

A CROSS TALK BETWEEN CELLULAR SIGNALING AND CELLULAR REDOX STATE DURING HEAT-INDUCED APOPTOSIS IN A RAT HISTIOCYTOMA

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Abstract—Increasing evidence provides support for oxidative stress to be closely linked to apoptosis. Reactive oxygen species (ROS) are thought to be involved in many forms of programmed cell death. Though heat shock is a universal phenomenon, BC-8, a macrophage-like cell line failed to mount a typical heat shock response. In the absence of heat shock proteins and functional p53, BC-8 cells undergo apoptosis through CD95 signaling. In the present study, we have investigated the role of ROS in the regulation of apoptosis in these cells. We show that cells transfected with hsp70 and functional p53 are resistant to heat-induced apoptosis through inhibition of CD95 expression and ROS induction. Furthermore, apoptosis in BC-8 cells resulted in two bursts of ROS generation, one correlated with heat stress and intracellular depletion of GSH and the other with Bax overexpression and cytochrome *c* release. Antioxidants could not protect these cells from heat-induced apoptosis and the death pathway seems to be dependent on initial signaling cascade subsequently altering the intracellular redox. Hence, our data suggest that ROS generation in BC-8 cells upon heat shock is facultative but not obligatory for apoptosis. © 2002 Elsevier Science Inc.

Keywords—Heat shock, Tumor cells, Apoptosis, Free radicals

INTRODUCTION

Hyperthermia damages cells and cell death is apparent at critical temperature load [1,2]. Recent studies indicated involvement of heat-mediated oxidative stress in hyperthermia-induced cytotoxicity [3,4]. Reactive oxygen species (ROS) have a major role in the mediation of cellular damage. Though the origin of ROS remains to be determined, mitochondria are thought to be the major source of ROS in vivo [5]. The production of ROS is associated with many forms of apoptosis [6] and also necrosis [7,8].

It has been shown in many model systems that cellular redox plays an essential role in cell survival and cellular signaling [5]. The primary target of redox regulation may be a sulphhydryl group (RSH) on cysteine residues, which is easily oxidized to form a disulphide bond (RSSR) [9]. Although inhibitors of cysteine transport leading to oxidative stress were reported [10], the exact relationship

between glutathione (GSH) depletion and ROS production during heat-induced apoptosis has not been explored.

ROS are also known to induce heat shock proteins that are critical for cellular thermoresistance and the development of thermotolerance [1,11]. Furthermore, ROS induce p53-mediated apoptosis through changes in mitochondrial membrane potential in a caspase-dependent and -independent manner [12] through the upregulation of CD95 death receptor [13]. Though the execution of CD95-induced cell death pathway is largely independent of ROS, the sole exposure of cells to hydrogen peroxide was sufficient to upregulate CD95 ligand expression [14]. Caspase-8 signals through the formation of a death-inducing signaling complex when CD95 is activated by CD95 ligand on the cell surface [15]. Caspase-8 activation can lead to two signaling events: (i) direct activation of downstream effectors, caspase-3, caspase-6, and caspase-7; and (ii) mitochondrial dysfunction through Bax translocation and cytochrome *c* release [16]. The redox components of the apoptotic signal transduction pathway have not yet been

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clearly identified. Cytochrome *c*, a protein that normally functions in redox reactions within the mitochondria, is a key component of apoptosome that activates apoptosis [17].

We have previously reported that a rat macrophage-like cell line, BC-8, failed to mount a typical heat shock response and hyperthermia-induced apoptotic cell death in these cells [18]. Furthermore, apoptosis is correlated with the surface expression of CD95 [19]. Since ROS act as mediators of hyperthermia-induced cellular damage, the present study is aimed at understanding the effect of hyperthermia in the regulation of intracellular ROS and redox state and their role in apoptosis signaling.

We report here that BC-8 cells, upon heat shock, release ROS in two bursts: one immediately after heat shock, which comes down by 45 min after heat shock, and another 1 h after heat shock during recovery at 37°C. The first ROS burst correlated with the surface expression of CD95 and caspase-8 activation, and the second burst with mitochondrial dysfunction as seen through Bax induction and cytochrome *c* release. BC-8 cells transfected with hsp70 and functional p53 (3B4 cells) blocked cytochrome *c* release and inhibited apoptosis and the associated changes in redox state in the absence of CD95 signaling. Our results also demonstrate that treatment of BC-8 cells with antioxidants and downstream caspase inhibitors could not give protection from heat-induced apoptosis. Thus, the initial apoptotic stimulus decides the fate of the cell.

MATERIALS AND METHODS

Cell culture and heat shock conditions

BC-8, a single-cell clone of AK-5 tumor [18] cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 50 µg/ml streptomycin. Exponentially growing cells were given heat shock at 42°C for 30 min in a Julabo water bath. After heat shock, the cells were allowed to recover at 37°C for different time intervals prior to analysis.

Transfection of BC-8 with hsp70 and wild-type p53 genes

The full-length hsp70 gene was PCR amplified from a plasmid clone and subcloned into the N-terminus of pEGFP expression vector (Clontech, Palo Alto, CA, USA). BC-8 cells were transfected with pEGFP-hsp70 plasmid and selected for stable transformants. After clonal selection, several clones were tested for GFP and hsp70 expression. All clones that expressed GFP also expressed hsp70 and were similar in doubling time and in general protein synthetic pattern. Hence, one repre-

sentative clone was selected and used in the present study [19]. Similarly, wild-type p53 was stably transfected into BC-8 cells, tested for p53 expression and tumorigenicity, and a representative clone (3B4) was selected for subsequent study [20].

Antisense CD95 oligo treatment

Antisense CD95 (5' TAC GAC CCG TAG ACC TGG GAG GAT GGA GAC CAA GAA 3') was synthesized for coding region from the nucleotides 1 to 36 of the mouse CD95 gene [21]. Cells were preincubated with 5 µg oligonucleotide per 5×10^5 cells for 16 h and then subjected to heat shock. Cell viability and induction of apoptosis were monitored at 37°C following heat shock.

Propidium iodide staining and comet assay

Heat-shocked cells were allowed to recover at different time intervals at 37°C, washed with phosphate-buffered saline, and fixed with 80% ethanol. Cells were stained with propidium iodide (Calbiochem, San Diego, CA, USA) reagent (50 µg/ml in 0.1% sodium citrate buffer containing 0.1% Triton X-100) and visualized under fluorescent microscope for DNA analysis. Comet assay was performed as described earlier [18] and the cells were photographed in a fluorescent microscope after staining with ethidium bromide.

DNA extraction and electrophoresis

Cells were fixed in ethanol, washed with phosphate-buffered saline, and suspended in citrate-phosphate buffer. DNA was extracted following the procedure described earlier [22,23], 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.

Estimation of intracellular glutathione

Intracellular GSH was estimated by DTNB-GSSG reductase assay where GSH is oxidized by 5'-5' dithiobis (2-nitrobenzoic acid) (DTNB) to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB) [24]. Total cell lysates from (10^6 cells) control and from cells following different time intervals after heat shock were subjected to DTNB-GSSG reductase assay and the reduced GSH was measured spectrophotometrically at 410 nm.

Estimation of intracellular ROS

Superoxide-induced reduction of ferricytochrome *c* to ferrocyanochrome *c* was monitored spectrophotometri-

cally at 550 nm [25]. Control and heat-shocked cells were suspended in complete phenol-red-free DMEM and plated in 96-well plates at 5×10^4 cells/well. Superoxide release was estimated in the presence of 80 μM cytochrome *c* with and without superoxide dismutase (SOD) (300 U/ml).

Release of cytochrome *c* into the cytosol

BC-8 cells (3×10^6 cells) before and after heat shock at different time intervals were harvested in PBS resuspended in 0.5 ml buffer A (20 mM HEPES.KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM PMSF, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin). Cells were homogenized in the same buffer in a Dounce homogenizer giving 40 strokes with B-type pestle. Lysates were transferred to Eppendorf tubes and centrifuged at 3.5 K for 10 min at 4°C. The supernatant was collected and further centrifuged at 12 K for 20 min to obtain mitochondrial fraction. The cytosol is obtained after ultracentrifugation at 65 K for 30 min. For analysis, 25 μg of protein was loaded per lane on 10% SDS-PAGE.

Immunoblot analysis

Total cell lysates (25 μg) prepared from control cells and cells heat shocked after prior treatment with antisense oligonucleotides were run on 10% SDS-PAGE, transferred onto a nitrocellulose membrane (Amersham, Braunschweig, Germany), blocked, and probed with appropriate antibody as described earlier [26]. The specific bands were visualized using a chemiluminescence kit (BM Chemiluminescence Western Blotting Kit) from Boehringer Mannheim (Mannheim, Germany). Antibodies for anti-hsp70 were procured from Stressgen (Victoria BC, Canada), anti-CD95 from Calbiochem, and anti-p53 from Boehringer Mannheim; anti-cytochrome *c*, anti-Bax, and anti-caspase-8 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Treatment with antioxidants and caspase inhibitors

The antioxidants, GSH, NAC, and vitamin E were added to cells in culture at different concentrations and incubated for 16 h. The optimal concentration was chosen based on maximal cell viability as assessed by trypan blue dye exclusion at maximal antioxidant concentration. Prior to heat shock, 2×10^6 cells were incubated to a final concentration of 20 mM GSH for 6 h, 1 mM NAC for 8 h, and 10 IU vit E for 6 h, and used for the present study. Caspase inhibitors DEVD and YVAD (Bachem, Bubendorf, Switzerland) were added to a final concen-

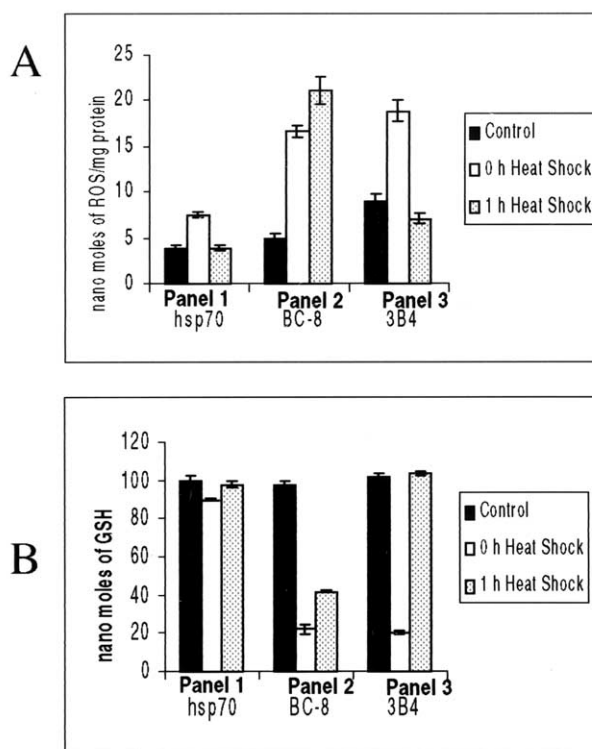


Fig. 1. (A) Release of intracellular ROS during heat shock-induced apoptosis in BC-8 cell types as measured by in vitro cytochrome *c* release assay. Panel 1: BC-8 transfected with hsp70; panel 2: parental BC-8; panel 3: BC-8 transfected with wild-type p53 (3B4). Each panel represents control, 0 h, and 1 h after heat shock. The amount of ROS is represented as nanomoles per milligram of protein ($n = 6$). (B) Estimation of intracellular-reduced glutathione (GSH) upon heat shock. GSH was estimated by DTNB-GSSG reductase assay and the amount of GSH was represented in nanomoles per 10^6 cells. Panel 1: BC-8 transfected with hsp70; panel 2: parental BC-8; panel 3: BC-8 transfected with wild-type p53. Each panel represents control, 0 h, and 1 h after heat shock ($n = 6$).

tration of 50 and 30 μM , respectively, and incubated for 8 h prior to heat shock.

RESULTS

ROS production upon heat shock

Exposure of BC-8 or 3B4 cells to heat shock resulted in an immediate increase in ROS production and a depletion in intracellular GSH levels (Figs. 1A and 1B, panels 2 and 3). ROS continued to be produced for 30 min following heat shock and reduced to control levels by 45 min in both types of cells. However, in BC-8 cells that undergo apoptosis upon heat shock, there was another increase in ROS levels after 1 h during recovery at 37°C (Fig. 1A, panel 2), which is not seen in 3B4 cells (Fig. 1, panel 3). Also, GSH levels returned to control level in 3B4 cells that are resistant to heat-induced apoptosis (Fig. 1B, panel 3) but not in BC-8 cells (Fig. 1B,

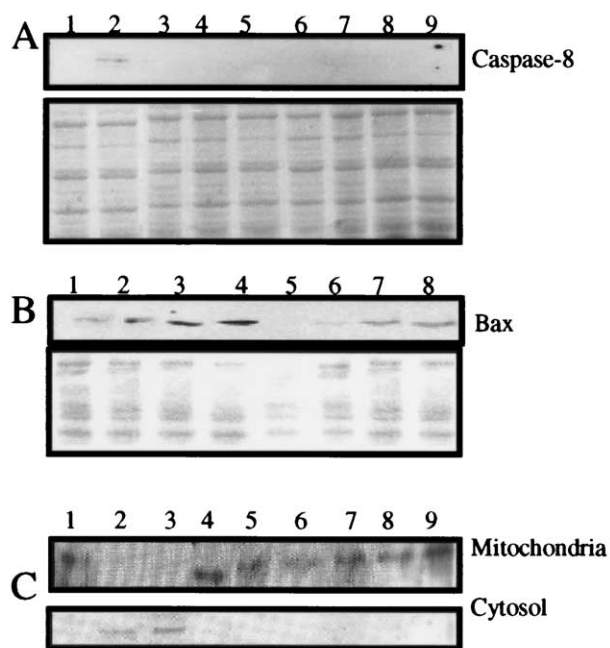


Fig. 2. (A) Immunoblot analysis of BC-8 cell types with active caspase-8 (p10) antibody. Lanes, 1–3 are parental BC-8 cells, control, 0 h, and 1 h after heat shock; lanes 4–6 are BC-8 transfected with hsp70, control, 0 h, and 1 h after heat shock; lanes 7–9 are BC-8 transfected with wild-type p53, control, 0 h, and 1 h after heat shock, respectively. Ponceu-S stained blot was shown below for equal protein loading. (B) Immunoblot analysis of cytosolic Bax expression in BC-8 cell types. Lanes 1–4 are parental BC-8 cells and lanes 5–8 are BC-8 transfected with wild-type p53. Only parental BC-8 showed induced expression of Bax upon heat shock, whereas BC-8 transfected with wild-type p53 showed only marginal expression. Ponceu-S stained blot was shown below for equal protein loading. (C) Mitochondrial release of cytochrome *c* in BC-8 cell types. Both mitochondrial and cytosolic cell extracts were subjected to immunoblot analysis with cytochrome *c* antibody. Lanes 1–3 are parental BC-8 cells, control, 1 h, and 2 h after heat shock; lanes 4–6 are BC-8 transfected with hsp70, control, 1 h, and 2 h after heat shock; lanes 7–9 are BC-8 transfected with wild-type p53, control, 1 h, and 2 h after heat shock. Note release of cytochrome *c* only in parental BC-8 cells. These experiments were repeated three times with similar results.

panel 2). The amount of ROS released immediately upon heat shock in BC-8 cells transfected with hsp70 is far less compared to untransfected BC-8 cells, and even that was reduced to normal levels during recovery (Fig. 1A, panel 1). Also, reduction in GSH levels seen in untransfected BC-8 cells upon heat shock is not seen in BC-8 cells transfected with hsp70 (Fig. 1B, panel 1).

Activation of caspase-8 during heat-induced apoptosis

Heat-induced downstream effector caspases were identified 4 h after heat shock during recovery at 37°C in BC-8 cells [18]. In order to identify the early executioners of CD95 ligation upon heat shock, total cell lysates after different time intervals were collected and immunoblotted with caspase-8 antibody. As seen in Fig. 2A,

BC-8 cells showed active caspase-8 at 0 h after heat shock (lane 2), but not after 1 h (lane 3). Active caspase-8 could not be detected either immediately after heat shock or after 1 h of recovery in hsp70- (lanes 5 and 6) and p53- (lanes 8 and 9) transfected cells. These results suggest involvement of hsp70 and wild-type p53 in the inhibition of caspase-8 activation, probably through the inhibition of surface expression of CD95 as shown earlier [18].

Modulation of Bax expression by p53 and hsp70

In order to understand how Bax expression regulated heat-induced apoptosis in BC-8 cells, expression of Bax protein was studied. Results presented in Fig. 2B show that BC-8 cells had a basal amount of Bax protein, which was further induced upon heat shock (lanes 2–4). Whereas in cells transfected with wild-type p53, no basal level of Bax was detected and the levels of induction were less upon heat shock. The Bax protein levels in hsp70-transfected cells remained unaltered upon heat shock (data not shown).

Induction of apoptosis is associated with cytochrome *c* release

To determine the role of mitochondria in the redox regulation in apoptosis, we separated mitochondria from cytosol and analyzed for cytochrome *c* release. By 1 h after heat shock, BC-8 cells showed release of cytochrome *c* from mitochondria into the cytosol (Fig. 2C, lanes 2 and 3), whereas cells transfected with hsp70 and p53 did not show cytochrome *c* release (lanes 5, 6, 8, and 9, respectively). Reports show that release of mitochondrial cytochrome *c* is regulated by Bax overexpression and can be inhibited by Bcl2 [27]. BC-8 cells do not show Bcl2 protein expression even after heat shock and the cells transfected with Bcl2 gene were found sensitive to heat-induced apoptosis.

Role of antioxidants in apoptosis

Antioxidants, GSH, NAc, and vitamin E were tested *in vitro* for apoptosis-inhibiting activity in BC-8 cells. Cells incubated with antioxidants show an increase in apoptotic cell death upon heat shock as seen in Fig. 3A. Even the combination of antioxidants could not give protection. Furthermore, cell death was found associated with DNA damage as seen by cell morphology and comet assay (Fig. 3B).

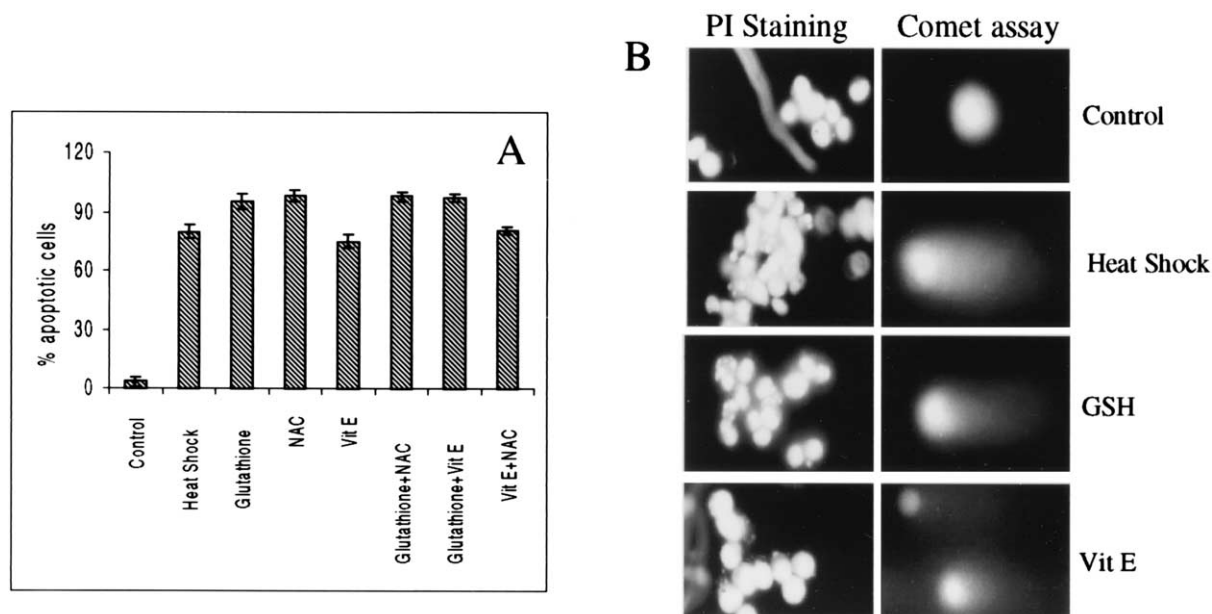


Fig. 3. (A) Role of antioxidants in heat-induced apoptosis. The percentage of apoptotic cells were represented in the presence and absence of antioxidants upon heat shock in parental BC-8 cells. E = vitamin E; N = N-acetyl cysteine; G = glutathione; HS = heat shock; and C = control. Note that antioxidants failed to protect cells from heat shock-induced and ROS-mediated apoptosis. Percent apoptosis was monitored by two persons each time ($n = 8$). (B) Single-cell gel electrophoresis of BC-8 cells upon heat shock and also in the presence of antioxidants. First panel shows the formation of apoptotic bodies as seen by propidium iodide staining; second panel shows the extent of DNA damage as seen by comet assay analysis.

Effect of antisense CD95 oligo on heat-induced apoptosis and ROS burst

To understand the effect of antisense CD95 oligo on heat-induced apoptosis and its role in the induction of ROS, BC-8 cells were incubated with antisense CD95 oligo for 16 h prior to heat shock. Samples from control and heat-shocked cells, analyzed for DNA damage and ROS generation, show apoptotic DNA ladder pattern in heat-shocked BC-8 cells (lane 2) but not in control BC-8 (lane 1) or in BC-8 cells preincubated with antisense CD95 oligo (lane 3). These results suggest inhibition of CD95 expression and, thereby, apoptosis of BC-8 cells upon heat shock by antisense CD95 oligo. Results of ROS generation in these samples presented in Fig. 4B show a drastic difference in ROS generation in the presence of antisense CD95 oligo as compared to the heat shock control. Increase in ROS at 0 h of heat shock is due to stress-induced generation, which increased further in cells going through apoptosis. Marginal increase of ROS from 0 time to 1 h after heat shock in antisense CD95-incubated cells could be probably due to the differential uptake of CD95 oligo by the cells.

DISCUSSION

Free radicals are chemical entities characterized by orbitals containing unpaired electrons. Although oxida-

tive radical stress is a potential inducer of apoptosis, the apoptotic machinery is subjected to a more complex redox regulation. Glutathione (GSH) is a major cellular reductant found in all eukaryotic cells [28] and cellular ROS is eliminated as GSH substrates [29]. GSH has been hypothesized to rescue cells from apoptosis by buffering an endogenously induced oxidative stress [30].

The estimation of intracellular GSH levels in BC-8 cells revealed that there is a drastic efflux of reduced glutathione (Fig. 1B) upon activation of apoptosis correlating with the increase in intracellular ROS levels (Fig. 1A). Cells transfected with hsp70 showed no change in intracellular GSH or ROS levels (Figs. 1A and 1B). These observations are in support of an earlier report by Macho et al. [31], which showed that GSH depletion is an early event of thymocyte apoptosis. The first ROS increase correlates with the surface expression of CD95 [18] and activation of caspase-8, and the second one correlated with induced Bax expression (Fig. 2B) and subsequent release of cytochrome *c* from mitochondria to cytosol (Fig. 2C).

The temporal coupling between GSH depletion and ROS production can probably be due to an initial ROS increase exclusively due to GSH depletion and apoptotic stimulus, causing the second elevation of ROS due to mitochondrial dysfunction. Depletion of GSH appears to be necessary for initial accumulation of ROS, since in

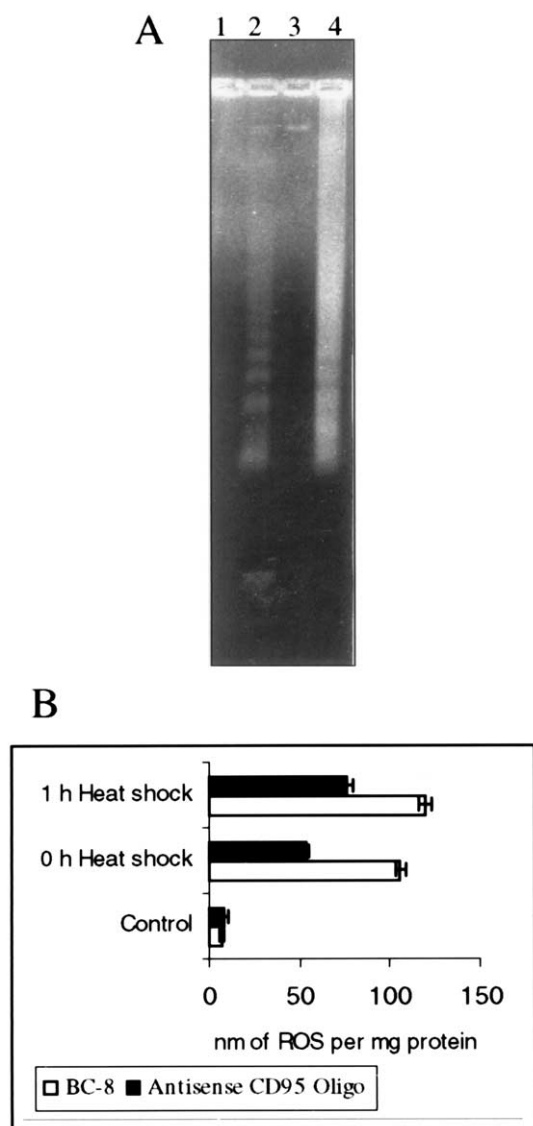


Fig. 4. (A) DNA fragmentation analysis of total genomic DNA. Lane 1: control parental BC-8 cells; lane 2: heat-shocked BC-8 cells; lane 3: BC-8 treated with antisense CD95 oligo; lane 4: BC-8 transfected with Bcl2. The experiment was repeated three times with similar results. (B) Release of intracellular ROS in the presence of antisense CD95 oligo in parental BC-8 cells as measured by in vitro cytochrome *c* assay. Heat-shocked BC-8 cells were used as positive control for ROS generation. The amount of ROS is represented as nanomoles per mg protein. Values are representative of four individual experiments ($n = 6$).

hsp70-transfected BC-8 cells there is no depletion of GSH and no production of ROS.

The thiol antioxidants have been shown to inhibit activation-induced cell death of T-cell hybridoma [32]. In contrast, our results show that GSH, NAc, or vitamin E are ineffective in inhibiting or delaying heat-induced apoptosis (Figs. 3A and 3B). Combination of antioxidants also could not give protection (Fig. 3A). This explains how caspase-8 activation leads to a mitochon-

dria-dependent pathway, even when Bcl2 is present upon CD95 signaling [33]. Hence, the initial caspase activation may prompt the cell to induce apoptosis or to follow a survival pathway.

Small heat shock proteins (HSPs) were shown to abolish the burst of intracellular ROS by TNF- α [34]. Samali and Cotter [35] reported that hsp70 protected cells against stimuli that cause DNA damage, UV irradiation, and serum withdrawal. Earlier we have reported inhibition of CD95 expression by hsp70 [19]. In the present study we show that hsp70 may also be regulating intracellular levels of GSH and ROS, thus inhibiting further damage to the mitochondria.

The tumor suppressor protein p53 is widely studied in both cell cycle [36,37] and apoptosis [38]. Although not strictly included in the category of redox-sensitive transcription factors, the expression of p53 is influenced by the redox phenomenon [9,39]. Evidence suggest that p53 induces cell death by a multitude of molecular pathways involving activation of target genes in a transcriptionally independent direct signaling [13].

Similar to parental BC-8 cells, BC-8 transfected with wild-type p53 also show GSH depletion and ROS release upon heat shock at 42°C for 30 min. However, after 1 h of recovery at 37°C both GSH and ROS levels became normal, suggesting a correlation between increase in ROS and depletion of GSH. Tan et al. [40] showed that in HT22 cells glutamate toxicity required depletion of GSH for the increase in ROS. Absence of second ROS burst in 3B4 cells that do not undergo apoptosis upon heat shock suggests that the second increase in ROS is probably due to mitochondrial damage and cytochrome *c* release.

Thus, the initial membrane signaling through CD95 ligation and early caspase activation are probably involved in ROS production via mitochondrial damage and subsequent cell death. Hsp70 abolished heat-induced ROS production and thereby cell death in BC-8 cells transfected with hsp70. Though there is an initial increase of ROS in wild-type p53-transfected BC-8 cells, further increase was not seen as the apoptotic pathway is not activated. These observations suggest that the cell's fate is determined by a cross talk between cellular signaling pathways and redox state through a complex regulatory mechanism where hsp70 and p53 play an important role.

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