Hyperthermia and protein aggregation
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CELLS OVEREXPRESSING HSP27 SHOW ACCELERATED RECOVERY FROM HEAT-INDUCED NUCLEAR PROTEIN AGGREGATION
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ABSTRACT

Protein denaturation/aggregation upon cell exposure to heat shock is a likely cause of cell death. In the nucleus, protein aggregation has often been correlated to inhibition of nuclear located processes and heat-induced cell killing. In Chinese hamster O23 cells made thermotolerant by a prior heating (20 min 44°C + 10 h 37°C) which induces the whole spectrum of heat shock proteins (hsp’s), the extent of nuclear protein aggregation during heat shock is reduced and the rate of recovery from aggregation after heat shock is enhanced. In contrast, a heat resistant Chinese hamster cell line overexpressing only hsp27 shows an unaltered sensitivity to formation of nuclear protein aggregates by heat, but shows the same enhanced rate of recovery from nuclear protein aggregation as thermotolerant cells. This suggests that accelerated recovery of protein aggregation could be partly responsible for hsp27-mediated thermoprotection.
5.1 INTRODUCTION

Thermal denaturation/aggregation of proteins is likely a major cause for the loss of cellular functions that eventually leads to cell killing after heat shock and is the triggering event responsible for the induction of the heat shock proteins (hsp's) [Hightower 1980, Bensaude et al. 1991, Kampinga 1993]. Accumulation of the hsp's, in turn leads to the acquisition by the cells of an enhanced capacity to sustain subsequent heat shock treatment. In these induced-thermotolerant (TT) cells, protein denaturation/aggregation is reduced or/and renaturation/ disaggregation rates after heat shock are enhanced. This has been illustrated in studies where the effect of heat shock on individual endogenous and foreign reporter enzymes was evaluated [Bensaude et al. 1991, Pinto et al. 1991, Kampinga 1993] as well as in studies where the aggregation of nuclear proteins was measured indirectly by evaluating the total protein content of isolated (TX-100 insoluble) nuclei [Kampinga 1993, Kampinga et al. 1987, 1989a, Borrelli et al. 1992]. This protection is likely due to the elevated levels of hsp’s, many of which have demonstrated protein chaperonin functions in vitro, that is a capacity to retard protein denaturation by heat or chemicals in vitro [Skowyra et al. 1990, Viitanen et al. 1990, Zeilstra-Ryalls et al. 1991, Wiech et al. 1992]. Several studies have shown that artificially increasing the expression of individual hsp’s by gene transfection to levels mimicking those found in TT, confers thermostability. In a previous study, we have obtained direct evidence that hsp70-mediated thermosistance may be related, at least in part, to the chaperonin function of hsp70. We found that cells overexpressing hsp70 showed, similarly to TT cells, a reduced formation of nuclear protein aggregates during heat shock as compared to their parent wild-type cells [Stege et al. 1994]. However, in these cells, in contrast to TT cells, recovery from these aggregates occurred at the same rate as in their parent wild-type cells [Stege et al. 1994]. These experiments suggested that hsp70 can reduce the rate of protein aggregation in situ, but may not contribute to the enhanced rate of recovery seen in TT cells which also express other hsp’s.

Constitutive or transient overexpression of hsp27 by transfection of the human [Landry et al. 1989], the mouse [Knauf et al. 1994] as well as the Drosophila hsp27 gene [Rollet et al. 1992] also confers heat resistance in Chinese hamster or mouse cells. These hsp’s are members of the family of small hsp’s, for which a protein chaperonin protective function has also been described recently [Horwitz 1992, Knauf et al. 1994, Jakob et al. 1993]. However, limited evidence for a similar chaperonin role in situ is available.

The aim of the current study was to investigate the putative contribution of hsp27 to the reduced rate of formation of heat-induced nuclear protein aggregates and the accelerated recovery from these protein aggregates as observed in thermostolerant cells. Using a thermoresistant cell line overexpressing only hsp27, we found, in contrast to our previous study with hsp70 overexpressing cells, that hsp27
overexpressors showed an unaltered sensitivity to formation of nuclear protein aggregates by heat, but had an enhanced rate of recovery from nuclear protein aggregation as compared to their control parental cells.

5.2 MATERIAL AND METHODS

O23 hamster cells and O23 cells transfected with the human hsp27 (2.2: [Landry et al. 1989]) were grown as monolayer cultures in Dulbecco-MEM (Gibco, Paisley, Scotland) supplemented with 10% foetal bovine serum (Gibco). Asynchronously, exponentially growing cells were used in all experiments. Hyperthermia was performed in precision waterbaths (± 0.1°C). To obtain thermotolerance, O23 cells were heated for 20 min at 44°C followed by 10 h at 37°C. Heat killing was analyzed by the colony forming assay. Immediately after heating the cells, they were trypsinized and plated at appropriate concentrations in petri dishes (Falcon, Etten-Leur, The Netherlands). After 8-10 days incubation at 37°C in a humidified CO₂ incubator, colonies were stained with 1% crystal violet. Colonies containing more than 50 cells were counted. Plating efficiencies were close to 100% for both O23 and 2.2 cells.

Nuclei were isolated as described before [Kampinga et al. 1989a, Stege et al. 1994]. Shortly, cells were lysed on the flask with a TX-100 lysis solution (0.1% TX-100; 5 mM Tris-HCl, pH = 8.0; 10 Mm NaCl) and scraped with a rubber policeman. After centrifugation (5 min 1500 rpm) the pellet was resuspended in the TX-100 lysis solution. After lysis TX-100 was removed by two times washing with TNMP (10 mM Tris-base, pH = 7.4; 10 mM NaCl; 5 mM MgCl₂; 0.1 mM phenylmethylsulphonylfluoride). The final TX-insoluble pellet (containing morphologically clean nuclei free of cytoplasmic capping) was resuspended in 0.4 ml TNMP. The nuclei were stained with 0.1 ml FITC (3 µg/ml final concentration) and 0.5 ml PI (35 µg/ml final concentration) to measure the relative nuclear protein content flow cytometrically. All isolation procedures were performed at 0-4°C. To determine nuclear protein aggregates, 10,000 nuclei were analyzed on a Becton Dickinson FACS STAR flow cytometer/sorter according to the method of Blair et al. [1979]. Nuclear protein content relative to control was determined by computing the mean of the FITC fluorescence distribution of nuclei from heated cells and dividing it by that of the nuclei isolated from control cells. Measurement were performed either immediately after heating the cells (nuclear protein aggregation) or 1-5 h thereafter (recovery). PI labelling of DNA was used to check for treatment-induced cell cycle redistribution that could affect FITC measurement. However, no such changes were observed (not shown).

5.3 RESULTS AND DISCUSSION
Transfection of the human hsp27 gene into hamster cells was shown to confer heat resistance, the extent of which correlating to the amount of human hsp27 expression [Landry et al. 1989]. From these series of clones, the most heat resistant clone 2.2 constitutively expressing the highest level of the transfected human hsp27 was selected. This clone expresses 12 ng human hsp27/µg total cellular proteins in addition to a normal level of 2 ng hamster hsp27/µg total protein [Landry et al. 1989]. Thus, in total the 2.2 clone contains a 7 fold increase in hsp27 levels compared to O23 cells. The results depicted in figure 5.1, confirm and extent upon earlier data [Landry et al. 1989] that cell death at elevated temperature is retarded in the 2.2 clone. This is true for heating for various periods at 44°C (figure 5.1b) as well as a constant time (1 h) at 42, 43, and 44°C (figure 5.1c). For comparison, survival of the parental O23 cells made thermotolerant by prior heating is shown: the permanent resistance against thermal cell killing of the 2.2 cells is comparable to the level of transient resistance that can be induced by a 20 min 44°C heat treatment of O23 cells given 10 h prior to the test challenge (O23-TT).

The increase in protein mass of isolated nuclei was used as a model end-point to assess the extent of protein denaturation/aggregation induced by heat in these cells. As it has been established for many cell lines, a “heat-dose” dependent increase in the FITC (protein) signal of the isolated nuclear (TX-100 insoluble) fraction can be observed also for the hamster O23 cells (figure 5.2a). The kinetics of this increase mirrors the cell survival curve (figure 5.1a). For O23-TT cells, nuclear protein aggregation appeared to be attenuated compared to non-TT cells for all time-temperature combinations tested (figure 5.2b). For the cells that constitutively overexpress the human hsp27, heat-induced protein aggregation occurred at the same rate as control non-TT cells (figure 5.2b), in spite of the fact that these 2.2 cells were almost as heat resistant as the O23-TT cells (figure 5.1b,c). So, it seems unlikely that hsp27 is involved in the protection against this type of damage as seen in thermotolerant cells [Borrelli et al. 1992, Stege et al. 1994, this report].

Recently, it was reported [Laszlo et al. 1993] that the heat resistance of hsp27 overexpressing clones also was not associated with any protection against heat-induced inhibition of total cellular protein or RNA synthesis. Also, these investigators did not find changes in the heat response of these cells in terms of the excess nuclear localization of hsp70/hsc70 as detected by in situ immunofluorescence. Similar data were obtained for rRNA synthesis and synthesis of specific mRNAs [Weber, L., personal communication]. Assuming that some kind of heat-induced denaturation (and subsequent aggregation) of proteins underlies the above mentioned biological effects of heat, these in situ data fit to our
observations that hsp27 cannot protect against heat-induced protein denaturation and subsequent aggregation.

Apparently, the chaperonin-like protection activity of hsp27 described in cell-free systems [Horwitz 1992, Knauf et al. 1994, Jakob et al. 1993, Merck et al. 1993] is not

**Figure 5.1** Effect of heat on the clonogenic ability of thermotolerant, normo-tolerant, and hsp27 transfected hamster cells. (a) Clonogenic ability of O23 cells after various exposure times to 42°C (circles); 43°C (triangles); and 44°C (squares). (b) Clonogenic ability of O23 cells (circles); heat-induced (20 min 44°C + 10 h 37°C) thermotolerant O23 cells (O23-TT: triangles); and hsp27 transfected hamster cells (clone 2.2: squares) after various exposure times to 44°C. (c) As b) but for 1 h exposure at 42°C, 43°C, or 44°C. The data are expressed relative to the plating efficiency of the untreated controls and are the mean values (± SEM) from three or more independent experiments.
involved in the protection of these cell activities and, in particular, the reduced intranuclear protein aggregation seen in TT rodent cells [Borrelli et al. 1992, Stege et al. 1994, this report] is not related to hsp27 function. It may instead be related to the (elevated) expression hsp70. In recent experiments, Rat-1 cells transfected with the human hsp70 gene showed protection against the formation of heat-induced nuclear protein aggregates which paralleled heat resistance at the survival level [Stege et al. 1994]. The reported [Skowyra et al. 1990] protection of DnaK (the prokaryotic hsp70 analogue) against thermal denaturation of RNA polymerase (using cell free approaches) is in agreement with these observations.

Figure 5.2 Heat-induced nuclear protein aggregation in thermotolerant, normo-tolerant, and hsp27 transfected hamster cells. (a) Time temperature dependency of the heat-induced nuclear protein aggregation in O23 cells; 42°C (circles); 43°C (triangles); and 44°C (squares). (b) Heat-induced nuclear protein aggregation as determined in O23 cells (circles); heat-induced (20 min 44°C + 10 h 37°C) thermotolerant O23 cells (O23-TT: triangles); and hsp27 transfected hamster cells (clone 2.2: squares) after 1 h exposure times at 42°C, 43°C, or 44°C. The data are expressed as the FITC signal of isolated nuclei relative to the signal of untreated controls and are the mean values (± SEM) from three or more independent experiments.

It has been demonstrated that cells, when returned to 37°C after heat shock are able to recover from the heat-induced nuclear protein aggregation [Roti Roti and Winward 1978, Kampinga et al. 1987, 1989a]. The rate of recovery was shown to be faster in TT cells for all cell lines tested so far. Also here, recovery from nuclear protein aggregation after 60 min 44°C heating was more rapid in TT than control O23 cells (figure 5.3). In accordance with earlier findings [Roti Roti and Winward 1978, Kampinga et al. 1987, 1989a], this was
also found when O23-TT were heated with more severe heat treatments leading to
the same initial aggregation as seen in the O23 (data not shown). Interestingly, the
hsp27 transfected cells also recovered more rapidly than O23 cells from this nuclear
protein damage, even after a heat treatment that induces the same initial increase as
in O23 cells (figure 5.3).

These data suggest that heat-induced elevated hsp27 expression in TT cells is
responsible for at least part of the accelerated recovery seen in TT cells [Kampinga et al. 1989a, Borrelli et al. 1992, Stege et al. 1994, this report: figure 5.3]. A faster
recovery from heat-inhibited RNA synthesis, rRNA synthesis, but not protein
syntheses [Laszlo et al. 1993, Weber, L., personal communication] also suggested a
role of hsp27 in recovery from heat damage to the nucleus and processes located
herein. Hsp27 might have a protein disaggregation activity that leads to accelerated
functional recovery of the aggregated proteins. A direct proof for such a chaperonin
activity of hsp27 is, however, lacking. It is equally possible that the observed
enhanced rates of recovery from nuclear heat damage is the result of the protective
action of hsp27 at another level of cellular activity. For example, hsp27 has been
reported to behave in vitro as an actin cap-binding protein and overexpression in vivo
was shown to result in the stabilization of actin filaments during stress [Lavoie et al.
A better maintenance of the actin cytoskeleton during heat shock may enable cells to repair the heat-induced nuclear damage more rapidly.

Summarizing, it was shown by using hsp27 overexpressing cells that elevated hsp27 levels do not attenuate nuclear protein aggregation (as seen in TT cells). However, higher levels of hsp27 is related to an accelerated rate of recovery form such aggregates. The more rapid recovery from nuclear protein damage might explain why these cells recover faster from heat-induced damage to nuclear functions [Laszlo et al. 1993]. So, elevated hsp27 in heat-induced thermotolerant cells may play a role in the observed faster recovery of thermotolerant cells from heat-induced nuclear protein aggregates [Kampinga et al. 1987, 1989a, Borrelli et al. 1992, Stege et al. 1994, this report] and nuclear functions [Laszlo et al. 1993]. Although the precise mechanism of faster recovery has not yet been fully elucidated, it could be part of the mechanism responsible for the heat resistant phenotype (clonogenic capacity) of hsp27 transfected as well as thermotolerant cells.

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