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Heat induced expression of CD95 and its correlation with the activation of apoptosis upon heat shock in rat histiocytic tumor cells

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Abstract The heat shock response is a universal phenomenon and is among the most highly conserved cellular responses. However, BC-8, a rat histiocytoma, fails to mount a heat shock response unlike all other eukaryotic cells. In the absence of induction of heat shock proteins, apoptotic cell death is activated in BC-8 tumor cells upon heat shock. We demonstrate here that stable transformants of BC-8 tumor cells transfected with hsp70 cDNA constitutively express hsp70 protein and are transiently protected from heat induced apoptosis for 6–8 h. In addition heat stress induces CD95 gene expression in these tumor cells. There is a delay in CD95 expression in hsp70 transfected cells suggesting a correlation between the cell surface expression of CD95 and the time of induction of apoptosis in this tumor cell line. Also expression of CD95 antigen appears to inhibit the interaction between heat shock factors and heat shock elements in these cells resulting in the lack of heat shock response.

Key words: CD95; Heat shock protein; Heat shock factor; Apoptosis

1. Introduction

The heat shock response or stress response is a universal phenomenon and is among the most highly conserved cellular responses [1]. It is triggered when organisms/tissues are exposed to or experience unfavorable conditions resulting in the synthesis of stress proteins or heat shock proteins [2].

Heat shock proteins (hsps) constitute a phylogenetically old cellular system providing increased resistance to a variety of cellular stress factors [3]. They (especially the inducible form of hsp70) function as chaperones facilitating the folding, unfolding and refolding of nascent or stress denatured proteins preventing their improper folding or further damage and degradation [4]. Depending upon the severity of the stress, cells either survive or if the stress is too severe and prolonged, face cellular death due to apoptosis or necrosis. Both cell survival and activation of the cell death process are based on cellular signalling systems either towards the expression of stress proteins or towards the activation of a cascade of molecular events leading to cell death [5].

Stress protein synthesis, which is regulated both at the transcriptional and the translational level, is triggered by the activation of heat shock transcription factor (HSF) by a signalling cascade. HSFs are constitutively present in the cytoplasm in a monomeric, non-DNA binding form and upon activation they are hyperphosphorylated, trimerized and translocated into the nucleus where they bind to specific sites on hsp gene promoter regions called heat shock elements (HSEs). Of the four related factors capable of specifically binding to heat shock elements, HSF1 has been shown to respond to signalling by stress factors including elevated temperature [6,7].

Though stress response and induction of stress proteins is a common phenomenon in all living systems, we have earlier reported that a rat histiocytic tumor cell line, BC-8, fails to mount a typical heat shock response and synthesize hsps [8]. In the absence of hsp synthesis, apoptotic cell death is activated in BC-8 cells upon heat shock. Several molecules like CD95, Bcl-2 and several classes of proteases have been shown to regulate apoptosis [9–11]. CD95 antigen, a type 1 transmembrane receptor present on a variety of normal and tumor cells [9], is a member of the signal transduction cascade inducing apoptosis in the presence of pro-apoptotic factors. It is shown to mediate apoptosis during elimination of unwanted cells during development, in the regulation of the immune system and during cytotoxic T-cell mediated cytotoxicity [12]. Binding of CD95 ligand or an agonistic anti-CD95 antibody induces apoptosis in CD95 bearing cells [13–14]. Several cysteine proteases were shown to be activated during apoptosis induced by CD95 [15]. The BC-8 tumor cells express CD95 ligand but the presence of CD95 receptor on the cell membrane could not be detected [16]. We report here that stable transformants of BC-8 tumor cells transfected with hsp70 cDNA constitutively express hsp70 protein and are transiently protected from heat induced apoptosis. We also show that heat stress induces CD95 expression in these cells and that there is a correlation between cell surface expression of CD95 and timing of the induction of apoptosis.

2. Materials and methods

2.1. Cell culture and heat shock condition

Rat histiocytic tumor AK-5, maintained as ascites, was adapted to grow in culture for several generations in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) in the presence of penicillin (100 U/ml) and streptomycin (50 μg/ml). BC-8 is a single cell clone derived from AK-5 tumor and possesses all the characteristics of the parent tumor. 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 [17]. Jurkat and rat hepatoma H411-E-C3, which were used as controls, were maintained in DMEM with 10% FCS and antibiotics as above.

2.2. Construction of pEGFP-hsp70 vector and transfections

The full length coding sequence of the hsp70 gene was PCR amplified from plasmid containing the full length cDNA, gel purified and ligated at the 3′-end of the pEGFP vector (Clontech) containing green

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fluorescent protein (GFP) under the control of the CMV promoter and a neomycin marker. After confirming the orientation and reading frame of the hsp70 gene in the plasmid, BC-8 cells were transfected with 20 μg of linearized pEGFP-hsp70 plasmid by electroporation [18]. Cells were subjected to six pulses of 150 V strength with 5 ms pulse duration using a Biojet cell fusion/electroporation apparatus. Subsequently the cells were incubated at 37°C for 5 min in the chamber and transferred to resealing medium and incubated at 37°C for another 30 min. The cells were washed gently and resuspended in DMEM–FCS. Twenty-four hours later, G418, at a concentration of 400 μg/ml, was added and cells were subjected to selection for 2 weeks. The surviving clones were expanded, single cell clones were isolated and checked for GFP and hsp70 expression.

2.3. Western blot analysis

Cell lysates from 5×10^5 cells/lane were run on 10% SDS–PAGE, transferred onto a nitrocellulose membrane, blocked and probed with appropriate antibody as described earlier [19] and the specific bands were visualized using a chemiluminescence kit from Boehringer Mannheim. Antibodies for hsp70 were from Stressgen, Canada and CD95 was from Calbiochem.

2.4. Gel mobility shift assay

Cell extracts prepared from control and heat shocked BC-8 cells were incubated with labeled oligonucleotides containing a canonical heat shock element (3′-nGAAn′ repeats synthesized in house) as described earlier [20]. Binding of HSF to HSE can be seen in the autoradiogram as a change in the mobility of the labeled oligo in the gel.

2.5. Propidium iodide staining and flow cytometry

Control and heat shocked BC-8 cells or hsp70 transfected cells were fixed immediately or after different intervals of recovery at 37°C in 70% ethanol, stained with propidium iodide reagent (50 μg in 0.1% sodium citrate containing 0.1% Triton X-100) and analyzed by flow cytometry as described earlier [21].

2.6. RNA isolation and Northern blotting

Total RNA from control and heat shocked BC-8 or hsp70 transfected cells was isolated using the acid guanidinium thiocyanate method [22]. RNA was run on a denaturing gel and transferred onto nylon membrane or dot blotted onto nylon membrane and probed with labeled CD95 cDNA or HSF1 cDNA [23].

2.7. DNA extraction and agarose gel electrophoresis

Cells were fixed in ethanol, washed with phosphate buffered saline (PBS) and suspended in citrate phosphate buffer. DNA was extracted following the procedure described earlier [24], electrophoresed on a 0.86% agarose gel at 2 V/cm for 16 h, stained with 5 μg/ml of ethidium bromide and visualized under UV light.

2.8. Confocal microscopy

Cells were fixed in 3.0% paraformaldehyde in PBS, washed with PBS and processed for direct green fluorescence under a laser confocal microscope.

3. Results

A variety of chemicals are known to induce apoptosis in BC-8 tumor cells. During the apoptotic process, cell fragmentation, activation of caspases, DNA ladder formation, etc. have been demonstrated earlier [25,26]. However, the initial trigger for the activation of apoptosis in BC-8 tumor cells is not clearly understood. CD95 ligand was shown to be expressed by BC-8 cells but the presence of CD95 antigen on the cell surface could not be detected. Activation of the apoptotic pathway by heat shock alone prompted us to study the mechanism of induction of apoptosis upon heat shock in these tumor cells.

3.1. Hsp70 transiently protects BC-8 cells from heat induced apoptosis

There is a strong correlation between induction of hsp70 synthesis and protection of cells from apoptosis [27]. To investigate whether inducible hsp70 protects BC-8 cells from undergoing apoptosis, cells were transfected with pEGFP-hsp70 plasmid and tested for the induction of apoptosis upon heat shock. As seen in Fig. 1A, transfected cells show green fluorescence indicating the expression of GFP and hsp70. The expression of hsp70 was also confirmed by immunoblot analysis. As shown in Fig. 1B in pEGFP-hsp70 transfected BC-8 cells, the amount of hsp70 appears to be more.

To study the protective effect of hsp70, control and transfected cells were given a heat shock and were allowed to

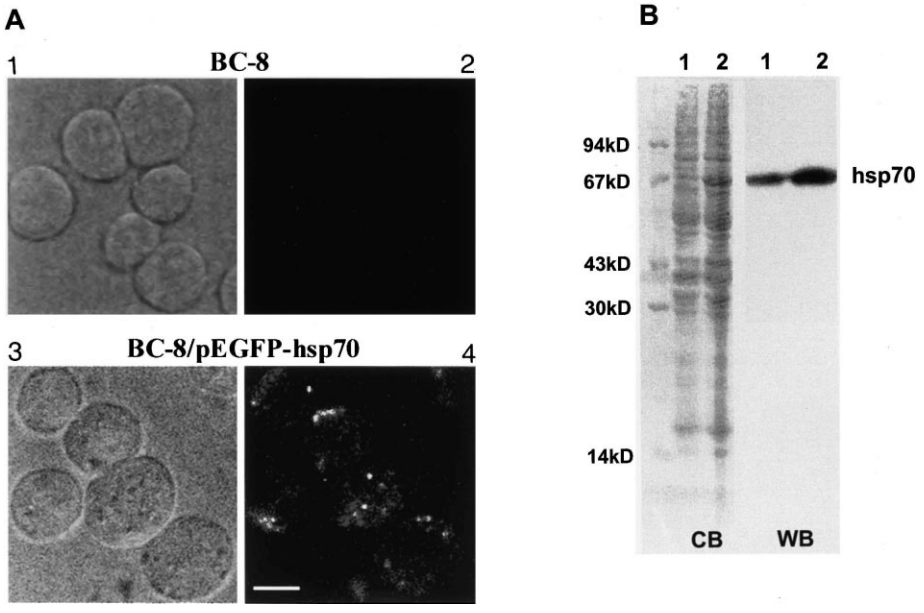

Fig. 1. Expression of GFP and hsp70 in BC-8 cells transfected with hsp70 cDNA. A: Panels 1 and 3, phase contrast. Panels 2 and 4, fluorescence. Bar indicates 10 μm. B: Western blot of control BC-8 and pEGFP-hsp70 cells with hsp70 antibody. Lanes 1, BC-8 control; 2, hsp70 transfected BC-8 cells. CB, Coomassie blue staining; WB, Western blot. Expression of hsp70 is more in the transfected clone.

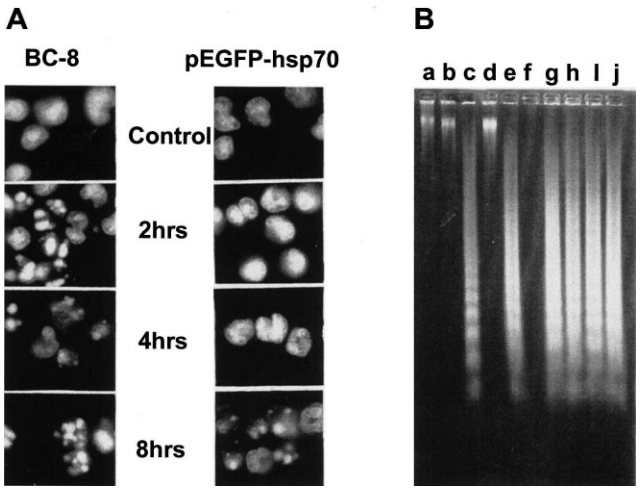

Fig. 2. Formation of apoptotic bodies upon heat shock. A: Panel 1, BC-8 control. Panel 2, pEGFP-hsp70 cells. Apoptotic body formation is delayed in transfected cells. B: Agarose gel electrophoresis of DNA extracted from control BC-8 and hsp70 transfected BC-8 cells. Samples from BC-8 and pEGFP-hsp70 were loaded in alternate lanes. Lanes a and b, control; c and d, 4 h; e and f, 6 h; g and h, 8 h; i and j, 12 h after heat shock.

recover at 37°C and monitored for apoptosis. As can be seen in Fig. 2A, even morphological observations suggest protection of BC-8 cells by hsp70. Fragmentation of cells in GFP-hsp70 transfected cells appears to be delayed by 6–8 h after heat shock suggesting partial protection by hsp70. To confirm these results, DNA was extracted from both types of cells after different periods of recovery following heat shock and analyzed for DNA fragmentation. Results presented in Fig. 2B clearly show protection of GFP-hsp70 transfected cells from apoptosis up to 6 h following heat shock. Subsequently, formation of apoptotic bodies and DNA laddering were visible even in GFP-hsp70 transfected cells.

3.2. Expression of CD95 antigen upon heat shock

In order to understand the mechanism of heat induced apoptosis in BC-8 cells, the expression of CD95 antigen was studied. Transcriptional activation of CD95 antigen was tested by probing the total RNA, from control and heat shocked BC-8 cells after specific times of recovery at 37°C, with CD95 cDNA. Results presented in Fig. 3A show that RNA hybridized with CD95 cDNA was present immediately after heat shock (lane b) and after 1 h of recovery only (lane c) suggesting transcriptional activation of the CD95 gene upon heat shock. Further, Western blot analysis of total cell lysates from control, heat shocked and hsp70 transfected BC-8 cells with CD95 antibody indicated translation of the newly synthesized mRNA into CD95 antigen. As shown in Fig. 3B, CD95 antigen could not be detected in control BC-8 cells (lane l) but can be seen immediately upon heat shock and even after 8 h of recovery at 37°C (lanes k–h). Interestingly, there is a delay in the expression of CD95 antigen in BC-8 cells transfected with the hsp70 gene (lanes b–e). The antigen was seen after 6 h of recovery after heat shock and its appearance coincides with the formation of apoptotic bodies seen microscopically. Absence of an immunoreactive antigen in the corresponding region in control and hsp70 transfected BC-8 cells and its presence in Jurkat cell lysates (lanes f and g) which are positive for CD95 expression confirm expression of CD95 antigen upon heat shock.

CD95 positive cells express the antigen on the cell surface to which the ligand binds leading to the activation of the signal transduction cascade for apoptosis. To study whether the CD95 antigen synthesized upon heat shock is expressed on the cell surface, control and heat shocked BC-8 cells, after different periods of recovery at 37°C, were fixed, incubated with the anti-CD95 antibody and analyzed by flow cytometry. Results presented in Fig. 3C show significant expression of CD95 antigen after 1 h of heat shock. With increasing time of recovery at 37°C, the percentage of cells expressing CD95 is reduced.

3.3. Lack of HSF–HSE interaction in BC-8 cells

Heat shock proteins are synthesized upon stress by the interaction of HSF1 with specific DNA sequences (HSEs) present in the promoters of heat shock genes. The BC-8 tumor cells do not express hsps upon heat shock [8]. To investigate

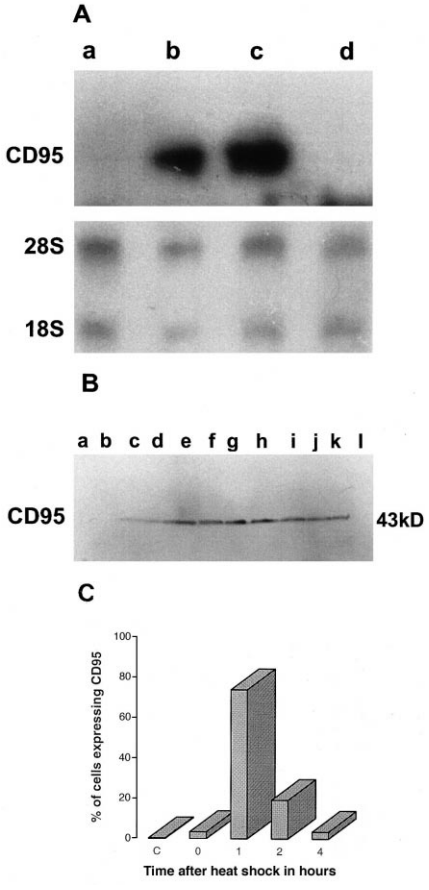

Fig. 3. A: Expression of CD95 in BC-8 cells upon heat shock. RNA isolated from control and heat shocked BC-8 cells was run on denaturing gels and probed with nick translated CD95 cDNA. Lanes a, control; b, immediately after heat shock; c, 1 h; d, 2 h after heat shock. B: Expression of CD95 antigen in heat shocked BC-8 cells. Total cell lysates were run on denaturing gels, blotted and probed with CD95 antibody. Lanes l–h, BC-8; a–e, pEGFP-hsp70 transfected BC-8; f and g, Jurkat cell lysates. l and a, control; k and b, 1 h; j and c, 2 h; i and d, 4 h; h and e, 8 h after heat shock. C: FACS analysis of control and heat shocked BC-8 cells stained with CD95 antibody and analyzed with FITC conjugated second antibody. The relative increase in fluorescence was scored.

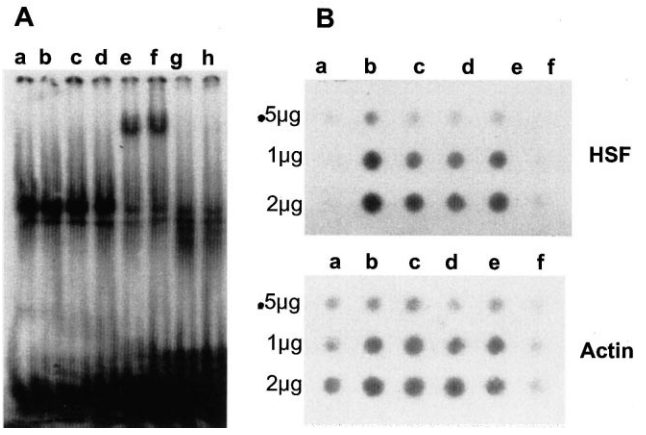

Fig. 4. Electrophoretic mobility shift assay with control and heat shocked BC-8 cells. Total cell lysates were incubated with labeled HSEs as described in Section 2 and run on PAGE. Lanes a–c, control BC-8, pEGFP-hsp70 and H411-E-C3 cells; d, pEGFP-hsp70 cells, 60 min after heat shock; e and f, H411-E-C3 cells; g and h, BC-8 cells, 30 and 60 min respectively after heat shock. H411-E-C3 cells are positive for heat shock response. B: Dot blot analysis of RNA from BC-8 cells with HSF1 cDNA. Panels a, control; b–f, 0 time, 2, 4, 6 and 8 h after heat shock.

whether the lack of hsp synthesis in BC-8 tumor cells is due to the lack of HSF–HSE interaction and to understand the effect of CD95 expression upon the heat shock response, cell lysates from control and heat stressed BC-8 cells after different times of recovery at 37°C were analyzed for HSE binding activity by gel mobility shift assay. Results presented in Fig. 4A clearly show a lack of HSF–HSE interaction upon heat shock. Rat hepatoma H411-E-C3 cells, which synthesize hsps upon heat shock [20], clearly show HSF1–HSE binding under the same conditions (lanes, e and f) thus demonstrating lack of HSE binding activity in BC-8 cell lysates following heat stress. To understand the transcriptional activation of the CD95 gene upon heat shock, total RNA from control and heat shocked BC-8 cells was dot blotted on nylon membranes and probed with rat HSF1 cDNA. Results presented in Fig. 4B indicate the activation of the HSF1 gene upon heat shock in these cells with maximum expression of HSF1 mRNA seen immediately after heat shock.

4. Discussion

Apoptosis is a physiological process defined by morphological alterations such as condensation of chromatin concomitant with cell shrinkage [29,30]. It is activated by different stimuli under a variety of conditions like during development for the elimination of unwanted cells, cytotoxic T-cell mediated cytotoxicity and in the regulation of the immune system [12]. The mechanism of activation of apoptosis and the role of various proteins during signal transduction and during the activation and execution stages of apoptosis have been areas of intense study in the past several years. In spite of the fact that there are some common events, the mechanism of activation of apoptosis appears to be different in different cell types. The quantity of inducible hsp70 molecules in a cell is one of the pivotal conditions determining cellular resistance to a variety of stress factors [31–33]. Hsp70 has been shown to provide cytoprotection during stress. Results presented in this study showing protection of BC-8 cells transfected with hsp70 from apoptosis for 6 h confirm the above data. The delay in the induction of apoptosis in these cells could be due to the presence of hsp70 with a cytoprotective function. However, the increase in the amount of hsp70 does not appear to be sufficient to protect BC-8 cells fully from heat induced apoptosis. Natural killer (NK) cells have been shown to induce apoptosis in target cells either through production of granzymes, tumor necrosis factor α or through CD95–CD95 ligand interactions [34]. Khar et al. have demonstrated that apoptosis in rat histiocytoma AK-5 is mediated by activated NK cells which show higher expression of granzyme B and CD95 L [14,15]. However, expression of CD95 antigen or CD95 specific transcripts could not be detected in apoptotic cells by Northern hybridization. We demonstrate here that in BC-8 tumor cells, heat shock induces CD95 gene expression (Fig. 3A). Immunoblot analysis of total cell lysates (Fig. 3B) of heat shocked BC-8 cells confirmed the presence of CD95 antigen. FACS analysis of whole cells stained with anti-CD95 antibody reveals the surface expression of CD95 upon heat shock in these cells. Since the CD95 ligand has already been shown to be expressed in BC-8 cells, expression of the CD95 antigen may be involved in triggering apoptosis in these cells upon heat shock.

Schett et al. [28] have demonstrated that activation of the CD95 pathway blocks heat shock induced activation of HSF1 and expression of hsp70. It is possible that immediate expression of CD95 in BC-8 cells upon heat shock inhibited HSF1 activation as HSF1–HSE binding activity could not be detected in BC-8 cells even after heat shock (Fig. 4A). Recently Zhang et al. have reported that lack of hsp expression in rat NB2 lymphoma cells following heat shock is probably due to proteolytic cleavage of HSF. Cysteine proteases, which are activated during apoptosis, are the enzymes that appear to be responsible for the proteolysis of HSF [35]. In view of this, the lack of HSF–HSE interaction in BC-8 cells upon heat shock could be due to the proteolysis of HSF. There is an increase in HSF1 RNA upon heat shock as demonstrated by dot blot analysis (Fig. 4B) but the lack of HSF–HSE interaction even after recovery at 37°C (Fig. 4A, lanes g and h) indicates that HSF1 mRNA is either not translated into protein or even if it is translated, the protein is probably degraded by the proteases activated during heat induced apoptosis.

Since the apoptotic pathway has already been activated upon heat shock, activation of HSF1 and hsp70 synthesis may be counterproductive to the cell as it gives resistance to apoptosis. Therefore, either the activation of nascent HSF must have been inhibited or HSF is degraded upon activation of the apoptotic pathway. The delay in CD95 expression in cells transfected with pEGFP-hsp70 indicates the protective role of hsp70 in BC-8 cells from heat induced apoptosis. The quantity of hsp70 present in pEGFP-hsp70 transfected cells is certainly more than that present in BC-8 cells (Fig. 1B) but the amount may not be sufficient to protect the cells fully from apoptosis following heat shock. The increased amount of hsp70 partially protecting heat shocked pEGFP-hsp70 transfected BC-8 cells from apoptosis and the delay in CD95 expression correlating with time of activation of apoptosis in these cells suggest a role for CD95 in the induction of apoptosis.

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