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Lack of heat shock response triggers programmed cell death in a rat histiocytic cell line

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Abstract Stress response is a universal phenomenon. However, a rat histiocytic cell line, BC-8, showed no heat shock response and failed to synthesize heat shock protein 70 (hsp70) upon heat shock at 42°C for 30 min. BC-8 is a clone of AK-5, a rat macrophage tumor line that is adapted to grow in culture and has the same chromosome number and tumorigenic potential as AK-5. An increase in either the incubation temperature or time or both to BC-8 cells leads to loss of cell viability. In addition, heat shock conditions activated apoptotic cell death in these cells as observed by cell fragmentation, formation of nuclear comets, apoptotic bodies, DNA fragmentation and activation of ICE-like cysteine proteases. Results presented here demonstrate that BC-8 cells cannot mount a typical heat shock response unlike all other eukaryotic cells and that in the absence of induction of hsps upon stress, these cells undergo apoptosis at 42°C.

Key words: Heat shock response; Heat shock protein; Apoptosis; BC-8 cell/rat histiocytoma

1. Introduction

Heat shock proteins (hsps) represent a highly conserved family of proteins whose expression is induced when cells/tissues experience stressful conditions [1,2]. The synthesis of hsps is tightly regulated both at the transcriptional and the translational level [2–4]. Hsps have been shown to have a chaperonin function and help other proteins to maintain or regain their native conformation by stabilizing them in partially unfolded states [5–7].

Induction of hsps was correlated with acquisition of thermotolerance to higher doses of stress [8–10] which may be lethal to cells. Samali and Cotter [11] have shown that heat shock treatment of cells increases their survival and resistance to apoptosis and the kinetics of development of resistance correlate with the kinetics of synthesis of hsps. Hsps have been associated with several functions and development of resistance to apoptosis appears to be one of them. However, their main function is to afford protection to cells during stress. When cells/tissues no longer experience stress, the synthesis of hsps drops back to normal levels. On the other hand, if the stress situation continues, hsps may not be able to protect the cells and at this stage, depending on the severity of the stress, the program for apoptosis or necrosis is activated leading to the cell death [12].

Cultured cells respond to changes in their environment in a graded fashion and at low levels of stress, the stress response is initiated [13]. Also cell lineage has a major influence on the cellular responses to stress, specifically heat stress, and the response is not universal in all cell types [14]. Considering this, we studied the effect of heat shock on BC-8 cells, a clonally derived rat histiocytoma AK-5 [15]. BC-8 cells have the same chromosome number and tumorigenic potential as AK-5, yet, they are adapted to grow in culture. BC-8 cells were used to avoid ambiguity in results due to tumor heterogeneity. We show that BC-8 cells are unable to mount a typical heat shock response unlike many eukaryotic cells and in the absence of induction of hsps upon stress, BC-8 cells are triggered to undergo apoptotic cell death. Heat shock-induced apoptotic death in BC-8 cells involves activation of caspases and the fragmentation of nuclear DNA.

2. Materials and methods

2.1. Cell culture and heat shock conditions

An AK-5 tumor which was maintained as ascites was adapted to grow for several generations in DMEM with 10% FCS in the presence of penicillin (100 U/ml) and streptomycin (50 μg/ml) at 37°C. A single cell clone of AK-5, called BC-8, has been used in these studies. Exponentially growing cells (1×10^6/ml) were given a heat shock at 42°C for 30 min. Subsequently, the heat-shocked cells were either processed immediately for analysis or allowed to recover at 37°C for an appropriate time prior to analysis. As a positive control for apoptosis, BC-8 cells were incubated with anti-AK-5 serum for 8 h. Serum from AK-5 tumor rejecting animals has been shown to induce apoptosis in these cells [16,17].

2.2. Western blotting

Cell lysates from 5×10^5 cells/lane were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, blocked and probed with anti-hsp70 antibody (Stressgen, Canada) and the bands were visualized using horseradish peroxidase-conjugated second antibody as described earlier [18].

2.3. RNA isolation, cDNA synthesis and PCR amplification

Total RNA from control and heat-shocked BC-8 cells was isolated using the acid guanidinium thiocyanate method [19]. RNA from both samples was reverse-transcribed to obtain cDNA and the cDNAs were amplified with primers for hsp27, hsp70 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [20].

2.4. Comet assay

A comet assay was performed as described earlier [21–23] and the cells were photographed in a fluorescent microscope after staining with ethidium bromide.

2.5. Propidium iodide staining and flow cytometry

Heat-shocked cells were allowed to recover at 37°C for 8 h, washed with phosphate-buffered saline (PBS), fixed in 70% ethanol, stained with propidium iodide (PI) reagent (Calbiochem, 50 μg in 0.1% sodium citrate containing 0.1% Triton X-100) and analyzed by flow cytometry [24].

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2.6. DNA extraction and electrophoresis

Cells were fixed in ethanol, washed with PBS and suspended in citrate-phosphate buffer. DNA was extracted following the procedure described earlier [16,25], electrophoresed on a 0.86% agarose gel at 2 V/cm for 16 h, stained with 5 μg/ml ethidium bromide and visualized under UV light.

3. Results

BC-8 cells were incubated at different temperatures (ranging from 37 to 45°C) for different durations (from 30 to 90 min) and were allowed to recover for 2–12 h at 37°C to establish the optimum conditions of heat shock response. The ideal heat shock conditions with 100% cell viability were found to be 42°C for 30 min followed by recovery at 37°C for 2 h. There was a steady decline in the cell survival either by increasing the temperature above 42°C or by increasing the incubation time beyond 30 min at 42°C. Therefore, these conditions were followed in all the experiments reported here.

3.1. Lack of induction of hsps upon heat shock

To study the pattern of hsp synthesis in BC-8 cells, total cell lysate of heat-shocked cells after 4 h of recovery (the time at which hsps are induced in other mammalian cells [26]) was analyzed on Western blots with anti-hsp70 antibody. Fig. 1a shows the protein profile of control and heat-shocked BC-8 cells stained with Coomassie blue. A low background level of hsp70 was detectable in non-stressed cells (Fig. 1b, lane 2) and there was no increase in its levels upon heat shock (lane 1). In order to understand whether the lack of hsp70 induction is due to the transcriptional failure of heat shock genes or due to the inability of the cells to translate hsp mRNA, total RNA from control and heat-shocked cells was reverse-transcribed (RT) and the cDNA obtained was PCR-amplified using primers that specifically amplify hsp27, hsp70 and GAPDH as a control. The GAPDH primers were designed to amplify 500 nucleotide region in the middle of the gene.

There was no amplification of the RT product with hsp27 and hsp70 primers (Fig. 2, lanes d–g), suggesting the absence of induction of these RNAs upon heat shock in BC-8 cells. Amplification of GAPDH from the same RT product (lanes b and c) shows that the RNA used for cDNA synthesis was intact and RT and PCR reactions worked. Western blot analysis demonstrating the lack of induction of hsp70 and the negative result of PCR with hsp primers in heat-shocked

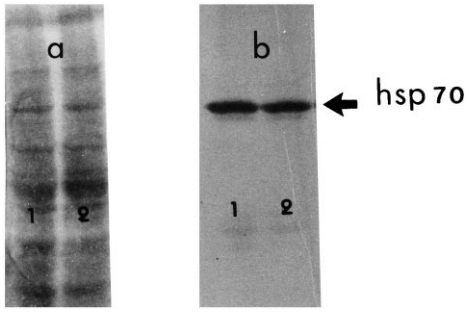

Fig. 1. (a) Protein profiles of heat-shocked (hs) and control cells. Total cell lysate was run on 10% SDS and the gel was stained with Coomassie blue. Lane 1, hs; lane 2, control. (b) Western blot analysis with anti-hsp70 antibody. Heat-shocked cells were allowed to recover for 4 h at 37°C and total cell lysate was analyzed on Western blots for induction of hsp70. Lane 1, hs; lane 2, control. (Note that there is no increase in the amount of hsp70 upon heat shock).

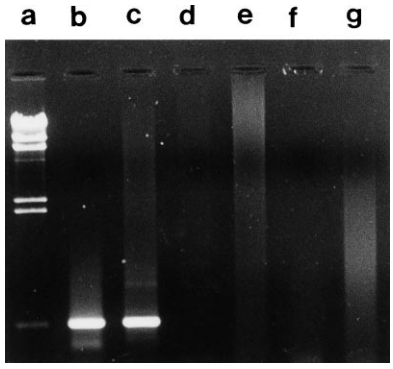

Fig. 2. PCR amplification of cDNA from control and heat-shocked BC-8 cells. Lanes: a, marker; b and c, amplification with control and heat-shocked cDNA with GAPDH primers; d and e, with hsp70 primers; f and g, with hsp27 primers. Note that there is no amplification of cDNA with hsp70 and hsp27 primers.

BC-8 cells clearly demonstrates the absence of induction of hsp27 and hsp70 mRNA in these cells.

3.2. Induction of apoptosis upon heat shock in BC-8 cells

Following heat shock treatment, BC-8 cells appeared normal and were viable after 2–3 h of recovery at 37°C (as judged by trypan blue dye exclusion). Upon longer incubation at 37°C, heat-shocked BC-8 cells showed membrane blebbing and formation of apoptotic bodies (Fig. 3A, b) as observed during apoptosis induced by anti-AK-5 serum factor (Fig. 3A, c). In order to confirm these observations, the heat-shocked BC-8 cells were analyzed for DNA fragmentation.

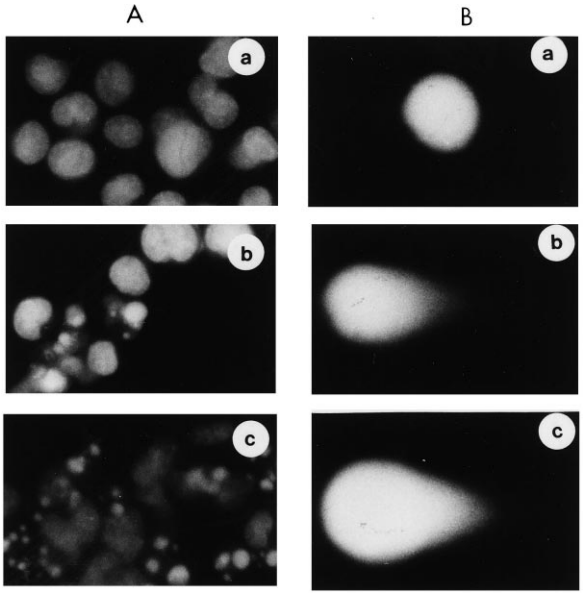

Fig. 3. (A) Formation of apoptotic bodies in BC-8 cells upon heat shock. Exponentially growing BC-8 cells were incubated at heat shock temperatures of 42° for 30 min and were allowed to recover at 37°C and were stained with propidium iodide. a: BC-8 cells at 37°C. b: Heat shock at 42°C for 30 min followed by recovery for 8 h at 37°C. c: BC-8 cells incubated overnight with an apoptotic inducer anti-AK-5 serum magnification 400×. (B) Comet morphology of control and heat-shocked BC-8 cells. BC-8 cells were processed as described in Section 2 for visualization of comets under neutral conditions, micro-electrophoresis was done for different durations and DNA was stained with ethidium bromide. a, control; b and c, electrophoresis for 5 and 8 min, respectively. Note the increase in comet length with increase in time of electrophoresis.

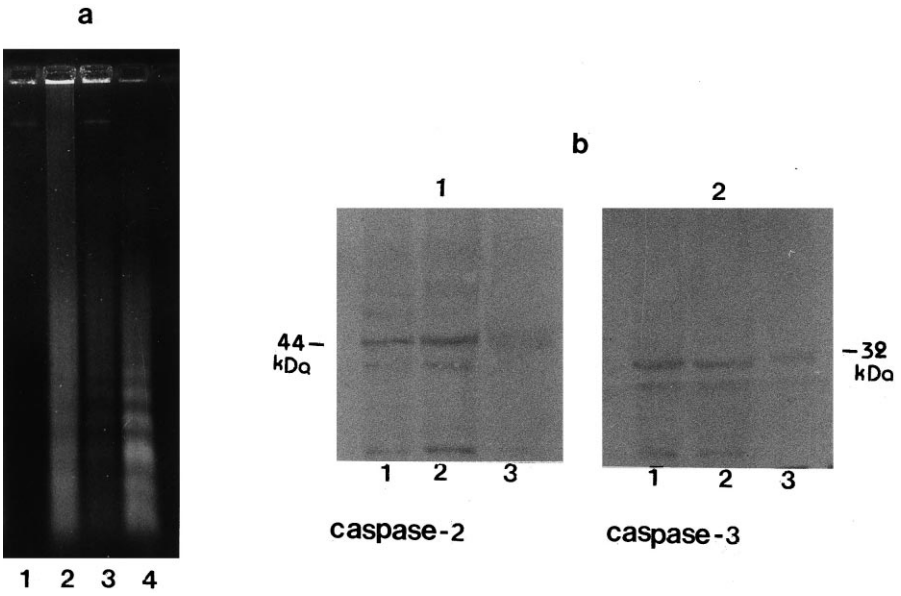

Fig. 4. (a) Induction of DNA fragmentation by heat shock. Agarose gel electrophoresis of DNA extracted from control, heat-shocked and cells treated with an apoptotic agent. Lanes: 1, control; 2, cells treated with an apoptotic agent; 3, immediately after heat shock and 4, after 8 h of recovery at 37°C. (b) Activation of caspases upon heat shock. BC-8 cells were given heat shock and were allowed to recover for 8 h. Total cell lysate was run on SDS-PAGE and processed for Western blot analysis with antibodies specific to caspase-2 (1) and caspase-3 (2). Lanes: 1, apoptosis induced by an apoptotic agent; 2, heat-shocked BC-8 cells and 3, control cells. Note the activation of caspase-2 and caspase-3 upon heat shock.

A comet assay, which detects breakage of DNA damage, is considered to be a hallmark of the apoptotic process. Results presented in Fig. 3B show the formation of comets in heat-shocked BC-8 cells, indicating fragmentation of the DNA (b, c) as is seen during apoptosis induced by the serum factor in BC-8 cells. An increase in the length of the comet tail with an increase in the duration of electrophoresis was also noticed (compare b and c), suggesting fragmentation of DNA upon heat shock.

3.3. Flow cytometric analysis of heat-shocked cells

Heat-shocked BC-8 cells were analyzed by flow cytometry after staining with PI. Results presented in Table 1 show a considerable increase in the percentage of sub G1 population upon heat shock and recovery as compared to the control cells. These observations also confirm activation of apoptosis upon heat shock in BC-8 cells.

3.4. Fragmentation of DNA upon heat shock

Appearance of a DNA ladder has been shown to be a biochemical signature for apoptotic cell death. To confirm the induction of apoptosis upon heat shock, DNA was extracted and fractionated on agarose gels. As shown in Fig. 4a, DNA extracted from cells after 8 h of recovery after heat shock clearly showed a DNA ladder (lane 4) typical of apoptosis. At the same time, there was no fragmentation of DNA immediately after heat shock (lane 3) or in the control cells (lane 1). Fragmentation of DNA in cells treated with an inducer of apoptosis is not complete but the fragmentation pattern can be seen (lane 2). These results further confirm the induction of apoptosis in BC-8 cells upon heat shock.

Table 1
Flow cytometric analysis of heat-shocked BC-8 cells

Time after heat shock	% Sub G1 population		
	Control	Heat shock	Apoptotic agent
0	2.1	8	–
4 h	–	27	–
8 h	–	44	66.5

3.5. Induction of caspases during heat shock-induced apoptosis

Caspases, a family of cysteine proteases which mediate specific proteolytic cleavage events, play a critical role during the execution phase of apoptosis. They are synthesized as inactive zymogens and are cleaved during activation [27,28]. Specifically, two of these cysteine proteases, caspase-2 and caspase-3, were characterized to be apoptotic markers in BC-8 cells [17]. To confirm that the apoptotic process of cell death is activated upon heat shock in BC-8 cells, activation of caspase-2 and caspase-3 was studied following heat shock using specific antibodies against these enzymes. Data from Western blot analysis of total cell lysates from control, heat-shocked cells and cells treated with an inducer of apoptosis were presented in Fig. 4b. Results show the appearance of the proteolytic fragments unique to caspase-2 (b1) and caspase-3 (b2), indicating the activation of these caspases upon heat shock.

4. Discussion

It has been well-established that both prokaryotic and eukaryotic cells, when exposed to elevated temperatures, mount a typical heat shock response resulting in the synthesis of a new set of proteins. Results presented in this study demonstrate that BC-8 cells fail to recover from the effect of heat shock.

Heat shock proteins have been shown to protect cells from undergoing apoptosis [29–31]. Among the hsps, the hsp70 family has been shown to play a prominent role. Mosser et al. [32] have shown that cells that constitutively overexpress

hsp70 resist apoptosis induced by ceramide. Accumulation of hsp70 in cultured cardiac cells was shown to protect them from stressful stimuli [33]. Also, Kim et al. [34] showed a correlation between accumulation of hsp70 and a protection from TNF-α-induced apoptosis in rat hepatocytes. He and Fox [35] have shown that the synthesis of hsp70 may protect cells from a subsequent heat dose in part by inhibiting the induction of apoptosis in HL-60 cells. All these studies suggest a strong correlation between hsp70 synthesis and induction of apoptosis.

Western blot analysis with hsp70 antibody revealed that BC-8 cells fail to induce hsp synthesis upon stress. A basal level of hsp70 could be detected in the Western blots under normal growth conditions in these cells but there was no further induction of hsp70 when cells were exposed to elevated temperatures. Data from RT-PCR of RNA isolated from heat-shocked BC-8 cells correlates these above observations. Lack of hsp70 induction might have triggered apoptosis in BC-8 cells upon heat shock. Fragmentation of nuclear DNA because of extensive double-stranded breaks which is characteristic of apoptosis was clearly visible in the comet assay. An increase in the length of the comet tail with an increase in time of electrophoresis confirmed fragmentation of DNA upon heat shock.

FACS analysis of heat-shocked BC-8 cells showed a considerable increase in the percentage of sub G1 population (Table 1), providing evidence for the induction of apoptosis upon heat shock. Appearance of a DNA ladder and activation of proteases, caspase-2 and caspase-3, further confirmed the onset of apoptosis upon heat shock in BC-8 cells.

It has been well-established that hsp70, with the aid of other polypeptides, helps in the recovery of partially denatured proteins and in removing the damaged, misfolded protein molecules [5]. Hsp-70 also binds to p53 [36], C-myc [37] and NF-kB regulatory complex subunits which are known to take part in the regulation of apoptosis [38,39]. It is possible that in BC-8 cells, some proteins that prevent apoptosis may be denatured during heat shock and cannot regain their native structure because of a lack of hsp70. Recently, Jaattela et al. [40], using human cervical carcinoma cells and WEHI-S murine fibrosarcoma cells that express different amounts of hsp70, showed that hsp70 rescues cells from apoptosis induced by staurosporine and doxorubicin and inhibits activation of cytosolic phospholipase A_2 and changes in the nuclear morphology, both of which are late caspase dependent events. The lack of chaperone activity of hsp70 in BC-8 cells due to their inability to mount a heat shock response is likely to trigger apoptosis in these cells.

Data presented in this study conclusively prove that heat shock induces apoptosis in BC-8 cells. How exactly hsp70 and other hsps prevent apoptosis needs further investigation and BC-8 cells represent an excellent experimental model to study the role of hsps in the inhibition of apoptosis and thereby prevention of death in these cells.

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