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EFFECT OF UKRAIN ON HUMAN LYMPHOMA CELL GROWTH WITH DIFFERENT EXPRESSIONS OF HEAT SHOCK PROTEIN 70 (HSP70)

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Summary: *The aim of this study was to determine the involvement of heat shock protein 70 (hsp70) in the protective mechanism of U-937 lymphoid cells against Ukrain-induced cell death. We compared the viability and susceptibility of human lymphoid cells U-937 and U-937/hsp70, which differed in their expression of hsp70, to apoptotic stimuli induced by the anticancer drug Ukrain. Ukrain is known to exhibit high cytotoxic and cytostatic activity against malignant, but not normal, cells. Ukrain at concentrations of 34.3, 51.4 and 68.5 μM was shown to decrease the growth rate of U-937 cells by about 3-fold compared with untreated cells, and approximately 55%-65% of the cell population died after 24 h incubation with Ukrain at these concentrations. U-937/hsp70 cells were more resistant to the same concentrations of Ukrain. A lower concentration of Ukrain 6.86 μM induced cell death in about 20% of both U-937 and U-937/hsp70 cells compared with control, and the mean living cell concentration in both cell types was found to decrease similarly: by 1.3-fold after 48 h incubation, and by 1.7-fold after 72 h. U-937 and U-937/hsp70 cells both demonstrated susceptibility to apoptosis induced by Ukrain 6.86 μM, whereas Ukrain at a concentration of 3.43 μM was shown to be nontoxic for U-937 and U-937/hsp70 cells. Treatment of cells with Ukrain at a concentration of 6.86 μM for 4 h was followed by decreased cathepsin B and L activity in both U-937 and U-937/hsp70 cells. The data presented in this paper indicate that hsp70 protected U-937 lymphoid cells from death induced by high concentrations of Ukrain.*

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Introduction

Heat shock proteins (hsp) are highly conserved proteins known to protect cells from adverse environmental, physical and chemical stresses by their ability to prevent protein aggregation and promote the

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refolding of denatured protein (1). The most remarkable property of hsp70 family members is their chaperonic activity, *e.g.,* their ability to bind damaged or newly synthesized proteins, to transport them through intracellular membranes and to assemble active protein structures (1). The protective function of proteins of the hsp70 family has been demonstrated in various cell and animal model systems. Cytoplasmic members of the hsp70 proteins protect myocardial and neural cells against ischemia-induced injury (2), and pancreatic islet cells against the noxious action of interleukin (IL)-1 beta (3) and others. Furthermore, hsp70 has been shown to defend cells against apoptosis-inducing factors (4).

Hsp70 can affect the apoptotic pathway at the levels of both cytochrome *c* release and initiator caspase activation (1). Selective deprivation of hsp70 with the aid of anti-sense mRNA was followed by increased apoptosis of breast carcinoma cells (5, 6). In view of the fact that hsp70 members are highly expressed in many tumor cell lines, these data suggest that they play an important role in tumor cell survival and may be considered as cancer-relevant anti-apoptotic proteins (5, 6). Hsp70 is abundantly expressed in malignant human tumors of various origins, whereas in normal cells its expression is mainly stress-induced. Furthermore, the expression of hsp70 correlates with increased cell proliferation, poor differentiation, lymph node metastases and poor therapeutic outcome in human breast cancer (7). High amounts of hsp70 in cases of melanoma (8) and colon cancer (9) also relate to a poor prognosis on individual patient survival.

In order to determine the involvement of hsp70 in cell protection from death we compared the viability and susceptibility of human lymphoid cells U-937 and U-937/hsp70, which differed in hsp70 expression, to apoptosis induced by treatment with the anticancer drug Ukrain. Ukrain, a semisynthetic compound of thiotepa and alkaloids isolated from *Chelidonium majus L.*, is an anticancer agent exhibiting high cytotoxic and cytostatic activities toward malignant but not normal cells (10). Ukrain has been shown to have high cytotoxic activity against more than 60 tumor cell lines *in vitro* (11).

Material and methods

Cells. Human promonocytic U-937 cells were purchased from the Russian Type Culture Collection (Institute of Cytology, RAN, St. Petersburg, Russia). U-937/hsp70 cells transfected with the hsp70 gene (overexpressing hsp70) were provided by the Laboratory of Cell Protection Mechanisms (Institute of Cytology, St. Petersburg, Russia). Cells were cultured at a density of 2-4 $\times$ 10^5 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 100 U/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma) and 10% fetal bovine serum (Sigma), in an atmosphere of 5% CO_2 at 37 °C.

Cell treatment. U-937 and U-937/hsp70 cells (2 $\times$ 10^5 per well) were treated with 3.43 or 6.83 μM of Ukrain (Nowicky Pharma, Vienna, Austria; molecular weight 1458.64) for 4, 24, 48 and 72 h, and with 34.3, 51.4 or 68.5 μM of Ukrain for 24 h. Cell growth rate was measured by counting cells with the aid of a Goryaeva camera. Cell viability after the addition of Ukrain was determined with trypan blue staining. Morphological analysis of apoptosis was performed using carmine staining (12). Cells with condensed chromatin and fragmented nuclei were scored. At least 200 cells in randomly selected microscopic fields were counted for each experimental condition. The percentage of apoptotic cells was calculated using the formula:

$$\text{total number of cells with apoptotic morphology} \times 100 / \text{total number of cells}$$

Cathepsin B and cathepsin L assay. Cathepsin B and L activities were measured in the control and in

Ukrain-treated U-937 and U-937/hsp70 cells, as described by Kirschke and Barrett (13). Cells were treated with 0.1% Triton X-100 (Sigma) before assay. Cathepsin B activity was determined against Z-Arg-Arg-NMec (Sigma) as a substrate. In brief, 100 μl of the cell lysates were added to 200 μl of 220 mM phosphate buffer pH 6.0, containing 5 mM dithiothreitol (DTT) and 2.5 mM ethylene diamine tetraacetic acid (EDTA), and incubated for 5 min at 37 °C. After the addition of 200 μl of substrate, giving a final concentration of 12.5 μM, the mixture was incubated for a further 30 min. The reaction was stopped by the addition of 2 ml of 0.1 M monochloroacetic acid with 0.13 M sodium hydroxide and 0.1 M acetic acid. Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Perkin-Elmer 650-10S fluorescence spectrophotometer (Hitachi, Ibaraki, Japan). Results were expressed as nanomoles of 7-(4-methyl)-coumarylamide (MCA) per milligram of protein per minute.

Cathepsin L activity was measured against Z-Phe-Arg-NMec as a substrate. To distinguish cathepsin B and L activities, the hydrolysis of Z-Phe-Arg-NMec was measured in the presence and absence of the selective inhibitor of cathepsin B, CA-074, at a final concentration of 0.5 μM. Briefly, 100 μl of the sample were preincubated for 5 min at 37 °C in 200 μl of acetic buffer (pH 5.5) containing 5 mM DTT and 2.5 mM EDTA. After the addition of the substrate, 200 μl of Z-Phe-Arg-NMec, additional incubation was carried out for 30 min. The reaction was terminated by the addition of 2 ml of 0.1 M monochloroacetic acid with 0.13 M sodium hydroxide and 0.1 M acetic acid. Fluorescence was read as described for cathepsin B. Specific activity was expressed in nanomoles of 7-MCA per milligram of protein per minute.

Statistical analysis. All results were expressed as a mean value ± SD. The data were calculated using Student's *t*-test, and a probability value of $p < 0.05$ was considered significant.

Results

Effect of Ukrain on cell growth and viability. In order to evaluate the effects of Ukrain on the growth rate and viability of human lymphoma cells with different expression of hsp70, U-937 and U-937/hsp70 cells—the latter transfected with the hsp70 gene—were incubated with Ukrain at concentrations of 3.43 and 6.86 μM for 24, 48 and 72 h, and with concentrations of 34.3, 51.4 and 68.5 μM for 24 h. Viability was analyzed using a trypan blue exclusion assay. Ukrain at concentrations of 34.3, 51.4 and 68.5 μM was shown to affect the viability of U-937 cells (Fig. 1). At 34.3, 51.4 and 68.5 μM, brief (24 h) exposure to Ukrain decreased the growth rate of U-937 cells by about 3-fold compared with untreated cells (Fig. 1A), and approximately 55%-65% of the cell population died (Fig. 1B). In the case of U-937/ hsp70, the reduction in cell viability was less marked (Figs. 1A and 1B): after 24 h incubation with 34.3, 51.4 and 68.5 μM of Ukrain, the mean dead cell values were 29%, 22% and 31% of the population, respectively, compared with the untreated control, and cell growth rate was reduced 1.4-, 1.9- and 2.1-fold, respectively (Fig. 1).

A concentration of Ukrain 6.86 μM induced cell death in both U-937 and U-937/hsp70 cell types by approximately 20% compared with control ($p < 0.05$). The mean cell concentrations in both cell types were found to decrease similarly: by 1.3-fold after 48 h and by 1.7-fold after 72 h. Comparison of cell susceptibility to apoptotic stimuli was performed under 6.86 μM Ukrain treatment for 24, 48 and 72 h (Fig. 2). U-937 and U-937/hsp70 cells demonstrated similar susceptibility to apoptosis in these conditions (Fig. 2). A concentration of 5 μg Ukrain in 1 ml of cell suspen-

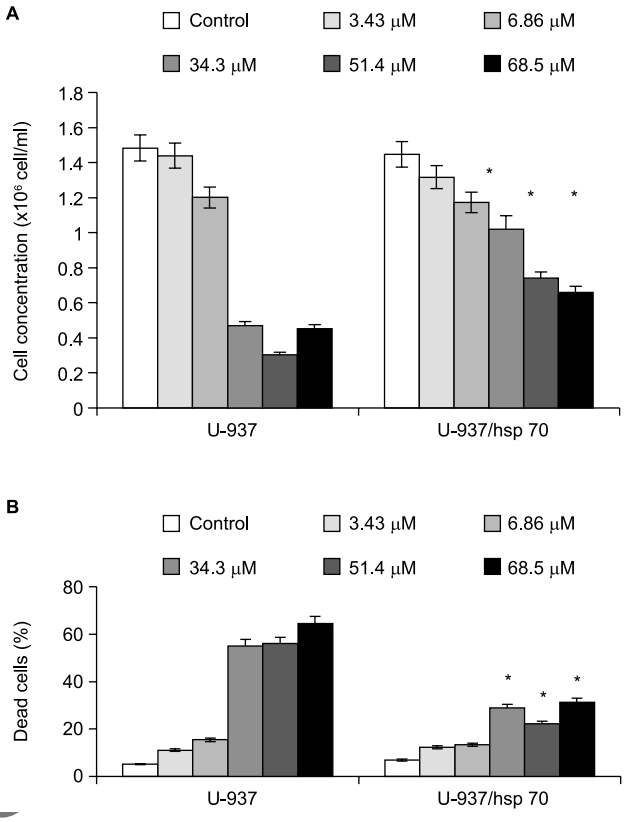

Fig. 1 Effect of different concentrations of Ukrain (3.43, 6.86, 34.3, 51.4 and 68.5 μM) on cell growth and viability of U-937 and U-937/hsp70 cells differing in hsp70 expression. The data represent the mean of three independent experiments ± SD. A: Living cell concentration; B: percentage of dead cells. *$p < 0.05$ compared with U-937 cells.

sion was determined as nontoxic for both U-937 and U-937/hsp70 cells (Fig. 1). Overall, it appeared that U-937/hsp70 cells with their higher hsp70 expression were more resistant to Ukrain treatment than U-937 cells with lower hsp70 expression.

Effect of Ukrain on lysosomal enzymes of U-937 and U-937/hsp70 cells. U-937/hsp70 cells were characterized by higher levels of cathepsin B and L activity compared to U-937 cells. The activity of cathepsin B and L in U-937/hsp70 cells was higher by 5- and 2-fold, respectively, than in U-937 cells (Fig. 3). Treatment with Ukrain at 6.86 μM for 4 h was followed by decreased cathepsin B and L activity in both U-937 and U-937/hsp70 cells (Fig. 3). However, incubation of U-937 and U-937/hsp70 cells with Ukrain 3.43 μM for 4 h did not induce any changes in cathepsin B or L activity (Fig. 3).

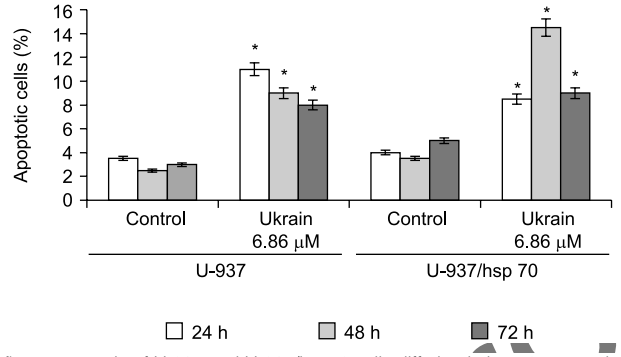

Fig. 2 Effect of Ukrain (6.86 μM) on apoptosis of U-937 and U-937/hsp70 cells differing in hsp70 expression. The data represent the mean of three independent experiments ± SD. *$p < 0.05$ compared with control.

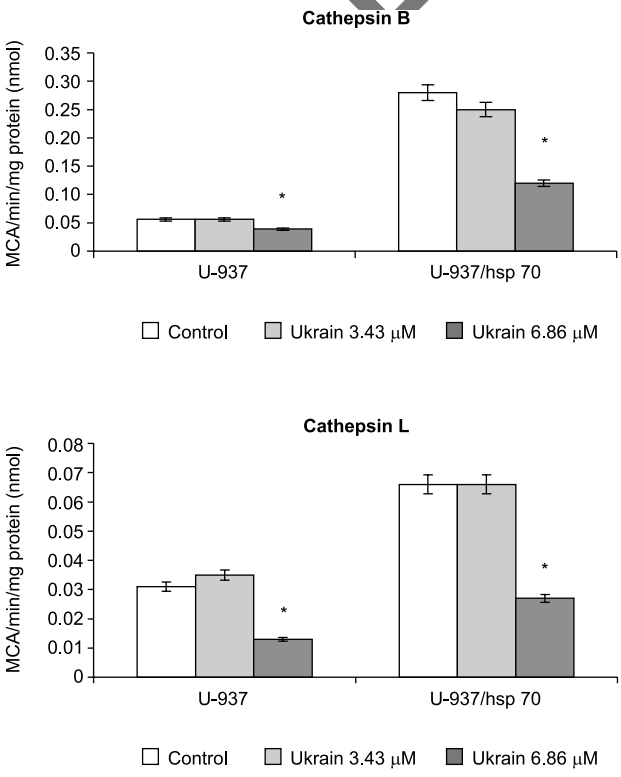

Fig. 3 Effect of Ukrain (3.43 and 6.86 μM) on cathepsin B and L activity in U-937 and U-937/hsp70 cells. *$p < 0.01$ compared with control (untreated cells). MCA: 7-(4-methyl)-coumarylamide.

Discussion

In the present study we analyzed the viability and susceptibility to apoptosis of human lymphoma cell lines U-937 and U-937/hsp70, the latter having a different basal content of hsp70. Cells were subjected to Ukrain treatment at various concentrations. Ukrain has been shown to exhibit high cytotoxic activity toward more than 60 tumor cell lines *in vitro* (10). Our experiments showed that cells expressing higher quantities of the hsp70 protein (U-937/hsp70) were more resistant to the cytotoxic and cytostatic effects of high doses of Ukrain (34.3, 51.4 and 68.5 μM) than those with lower levels of hsp70 (U-937). At a concentration of 6.86 μM, Ukrain had a similar effect on the cell growth rate and viability of U-937 and U-937/hsp70 cells, and the number of apoptotic cells in both cell types following 6.86 μM Ukrain treatment was also the same. This suggests that the viability and susceptibility to apoptosis of U-937 lymphoma cells treated with Ukrain 6.86 μM is not dependent on their hsp70 content. A concentration of Ukrain 3.43 μM did not affect cell viability or the number of apoptotic cells.

The mechanisms responsible for the antitumor effects of high concentrations of Ukrain are perhaps connected with its ability to inhibit DNA, RNA and protein synthesis, as demonstrated in various malignant cell lines at relatively high concentrations of Ukrain (10). The apoptosis in U-937 and U-937/hsp70 cells induced by Ukrain at a relatively low concentration (6.86 μM) is probably not dependent on their hsp70 content.

Elevated levels of hsp70 are thought to play a crucial role in the induction of antiapoptotic mechanisms (4). It has been shown that murine fibrosarcoma WEHI-S cells transfected with the human hsp70 gene were more resistant to various apoptosis-inducing drugs. Overexpression of hsp70 and hsp90 in transfected neuronal cells protected them from thermal stress, but not from programmed cell death induced by serum deprivation or nerve growth factor withdrawal. The protective effect mechanism of hsp70 in apoptosis is thought to stem from its chaperonic activity, by which the protein hsp70, with the aid of other polypeptides, takes part in the recovery of partially denaturized proteins and in the removal of damaged protein molecules (14). Additionally, a growing body of data indicates that hsp70 and its constitutive analog, hsc70, can bind mature proteins p53, c-Myc, and nuclear factor (NF)-kappa B regulatory complex subunits (14), which are known to take part in the regulation of apoptosis. On the other hand, extracellular hsp70 induces natural killer (NK) activity (14, 15), and plasma membrane-bound hsp70 renders tumor cells more sensitive to lyses mediated by NK cells (15).

Lysosomal cysteine proteases, such as hsp, exhibit a dual action on cancer cells. On one hand, cathepsins B, L and D are involved in the process of cancer invasion (16). Altered regulation, intracellular trafficking and increased expression of mRNA, protein and activity levels have been observed in neoplastic tissues (16). On the other hand, the cysteine proteases cathepsins B and L are known to play an important role in apoptosis of tumor cells. Some anticancer drugs are able to induce apoptosis related to cysteine (cathepsin B) and aspartic (cathepsin D) proteases. Release of cathepsin B, L and D from lysosomes has been shown to be an initial stage in non-caspase apoptosis (4). In our investigation, decreased cathepsin B and L activity in U-937 and U-937/hsp70 cells under treatment with Ukrain 6.86 μM apparently resulted from the ability of Ukrain to inhibit protein synthesis. It was also found that there was significantly higher cathepsin B and L activity in U-937/hsp70 cells, with their higher level of hsp70 expression, compared to U-937 cells. It is unclear whether this was connected with their enhanced expression of hsp70 protein.

One can therefore conclude that U-937 histiocytic lymphoma cells with enhanced levels of hsp70 expression were more resistant to the cytotoxic and cytostatic effects of high doses of Ukrain.

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