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Induction of the small stress protein, hsp25, in Ehrlich ascites carcinoma cells by anticancer drugs

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Abstract

Treatment of in vitro cultured Ehrlich ascites carcinoma cells with cisplatin, daunomycin, doxorubicin, cytosine arabinoside, 3'-fluorodeoxythymidine, colchicine and vincristine in cytostatically effective concentrations results in significantly increased levels of the small stress protein, hsp25, as analyzed by immunoblotting. However, no induction of hsp25 could be detected after treatment of the tumour cells with 5-fluorouracil, aminopterin, amethopterin, mithramycine and cyclophosphamide. None of these cytostatic drugs induces hsp70.

Key words: Stress protein; Heat shock; Anticancer drug; Tumour cell

1. Introduction

Exposure of cells to heat shock and various chemicals results in synthesis and accumulation of different classes of stress proteins, which also correlates with the development of a transient state of cross-resistance to other stress inducing agents (for reviews see [1–3]). Generation of resistance is one of the problems in cancer therapy. Besides metabolic degradation of cytostatics and development of multi-drug resistance of cells by activation of the *mdr-1* gene, stress proteins may also be involved in acquired resistance of tumor cells against anti-neoplastic treatments. In a previous paper [4] we reported on the induction of the small stress protein, hsp25, in Ehrlich ascites carcinoma cells by cisplatin. Because of the possible clinical importance of this finding, we tested further anticancer drugs which interfere with different biochemical pathways or act on molecular targets involved in cell proliferation, for their ability to induce the mammalian stress proteins, hsp25 and hsp70.

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Abbrevations: DMEM, Dulbeccos's modified Eagle's medium; EAC, Ehrlich ascites carcinoma; FCS, fetal calf serum; hsc70, constitutively expressed 70 kDa heat shock protein; hsp, heat shock, or more generally, stress inducible proteins (hsp25 murine, hsp27 human low molecular stress protein; hsp70 inducible stress protein of the high molecular family); SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

2. Experimental

2.1. Cultivation of EAC cells

Ehrlich ascites carcinoma cells (EAC cells) were cultured in 5 ml DMEM supplemented with 15% heat inactivated FCS in a humified CO_2 incubator. $2 \cdot 10^6$ cells per 20 ml flask were seeded in the assays with drugs which were applied at final concentrations (see Fig. 1) that resulted in 100% inhibition of cell proliferation. In the controls without drugs, starting with $1 \cdot 10^5$ cells, the increase in cell number during the total incubation time of 72 h was about 20-fold. According to this protocoll, the final number of cells at the end of incubations was about $2 \cdot 10^6$ both in the controls and in the assays with drugs. Cells were incubated at 37°C at first for 48 h without (controls) or in the presence of the respective drug. Thereafter, drugs were washed out and then the cells cultured for a further 24 h period for recovery before being processed for hsp25 and hsp70 detection by immunoblotting. Heat shock induction of hsp25 and hsp70 was performed by incubation of EAC cells for 1 h at 41.0°C, followed by a 4 h recovery period at 36.8°C and a second heat shock for 2 h at 42.5°C.

2.2. Western blot analysis of hsp25 and hsp70

EAC cells were lysed in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 3% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.1% (w/v) Bromophenol blue) by boiling for 5 min. Proteins (50 μg per lane, equivalent to $5 \cdot 10^5$ cells) were separated by SDS-PAGE [5]. After electrophoresis, Western blotting was performed as described [6,7]. Anti-hsp25 antibodies were raised in rabbits against mouse hsp25 as described [6,7]. Anti-hsp70 monoclonal antibodies, specific for the inducible form (SPA-810AP; clone C92F3A-5), and anti-hsc70 antibodies for the constitutive form (SPA-815; clone 1B5) were purchased from StressGen Biotech. Corp., Vic., Canada.

2.3. Anticancer drugs

Amethopterin (methotrexate), aminopterin, cisplatin (*cis*-diamminedichloroplatinum), cyclophosphamide, daunomycin (daunorubicin), doxorubicin (adriablastin, adriamycin), mithramycine and vincristine were from Sigma, colchicine from Fluka, cytosine arabinoside from Ferak, 5-fluorouracil from La Roche; 3'-fluorodeoxythymidine was synthezised and supplied by Dr. M. v. Janta-Lipinsky (Max-Delbrück-Center, Berlin).

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3. Results

Cultured EAC cells in the exponentially growing phase contain hsc70, the constitutive form of the hsp70 stress protein family (data not shown), but they do not express hsp25 and hsp70 in measurable amounts (Fig. 1, lane 1). Exposure of EAC cells to heat shock (see section 2) leads to significant induction of hsp25 and hsp70 (Fig. 1, lanes 2 and 15), while the amount of hsc70 is not increased above the constitutive level (not shown). Cisplatin, doxorubicin, daunomycin, cytosine arabinoside, 3′-fluorodeoxythymidine, colchicine and vincristine induce significant expression of hsp25 at concentrations required to achieve complete inhibition of cell proliferation (Fig. 1, lanes 3–9). However, no induction of hsp25 could be detected for 5-fluorouracil, aminopterin, amethopterin (methotrexate) and mithramycine. Cyclophosphamide, which exerts its cytostatic effect after in vivo transformation, does not induce hsp25 in in vitro cultured EAC cells. Furthermore, hsp25 could not be dedected in EAC cells after exposure to 1.0 Gy ^{60}Co generated gamma rays (5 Gy/min dose rate) as applied in human cancer radiotherapy (S. Oesterreich and G. Erzgräber, data not shown). None of the tested cytostatic substances induces hsp70 (Fig. 1).

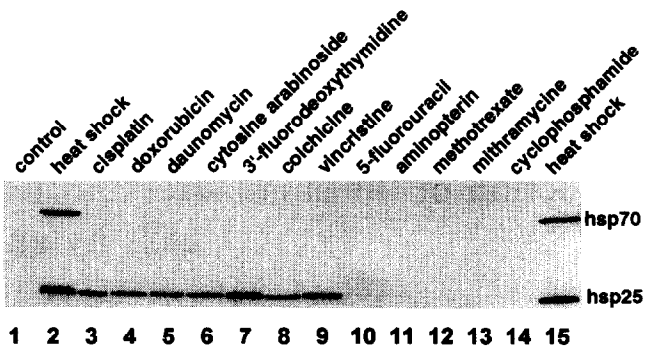

Fig. 1. Stress protein response of in vitro cultured Ehrlich ascites carcinoma cells to heat shock and to anticancer drugs at concentrations resulting in about 100% inhibition of cell proliferation. Analysis by immunoblotting. Lanes: 1, control (untreated cells); 2, heat shocked cells (see section 2); 3, $2 \cdot 10^{-6}$ M cisplatin; 4, $3 \cdot 10^{-7}$ M doxorubicin; 5, $3 \cdot 10^{-7}$ M daunomycin; 6, $1 \cdot 10^{-6}$ M cytosine arabinoside; 7, $1 \cdot 10^{-4}$ M 3′-fluorodeoxythymidine; 8, $1 \cdot 10^{-5}$ M colchicine; 9, $1 \cdot 10^{-5}$ M vincristine; 10, $1 \cdot 10^{-7}$ M 5-fluorouracil; 11, $5 \cdot 10^{-8}$ M aminopterin; 12, $5 \cdot 10^{-8}$ M methotrexate; 13, $1 \cdot 10^{-6}$ M mithramycine; 14, $5 \cdot 10^{-6}$ M cyclophosphamide; 15, heat shock (as in lane 2).

4. Discussion

We have shown in the present communication that the response of EAC cells to various anticancer drugs differs with respect to expression of hsp25 and hsp70. Drugs that induce hsp25 do not induce expression of hsp70. Possibly, the genes of the two heat shock proteins are the subject of different types of regulation of their activity in response to various chemical agents, which possibly generate different activating processes.

A further important point of the present investigation is the finding that certain drugs induce hsp25, but others do not. This raises the question about correlations between the mode of biochemical actions resulting in cytostatic effects and the stimulation of hsp25 expression. It is apparent that effective drugs interfere directly with processes at the level of DNA replication in the S phase (cisplatin, doxorubicin, daunomycin, cytosine arabinoside, 3′-fluorodeoxythymidine) or with the microtubule system arresting cells in the M phase of the cell cycle (colchicine, vincristine), whereas drugs which act cytostatically by interfering with nucleotide metabolism in the G_1 phase (5-fluorouracil, aminopterin, amethopterin) do not induce hsp25. Cisplatin, as well as doxorubicin and daunomycin, form adducts with DNA resulting in inhibition of template properties of DNA. Cytosine arabinoside interferes with DNA replication after phosphorylation to the corresponding triphosphate as a competitive inhibitor of DNA polymerase with respect to dCTP. 3′-Fluorodeoxythymidine inhibits elongation of the nascent DNA chain at the template by preventing the formation of phosphodiester bonds. Following the rules mentioned above, mithramycine, which binds noncovalently and non-intercalatively to GC base pairs of DNA, should also properly induce hsp25. That it does not do so is makes it an exception to the proposed suggestion. As shown, cytostatics acting in the G_1 phase do not induce hsp25. 5-Fluorouracil inhibits conversion of deoxyuridylic acid (dUMP) to deoxythymidylic acid (dCMP) by thymidylate synthase (EC 2.1.1.45), and both aminopterin and amethopterin are efficient as inhibitors of dihydrofolate reductase (EC 1.5.1.3). Studies are under way to examine activation of heat shock elements and transcription factors in cells treated with various cancerostatics.

A third aspect to be discussed concerns correlations between the synthesis of the small mammalian stress protein and the development of resistance against anticancer drugs. For instance, Ciocca et al. [8] have shown that human breast cancer cells expressing high levels of hsp27 after heat shock are more resistant to doxorubicin treatment than non-stressed cells, while heat shock did not confer cross-resistance to other anticancer drugs such as 5-fluorouracil, methotrexate, colchicine and cisplatin. Resistance to vincristine-inflicted cytotoxicity and microtubule destruction in heat shocked rat brain tumour cells has been described by Lee et al. [9]. In experiments with Chinese hamster cells, Hahn et al. [10] found that thermotolerant cells were resistant to doxorubicin, and, in contrast to the findings of Ciocca et al. [8], also to cisplatin. Huot et al. [11] described acquired thermoresistance of Chinese hamster cells following transfection with a plasmid containing the structural gene of

human hsp27. Cells over-expressing hsp27 were also found to be more chemoresistant in response to doxorubicin, daunorubicin, colchicine, and vincristine, but not to 5-fluorouracil. Oesterreich et al. [12] found that human breast cancer cells, over-expressing hsp27 after transfection with hsp27–cDNA displayed elevated resistance to doxorubicin. When these findings of other authors and the results of our studies are considered it is apparent that thermoresistant cells are also chemoresistant against such cancerostatics (doxorubicin, daunomycin, cisplatin, colchicine, vincristine) which induce hsp25/hsp27, but not against drugs which do not increase hsp25 (5-fluorouracil, methotrexate). The induction of stress proteins is considered to be correlated with thermotolerance and also transient states of cross-tolerance to cytostatic drugs. This may have considerable clinical importance in the treatment of tumors by chemotherapy, irradiation and hyperthermia as clinical studies on the combination of various therapeutic modalities become more frequent.

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