The mechanism whereby heat shock induces apoptosis depends on the innate sensitivity of cells to stress

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Abstract The cellular response to heat shock (HS) is a paradigm for many human diseases collectively known as “protein conformation diseases” in which the accumulation of misfolded proteins induces cell death. Here, we analyzed how cells having a different apoptotic threshold die subsequent to a treatment with HS. Cells with a low apoptotic threshold mainly induced apoptosis through activation of conventional stress kinase signaling pathways. By contrast, cells with a high apoptotic threshold also died by apoptosis but likely after the accumulation of heat-aggregated proteins as revealed by the formation of aggresomes in these cells, which were associated with the generation of atypical nuclear deformations. Inhibition of the proteasome or expression of an aggregation prone protein produced similar nuclear alterations. Furthermore, elevated levels of chaperones markedly suppressed both HS-induced nuclear deformations and apoptosis induced upon protein aggregation whereas they had little effect on

stress kinase-mediated apoptosis. We conclude that the relative contribution of stress signaling pathways and the accumulation of protein aggregates to cell death by apoptosis is related to the innate sensitivity of cells to deadly insults.

Keywords Apoptosis · Aggresome · Heat shock · Huntingtin · Nuclei morphology · Proteasome

Introduction

A number of human degenerative diseases emerge as a result of genetic alterations that cause proteins to misfold and form aggregates. The accumulation of misfolded proteins generates a proteotoxic stress that ultimately induces cell death through a variety of mechanisms, such as apoptosis and autophagy (Bredesen et al. 2006). However, the basics that guide the decision to die by means of one mechanism or the other have yet to be defined.

Studies on the cellular response to heat shock (HS) contributed to the understanding of how cells cope with proteotoxicity. The observation that transcription of molecular chaperones, or heat shock proteins (HSPs), was induced upon HS was the first example of the existence of an adaptive cellular response to stress (reviewed in Nadeau and Landry 2007). It is now widely accepted that increased amount of one or more HSPs in the cell promotes its effective recovery and survival under stressful conditions, including heat shock (Kregel 2002). Accordingly, the accumulation of Hsp70, for example, has been shown to be essential for HS-induced thermotolerance (Gabai et al. 2000). Indeed, high levels of Hsp70 prevent protein aggregation and markedly improve survival of thermotoler-

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ant cells in response to HS. HSPs act as molecular chaperones and prevent protein aggregation by binding to exposed hydrophobic domains on denatured proteins in order to assist their proper refolding or target them for degradation through the ubiquitin–proteasome system (Glover and Lindquist 1998; Hohfeld et al. 2001). Overexpression of Hsp70 can also prevent irreversible degradation and assist in the refolding of denatured reporter enzymes (Michels et al. 2000). Moreover, the ability of Hsp70 to prevent or delay the formation of cytotoxic mutant protein aggregates in vivo has been documented in degenerative diseases (Cummings et al. 2001; Auluck et al. 2002; Bailey et al. 2002). Hence, HSPs can reduce protein aggregation regardless of how they are generated.

The molecular basis of HS-induced toxicity is not well understood. In conformational diseases, proteotoxicity may result from the loss of function of critical proteins as a consequence of their denaturation as well as from a gain of toxic functions by the denatured proteins (Bucciantini et al. 2002; Bennett et al. 2005). In yeast, survival after HS depends mainly on the ability of the cells to degrade the damaged proteins, implying a gain of toxic functions of the misfolded proteins rather than a loss of essential functions (Friant et al. 2003).

Here, we present evidence that HS can induce apoptosis through two initiating events. The first event involves mitogen-activated protein kinase (MAPK) signaling and proceeds rapidly after HS in cells with a low apoptotic threshold. The second initiating mechanism, observed mostly in cells with a higher apoptotic threshold, is based on the accumulation of damaged proteins and leads to apoptosis only many hours (>8 h) after HS. We demonstrate that HS-induced protein aggregation, proteasome inhibition, or overexpression of poly-Q containing proteins, all initiated nuclear deformations around an aggresome in the nuclear fold and that these nuclear deformations are features associated with apoptosis as a consequence of the accumulation of protein aggregates.

Materials and methods

Reagents

Cisplatin, SB203580, LY294002, 4-6-diamidino-2-phenylindole (DAPI), 4-hydroxytamoxifen (OHT), MG132, and hydrogen peroxide (H₂O₂) were purchased from Sigma Chemicals (St. Louis, MO, USA). *N*-Benzylloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was from Enzyme System Products (Livermore, CA, USA). Hypo-osmolar buffer and CO₂-independent culture medium were purchased from Brinkmann (Mississauga, ON, Canada) and Gibco (Burlington, ON, Canada), respectively.

Antibodies

The rabbit antibody against cleaved caspase-3 was obtained from Cell Signaling Technology (Beverly, MA, USA). The mouse anti-HA (HA.11) antibody was from Babco (Richmond, CA, USA). The anticytochrome c antibody 6H2.B4 was from BD Biosciences (Mississauga, ON, Canada). Anti-Bax antibody (clone N-20) was from Santa Cruz (Santa Cruz, CA, USA). Anti-Vimentin clone V9 and anti- γ -tubulin clone GTU-88 were from Sigma. Anti-Myc (9E10) is a mouse monoclonal antibody recognizing the EQKLISEEDL peptide sequence from the human c-Myc protein (ATCC). The Alexa Fluor 594 goat antimouse IgG was from Molecular Probes (Burlington, ON, Canada). The horseradish peroxidase conjugated antimouse IgG and antirabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Human Fas-activating antibody (clone CH11) was from Upstate Cell Signaling Solutions (Lake Placid, NY, USA).

Plasmids

Bcl-2-GFP was expressed using pEGFP-C3-hBcl2 plasmid (Wolter et al. 1997). pcDNA3-Ha-Ask1-KM plasmid was used to express a kinase-inactive variant of Ask1 (Chang et al. 1998). The plasmid expressing H2A-GFP (pEGFP-C1-H2A) has been described previously (Perche et al. 2000). Lamin B1 was expressed as a GFP-tagged protein using a pEGFP-hLMNB1 plasmid (Moir et al. 2000). The plasmids pHD 23, 43, and 74Q-HA encoded an HA-tagged Huntingtin (Htt) exon1 fragment with 23, 43, and 74 CAG (glutamine) repeats, respectively, and were generously provided by D.C. Rubinsztein (Wytttenbach et al. 2000). Myc-tagged ubiquitin expressed in a pCW7 plasmid was a kind gift from R.R. Kopito (Ward et al. 1995). The pCIN-Fas plasmid allows expression of the Fas receptor (Charette et al. 2000).

Cell culture and cell line

Rat1/c-MycERTM cells (Rat1-Myc cells) express an inducible fusion protein made of human c-Myc and a modified murine estrogen receptor responsive to OHT. The MycER protein was activated by adding OHT to the culture medium at a final concentration of 100 nM for 16 h. Cells expressing a nonfunctional truncated form of c-Myc (106–143) fused to the aforementioned estrogen receptor (Rat1-control cells) were also used as a control for c-Myc activity (Littlewood et al. 1995). The cells were cultured in modified Eagle's medium containing NaHCO₃ (2.2 g/l) and supplemented with 10% fetal bovine serum (FBS). Cells were selected for MycER or MycER expression by adding puromycin to the culture medium at a final

concentration of 5 g/ml. Puromycin was removed 24 h before experiments were performed. Human embryonic kidney 293 cells (HEK293), mouse embryo fibroblasts (MEF), and HeLa cells were cultivated in Dulbecco's modified Eagle's medium supplemented with NaHCO_3 (2.2 g/l), 4 mM L-glutamine, 4.5 g/l glucose, and 10% fetal bovine serum. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2 .

Transfection

Rat1 cells were transfected by electroporation (100 V, 100 ms, 12 pulses) in an electro square porator ECM830 (BTX, San Diego, CA, USA) containing 1×10^6 cells and 50 μl hypoosmolar buffer. Cells were then kept for 6 h in CO_2 -independent medium followed by 24 h in α -minimal essential medium supplemented with 10% FBS. When required, OHT was added 30 h after transfection. HEK293 cells were transfected as described previously (Landry et al. 1989).

Heat shock treatment

Cell culture flasks were sealed with parafilm and incubated in thermoregulated water bath for the times and at the temperatures indicated on the figures.

Immunocytochemistry

Cells were washed twice with a phosphate-buffered saline (PBS) solution and fixed in PBS containing 3.7% formaldehyde for 15 min. Cells were then incubated for 60 min at room temperature in PBS containing 0.1% saponin and 3% bovine serum albumin and the indicated primary antibody was then added for a further 60 min at 37°C. Cells were washed three times in PBS containing 0.1% saponin and 3% bovine serum albumin and incubated for 60 min at 37°C in PBS containing 0.1% saponin and 3% bovine serum albumin containing the appropriate secondary antibody and DAPI (2.5 g/ml). Observations were performed with a Nikon Eclipse TE600 upright microscope (Tokyo, Japan) equipped with a 60 $\times$ 0.85 NA objective by counting at least 300 different cells in 15 random microscopic fields per dish. All experiments were composed of triplicates and were repeated at least three times.

Western blot analysis

Cells were seeded at 5×10^5 cells per 6-cm dishes. After treatment, they were washed twice with ice-cold PBS and scraped off the culture dishes in lysis buffer. The cell extracts were boiled 5 min, separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane. To reveal Myc-tagged ubiquitin, the membranes were boiled in a water bath for 5 min before

Western blotting. The membranes were blocked with 5% (w/v) milk powder in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated with the appropriate antibodies diluted in 5% (w/v) bovine serum albumin in TBS-T for 1 h at room temperature. The horseradish peroxidase-conjugated secondary antibodies were incubated 1 h at room temperature in 5% milk powder in TBS-T. Peroxidase activity was visualized with the Supersignal Chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Live cell analysis

Cells cultivated in 3 cm Petri dishes were placed into a temperature-controlled (37°C) incubation chamber enclosing a Nikon Eclipse TE2000 inverted microscope (Tokyo, Japan) equipped with a 40 $\times$ objective shielded from ambient light and exposed to 5% CO_2 /95% humidified air. Images were taken every 30 min for 24 h in at least six fields per dish for a total of at least 60 cells. All experiments were performed at least in duplicate.

Clonogenic survival

Cells were treated in the exponential growth phase. Immediately after treatment, cells were trypsinized and plated at the appropriate dilution in triplicate in order to have approximately 50–200 viable cells per dish (Huot et al. 1996). Relative survival was obtained by counting the number of colonies of 50 cells or more 9 days later. The data were adjusted according to the plating efficiency obtained with the appropriate control.

Results

HS induces nuclear alterations that are morphologically distinct from apoptosis

HS induces nuclear alterations that are in marked contrast with those accompanying apoptosis (Fig. 1a). After HS, the nuclei of HEK293 cells underwent severe deformation often becoming crenated or crumpled, a feature clearly distinguishable from the typical fragmented and condensed nuclei observed after stimulating the extrinsic pathway of apoptosis downstream of the death receptor Fas. HS-induced nuclear deformation was very rapid reaching a maximum by 2 h after a treatment at 44°C for 120 min (Fig. 1b). Similar effects were observed in HeLa cells (Fig. 1a). In these cells, cisplatin induced apoptosis that resulted in the appearance of fragmented nuclei starting at 8 h. HS induced a rapid nuclear deformation process that peaked at around 6 h (data not shown). Typical HS-induced

Fig. 1 HS caused nuclear deformation. **a** Pictures of nuclei of different cell types after different treatments. For HS treatment, HEK293 and MEF cells were exposed to HS at 44°C for 120 and 45 min, respectively, followed by incubation at 37°C for 24 h. Rat1-Myc and HeLa cells were exposed to a HS treatment for 45 min at 43.5°C followed by incubation at 37°C for 6 h. For apoptotic stimuli, HEK293 cells ectopically expressing Fas receptor were treated with Fas antibody (100 ng/ml) for 16 h. Rat1-Myc and HeLa cells were exposed to cisplatin (35 μ M) for 6 and 16 h, respectively. After treatment, cells were fixed and stained with DAPI to visualize nuclei. **b** HEK293 cells were exposed to HS at 43.5°C for 45 min followed by different incubation time at 37°C before fixation and analysis. The percentage of nuclear alterations was determined microscopically. **c**, **d** Rat1-Myc and Rat1-control cells were exposed to HS at 43.5°C for 45 min (**c**) or for the indicated time (**d**) followed by 0–8 (**c**) or 6 h (**d**) at 37°C before fixation and analysis

nuclear alterations were also observed in MEF cells (Fig. 1a) suggesting that nuclear deformation was a general reaction of the nucleus to HS treatment.

HS did not induce significant apoptotic morphology in any of the above cell lines, at least at early times and under the conditions tested as compared to the apoptotic inducers (Fig. 1a). HS-induced nuclear alterations observed in sensitized Rat1-Myc fibroblasts were compared to those obtained in their transformation defective counterpart, the Rat1-control cells. Addition of OHT to the culture medium deregulates c-Myc expression and markedly sensitizes Rat1-Myc cells to apoptosis (Littlewood et al. 1995). By contrast, Rat1-control cells express a transcriptionally inactive deletant of c-Myc and are not responsive to OHT. As expected, a deregulation of c-Myc expression substan-

tially sensitized Rat1-Myc cells to HS-induced apoptosis. Eight hours after HS, nuclear fragmentation typical of apoptosis was seen in about 40% of the Rat1-Myc cells whereas no apoptotic nuclei were observed in Rat1-control cells under the same conditions (Fig. 1c). Nevertheless, HS induced, in both Rat1 cells, nuclear deformations very similar to those observed in the HEK-293 cells (Fig. 1a). The induction of nuclear deformation was more rapid than the induction of nuclear fragmentation, reaching a maximum of 60% to 80% 4 h after treatment in both Rat1-Myc and Rat1-control cells (Fig. 1d). Both apoptosis and HS-specific nuclear deformation were induced in a dose-dependent manner (Fig. 1d).

Since both classical apoptotic nuclei and HS-induced deformed nuclei could be seen in the same microscopic

field 6 h after HS in Rat1-Myc cells, the latter cells were therefore useful to characterize simultaneously the biochemical differences between the two morphological alterations. First, the cells were labeled with DAPI, to stain their nuclei, and with antibodies against cleaved caspase-3 (Fig. 2a), cytochrome c, or activated Bax (Fig. 2b). In all cells undergoing typical apoptosis, whether induced by cisplatin or HS, Bax was activated, caspase-3 was cleaved, and cytochrome c was released from mitochondria to cytoplasm (arrowheads in Fig. 2a, b). In contrast, neither of these phenomena was observed in cells with the HS-induced nuclear deformation (arrows in Fig. 2a, b).

Another feature of apoptosis is the destruction of the nuclear envelope (Broers et al. 2002). To test whether the nuclear envelope was altered after HS, the cells were transfected with a construct expressing laminB-GFP (Moir et al. 2000). No breakdown of the nuclear envelope was observed in the control nuclei (CTL, Fig. 2c, left panel) and in the deformed nuclei after HS (Fig. 2c, middle panel) but it was completely broken down in fragmented nuclei (Fig. 2c, right panel).

The Bcl-2 protein family plays a critical role in pro- or anti-apoptotic processes. Overexpression of the anti-apoptotic Bcl-2 protein, which acts by antagonizing the action of the pro-apoptotic Bax protein, had no effect on the HS-induced nuclear deformation. However, it blocked very efficiently HS-induced apoptosis (Fig. 3a) suggesting that Bcl-2 blocked the consequence of HS induced damage but not the damage by itself. Similarly, z-VAD-fmk, a general inhibitor of caspases, blocked HS-induced apoptosis but not the HS-induced nuclear deformation (Fig. 3c).

We previously showed that the MAP3 kinase Ask1 and the MAPK p38 play important roles in the apoptotic process upstream of the mitochondria (Desbiens et al. 2003). To analyze whether they would be implicated in the HS-induced nuclear deformation, Rat1-Myc cells were transfected with a dominant-negative form of Ask1 (AskKM) or treated with SB203580, an inhibitor of p38 (Fig. 3b). Both interventions led to substantial diminution of apoptotic nuclear fragmentation 6 h after HS. However, they did not affect the nuclear deformation typical for HS suggesting that the signaling pathways regulated by Ask1 and p38 are not involved in the regulation of this phenotype. Taken together, all these results showed that the molecular mechanisms underlying the induction and the development of HS-induced deformed nuclei were upstream of the activation of stress kinases and mitochondrial events and independent of caspase activation.

The HS-induced nuclear deformation may culminate in apoptosis at later time points

HS did not induce noticeable apoptosis in the Rat1-control cells until at least 8 h posttreatment (Fig. 1c). Nevertheless,

Fig. 2 Nuclear deformation caused by HS is not apoptosis. **a, b** Rat1-Myc cells were exposed to HS for 43.5°C for 45 min followed by 6 h at 37°C or exposed to cisplatin (35 μ M, 6 h). Cells were fixed and stained with an antibody against the cleaved form of caspase-3 (**a**) or with antibodies recognizing cytochrome c and the activated form of Bax (**b**). Nuclei were visualized by DAPI staining. *Arrowheads* indicate fragmented apoptotic cells; *arrows* indicated deformed nuclei. **c** Cells were transfected with laminB-GFP and exposed to HS as in **a** or not. Cells were fixed and images were taken on a confocal microscope

Fig. 3 HS-induced nuclear deformation is not caused by MAP kinase signaling. **a** Rat1-Myc cells were transfected with GFP or Bcl2-GFP and then treated with HS at 43.5°C for 45 min and 6 h at 37°C. Cells were fixed and nuclei visualized by staining with DAPI. The percentage of nuclear alterations was determined microscopically in the GFP-positive cells. Data are means $\pm$ SD of two independent experiments done in duplicate. **b** Rat1-Myc cells were transfected with pcDNA3 (*Ctl*) or with AskKM-Ha and treated with HS as in **a**. Where indicated, SB203580 (+SB) was added 1 h before HS at a final concentration of 10 μ M. Cells were fixed and nuclear alterations were determined microscopically in cells positive for the Ha-tag after DAPI and Ha staining. Data are means $\pm$ SD of at least two independent experiments done in duplicate. **c** Rat1-Myc cells were treated with HS as in **a**. z-VAD-fmk (100 μ M) was added 1 h before HS (*HS+zVAD*). Cells were fixed and nuclei visualized by staining with DAPI. Nuclear alterations were determined microscopically

visualization of nuclear morphology (Fig. 4b) and the observation of individual cells by video microscopy over a period of 1 or 2 days. Such analyses revealed that the HS-induced nuclear deformation occurred in almost all individual cells after HS (Fig. 4b, Fig. S1, and data not shown). In both cell types, 99% of the nuclei started to show nuclear indentations within 1 to 4 h after HS. The indentations became more prominent with time leading in extreme cases to the formation of multilobated nuclei. The process was very dynamic, the indentations going in and out rapidly (Fig. S2), explaining why only 60–80% of deformed nuclei were detected in fixed cells. In the Rat1-control cells, the HS-induced nuclear deformation was a reversible process. Indeed, 50% of the nuclei became normal 10–20 h after HS (Fig. S1). All other cells finally developed apoptosis between 8 and 16 h post-HS. In contrast, apoptosis in Rat1-Myc cells developed earlier occurring mostly between 1 to 8 h after HS (Fig. 4c and Fig. S3). This is also clearly demonstrated by comparing Figs. S2 and S3. Cumulative apoptosis peaked at 99% in the Rat1-Myc cells compared to 50% in the Rat1-control cells. This latter result was in agreement with the data obtained by the colony formation assay mentioned above. In control conditions, we observed less than 6% apoptosis after 24 h in either cell lines. The nuclear fragmentation observed in Rat1-control cells corresponded to classical apoptosis since it was blocked by the caspase inhibitor z-VAD-fmk (data not shown).

All together, these results showed that HS initiates two apoptotic processes. One is rapidly induced, depends on cell signaling, and is observed only in apoptosis-sensitive cells (Rat1-Myc cells). The other one is slower and observed in both Rat1 cells.

Thermotolerant cells are protected against HS-induced nuclear deformation and apoptosis

A major feature of HS is the massive induction of HSPs expression, whose role is to protect cells against HS-induced cell death (Lindquist 1986). Thus, we first tested

a colony formation assay showed that only 40% of the Rat1-control cells survived after HS compared to 0.8% for the Rat1-Myc cells (Fig. 4a).

To analyze more precisely the fate of Rat1 cells for a long period after HS, Rat1-Myc and Rat1-control cells were transfected with a plasmid expressing histone2A-GFP (Perche et al. 2000). This marker allowed the live

Fig. 4 Live imaging analysis reveals that Rat1-control cells die by apoptosis occurring at later time. **a** Rat1-Myc (*myc*) and Rat1-control cells (*control*) were treated at 43.5°C for 45 min. Nine days after incubation at 37°C, survival was measured by counting the number of colonies. Data are means of one experiment done in triplicate. **b**, **c** Rat1-Myc (*Myc*) and Rat1-control (*control*) cells were transfected with H2A-GFP and treated at 43.5°C for 45 min and then nuclei morphology was monitored by live

fluorescent microscopy imaging for 24 h. In **b**, a representative field for both Rat1-Myc and Rat1-control is shown at different times after HS. Numbers identify cells in each field. The color of the numbers indicates the nucleus status: white normal nuclei, green deformed nuclei, red condensed or fragmented nuclei. Curves shown in **c** are the average of two independent experiments where about 30 up to 60 cells were analyzed for each condition in each experiment

whether a mild HS pretreatment, known to induce the whole spectrum of HSPs (data not shown) and to make cells thermotolerant, would have the capacity to block the slower apoptotic process induced by a second severe HS. Rat1-control cells pretreated with a 20-min treatment at 43.5°C were extremely resistant for both HS-induced nuclear deformation and apoptotic nuclear fragmentation (Fig. 5a, b). These results suggested that the HSP expressed in the primed cells could block the initial nuclear deformation and that this nuclear deformation likely leads to apoptosis.

We then tested if thermotolerance induced by a prior HS could also block the HS-induced nuclear deformation and rapid apoptosis in Rat1-Myc cells. Primed Rat1-Myc cells expressed the same level of HSP as primed Rat1-control cells (data not shown) and, like these cells, also became resistant to HS-induced nuclear deformations and apoptosis (Fig. 6a). However, primed Rat1-Myc cells remained fully sensitive to HS-induced rapid apoptosis (Fig. 6a). A colony

formation assay showed that primed Rat1-Myc cells increased their survival level after a severe HS by more than 30-fold (Fig. 6b). These results suggested that apoptosis could be induced by two different initiating processes. The first one depended on the prime activation of stress kinases and mitochondrial apoptotic signaling. This signaling events induced by HS were responsible for the apoptosis observed rapidly after HS (<8 h) in Rat1-Myc cells. This rapid induction of apoptosis was not influenced by the thermotolerant state of the cells. In contrast, the second wave of apoptosis observed at later time points after HS (>8 h) especially in Rat1-control cells was inhibited by a prime HS inducing HSPs expression. This was further supported by the results showing that induction of thermotolerance had no protective effect on cisplatin-induced apoptosis and loss of colony formation (Fig. 6a, b). In fact, HS-primed cells even had a greater cisplatin sensitivity than naïve cells

Fig. 5 Thermotolerance blocks HS-induced nuclear deformation and apoptosis occurring at later time in Rat1-control cells. **a, b** Rat1-control cells were transfected with H2A-GFP. Cells were then treated in three different ways before analysis by live imaging: Cells were heated for 45 min at 43.5°C (*HS*); cells were first treated with a mild HS at 43.5°C

for 20 min followed by incubation at 37°C for 6 h and then heated again (*TT-HS*) or not (*TT-Ctl*) for 45 min at 43.5°C. Nuclear deformation was calculated at 4 h (**a**) and apoptosis was followed for 24 h (**b**). Data shown in this figure are the average of two independent experiments where about 25 up to 60 cells were analyzed for each condition in each experiment

Protein aggregation triggers HS-like nuclear deformation

The main effect of heat damage is accumulation of aberrant proteins. It has been shown that saturation of the cell's capacity to eliminate altered proteins through degradation by the proteasome is a major reason for toxicity (Friant et al. 2003). The above finding that the accumulation of HSP after HS priming can block the induction of the HS-induced nuclear deformation suggested that accumulation of aggregated proteins might be the cause of these nuclear

alterations. We first aimed at confirming whether HS induced a major increase in the level of ubiquitinated proteins as a sign of increased accumulation of aberrant proteins. Cells were transfected with a myc-tagged ubiquitin vector and the level of protein-ubiquitin conjugates was evaluated by Western blot. In cells treated only with the vehicle (DMSO), myc-ubiquitin (myc-Ub) was recovered not only as free myc-Ub migrating at 16 kDa but also as high molecular weight ubiquitin-conjugated products (>200 kDa; Fig. 7a). Inhibition of the proteasome with

Fig. 6 Mild HS protects only against HS-induced apoptosis occurring at later time but not against HS-induced rapid occurring apoptosis or cisplatin-induced apoptosis. **a** HSP expression was induced (*TT*) or not (*CTL*) in Rat1-Myc cells with a heat shock of 20 min at 43.5°C followed by an incubation of 5 h at 37°C. Cells were then treated with 50 μM cisplatin for 5 h (*Cis*) or heat shocked for 45 min at 43.5°C followed by incubation at 37°C for 5 h (*HS*). The nuclei were stained

with DAPI and analyzed by fluorescence microscopy. Data are means ± SD of at least two independent experiments done in duplicate. **b** Control (*CTL*) or preheat-shocked (*TT*) Rat1-Myc cells were treated with 50 μM cisplatin (*Cis*) for 5 h or heat shocked for 120 min at 43.5°C (*HS*). Nine days after incubation at 37°C, survival was measured by counting the number of colonies. Data are means of one experiment done in triplicate

Fig. 7 Inhibition of the proteasome by MG132 causes the same nuclear alterations as HS. **a** HEK293 cells were transfected with pCW7 plasmid to allow Myc-ubiquitin expression and treated with DMSO for 16 h, HS at 44°C for 120 min, or treated with MG132 (5 μ M) for 5 or 16 h. Cellular extracts were analyzed by Western blot using an antibody recognizing the myc tag. **b** HEK293 and Rat1-

control cells were exposed to MG132 (5 μ M) for 16 or 6 h, respectively. Cells were fixed and nuclei were stained with DAPI. **c** Rat1-Myc and Rat1-control cells were exposed to MG132 (5 μ M) for 6 h. The percentage of nuclear alterations was determined microscopically after cell fixation and DAPI staining of the nuclei. Data are means $\pm$ SD of two independent experiments done at least in duplicate

MG132 led to the accumulation of high molecular weight conjugates whereas the concentration of free ubiquitin decreased. Likewise, HS led to the accumulation of a large quantity of polyubiquitinated products and the disappearance of the free ubiquitin pool within 24 h (Fig. 7a). These results confirmed, as previously described (Friant et al. 2003; Salomons et al. 2009), that HS induced an accumulation of nondegradable aggregated proteins, which may lead to an overload or an inefficiency of the proteasome.

If accumulation of aggregated proteins was responsible for inducing the nuclear deformation observed after HS treatment, one would expect that this morphological alteration of the nucleus would be also induced by MG132. Indeed, MG132 treatment induced nuclear alterations similar to those induced by HS in both HEK293 and Rat1-control cells (Fig. 7b) and led to nuclear deformation as observed after HS and to apoptosis-like nuclear fragmentation in Rat1-control cells (Fig. 7c). These results strongly suggested that aggregation of damaged proteins was responsible for the nuclear deformation caused by HS.

To test this hypothesis, the localization of aggregated proteins in cells with HS- or MG132-induced deformed nuclei was investigated. It has been described that aggregated proteins often accumulate in cells as aggresomes which localize at the MTOC and are often wrapped by intermediate filaments where vimentin is the most consistent component (Johnston et al. 1998). The staining of

vimentin in Rat1-control and HEK293T cells treated with a HS or with MG132 showed that the vimentin network was disrupted and that vimentin accumulated at the perinuclear region. Moreover, vimentin was often localized in the nuclear invagination found in the deformed nuclei (Fig. 8). Also, γ -tubulin was found in the nuclear indentation after treatment with HS or with MG132 in Rat1-control cells suggesting that the MTOC was found in this part of the cells. Interestingly, as shown in Fig. 8d, mitochondria as evidenced by cytochrome c staining also clustered in or near the HS-induced nuclear invagination of laminB-GFP transfected cells. This is consistent with previous observations demonstrating that mitochondria accumulate near the aggresomal structure (Johnston et al. 1998; Waelter et al. 2001) and that centrosomes are affected by HS (Hut et al. 2005). These results suggested that aggregated materials following HS might accumulate near the nucleus and induce the typical nuclear distortion.

This theory was directly tested by studying the consequences of overexpressing an aggregation-prone protein in the cells. The accumulation of such a protein should have upon aggregation the same effect on the nuclear structure as HS. The cells were thus transfected with different forms of the Htt protein corresponding to exon 1 of Htt coupled with varying length of a polyglutamine stretch. Abnormal Htt variants with a long polyglutamine stretch have been shown to aggregate and correlate with the pathogenesis of

Fig. 8 HS and proteasome inhibition lead to the accumulation of vimentin in the nuclear invagination. **a, c** Rat1-control cells were exposed to HS (43.5°C, 45 min followed by 6 h at 37°C) or exposed to MG132 (5 μ M) for 6 h. **b** HEK293T cells were exposed to HS (43.5°C, 45 min followed by 6 h at 37°C) or exposed to MG132 (5 μ M) for 16 h. Cells were fixed and stained with antibodies recognizing vimentin (**a, b**) or γ -tubulin (**c**). Nuclei were visualized after DAPI staining. Photos were taken by immunofluorescence imaging. **d** Cells were transfected with laminB-GFP and exposed to HS as in **a**. Cells were fixed, stained with anti-cytochrome c (Cyt. c) antibody. Images were taken on a confocal microscope

Huntington's disease (Waelter et al. 2001). Htt with a short stretch of glutamine, Htt23Q, was found with a diffuse distribution in the cytoplasm and produced nearly no morphological disturbances (Fig. 9). In contrast, Htt43Q and 74Q, with stretch of 43 and 74 glutamines, respectively, formed in many cells small aggregates that accumulated at the periphery of the nucleus (Fig. 9). In more than 20% of these cells, the aggregates induced nuclear deformations that were very similar to those induced by HS. Htt74Q induced nuclear alterations in a higher proportion of the cells than Htt43Q, likely because Htt74Q has a higher potential of aggregation than Htt43Q. Importantly, in a majority of the cells where Htt43Q and 74Q caused nuclear deformations, the aggregated Htt proteins were found in the invagination of the deformed nuclei (Fig. 9b). Moreover, overexpression of Htt74Q in Rat1-control cells led to the deformation of their nuclei, which culminates in apoptosis in a proportion of 67% as determined by live imaging (data not shown). These results showed that the accumulation of aggregated proteins, at the nuclear periphery, causes a nuclear deformation which, most of the time, forecast the apoptotic death of the affected cell.

Discussion

In this study, we show that HS can induce two different apoptotic processes. The first one is rapid and involved the activation of MAPKs and of the intrinsic apoptotic pathway. The second is slower and likely depends on the toxicity caused by the accumulation of aggregated proteins. Moreover, these protein aggregates induce a nuclear deformation in all the studied cell lines. Globally, our results give new insights on how cells die upon severe HS treatment and describe a new morphologic clue to determine whether a cell is affected or not by a proteotoxic stress.

Different HS-induced apoptotic mechanisms depending on the cellular context

The cellular response to stresses is tightly related to the context in which the cells are at the time of the stress. For example, cells containing high concentrations of antioxidants proteins have a better protection against oxidative stress as it is the case of cancer cells expressing high levels

Fig. 9 Accumulation of aggregated Htt leads to nuclear alterations. HEK293 cells were transfected with different length of polyglutamine stretches of Ha-tagged Huntingtin (23Q, 43Q, 74Q). The percentage of nuclear alterations in the transfected cells was determined microscopically in cells with diffuse and with aggregated Huntingtin (**a**) and photos were taken (**b**). Data are means $\pm$ SD of two independent experiments done in triplicate

of thioredoxin (Maulik and Das 2008). Contrarily, the expression of some proteins can render cells more stress sensitive. Deregulation of the oncoprotein c-Myc is a good example. The cells used in this study, the Rat1-Myc cells, have a deregulated expression of c-Myc and are more sensitive to apoptosis. c-Myc triggers a proapoptotic

mitochondrial destabilizing activity (Evan et al. 1992; Juin et al. 2002). More precisely, recent published data showed that in response to cytotoxic drugs, c-Myc can promote Bax oligomerization in the outer mitochondrial membrane of the mitochondria to form channels that facilitate cytochrome c release (Cao et al. 2008). Here, HS rapidly induced apoptosis in the Rat1-Myc cells. As revealed by the protective effect of Bcl-2, HS-induced rapid cell death observed in Rat1-Myc cells was mainly caused by activation of the classical mitochondrial pathway. Furthermore, the protective effects of both AskKM and p38 inhibitor, SB203580, suggested that the mitochondrial-dependent apoptotic pathway needed prior activation by MAPK pathway before inducing apoptosis. These results are in line with a previous study showing that the Ask1-p38-Bax pathway is involved in the cisplatin-induced apoptosis in Rat1-Myc cells (Desbiens et al. 2003). More interestingly, when HSPs expression was increased in thermotolerant Rat1-Myc cells, the slower but not the rapid HS-induced apoptosis was blocked. This supports our hypothesis that the fast-induced apoptotic cell death observed in Rat1-Myc cells involves initiating events and downstream mechanisms different from those regulating the slower HS-induced apoptosis.

The analysis of Rat1-control cells response to HS showed that they were sensitive to a slower process leading to apoptosis. HS treatment on Rat1-control cells, as for the Rat1-Myc, induced the development of the nonapoptotic nuclear deformations, which could not be prevented by inhibition of the classical apoptotic pathways but depended mainly on the accumulation of aggregated proteins. The proportion of these protein aggregates exceeded the renaturation/degradation capacity of the cell, which then led to the late activation of the apoptotic process. But how could protein aggregates lead to apoptosis in the cells?

When the rate of accumulation of damaged or aggregated proteins overloads the rate of degradation by the ubiquitin–proteasome system, mimicked with proteasome inhibitor treatments or expression of disease-derived aggregating proteins, aggresomes can form (Johnston et al. 1998; Wigley et al. 1999) by a process in which small protein aggregates, called microaggregates, are actively transported toward the perinuclear region on microtubules (Kopito 2000). It has been proposed that the formation of aggresomes would rather be cytoprotective than cytotoxic because microtubule-disrupting agents prevent aggresome formation and at the same time enhance the toxicity of polyglutamine protein aggregates (Fortun et al. 2003). However, one can expect that when a cell is exposed to a stress that leads to an important accumulation of aggregated proteins, the formation of an aggresomal structure might not be sufficient to buffer all these aggregating proteins. Consequently, the observation of different parameters

linked to the formation of an aggresome should be considered as a sign that the cell is facing a potentially lethal situation. Moreover, after HS, the accumulation of aggregated proteins is probably more rapid than in the case of a treatment with MG132 or when a mutant protein is overexpressed. This much faster accumulation of misfolded proteins may lead to the impairment of the transport of protein aggregates at the aggresome due to cytoskeletal damages. This could lead to a totally uncoordinated aggresome formation and consequently causes a cytotoxic effect rather than cytoprotective effect.

Nevertheless, we cannot rule out that HS might affect the integrity of centrosomes (Vidair et al. 1993; Hut et al. 2005). In this case, division errors would occur and multiple spindle poles would become apparent. However, since we did not observe multiple spots of γ -tubulin in HS-treated Rat1 cells, centrosomes are likely unaffected in our system. On the other hand, the observation by Hut and colleagues that recruitment of hsp70 to centrosomes delays mitosis is supported by our data that HS-treated Rat1-control cells have a much longer mitotic delay relative to Rat1 control cells not subjected to HS (data not shown).

We showed that depending of the cellular context, cells died through the activation of apoptotic processes by using different paths in the cell. In both cell lines, the observed nuclear deformation suggested that the aggregated material was accumulating in the perinuclear region. The more rapidly occurring cell death seen in the Rat1-Myc cells, which were primed for apoptosis, occurred subsequent to the activation of MAPK pathways involving Ask1-p38-Bax that “sensed” the HS stress suggesting that the threshold for apoptosis was lowered by the expression of c-Myc in these cells and was overriding any possible reparation program. On the other hand, our results demonstrate that in cells with higher apoptotic threshold, severe HS led to the accumulation of aggregating proteins leading to the appearance of different aggresome features such as vimentin relocation, mitochondria clustering, and nuclear deformation. Whatever cell death is triggered through the formation of the aggresome per se or through the accumulation of unfolded proteins (whether aggresome formation is or is not a prosurvival event), it seems clear that HS-induced protein aggregation is a key element in the later occurring apoptosis. This is supported by the fact that HSP expression, undoubtedly known to be involved in the elimination of unfolded aggregating proteins, was enough to block both the manifestation of unfolded protein accumulation like nuclear deformation and cell death.

In conclusion, our results clearly indicate that severe HS treatment leads to the formation of an aggresomal structure comparable to the one seen by the expression of abnormal variants of Htt or other disease-derived aggregating proteins in all cells tested. Because HS, MG132 treatment, and Htt

expression led all to the same typical nuclear deformation in different cell lines, we propose to include nuclear deformation as a new distinctive feature of the formation of toxic protein aggregates.

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References

- Auluck PK, Chan HY, Trojanowski JQ, Lee VM, Bonini NM (2002) Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. *Science* 295:865–868
- Bailey CK, Andriola IF, Kampinga HH, Merry DE (2002) Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. *Hum Mol Genet* 11:515–523
- Bennett EJ, Bence NF, Jayakumar R, Kopito RR (2005) Global impairment of the ubiquitin–proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. *Mol Cell* 17:351–365
- Bredesen DE, Rao RV, Mehlen P (2006) Cell death in the nervous system. *Nature* 443:796–802
- Broers JL, Bronnenberg NM, Kuijpers HJ, Schutte B, Hutchison CJ, Ramaekers FC (2002) Partial cleavage of A-type lamins concurs with their total disintegration from the nuclear lamina during apoptosis. *Eur J Cell Biol* 81:677–691
- Bucciantini M, Giannoni E, Chiti F et al (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 416:507–511
- Cao X, Bennett RL, May WS (2008) c-Myc and caspase-2 are involved in activating Bax during cytotoxic drug-induced apoptosis. *J Biol Chem* 283:14490–14496
- Chang HY, Nishitoh H, Yang X, Ichijo H, Baltimore D (1998) Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science* 281:1860–1863
- Charette SJ, Lavoie JN, Lambert H, Landry J (2000) Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol* 20:7602–7612
- Cummings CJ, Sun Y, Opal P et al (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum Mol Genet* 10:1511–1518
- Desbiens KM, Deschesnes RG, Labrie MM, Desfosses Y, Lambert H, Landry J, Bellmann K (2003) c-Myc potentiates the mitochondrial pathway of apoptosis by acting upstream of apoptosis signal-regulating kinase 1 (Ask1) in the p38 signalling cascade. *Biochem J* 372:631–641
- Evan GI, Wyllie AH, Gilbert CS et al (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 69:119–128
- Fortun J, Dunn WA Jr, Joy S, Li J, Notterpek L (2003) Emerging role for autophagy in the removal of aggresomes in Schwann cells. *J Neurosci* 23:10672–10680
- Friant S, Meier KD, Riezman H (2003) Increased ubiquitin-dependent degradation can replace the essential requirement for heat shock protein induction. *EMBO J* 22:3783–3791
- Gabai VL, Yaglom JA, Volloch V et al (2000) Hsp72-mediated suppression of c-Jun N-terminal kinase is implicated in development of tolerance to caspase-independent cell death. *Mol Cell Biol* 20:6826–6836

- Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* 94:73–82
- Hohfeld J, Cyr DM, Patterson C (2001) From the cradle to the grave: molecular chaperones that may choose between folding and degradation. *EMBO Rep* 2:885–890
- Huot J, Houle F, Spitz DR, Landry J (1996) HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer Res* 56:273–279
- Hut HM, Kampinga HH, Sibon OC (2005) Hsp70 protects mitotic cells against heat-induced centrosome damage and division abnormalities. *Mol Biol Cell* 16:3776–3785
- Johnston JA, Ward CL, Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* 143:1883–1898
- Juin P, Hunt A, Littlewood T, Griffiths B, Swigart LB, Korsmeyer S, Evan G (2002) c-Myc functionally cooperates with Bax to induce apoptosis. *Mol Cell Biol* 22:6158–6169
- Kopito RR (2000) Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol* 10:524–530
- Kregel KC (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 92:2177–2186
- Landry J, Chretien P, Lambert H, Hickey E, Weber LA (1989) Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *J Cell Biol* 109:7–15
- Lindquist S (1986) The heat-shock response. *Annu Rev Biochem* 55:1151–1191
- Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI (1995) A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* 23:1686–1690
- Maulik N, Das DK (2008) Emerging potential of thioredoxin and thioredoxin interacting proteins in various disease conditions. *Biochim Biophys Acta* 1780:1368–1382
- Michels AA, Kanon B, Konings AW, Bensaude O, Kampinga HH (2000) Cycloheximide- and puromycin-induced heat resistance: different effects on cytoplasmic and nuclear luciferases. *Cell Stress Chaperones* 5:181–187
- Moir RD, Yoon M, Khuon S, Goldman RD (2000) Nuclear lamins A and B1: different pathways of assembly during nuclear envelope formation in living cells. *J Cell Biol* 151:1155–1168
- Nadeau SI, Landry J (2007) Mechanisms of activation and regulation of the heat shock-sensitive signaling pathways. *Adv Exp Med Biol* 594:100–113
- Perche PY, Vourc'h C, Konecny L, Souchier C, Robert-Nicoud M, Dimitrov S, Khochbin S (2000) Higher concentrations of histone macroH2A in the Barr body are correlated with higher nucleosome density. *Curr Biol* 10:1531–1534
- Salomons FA, Menendez-Benito V, Bottcher C, McCray BA, Taylor JP, Dantuma NP (2009) Selective accumulation of aggregation-prone proteasome substrates in response to proteotoxic stress. *Mol Cell Biol* 29:1774–1785
- Vidair CA, Doxsey SJ, Dewey WC (1993) Heat shock alters centrosome organization leading to mitotic dysfunction and cell death. *J Cell Physiol* 154:443–455
- Waelter S, Boeddrich A, Lurz R, Scherzinger E, Lueder G, Lehrach H, Wanker EE (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. *Mol Biol Cell* 12:1393–1407
- Ward CL, Omura S, Kopito RR (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 83:121–127
- Wigley WC, Fabunmi RP, Lee MG, Marino CR, Muallem S, DeMartino GN, Thomas PJ (1999) Dynamic association of proteasomal machinery with the centrosome. *J Cell Biol* 145:481–490
- Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 139:1281–1292
- Wytenbach A, Carmichael J, Swartz J, Furlong RA, Narain Y, Rankin J, Rubinsztein DC (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A* 97:2898–2903