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Thermotolerance and cell death are distinct cellular responses to stress: dependence on heat shock proteins

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Abstract We tested the hypothesis that heat shock protein (Hsp) induction and cell death are mutually exclusive responses to stress. Despite activation of heat shock transcription factor 1 at temperatures ranging from 40 to 46°C, Hsp72 and Hsp27 were not induced above 42°C. Moreover, cells underwent apoptosis at 44°C and necrosis at 46°C, with mitochondrial cytochrome *c* release at both temperatures. However, only apoptosis was associated with caspase activation. Treatment of cells with z-VAD-fmk prior to heat shock at 44°C failed to restore Hsp induction despite inhibition of heat-induced apoptosis. Furthermore, accumulation of Hsps after incubation at 42°C rendered the cells resistant to apoptosis. These results suggest that lack of Hsp induction is the cause rather than the consequence of cell death.

Key words: Apoptosis; Caspase; Heat shock protein; Heat shock factor 1; Necrosis; Thermotolerance

1. Introduction

Exposure of cells to transient, non-lethal elevations in temperature activates cellular stress responses and induces a state of thermotolerance, which renders the cells resistant to subsequent lethal insults [1–3]. Several groups have shown that thermotolerant cells are less sensitive to cytotoxicity induced by hyperthermia, growth factor withdrawal, heavy metals, radiation and anti-cancer drugs (for review, see [4,5]). Thermotolerance is associated with the synthesis and accumulation of a family of highly conserved proteins referred to as heat shock proteins (Hsps). The primary mammalian Hsps include proteins with molecular weights of 110, 90, 70, 60, 40 and 27 kDa (for review, see [6,7]). The inducible transcription of vertebrate *hsp* genes is mediated by a family of heat shock factors (HSFs) that function as the molecular links between an environmental stress and Hsp induction (for review, see [8]). Upon activation, HSFs translocate into the nucleus where they bind to the heat shock responsive elements (HSEs) and activate the transcription of *hsp* genes (for review, see [9,10]).

Apart from the induction of thermotolerance, heat shock can also cause a loss of cell viability if cellular defense mechanisms are not sufficient to cope with the stress. This is particularly obvious when the temperature increases well above that of the normal environment and/or exposure time is prolonged. In general, cell death follows two distinct pathways, apoptosis or necrosis. Necrosis is a passive form of cell death occurring when the level of damage to a cell is such that it cannot initiate and execute the apoptotic program. Necrosis involves lysis of the damaged cell and the release of its contents into the surrounding environment [11]. Apoptosis, on the other hand, is a highly regulated process involving condensation of nuclear chromatin, cytoplasmic shrinkage, membrane blebbing and externalization of phosphatidylserine, nuclear fragmentation and, finally, formation of apoptotic bodies (for review, see [12]). Apoptosis is associated with activation of the caspase family of proteases, which consists of at least 14 caspases in mammalian cells. Caspases are synthesized as inactive precursor molecules (pro-caspases) and are converted by proteolytic cleavage to active enzymes [13]. A large number of proteins have been identified as caspase substrates in apoptotic cells [14]. Cleavage of specific target proteins has been proposed to either activate death effector molecules or trigger the structural changes characteristic of apoptotic cells.

Emerging evidence places the mitochondrion as a central player in the activation or amplification of the caspase cascade via release of cytochrome *c* and other intermembrane space proteins (for review, see [15]). It has been demonstrated that following its release from the mitochondria, cytochrome *c* binds to Apaf-1, forming a caspase activating protein complex, in the presence of dATP [16–18]. In this study, we examined cellular responses to heat shock at various temperatures, in particular those events that dictate the survival or death of cells after heat shock.

2. Materials and methods

2.1. Cell culture and treatment of cells

Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. For heat shock experiments, the cell density was adjusted to 10^6 cells/ml. The required numbers of cells were placed in culture flasks, which were then sealed by wrapping parafilm around their lids. The flask was immersed in a thermostatted water bath at the indicated temperatures (±0.5°C) for 1 h. After the incubation period, cells were resuspended in fresh medium and incubated at 37°C for various times. For inhibition of apoptosis, cells were pre-incubated for 30 min with 20 μM benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl)fluoromethyl ketone (z-VAD-fmk), purchased from Enzyme Systems Products (Livermore, CA, USA) prior to induction of apoptosis.

2.2. Western blot

Protein samples (15–20 μg protein per lane) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transblotted onto nitrocellulose. Western blotting

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was performed using mouse monoclonal antibodies to Hsp72 and Hsp27 (StressGen Biotechnologies), mouse monoclonal antibodies to cytochrome *c* (gift from Dr Ronald Jemmerson), rat monoclonal antibody against Hsc70 (StressGen Biotechnologies), rabbit polyclonal antibodies to HSF1 [19] and caspase-3 (gift from Dr Donald Nicholson). The secondary antibodies conjugated to horseradish peroxidase were obtained from Pierce. Protein bands were visualized using the ECL Western Blot Detection kit from Amersham (Buckinghamshire, UK).

2.3. Cell viability and morphology

Cell viability was determined by the ability of cells to exclude trypan blue. Cell morphology was evaluated by staining cytocentrifuge preparations with RapiDiff II (Paramount reagents, UK). Apoptotic and necrotic cells were identified as described previously [20].

2.4. Gel mobility shift assay

Whole cell extracts (15 μg) were incubated with a ^{32}P-labelled oligonucleotide corresponding to the proximal HSE of the human *hsp70* promoter and the protein-DNA complexes were analyzed on a native 4% polyacrylamide gel as described previously [21]. The synthetic oligonucleotide was ^{32}P-labelled with T4 polynucleotide kinase (Promega).

2.5. Measurement of mitochondrial transmembrane potential

The changes in mitochondrial transmembrane potential were detected using tetramethylrhodamine ethyl ester perchlorate (TMRE, Molecular Probes), as previously described [22]. Changes in fluorescence were detected using a FACScan flow cytometer (Becton Dickinson).

2.6. Detection of cytochrome c release

Cells were washed in PBS and cytosolic and mitochondria-enriched fractions were separated as previously described [22]. After determination of the protein concentration, the samples were frozen at −70°C until further analysis by a Western blot.

3. Results

3.1. Induction of Hsps and cell death are temperature-dependent cellular responses to thermal stress

In order to determine the effect of heat shock at various temperatures on induction of Hsps and cell survival, Jurkat cells were incubated at temperatures ranging from 37 to 46°C for 1 h. The cells were allowed to recover for 6 h at 37°C to

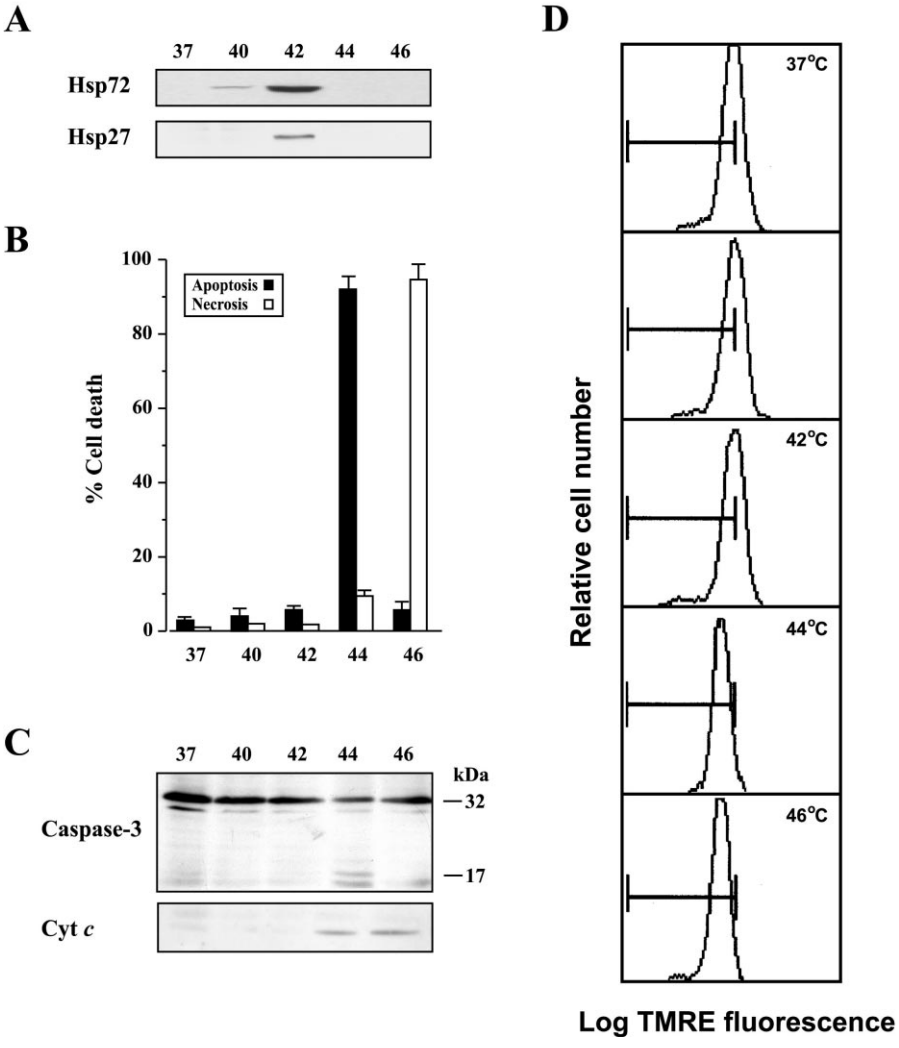

Fig. 1. Temperature-dependent induction of Hsps and cell death. Jurkat cells were incubated at the indicated temperatures for 1 h and allowed to recover at 37°C for 6 h. (A) Equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against Hsp72 or Hsp27. (B) The percentage of cell death was assessed by morphological examination of stained cytospin preparations, from three independent experiments. The results are presented as mean percentage ± S.E.M. (C) Western blot analysis of the cleavage pattern of caspase-3 using anti-p17 antibody and distribution of cytochrome *c* in the cytosolic fractions of control and heat-shocked cells by subjecting protein samples to SDS-PAGE. (D) Flow cytometric analysis of $\Delta\Psi_m$ of cells using TMRE (25 nM).

permit synthesis and accumulation of Hsps. Samples were prepared for Western blotting and morphological analysis. Western blot analysis demonstrated that Hsp72 expression was induced following incubation at 40°C and, to a much greater extent, at 42°C (Fig. 1A). The expression of Hsp27 was induced at 42°C only (Fig. 1A). In contrast, no increase in the level of Hsps was detected in cells heat-shocked at 44 or 46°C (Fig. 1A). The level of Hsc70, the constitutively expressed member of the Hsp70 family, remained unaffected by heat stress and was used as an internal control for equal loading of gels (data not shown).

Exposure of the cells to temperatures greater than 42°C caused cell death, the mode of which was determined by morphological criteria. Following heat shock at 44°C, there was a predominance of apoptotic cells in the cultures while at 46°C, the cells were primarily necrotic (Fig. 1B). At 44°C, there was a marked increase in pro-caspase-3 cleavage to the 17 kDa fragment, indicative of apoptosis, while at 46°C, there was no apparent caspase activation (Fig. 1C).

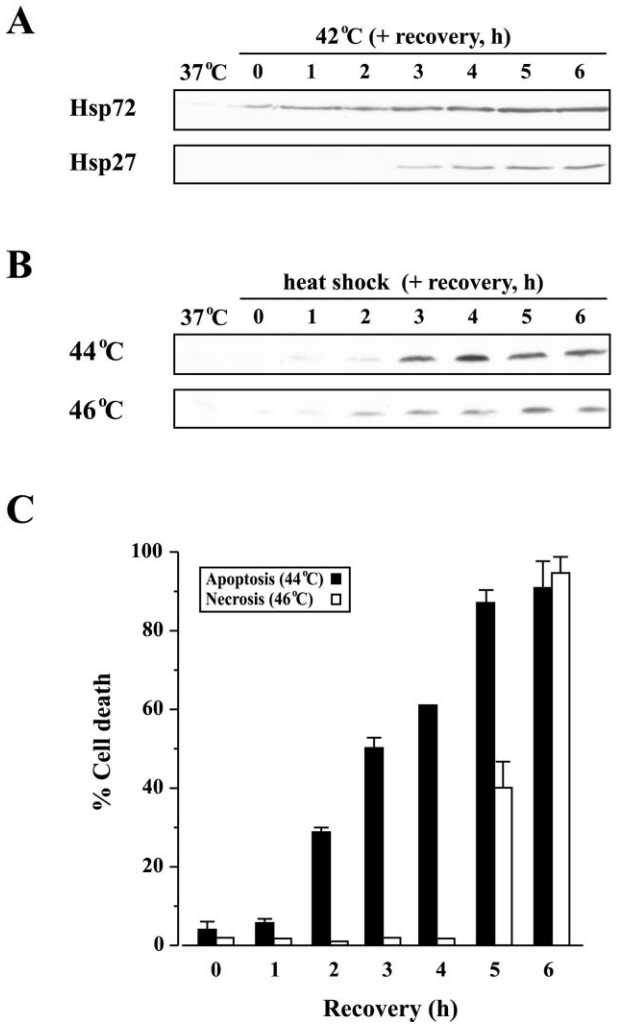

Fig. 2. Kinetics of induction of Hsps and cell death. Cells were incubated at the indicated temperatures for 1 h and allowed to recover at 37°C for 0–6 h. (A) Western blot analysis of Hsp72 and Hsp27 levels in cells heat-shocked at 42°C. (B) Western blot analysis of cytochrome *c* in the cytosolic fractions of control (37°C) and heat-shocked (44 and 46°C) cells. (C) The percentage of cell death was assessed by morphological examination of stained cytospin preparations, from three independent experiments. The results are presented as mean percentage ± S.E.M.

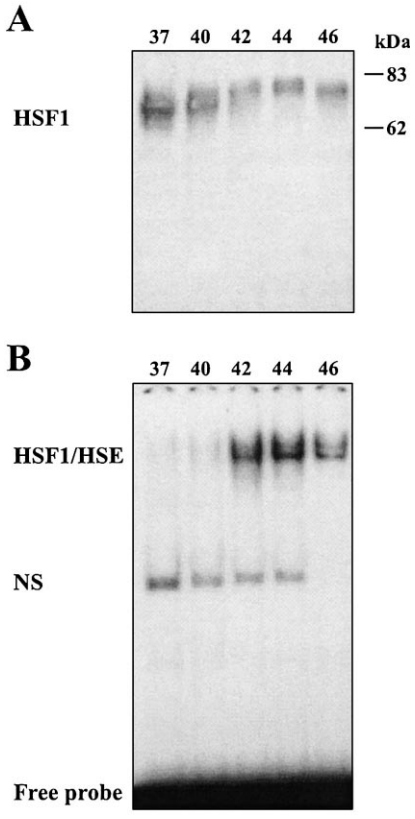

Fig. 3. Activation of HSF1 in response to modest and severe heat stress. Jurkat cells were incubated at indicated temperatures for 1 h and whole cell extracts were prepared. (A) Western blot analysis with specific antibodies against HSF1. (B) The DNA-binding activity of HSF1 was examined by subjecting 15 μg of whole cell extract to a gel mobility shift assay using a HSF-specific ^{32}P-labelled oligonucleotide probe (HSE). HSF/HSE complex, non-specific DNA-binding activity (NS) and free unbound HSE oligonucleotide probe are all indicated in the figure.

Apoptosis and necrosis can be induced as a result of mitochondrial damage and both forms of cell death have been associated with alterations in the mitochondrial membrane potential ($\Delta\Psi_m$) and/or cytochrome *c* release, although only apoptosis is associated with caspase activation. To determine if heat-induced cell death (apoptosis and necrosis) coincides with alterations in $\Delta\Psi_m$, we used the potential-sensitive probe TMRE. Flow cytometric analysis of $\Delta\Psi_m$ of cells incubated at 37–46°C demonstrated that thermal treatment at 44–46°C resulted in a drop in $\Delta\Psi_m$, which was not observed after treatment at a lower temperature (Fig. 1D). Western blot analysis of the cytosolic fraction prepared from cells incubated at the indicated temperatures demonstrated the release of cytochrome *c* from mitochondria only after lethal insult (Fig. 1C). The early loss of $\Delta\Psi_m$ and cytochrome *c* release from mitochondria may be an indication that heat shock at lethal temperatures induces permeability transition. However, the inhibitors of permeability transition, cyclosporin A and bongkrekic acid, could block neither event (data not shown). This observation suggests that the mitochondrial alterations were independent of permeability transition.

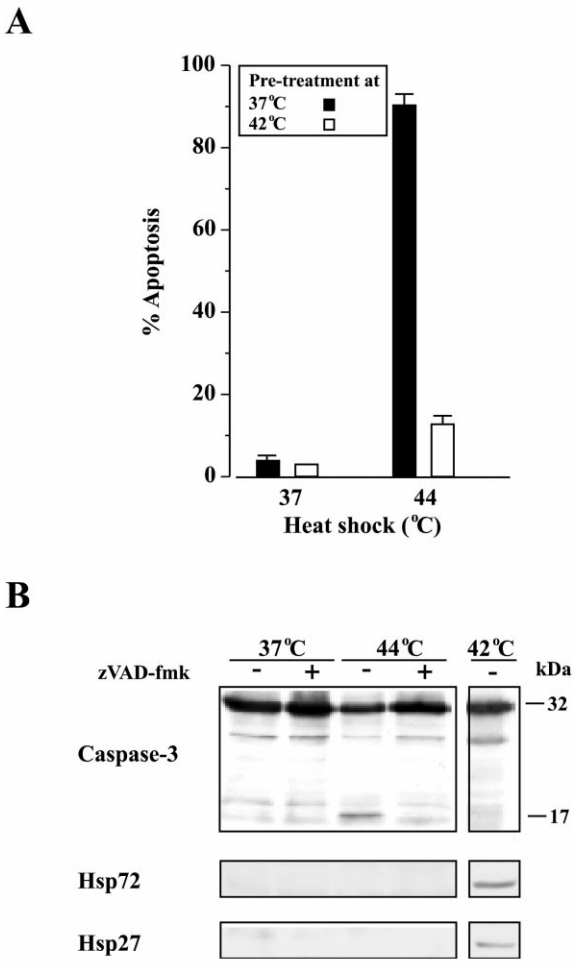

Fig. 4. Inhibition of Hsp induction leads to cell death. (A) Cells were heat-shocked at 42°C for 1 h and allowed to recover at 37°C overnight to allow for accumulation of Hsps. Control and heat-shocked cells were challenged by incubation at 44°C for 1 h and allowed to recover at 37°C for a further 6 h. Cytospin samples were then prepared for assessment of apoptotic morphology. (B) Cells were maintained at 37°C or heat-shocked at 44°C for 1 h to induce apoptosis in the presence or absence of the general caspase inhibitor z-VAD-fmk (20 μM). z-VAD-fmk was included in the medium for 30 min prior to heat shock and was maintained in the medium during heat shock and the 6 h recovery period. Samples of cells heat-shocked at 42°C were included as control to demonstrate a lack of caspase-3 cleavage in the presence of Hsp induction. Protein samples were analyzed by Western blotting for caspase-3 cleavage and the expression of Hsp72 and Hsp27 as indicated.

3.2. Kinetics of responses to thermal stress

A time-course study of induction of Hsps, apoptosis and necrosis was then carried out. As demonstrated in Fig. 2A, Hsp72 levels were rapidly induced after 1 h heat shock at 42°C and increased over time, while an increase in Hsp27 level was detectable after 3 h recovery (Fig. 2A). A time-course study of cytochrome *c* release from mitochondria following heat shock at 44 and 46°C (to induce apoptosis and necrosis, respectively) demonstrated cytosolic appearance of cytochrome *c* after 1–2 h recovery with further accumulation during the next several hours (Fig. 2B). Caspase activation due to heat shock at 44°C followed the same kinetics as that of cytochrome *c* release (data not shown). However, apoptotic nuclear condensation appeared after 2 h recovery (Fig. 2C). Evidence of necrosis and loss of plasma membrane integrity occurred after 5–6 h recovery (Fig. 2C). Thus, the early features of apoptosis and necrosis occurred at least 1 h later than Hsp72 induction.

3.3. Lack of Hsp induction during cell death is not due to inactivation of HSF1

We hypothesized that the lack of Hsp induction at lethal temperatures may have been due to proteolysis of HSF1 and/or loss of its DNA-binding activity. In order to investigate these possibilities, we monitored HSF1 levels and activity in our samples. Western blot analysis clearly demonstrated a lack of proteolytic degradation of HSF1 (Fig. 3A). In addition, HSF1 was equally phosphorylated at 42–46°C as determined by the slower mobility of the protein band on SDS-PAGE (Fig. 3A). The phosphorylation-dependent slower migration of HSF1 at temperatures above 37°C is consistent with previous reports [23,24]. Although HSF1 contains several potential caspase cleavage sites, it does not appear to be cleaved by caspases during heat-induced apoptosis (Fig. 3A). Moreover, analysis of HSF1 activity by gel mobility shift assay demonstrated a prominent DNA-binding activity at 42–46°C (Fig. 3B). Therefore, the lack of Hsp induction at high temperatures was not due to degradation or loss of HSF1 activity.

3.4. Cell death at high temperatures is due to the lack of Hsp induction

In order to determine whether lack of Hsp induction was the cause or a result of cell death, we took two different approaches. The first involved pre-conditioning of cells at 42°C to allow for Hsp induction, after which we determined whether conditioned cells resisted apoptosis induced by incubation at 44°C. As demonstrated in Fig. 4A, pre-conditioning of cells at 42°C significantly blocked apoptosis induced by a subsequent heat shock at 44°C, suggesting that the induction of Hsp27 and Hsp72 can block apoptosis. Secondly, cells were treated with the broad-spectrum caspase inhibitor z-VAD-fmk prior to heat shock at 44°C to determine whether inhibition of apoptosis could restore Hsp induction. Although pretreatment of cells with z-VAD-fmk inhibited caspase activity and morphological features of apoptosis, it failed to restore Hsp induction in these cells (Fig. 4B, data not shown). These results suggest that a lack of Hsp induction at high temperatures is the cause, rather than a result, of cell death.

4. Discussion

In this paper, we have presented data to demonstrate that exposure of cells to elevated temperatures results in either induction of Hsps and acquired thermotolerance or in cell death, depending on the temperature. Our results indicate that the induction of Hsps/thermotolerance or cell death appear to be mutually exclusive cellular responses to stress as previously suggested [20]. Induction of Hsps in response to stress stimuli has been shown to not only serve as a macromolecular repair mechanism, but also as a defensive strategy against a subsequent challenge [25]. Indeed, we have previously demonstrated that expression of Hsp72 and/or Hsp27 increases resistance of U937 cells to apoptosis [26].

The reason behind the lack of Hsp induction at 44 or 46°C (i.e. under conditions which lead to apoptosis or necrosis,

respectively) is not clear. Nevertheless, in our studies, we have excluded proteolysis or the loss of DNA-binding activity of HSF1 as possible explanations. The lack of proteolytic cleavage of HSF1 during apoptosis was also recently reported in a model of CD95-mediated cell death [27]. It has been reported previously that both apoptosis and necrosis are associated with the cleavage/degradation of ribosomal RNA [28]. Thus, the lack of Hsp induction may be explained by the lack of translation at these extreme temperatures. High temperatures (44–46°C) may also affect other steps involved in protein synthesis, thus delaying or completely aborting de novo protein synthesis [29,30]. The time-course studies carried out here demonstrate that the rate of Hsp induction is faster than that of cell death. This observation excluded the possibility that the cells die too fast at high temperatures to allow for Hsp induction. This conclusion was further supported by the fact that inhibition of apoptosis by z-VAD-fmk did not affect Hsp induction over the 6 h experimental observation period.

If cells were capable of producing Hsps at high temperatures (44 and 46°C), thus enabling them to withstand this stress, the outcome for a multicellular organism is likely to be detrimental. Extreme elevations in temperatures could cause deleterious injuries like DNA damage leading to mutations [31]. Therefore, inhibition of Hsp induction may serve as a cellular strategy to ensure that repair of cells with severe injuries does not occur. In conclusion, these data show that cell death, by either apoptosis or necrosis, and Hsp induction are mutually exclusive events. This effect is in agreement with the hypothesis of a reciprocal relationship between cell death and Hsp induction [20].

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References

[1] Li, G.C. and Hahn, G.M. (1980) Cancer Res. 40, 4501–4508.
[2] Li, G.C. and Werb, Z. (1982) Proc. Natl. Acad. Sci. USA 79, 3218–3222.
[3] Parsell, D.A. and Lindquist, S. (1993) Annu. Rev. Genet. 27, 437–496.
[4] Samali, A. and Orrenius, S. (1998) Cell Stress Chaperones 3, 228–236.
[5] Jäättelä, M. (1999) Exp. Cell Res. 248, 30–43.
[6] Moseley, P.L. (1997) J. Appl. Physiol. 83, 1413–1417.
[7] Lindquist, S. and Craig, E.A. (1988) Annu. Rev. Genet. 22, 631–677.
[8] Morimoto, R.I., Kline, M.P., Bimston, D.N. and Cotto, J.J. (1997) Essays Biochem. 32, 17–29.
[9] Wu, C. (1995) Annu. Rev. Cell Dev. Biol. 11, 441–469.
[10] Morimoto, R.I. (1998) Genes Dev. 12, 3788–3796.
[11] Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. (1972) Br. J. Cancer 26, 239–257.
[12] Johnson Webb, S., Harrison, D.J. and Wyllie, A. (1997) Adv. Pharmacol. 41, 1–34.
[13] Thornberry, N.A. and Lazebnik, Y. (1998) Science 28, 1312–1316.
[14] Stroh, C. and Schulze-Osthoff, K. (1998) Cell Death Differ. 5, 997–1000.
[15] Kroemer, G., Dellaporta, B. and Resche-Rigon, M. (1998) Annu Rev. Physiol. 60, 619–642.
[16] Liu, X., Kim, N.C., Yang, J., Jemmerson, R. and Wang, X. (1996) Cell 86, 147–157.
[17] Zhou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Cell 90, 405–413.
[18] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[19] Holmberg, C.I., Illman, S., Kallio, M., Mikhailov, A. and Sistonen, L. (1999) (in preparation).
[20] Gorman, A.M., Heavey, B., Creagh, E., Cotter, T.G. and Samali, A. (1999) FEBS Lett. 445, 98–102.
[21] Mosser, D.D., Theodorakis, N.G. and Morimoto, R.I. (1988) Mol. Cell. Biol. 8, 4736–4744.
[22] Samali, A., Cai, J., Zhivotovsky, B., Jones, D.P. and Orrenius, S. (1999) EMBO J. 18, 2040–2048.
[23] Sarge, K.D., Murphy, S.P. and Morimoto, R.I. (1993) Mol. Cell. Biol. 13, 1392–1407.
[24] Cotto, J.J., Kline, M. and Morimoto, R.I. (1996) J. Biol. Chem. 271, 3355–3358.
[25] Henics, T., Nagy, E., Oh, H.J., Csermely, P., von Gabain, A. and Subjeck, J.R. (1999) J. Biol. Chem. 274, 17318–17324.
[26] Samali, A. and Cotter, T.G. (1995) Exp. Cell Res. 223, 163–170.
[27] Schett, G., Steiner, C.W., Groger, M., Winkler, S., Graninger, W., Smolen, J., Xu, Q. and Steiner, G. (1999) FASEB J. 13, 833–842.
[28] Samali, A., Gilje, B., Døskeland, S.O., Cotter, T.G. and Houge, G. (1997) Cell Death Differ. 4, 289–293.
[29] Sciandra, J.J. and Subjeck, J.R. (1984) Cancer Res. 44, 5188–5194.
[30] Black, A.R. and Subjeck, J.R. (1989) J. Cell Physiol. 138, 39–49.
[31] King, M.T. and Wild, D. (1983) Mutat. Res. 111, 219–226.