Resistance of Ehrlich tumor cells to apoptosis can be due to accumulation of heat shock proteins

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Abstract Previously we have found that stationary Ehrlich ascites carcinoma (EAC) cells in vivo accumulated heat shock proteins (HSPs) and became resistant to necrotic death induced by prolonged energy deprivation of hyperthermia. Here we report that apoptotic death induced by nutrient starvation, transient ATP depletion, heat shock and a microtubule-disrupting drug, vinblastine, was also suppressed in stationary EAC cells comparing with exponential cells. When exponential (sensitive) cells were subjected to short-term heating with recovery to accumulate inducible form of HSP70, they also became resistant to all of the employed apoptosis-inducing exposures, and an inhibitor of cytosolic protein synthesis, cycloheximide, prevented acquisition of the resistance. It is suggested that in vivo accumulation of HSPs in stationary tumor cells can be endogenous protective device against apoptotic death induced by starvation or some anticancer treatments.

Key words: Apoptosis; DNA fragmentation; Heat shock proteins; Ehrlich ascites carcinoma

1. Introduction

Apoptosis is the main mechanism of cell elimination during embryogenesis, senescence, and under other various physiological exposures (e.g. hormones, growth factor withdrawal, etc.) [1–3]. At the same time, many non-physiological damaging factors (irradiation, heat shock, ischemia, oxidative stress) can result in apoptotic death at low doses and necrotic death at higher doses [4,5]. These data suggest that some biochemical events causing cell death may be common both for apoptosis and necrosis. As we have previously found, one of the earliest cell injuries under various stresses is the cytoskeletal protein (in particular, actin) aggregation, and the rate of necrotic death well correlates with the level of this aggregation [6,7]. Since prevention of protein aggregation within a cell is one of the important functions of heat shock proteins (HSPs), rise in HSP level was found to protect cells from necrosis induced in vitro by hyperthermia and energy deprivation [8,9]. In vivo, correlation between HSP expression and protein of heart and brain from ischemia-induced necrosis was also reported (see [10,11] for review). These data indicate that HSPs can save mammalian cells from necrotic death, at least in some cases.

There are some proteins which are known to affect apoptosis. For example, expression of bcl-2 can rescue cells from such different apoptosis-inducing agents as radiation, heat shock, chemotherapeutic drugs, tumor necrosis factor, azide, etc. (see [12] for review), and mutation or deletion of p53 blocked apoptosis induced by different DNA-damaging exposures (e.g. radiation, 5-fluorouracil, etoposide, adriamycin) [13]. However, the role of HSPs in apoptotic death has not been fully evaluated.

Recently we have observed that cytoskeletal protein aggregation in EAC cells is accompanied by DNA fragmentation, a hallmark of apoptosis [14]. Therefore, the question arises as to whether excess HSPs can protect cells also from apoptotic death? To our knowledge, there was one report where this issue was addressed and accumulation of HSP70 was found to prevent hyperthermia-induced apoptosis in a human leukemia cell line, although no other apoptosis-inducing agents were examined and the mechanism of the protection was not studied [15].

In our previous work we observed that resistance to necrosis induced by energy deprivation, hyperthermia and oxidative stress in stationary EAC cells in vivo can be due to accumulation of the major cytosolic HSPs (HSP70, HSP90 and HSP27) [16]. The goal of this work was to study the resistance of these cells to apoptotic death and to evaluate a possible involvement of HSPs in this resistance.

2. Materials and methods

2.1. Cell culture

EAC cells were inoculated into C57BL6 mice once a week (10^7 cells per mouse). For experiments, the cells were isolated from peritoneal cavity either on the 5th day after inoculation (exponential phase of growth) or on the 8th day (stationary phase) [16].

2.2. Cytotoxic treatments

After washing of cells with PBS they were resuspended either in DMEM with 10 mM HEPES (control) or subjected to starvation (PBS) and various stresses: (i) transient energy deprivation (2 μM of CCCP for 1 h with subsequent addition of glucose) [14]; (ii) hyperthermia (44°C for 30 min in a water bath); (iii) microtubule-disrupting agent, vinblastine (10 μg/ml). During subsequent 5 h of incubation, cell blebbing and plasma membrane permeability were determined using phase-contrast microscopy and trypan-blue staining as described earlier [14].

2.3. Assessment of apoptosis

Morphological quantification of chromatin condensation was carried out on methanol-fixed cells by staining with hematoxilin-eosin and conventional light microscopy. Cells with condensed nuclei and shrinked cytoplasm were considered as apoptotic. DNA fragmentation was determined essentially as described previously [14]. Briefly, cells were lysed with 0.2% Triton X-100, 4 mM Tris-HCl, 1 mM EDTA (pH 7.5) for 5 min, and after centrifugation at 6,000 × g for 15 min the

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMEM, Dulbecco's modified Eagle's minimal essential medium; EAC, Ehrlich ascites carcinoma; HSP, heat shock protein; PBS, phosphate-buffered saline.

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fraction of fragmented (supernate) DNA was evaluated fluorimetrically using Hoechst-33258 (2 μM) [17]. Flow cytometric analysis to identify cellular DNA content and cell size was performed in hypotonic buffer as described [18]. Cell pellets were suspended in hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100), and cells were analyzed by the use of a FACS Vantage (Becton and Dickinson, Mountain View, CA) with Cell Fit software.

2.4. DNA isolation and agarose gel electrophoresis

Cellular DNA was isolated using chlorophorm/isoamyl alcohol (24:1) extraction and ethanol precipitation. Samples were electrophoresed through 1.5% agarose gel at 90 V for 3 h, and DNA patterns were visualized under UV light by ethidium bromide staining.

2.5. HSP induction and immunoblotting

For HSP induction exponential EAC cells were heat-shocked at 44°C for 10 min in DMEM and then recovered at 37°C for 3 h to accumulate HSPs. Electrophoresis of cell lysates and immunoblotting with monoclonal antibodies C92 to inducible form of HSP70 (kindly provided by Prof. W. Welch, San Francisco, USA) was performed as described earlier [8].

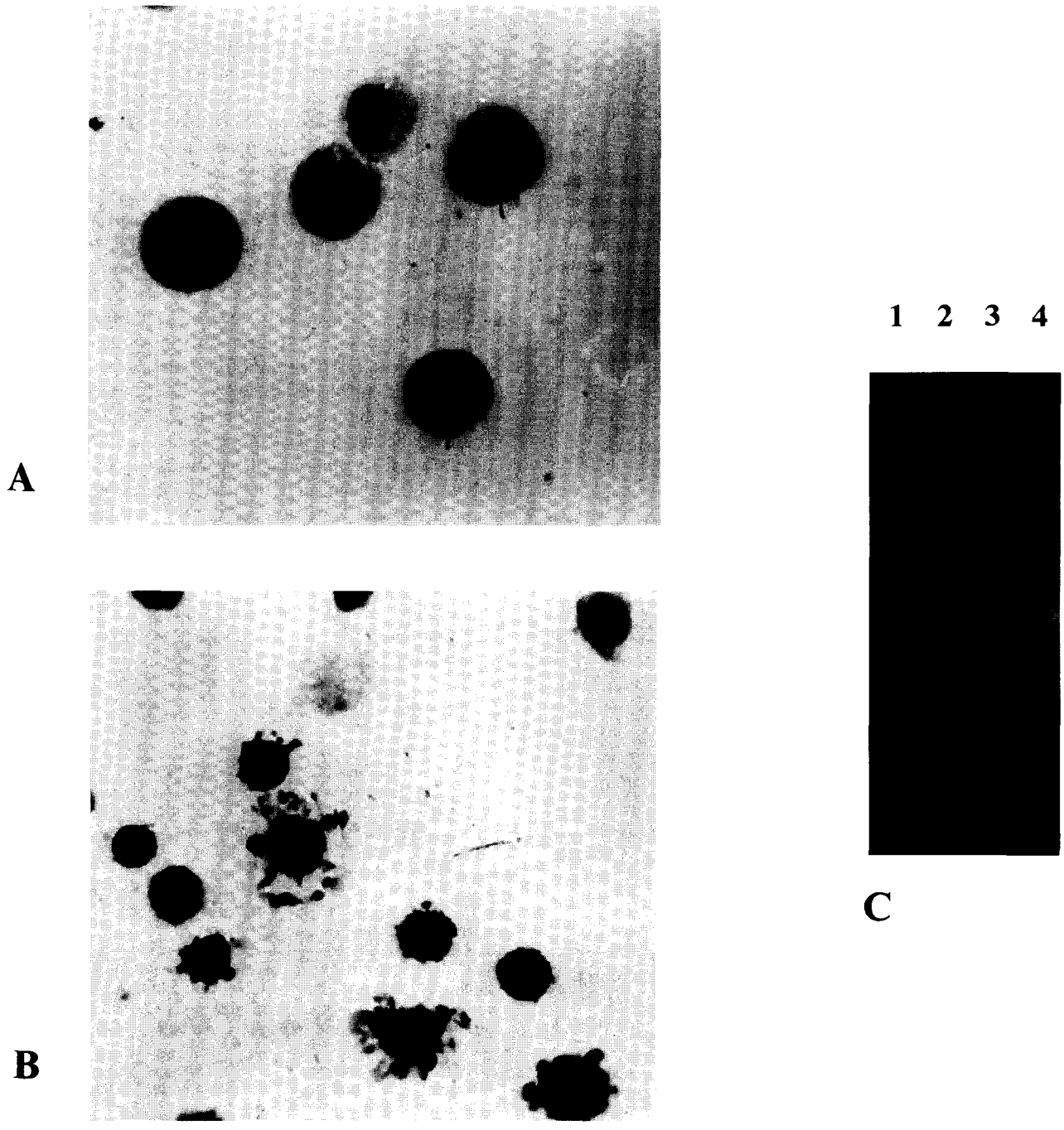

Fig. 1. Morphology (A,B) and DNA fragmentation (C) of EAC cells during apoptosis. Exponential EAC cells were stained with hematoxilin-eosin after incubation for 3 h following heat shock (44°C, 30 min; B) or without heat shock (control; A). Cell shrinkage, chromatin condensation and blebbing are clearly seen in heat-shocked EAC cells. Agarose gel electrophoresis (C) of total cellular DNA after 3 h of EAC cell incubation in a rich medium (DMEM) without treatment (1) or subjected to starvation (2), transient ATP depletion (3), or heat shock (4).

3. Results

Previously we have observed that non-proliferating EAC cells became resistant to necrotic death under energy deprivation which can occur in stationary phase of their growth in vivo [16]. Besides necrotic death tumor cells may undergo apoptotic death. When EAC cells were incubated in a rich medium (DMEM), neither apoptosis nor necrosis (as judged by Trypan blue staining) were found during the first 1–6 h of incubation under number of treatments including hyperthermia (44°C, 0.5–1 h), respiratory inhibitor (rotenone) or uncoupler of oxidative phosphorylation (CCCP), and a microtubule-disrupting

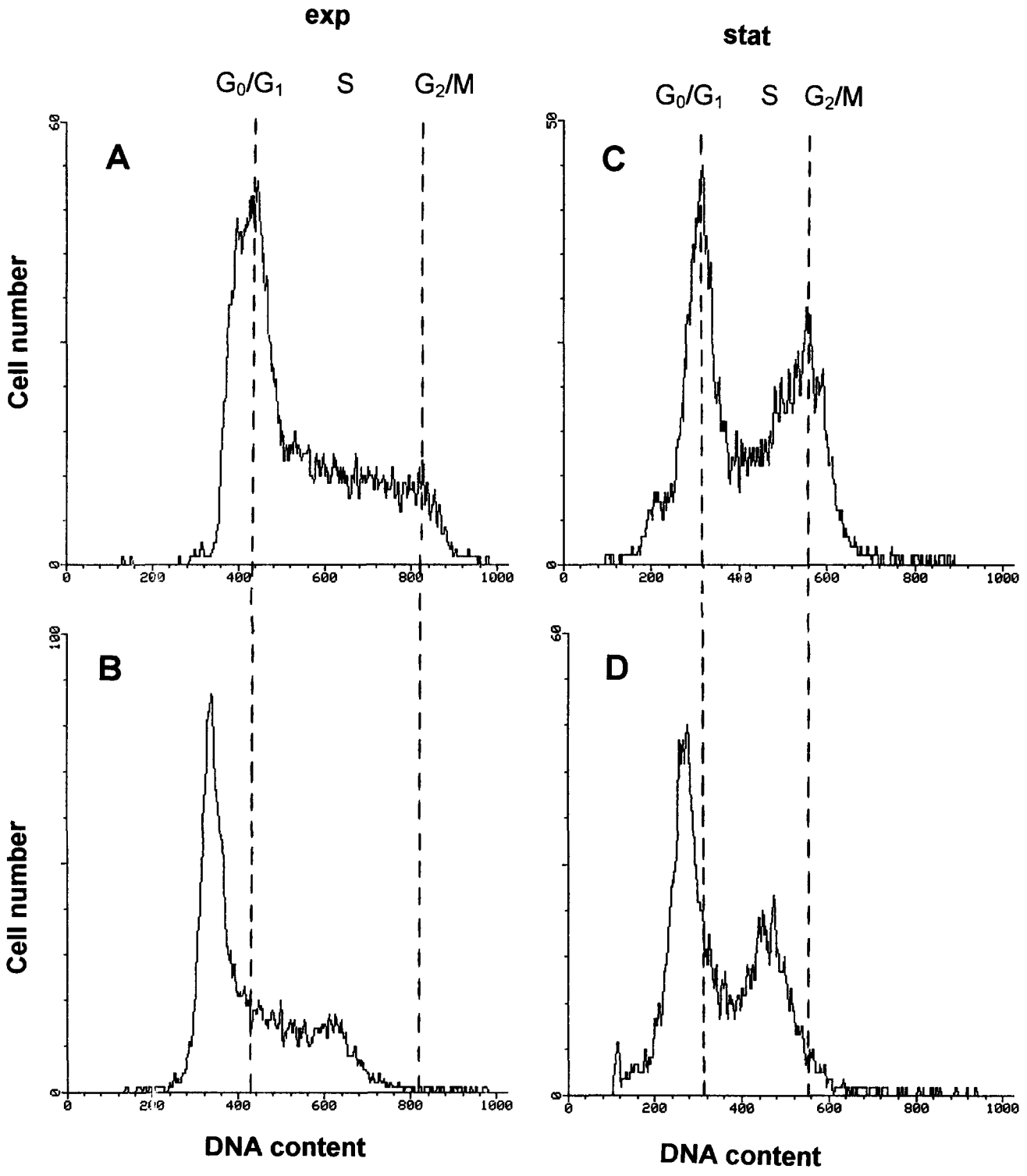

Fig. 2. Changes in DNA content of exponential (exp) and stationary (stat) EAC cells during apoptosis. DNA fluorescence histograms of propidium iodide-stained exponential (A,B) or stationary (C,D) EAC cells incubated for 5 h after heat shock (B,D) or without stress (A,C). Relative DNA content in all cell cycle phases decreased after heat shock more significantly in exponential than in stationary cells. Stationary cells have also increased G_2/M fraction (33%) comparing with exponential cells (4%).

agent, vinblastine (data not shown). However, in a nutrient-free medium (PBS), which may simulate conditions of starvation occurring in late stages of tumor growth, all typical singes of apoptotic cell death, namely cell shrinkage, blebbing, chromatin condensation and DNA fragmentation were clearly seen (Fig. 1). Moreover, such stresses as transient ATP depletion, hyperthermia and treatment with vinblastine markedly accelerated apoptotic death in starved exponential EAC cells (Table 1). At the same time, stationary EAC cells were found to be much more resistant to apoptotic death under all the conditions used (Table 1). No increase in both necrotic and apoptotic death was observed in EAC cells during their stationary phase of growth in vivo (8–11 days after inoculation) (data not shown). Flow cytometric analysis of cell death in exponential and stationary EAC revealed that cellular DNA content in exponential cells decreased more markedly than in stationary cells (Fig. 2); in addition, this decrease in cellular DNA was accompanied by diminution of cell size (forward light scatter, data not shown). These data indicate that the observed death pattern of EAC cells (namely, chromatin condensation, blebbing, cell shrinkage, decrease in DNA content) are hallmarks of apoptotic death, although there are no clear internucleosomal DNA fragmentation (Fig. 1) and appearance of subdiploidal DNA peak (Fig. 2) as it was observed in lymphoid cells [1,3].

One of the possible explanations of the above findings may be decreased level or activity of the apoptotic effectors, namely proteases and endonucleases [2], in stationary cells. To test this possibility, EAC cells were treated with a detergent, Triton X-100, which was shown to induce rapid DNA fragmentation and chromatin condensation in carcinoma cells [19]. When EAC cells were treated with Triton X-100 (0.01%), both exponential and stationary cells exhibited about the same DNA fragmentation (50%) and blebbing after 1 h of incubation (data not shown). In addition, as we found previously, more prolonged incubation with CCCP (2–3 h) without glucose or under hyperthermic conditions (44°C, 1 h) could induce blebbing and DNA fragmentation in stationary EAC cells, whereas in exponential cells such treatments resulted in necrotic death [8,14]. These data indicate that stationary cells are not defective in apoptotic machinery.

Another reason for resistance of stationary EAC to apoptosis may be associated with defense against stress-induced damage causing apoptosis. This decreased damage may be linked, in particular, with accumulation of HSPs. If it is true, sensitive (exponential) cells can be converted to resistant ones simply through induction of HSPs in them. For this purpose, exponential cells were heated for 10 min at 44°C with subsequent recovery for 3 h to accumulate the inducible form of HSP70 (HSP68) (Fig. 3) and make them thermotolerant [8,9]. Thereafter, these cells as well as control ones (without heat shock) were exposed to the above stresses to induce apoptosis. As one can see from Table 2, preliminary heat shock with recovery did protect exponential EAC cells from apoptotic death rendering them nearly as resistant as the stationary cells (cf. Table 1). In contrast, heat shock without recovery or prevention of HSPs accumulation by inhibition of cytosolic protein synthesis with cycloheximide (50 μM) did not rescue exponential EAC cells from apoptosis (data not shown). Therefore, resistance of stationary EAC cells to apoptotic death can be due to accumulation of HSPs in these cells (Fig. 3).

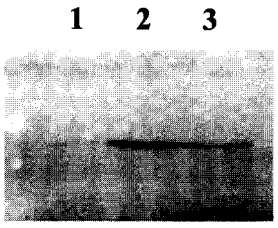

Fig. 3. Blotting and immunoperoxidase staining of total cell lysates from exponential (1), heat-shocked (thermotolerant) exponential (2) and stationary EAC cells (3) with specific monoclonal antibodies to inducible form of HSP70 (HSP68). Equal numbers of cells (10^6) were applied to each track.

4. Discussion

Summarizing, in this work we demonstrate: (i) apoptotic death of EAC cells can be induced by starvation, transient ATP depletion, hyperthermia and vinblastine; (ii) stationary cells with elevated HSP content are much more resistant to apoptotic death than exponential cells; (iii) HSP accumulation in exponential cells rendered them resistant to apoptosis.

Table 1
Apoptotic death of exponential (exp) and stationary (stat) EAC cells subjected to stresses

Exposures	Cell state	Blebbing (%)		Apoptotic nuclei (%)	DNA fragm. (%)		TB staining (%)	
		1 h	3 h	3 h	3 h	5 h	3 h	5 h
(1) Starvation	exp	18	17	8	10	14	13	20
	stat	3*	4*	0*	4*	5*	8	8*
(2) + heat shock	exp	13	20	29	14	26	29	40
	stat	7	12*	1*	5*	6*	12*	23*
(3) + ATP deplet.	exp	68	0**	14	17	28	23	23
	stat	41*	0**	0*	6*	11*	9*	14*
(4) + vinblastine	exp	38	15	29	10	24	22	32
	stat	4*	2*	1*	5*	10*	8*	8*

Mean data from 3–5 experiments are shown; *$P < 0.05$ by Student's t-test between stationary and exponential cells. **Blebbing was reversed after 2 h of incubation with glucose. EAC cells were incubated in PBS only (starvation), heat-shocked at 44°C for 30 min, exposed to CCCP (2 μM) for 1 h with subsequent addition of 10 mM glucose (transient ATP depletion), or treated with vinblastine (10 μg/ml). Blebbing, apoptotic nuclei (chromatin condensation), DNA fragmentation (fragm.) and trypan blue (TB) staining were determined as described in section 2.

Table 2
Protection of thermotolerant (TT) exponential EAC cells with elevated HSP content from apotic death

Exposures	Cell state	Blebbing (%)		DNA fragm. (%)		TB staining (%)	
		3 h	5 h	3 h	5 h	3 h	5 h
(1) Starvation	cont	13	22	8	9	13	26
	TT	3*	9*	2*	5*	8	16*
(2) + heat shock	cont	6	26	20	40	21	68
	TT	3	4*	5*	8*	19	35*
(3) + ATP deplet.	cont	0**	–	15	36	16	18
	TT	0**	–	4*	26*	10	10*
(4) + vinblastine	cont	8	12	10	17	15	47
	TT	2*	6*	8*	5*	10	21*

Mean data of 3 experiments are shown; *$P < 0.05$ by Student's t-test comparing with control (cont). **Blebbing was reversed after 2 h of incubation with glucose. Exponential EAC cells were subjected to heat shock (44°C, 10 min), recovered for 3 h at 37°C to accumulate HSP70 (see Fig. 3) and then exposed to apoptosis-inducing agents; control cells were treated in the same way, but without heat shock. All represented parameters were measured as described in section 2.

The first question is whether accumulation of HSPs rather than other proteins with anti-apoptotic activity (e.g. bcl-2) is responsible for the resistance of thermotolerant cells to apoptosis. As we previously found by autoradiography, heat shock treatment of EAC cells employed in this study (44°C, 10 min) did not induce synthesis of other proteins besides HSPs [9]; in addition, bcl-2 was not detected by immunoblotting either in thermotolerant or stationary EAC cells (Gabai et al., unpublished data).

The second question is which HSP can confer tolerance to apoptosis. Previously a protective role of HSP70 accumulation under hyperthermia-induced apoptosis was demonstrated in a human leukemia cell line [15], and quercetin-induced apoptosis in various tumor cell lines was associated with down-regulation of HSP70 expression [20]. Although studies with transfected HSPs are necessary for direct demonstration of HSP-mediated protection against apoptosis, our results together with the data of the above-mentioned works suggest this novel function of HSPs.

Mechanism of HSP-mediated protection from apoptosis has not been investigated, but several suggestions can be made. First of all, the main function of HSPs is prevention of protein aggregation within cells. Both hyperthermia and ATP depletion were found to induce protein aggregation, and suppression of the aggregation in stressed cells with the elevated HSP content [9,16] may be responsible for the protection from these apoptosis-inducing exposures. Suggested suppression of actin aggregation by HSPs [21] is of special importance since G-actin (but not F-actin) is a potent inhibitor of DNase I which can be involved in apoptotic DNA fragmentation [22–24].

Maintenance by excess HSPs of other cytoskeletal constituents, microtubules and intermediate filaments, also may be important for their anti-apoptotic function. Indeed, previously apoptotic death induced by microtubule-damaging drugs (colchicine, vinblastine) was demonstrated in murine thymoma cells [25] and rat hepatocytes [26], whereas HSP70 accumulation was found to confer resistance to vincristine (a vinblastine analog) in rat brain tumor cells [27]. Although in the latter study the mode of death has not been examined, here we demonstrated acceleration of EAC cell apoptosis by vinblastine and the protective effect of HSP70 accumulation.

Another reason for the anti-apoptotic effect of HSPs may be protection of the chromatin from the attack of proteases and nucleases involved in apoptosis. Various stresses may induce unfolding of intracellular proteins including proteins of the chromatin, and well-known HSP70 translocation into nucleus during stresses may serve for the stabilization of the chromatin structure.

In our opinion, anti-apoptotic effect of HSPs may be rather wide and include, besides the above described, such diverse damaging factors as transient ischemia, oxidative stress, UV-radiation and tumor necrosis factor (TNF). For example, involvement of HSP70 in protection of ischemic brain was suggested [11], whereas transient ischemia can result in apoptotic death of neurons [28]. Both HSP70 and HSP27 were found to be protective against necrosis caused by H_2O_2 and TNF [29,30]. At the same time, it was shown that low doses of oxidants are able to bring about apoptotic death [4,31], therefore, it is very probable that HSPs may perform an important protective function against oxidant-induced apoptosis.

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