Abstract. Cancer cells are continuously challenged by adverse environmental factors including hypoxia, metabolite restriction, and immune reactions, and must adopt diverse strategies to survive. Heat shock protein (Hsp) 70 plays a central role in protection against stress-induced cell death by maintaining protein homeostasis and interfering with the process of programmed cell death. Recent findings have suggested that Hsp70 acetylation is a key regulatory modification required for its chaperone activity, but its relevance in the process of programmed cell death and the underlying mechanisms involved are not well understood. In this study, we sought to investigate mechanisms mediated by Hsp70 acetylation in relation to apoptotic and autophagic programmed cell death. Upon stress-induced apoptosis, Hsp70 acetylation inhibits apoptotic cell death, mediated by Hsp70 association with apoptotic protease-activating factor (Apaf)-1 and apoptosis-inducing factor (AIF), key modulators of caspase-dependent and -independent apoptotic pathways, respectively. Hsp70 acetylation also attenuated autophagic cell death associated with upregulation of autophagy-related genes and autophagosome induction. Collectively, these results suggest that the acetylation of Hsp70 plays key regulatory roles in cell death pathways as well as in its function as a chaperone, together enabling cellular protection in response to stress.

Introduction

Cancer cells are confronted with diverse environmental stresses including hypoxia, nutrient deprivation, and pH changes caused by metabolic byproducts and the tumour microenvironment (1-3). Stress factors induce diverse apoptotic signaling in cells, in which various pro-apoptotic proteins can be activated. To overcome the apoptotic response, cancer cells develop diverse ways to inhibit apoptotic signaling (2,4,5). Furthermore, these signaling alterations can also allow cancer cells to resist chemo- or radiotherapeutic challenge (6).

The heat shock protein (HSP) 70 chaperone system is upregulated in many cancers and facilitates the refolding or degradation of proteins that are denatured as a result of stress (7-9). In addition, Hsp70 can also directly interfere with apoptosis pathways to protect cells (10-12). The diverse protective mechanisms of Hsp70 are known to confer resistance to some forms of stress-induced cell death.

Several pro-apoptotic proteins that are directly inhibited by Hsp70 have been reported. Apoptotic protease-activating factor 1 (Apaf-1) and apoptosis-inducing factor (AIF), key modulators of caspase-dependent and -independent apoptotic pathways, respectively. Hsp70 acetylation also attenuated autophagic cell death associated with upregulation of autophagy-related genes and autophagosome induction. Collectively, these results suggest that the acetylation of Hsp70 plays key regulatory roles in cell death pathways as well as in its function as a chaperone, together enabling cellular protection in response to stress.
Recently, Hsp70 was reported to be acetylated by acetyltransferase arrest defective (ARD) 1 (Naa10, N-acetyltransferase 10) and this acetylation contributes to its protective function under cellular stress (19). Here, we sought to investigate the molecular mechanisms of Hsp70 acetylation-mediated cellular protection.

Materials and methods

Cell culture and stimulation. SH-SY5Y, HeLa, and HEK293T cells were obtained from the American Type Culture Collection (ATCC). Cultured cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO2 humidified atmosphere at 37°C. To induce cellular stress, cells were treated with 1 µg/mL doxorubicin or 0.3 mM H2O2 for 24 h.

Plasmid construction. Full-length cDNAs for human Hsp70 (Genbank: NM_005345.5) and human ARDI (Genbank: NM_003491.3) were generated by PCR and subcloned into pCDNA3.1 (FLAG-ARD1) or pEGFP-C3 (GFP-Hsp70) vectors for cellular expression. For the construction of stable cell lines, cDNA constructs for Hsp70 and ARDI were co-inserted into the pIRES vector, purchased from Clontech.

Transfection. Transfection was carried out as described previously (20). HEK293T cell transfection used polyethyleneimine (PEI) at a ratio of 4:1 (µL PEI/mg plasmid DNA). Transfection was carried out as described previously (20). HEK293T cell transfection used polyethyleneimine (PEI) at a ratio of 4:1 (µL PEI/mg plasmid DNA) according to the manufacturer's instructions. Transfected cells were collected and LDH activity was determined with an LDH assay kit (DoGen). Total cellular LDH activity was measured by solubilizing the cells with 0.2% Triton X-100.

Immunoblotting and immunoprecipitation. Proteins were extracted using lysis buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100 and a protease inhibitor cocktail (Roche). Then, 20 µg of cell extract was used for immunoblotting. For immunoprecipitation, 1 µg of protein was incubated with a corresponding antibody conjugated to A or G beads (Upstate) overnight at 4°C. Beads were washed three times with washing buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl and 0.1 mM EDTA. Following SDS-PAGE, membranes were immunoblotted using the corresponding primary antibody overnight at 4°C. The amount of lactate dehydrogenase (LDH) released from the cells into the medium was measured by solubilizing the cells with 0.2% Triton X-100.

Statistical analysis. Results are expressed as the means ± SD P-values were calculated by applying the two-tailed Student's t-test. A difference was considered statistically significant at P<0.05.

Results

Hsp70 acetylation protects cancer cells from doxorubicin-induced cell death. Hsp70 acetylation at residue K77 has been previously reported to protect cells from stress. To confirm the protective effect of Hsp70 acetylation, we treated Hsp70 wild-type (WT) and K77R mutant-expressing SH-SY5Y cells with doxorubicin and analyzed cell viability. Consistent with a previous report, overexpression of wild-type Hsp70 protected cells against doxorubicin-induced cell death, but this protective effect was eliminated by the presence of the K77R mutation (Fig 1A). The acetylation of Hsp70 at K77 is mediated by acetyltransferase ARD1 (19). To further validate the relevance of ARDI in the protective function of Hsp70, ARDI WT and a dominant-negative mutant (DN) that does not harbor acetyltransferase activity was co-expressed with Hsp70 constructs in SH-SY5Y cells, and cell viability was assessed after doxorubicin treatment (21). As expected, co-expression of the dominant-negative mutant ARDI abolished the protective effect of Hsp70 WT (Fig. 1B), indicating that ARDI-mediated Hsp70 acetylation protects cancer cells from doxorubicin-induced cell death.
Hsp70 acetylation inhibits caspase-dependent apoptosis.

To further investigate the underlying mechanisms of Hsp70 acetylation-mediated cellular protection, we first analyzed caspase-dependent apoptosis. Doxorubicin treatment induced cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), which are hallmarks of apoptosis. (Fig. 2A). Consistent with the doxorubicin-induced cell death shown in Fig. 1, Hsp70 WT overexpression prevented doxorubicin-induced cleavage of caspase-3 and PARP, whereas the presence of the K77R mutation abolished the protective effect. Moreover, co-expression of the dominant-negative mutant ARD1 diminished Hsp70 acetylation-mediated inhibition of PARP and caspase cleavage (Fig. 2B).

Since Hsp70 is reported to inhibit apoptosome formation via binding with Apaf-1, a key molecule of caspase-dependent apoptosis, we next investigated whether acetylation at K77 can
modulate Apaf-1 binding. Hsp70 WT or the K77R mutant was overexpressed in HEK293T cells and their affinity to Apaf-1 was assessed by co-immunoprecipitation. Consistent with previous reports, Hsp70 wild-type was co-immunoprecipitated with Apaf-1 in cell extracts. Interestingly, however, mutation at K77 abrogated its binding to Apaf-1, implying that Hsp70 acetylation contributes to Hsp70/Apaf-1 association, subsequently leading to inhibition of functional apoptosome assembly and caspase activation (Fig. 2C). These results suggest that Hsp70 acetylation enables the association of Apaf-1 with Hsp70 and prevents apoptosis.

Hsp70 acetylation prevents caspase-independent apoptosis. Another cell death pathway in which Hsp70 is involved is AIF-dependent apoptosis. Hsp70 inhibits caspase-independent cell death by sequestering AIF and blocking its induction of apoptosis. To examine its relevance to caspase-independent cell death by sequestering AIF and blocking its induction of AIF-dependent apoptosis. Hsp70 inhibits caspase-independent cell death. Collectively, these findings indicate that Hsp70 acetylation may play a role in the prevention of autophagic cell death.

We also analyzed microtubule-associated protein light chain 3 (LC3) to monitor autophagic induction. Upon autophagy, the unconjugated cytosolic form of LC3-I is converted to the phosphatidylethanolamine-conjugated form of LC3-II that forms the autophagosomal membrane (26,27). Therefore, the transition of LC3 from a diffusive cytoplasmic pattern to the punctated membrane pattern is a hallmark of autophagy induction, indicating the formation of autophagic vacuoles (27). When compared to WT, the Hsp70 mutant caused an increase in LC3 expression and perinuclear autophagic vacuole formation (Fig. 4B). These results suggest that Hsp70 acetylation may play a role in the prevention of autophagic cell death.

Discussion

In response to stress, Hsp70 acetylation on K77 residue facilitated Hsp70 interaction with Apaf-1 and AIF, and inhibited Apaf-1 and AIF-dependent apoptosis. Moreover, acetylation of Hsp70 attenuated autophagy, observed via Atg12-Atg5 complex formation, Beclin-1 expression and perinuclear LC3 puncta formation, resulting in the inhibition of autophagic cell death. Taken together, our results suggest that Hsp70 acetylation inhibits cell death by at least three different mechanisms: i) attenuation of caspase-dependent pathways by interacting with Apaf-1 and blocking apoptosome formation, ii) inhibition of caspase-independent pathways by interacting with AIF and preventing its nuclear translocation, and iii) attenuation of autophagic cell death (Fig. 5).

Previously, Hsp70 acetylation induced by cellular stress was reported to increase its protein refolding chaperone activity. This is mediated by increased association of Hsp70 with co-chaperones assisting protein refolding such as Hsp90...
and Hop (19). In addition to co-chaperone binding, in this study, Hsp70 acetylation facilitated binding of pro-apoptotic proteins as well, implying a broad impact for Hsp70 acetylation on its overall functionality.

Autophagy, together with HSP systems, represents a major protein quality control system. To cope with stress-induced cell damage, Hsp70 maintains protein homeostasis primarily by facilitating protein refolding and prevent aggregation, while autophagy results in protein and whole-organelle degradation. However, the role of autophagy in cell death and survival has long been controversial (3,28). It has been accepted as a cell survival mechanism in response to cellular stresses like starvation. However, recent molecular approaches have provided evidence that autophagy contributes to programmed cell death (29,30). Hsp70 has been suggested to play a crucial role in autophagy regulation, although the underlying mechanisms need further investigation (18,22). This study elucidates Hsp70 acetylation as a new regulatory mechanism in autophagic induction and also adds evidence for the contribution of autophagy to programmed cell death. Furthermore, it also suggests the possible linkage between HSP and the autophagy system mediated by Hsp70 acetylation, although the precise causality in physiological signals and underlying mechanisms requires further investigation.

How Hsp70 acetylation at K77 can affect its target protein affinity is another issue that needs to be addressed. The nucleotide binding domain (NBD) of Hsp70 that contains K77 is required for Hsp70/Apaf-1 interaction, whereas the
Hsp70/AIF interaction appears to be independent of NBD (13). Previously, we suggested that K77 acetylation in NBD may induce allosteric conformational changes in other domains of Hsp70, resulting in overall changes to target protein binding. The significant location of acetylation site K77 at interdomain contacts increases the interesting possibility that acetylation may modulate the Hsp70 conformational changes important for its protein domain interactions and overall activity. Although detailed studies are needed to elucidate the exact mechanisms involved, our results provide insight into the acetylation-mediated allosteric regulation of Hsp70.

In conclusion, we have described cancer cell survival mechanisms mediated by Hsp70 acetylation under stress. The findings offer rationale for the development of an Hsp70 inhibitor that minimizes disturbance of the normal cellular function of Hsp70. Regulation of Hsp70 K77 acetylation might be helpful in treating various diseases that involve Hsp70, including cancer, inflammatory diseases and neurodegenerative diseases.

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