with 10% calf serum under 5% CO_2 atmosphere at 37 °C. The cultures were passed every day at a concentration of 5×10^5 cells/ml.

DDP was synthesized as described elsewhere (Spassovska *et al.*, 1981). DDP was dissolved immediately before use in water to obtain stock solutions. Each of these solutions was then used at 1% concentration in the experiments. Exponentially growing cells were treated with different concentrations of *cis*platin for different periods of time and counted thereafter hemocytometrically. The number of dead cells was determined by staining with trypan blue. The mean of triplicate determinations of three independent experiments and the standard deviation was calculated.

Production and characterization of monoclonal antibodies, specific for human HSP90, has been previously described (Kyurkchiev *et al.*, 1985, Kenderov *et al.*, 2000).

Indirect immunofluorescence was performed as follows: The cell suspension was washed in phosphate buffered saline (PBS), fixed and permeabilized by exposure to absolute methanol at -4^0C for 10 min. The primary antibody (3F12, hybri-

doma supernatant) was added to the cells and they were incubated overnight at 4 °C. After rinsing in PBS, the cells were incubated for 1 h with FITC-conjugated rabbit antimouse IgG (Sigma Chemicals, USA) diluted 1:50 with PBS containing O.2% BSA, 0.1% NaN_3, 0.1% Triton X-100. After 45-min rinse in PBS, the cells were mounted in 90% glycerol in PBS and analyzed in a Zeiss epifluorescence microscope equipped with interference filters for FITC. Fotomicrografs were recorded using a Zeiss MC-100 camera system and Kodak T-MAX 400 film.

The avidin-biotin-peroxidase (ABC) technique of Hsu *et al.* (1981) was applied.

Controls for the light immunocytochemistry were performed as follows: (a) the first antibody was omitted or replaced with normal mouse serum; (b) the ABC procedure was omitted; (c) incubation with control Mab (5G5 which recognized 59 kDa antigen (Russinova *et al.*, 1994) instead of the primary antibody).

Densitometer measurements were performed using a Shimadzu dual-wave length TLC Scanner CS, 930. The mean optical density of treated cells (15–20 cells) was determined on the film by a dual-wave length measurement (690 nm and 500 nm) using absorption-transmission photo mode program, and expressed as a percent of that of control cells (100%).

Acknowledgements

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