CHAPTER 9

INVOLVEMENT OF HEAT SHOCK PROTEINS IN PROTECTION OF TUMOR CELLS FROM GENOTOXIC STRESSES

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Abstract: Anti-apoptotic functions of heat shock proteins Hsp70 and Hsp27 are well established. However, radiation and genotoxic antineoplastic drugs at clinically relevant doses induce apoptosis mostly in lymphoid cells, while in epithelial tumors they evoke different type of response, mainly senescence and mitotic catastrophe, which leads to loss of clonogenic potential of cells. Here we review old and new data showing that upregulation of Hsp27 or Hsp70 levels protect various tumor cell lines from gamma- and UV-radiation and genotoxic anti-neoplastic drugs. Accordingly, downregulation of Hsp27 or Hsp70 levels by antisense or siRNA sensitizes tumor cells to these agents. Importantly, protection and sensitization by modulation of Hsp27 or Hsp70 levels were manifested not only by modulation of apoptosis, but by clonogenic survival as well, and recent data indicate that these Hsps can suppress also drug-induced senescence. Several studies demonstrated that intrinsic and acquired chemo- and radioresistance in tumor cell lines and in patients with certain forms of cancers can be associated with upregulation of Hsp27 and/or Hsp70. Possible mechanisms of Hsp-induced protection, in particular, modulation of p53-dependent and p53-independent DNA-damaging signaling pathways, are discussed

Keywords: Hsp27, Hsp70, radio- and chemoresistance, senescence, clonogenic survival, apoptosis, p53

INTRODUCTION

Radiation and genotoxic drugs are still the most common agents in treatment of various forms of cancer. However, despite the progress in chemo-and radiotherapy, resistance of tumor cells to the treatment is the main obstacle in cancer cure. Therefore, elucidation of mechanisms of tumor cell resistance to DNA-damaging agents will help to find new drugs or their combination. Among various endogenous factors of tumor radio-and

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S.K. Calderwood, M.Y. Sherman and D.R. Ciocca (eds.), Heat Shock Proteins in Cancer, 169–189.

chemoresistance, heat shock proteins (Hsps) apparently play an important role (See *Ciocca et al*, Chapter 2, this volume). As discussed elsewhere in this book, various tumors express higher levels of Hsps comparing to normal tissues, and overexpression of Hsps is regarded as important anti-apoptotic mechanism during tumor development. Hsps may also protect cancer cells from harmful factors of tumor microenviroment like hypoxia, or immune attack (TNF, FAS). Anti-apoptotic function of Hsps, especially Hsp70, is often considered as a major factor in chemoresistance of human cancers (See: *Brunet*, Chapter 11). However, no clear correlation was found between pro- or anti-apoptotic markers and chemo- radioresistance of most common solid tumors. Furthermore, despite numerous efforts, only a few drugs directly modifying apoptotic pathway are currently in clinical trials. Such disappointing results apparently lie in overestimation of role of apoptosis in cancer cell death upon conventional drug and radiation treatment. It is now becoming obvious that apoptosis is the major form of cell demise only in lymphoid cells, whereas in most common epithelial tumors (e.g., breast or prostate) clinically relevant doses of radiation or genotoxic drugs do not cause any apoptosis. Instead, as it was demonstrated during last years, they may cause growth arrest leading to DNA repair and cell survival, or, if DNA repair is unsuccessful, it led to premature senescence, or mitotic catastrophe (see ref (Roninson, 2003; Roninson et al., 2001; Schmitt, 2007) for review) (Figure 1). Numerous *in vitro* studies clearly demonstrated that senescence and/or mitotic catastrophe rather than apoptosis is the main cause of elimination of epithelial tumor cells (Schmitt, 2007). Although mitotic catastrophe is difficult to assess *in situ*, markers of senescence (beta-galactosidase staining) was indeed found in patients undergoing chemotherapy (Roninson, 2003; Schmitt, 2007). As a combined measure of different modes of cell death, the most reliable method is apparently clonogenic assay, since it measures ability of a cell to divide and form a colony. Therefore, this assay is basically independent on the way how

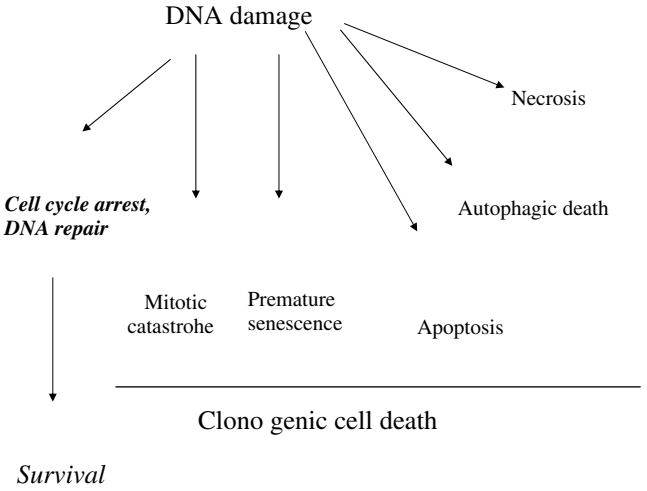

Figure 1. Responses of a cell to DNA damage (see text for explanations)

a cell is killed, e.g., by apoptosis, necrosis, autophagic cell death, mitotic catastrophe, or some unknown at present time mechanism, and senescent cell can not form colonies as well (Figure 1). It is not surprising that in many cases inhibition of apoptosis of tumor cells does not preserve their clonogenic ability since irreversibly damaged cells die by other mechanisms (see, e.g. (Zhang et al., 2006)). Invented more than five decades ago, this assay is still a gold standard for evaluation of cell sensitivity to radiation and various drugs, and there is a good correlation between clonogenic ability of cells *in vitro* and tumor response *in vivo*. In this chapter we discuss some old and new data regarding role of Hsp27 and Hsp70 in chemo- and radioresistance of tumor cells.

HSP27 AND RESISTANCE OF CELLS TO GENOTOXIC STRESSES

The first works addressing the role of Hsp27 in resistance to genotoxic drugs were performed during the early 90s (See: *Arrigo*, Chapter 4). Hout et al found that overexpression of Hsp27 conferred resistance to doxorubicin, as well as other drugs such as colchicine, vincristine, actinomycin D, hydrogen peroxide and sodium arsenite (Huot et al., 1991). The extent of doxorubicin resistance was proportional to the levels of Hsp27 in different clones. As a result of this work, Hsp27 was proposed as a determinant of clinical resistance to antineoplastic drugs. Other works from several groups have confirmed these findings and it is now clear that Hsp27 plays a role in acquisition of resistance to doxorubicin. This drug exerts its cytotoxic action via inhibition on topoisomerase II and subsequent generation of lethal double strand breaks in the DNA. Other inhibitors of topoisomerases are used in the clinics are etoposide (inhibitor of topoisomerase II) and camptothecin (inhibitor of toposiomerase I), and Hsp27 has been highlighted as a molecule conferring chemoresistance in both cases (Table 1). In addition to chemotherapeutic drugs, radiation is the most common agent in treatment of various forms of cancer, and several reports indicate that increased levels of Hsp27 can confer radioresistance to various cell types (Table 1). Below we will discuss available data as well as some new insights regarding the mechanisms of protection of Hsp27 from most studied agents, topoisomerase II inhibitors and ionizing radiation.

The cellular response to genotoxic stressors, either to die or not to die, may largely depend on the extent of the damage in the DNA. It is therefore logical to suggest that the protective role of Hsp27 could be due to an ability to reduce the genotoxic burden in the cells. This could be achieved either by decreasing the damage per se or increasing the repair capability of cells. In this sense, it was showed that thermotolerant cells accumulated less DNA damage after doxorubicin and repaired DNA aberrations more efficiently than non-thermotolerant counterparts as assessed by alkaline comet assay (Nadin et al., 2003), a method to determine overall DNA damage (single and double strand breaks etc). It is important to stress, however, that thermotolerant cells overexpress a cohort of Hsps, not only Hsp27, and therefore it is difficult to draw conclusions regarding the role of particular Hsps in DNA repair mechanisms from this experiment.

Table 1. Effects of modulation of Hsp27 levels on response to DNA damaging agents

Treatment	Cell line	Hsp27 levels modulation	Effect/assay	Reference
Doxorubicin (topoisomerase II inhibitor)	MCF-7 human breast carcinoma	Heat shock	Increase in colony forming ability in soft-agar	(Ciocca et al., 1992)
		Antisense	Decrease in colony forming ability in soft-agar	(Oesterreich et al., 1993)
	MDA-MB-231 human breast carcinoma	Heat shock	Increase in colony forming ability in soft-agar	(Ciocca et al., 1992)
		Overexpression	Increase in colony forming ability in soft-agar	(Oesterreich et al., 1993)
		Overexpression	Decrease in apoptosis (DNA laddering, cell morphology)	(Hansen et al., 1999)
	HCT116 human colon carcinoma	siRNA	Decrease in clonogenic survival, increase in senescence	O'Callaghan-Sunol et al, submitted
	Chinese hamster ovary	Overexpression	Increase in clonogenic survival	(Huot et al., 1991)
Etoposide (topoisomerase II inhibitor)	U937 human leukemia	Overexpression	Decrease in apoptosis (Hoescht staining)	(Parcellier et al., 2003)
	TF-1 human erythroleukemic cell line	siRNA	Increase in apoptosis (anexin V/PI staining)	(Schepers et al., 2005)
Camptothecin (topoisomerase I inhibitor)	Wehi-s murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT)	(Samali and Cotter, 1996)
Cisplatin(crosslinker)	HT-29 human colon carcinoma	Overexpression	Decrease in apoptosis	(Garrido et al., 1997)
	Chinese hamster ovary	Overexpression	Increase in clonogenic survival	(Fortin et al., 2000)
Ionizing radiation	L929 murine fibroblast	Overexpression	Increase in clonogenic survival; decrease in apoptosis (DNA fragmentation)	(Park et al., 2000a)

(*continued*)

Table 1. (*continued*)

Treatment	Cell line	Hsp27 levels modulation	Effect/assay	Reference
			Decrease in apoptosis	(Cho et al., 2001)
			Increase in clonogenic survival	(Yi et al., 2002)
			Increase in clonogenic survival; decrease inapoptosis (DNA fragmentation)	(Cho et al., 2002)
	Mouse RIF (radiation induced fibrosarcoma)	Overexpression	Clonogenic survival Apoptosis (chromatin condensation)	(Lee et al., 2002)
	MEF wt and Hsf1-/-	Overexpresion	Decrease in apoptosis (PARP cleavage TUNEL assay), necrosis (PI staining); increase in clonogenic survival.	(Kabakov et al., 2006)
	Jurkat T-cell leukemia cells	Overexpression	Decrease in apoptosis (DNA fragmentation and caspase activation)	(Lee et al., 2005)
	DU145 human prostate cancer	Antisense	Decrease in clonogenic survival	(Teimourian et al., 2006)
UVC	HCT116 colon carcinoma cells	siRNA	Decrease in clonogenic survival	Gabai et al, manuscript in preparation
	human embryo-derived fibroblastic cell line	Antisense	Decrease in clonogenic survival	(Wano et al., 2004)

Note: No protection by Hsp27 upregulation from gamma-radiation: (Ekedahl et al., 2003; Fortin et al., 2000)

Wano et al (Wano et al., 2004) established a clear protective role of hsp27 to UVC induced damage. They demonstrate that overexpression of Hsp27 resulted in more efficient clearance of toxic motifs –thymidine dimers and (6-4)photoproducts- in response to damage induced by UV light. Finally, Kabakov et al showed by alkaline and neutral comet assays that overexpresion of Hsp27 lead to decreased initial DNA damage upon ionizing radiation (Kabakov et al., 2006). It is important to note that whereas alkaline comet assay measures all types of breaks in the DNA, neutral comet measures only double strand breaks. In this work, the protective effect of Hsp27 was accompanied with suppression of p53 accumulation after exposure to radiation, suggesting that reduction in p53 activation may be a consequence of attenuation of its upstream activator, DNA damage (Kabakov et al., 2006).

Data in our lab (Callaghan-Sunol et al, submitted) has shown that downregulation of Hsp27 by siRNA in human colon carcinoma sensitized cells to low doses of doxorubicine. This phenomenon was associated with exacerbated senescence upon doxorubicin treatment in Hsp27-depleted cells (Figure 2A). Moreover, downregulation of Hsp27 on its own lead to appearance of senescent phenotype, indicating that Hsp27 can protect cancer cell lines from endogenous as well as drug-induced senescence. It is now well established that DNA damage can trigger the activation senescence programs, in particular, through p53/p21 pathway. We did not observe

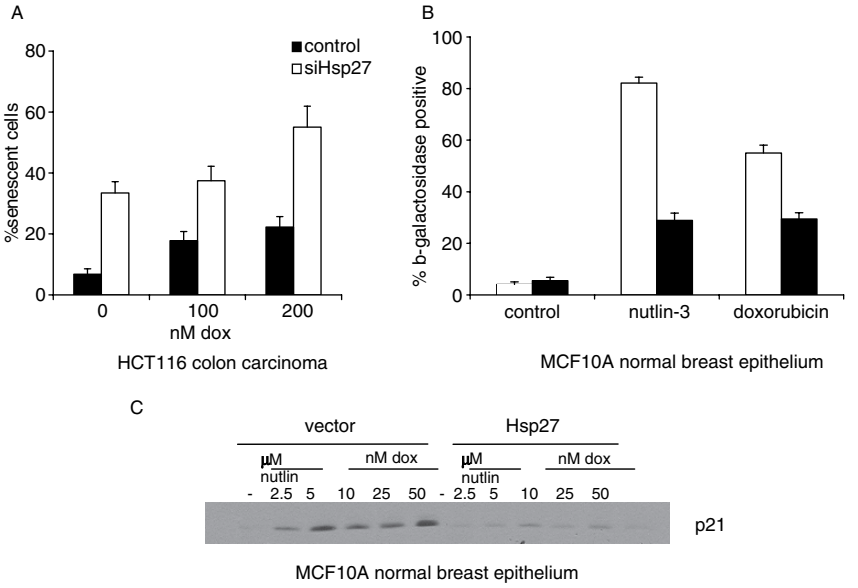

Figure 2. Hsp27 inhibits senescence induced by doxorubicin (colon carcinoma HCT116 cells and normal breast epithelial cells MCF10A) and nutlin-3 (MCF10A) by suppression of p53-p21 cascade. **A.** Percentage of senescent cells (enlarged flat cells) upon treatment with doxorubicin in control and Hsp27-depleted HCT116 cells. **B.** Percentage of senescent cells (as b-galactosidase positive cells) upon doxorubicin and nutlin-3 treatment in MCF10A control and overexpressing cells. **C.** Accumulation of p21 upon doxorubicin and nutlin-3 treatment in MCF10A control and Hsp27 overexpressing cells

either appearance of DNA damage or activation of upstream components of the DNA damage response upon depletion of Hsp27, indicating that depletion of Hsp27 per se does not lead to genotoxic stress. We did observe, however, activation of p53 and accumulation of p21, suggesting that high levels of Hsp27 in cancer cells suppress p53 activation. Moreover, we found that overexpression of Hsp27 in human normal breast epithelial cells protected cells from senescence induced by doxorubicin (Figure 2B). This effect of hsp27 was accompanied by suppression of p53 activation (accumulation of p21) upon treatment with doxorubicin (Figure 2C). Interestingly, similar effects were found when cells were treated with nutlin-3 (Figure 2B, C), a small molecule that inhibits Mdm2 and therefore activates p53 without generating DNA damage, indicating that in our cellular systems Hsp27 contributes to survival of cells by suppression DNA damage response at the level of p53, independently of DNA damage.

In addition to decrease in the genotoxic burden and suppression of p53 response, Hsp27 cellular levels have been shown to modulate several survival pathways. Here we will focus on the role of Hsp27 in the regulation of NF-κB, PKCδ and p38 and c-jun kinases and what consequences it might have for the survival of cells.

Works from two independent labs have shown that overexpression of Hsp27 results in downregulation of IκB and therefore, higher activity of the transcription factor NF-κB, a well-known pro-survival factor. According to Parcellier et al. this effect is the result of enhanced activation of the proteasomal activity in Hsp27 overexpressing cells when treated with etoposide or TNFα (Parcellier et al., 2003). Hsp27 mediates the degradation of IκB by facilitating the delivery of phosphorylated form of IκB to the proteasome when cells are treated with the above mentioned stressors, accelerating its degradation. This effect appears to be specific for Hsp27, since other chaperones that confer resistance to TNFα or etoposide, like Hsp70, do not seem to bind to phospho-IκB (Parcellier et al., 2003). In another work, Yi et al. address role of NF-κB hyperactivation by Hsp27 in cellular protection from ionizing radiation (Yi et al., 2002). They showed that overexpression of Hsp27 increased transcriptional activity of NF-κB that, in turn, lead to accumulation of MnSOD, a superoxide scavenger. Since ionizing radiation exerts its detrimental effects on cell viability partially via the generation of reactive oxygen species (ROS), it is natural to suggest that increased levels of MnSOD can contribute to the protective effects of Hsp27 (Figure 3).

PKCδ is a member of the protein kinase C family, and it is associated with suppression of cell cycle progression and activation of apoptosis. Modulation of this protein, therefore, may be crucial to dictate the fate of cells, especially lymphoid, upon treatment with genotoxic agents. In Jurkat cells, overexpression of Hsp27 lead to suppression of PKCδ activation and reduced generation of ROS upon treatment with ionizing radiation (Lee et al., 2004) (Lee et al., 2005). In another work by the same authors, reduced activation of PCKδ upon Hsp27 overexpression was attributed to decreased ROS content in murine fibroblasts, most likely due to increased levels of MnSOD (see above) in the cell. Suppressed activation of PKCδ resulted in reduced activation of ERKs, and this suppressed activation of ERKs was suggested to be responsible for protective effects of Hsp27 (Cho et al., 2001; Cho et al., 2002). Although it is generally understood that activation of ERKs leads to cell survival,

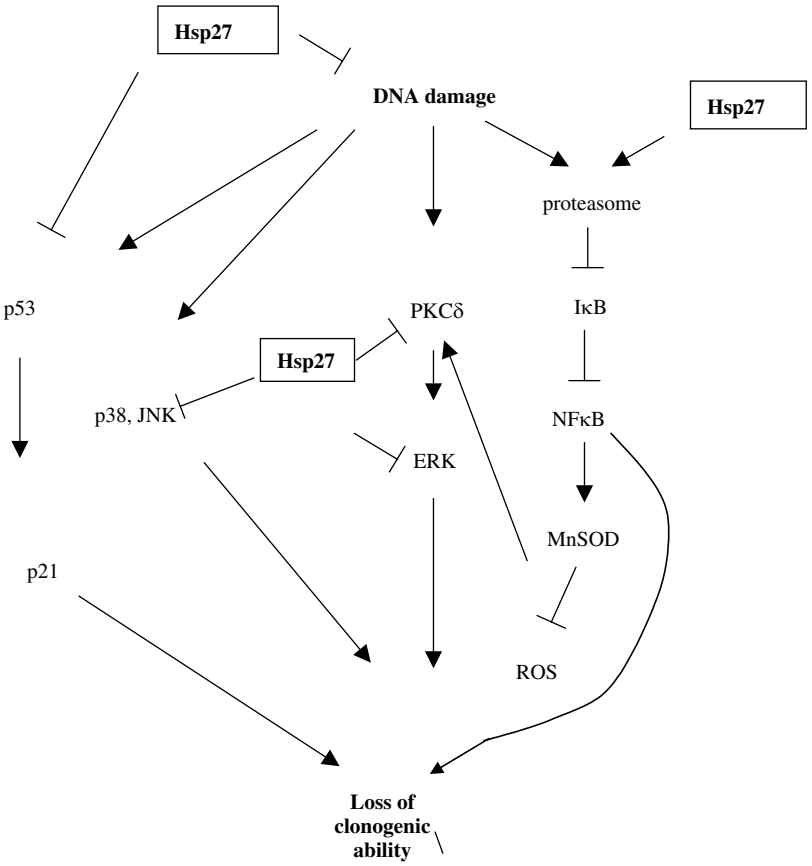

Figure 3. Suggested mechanisms of protection from genotoxic stresses by Hsp27 (see text for details)

it now accepted that in some scenarios hyperactivation of this MAP kinase cascade can be detrimental for cell viability. The ability of Hsp27 to modulate MAP kinase signaling has also been demonstrated for other two kinases in the same family: p38 and JNK. It was shown that treatment of leukemic cells with etoposide lead to apoptotic cell death characterized by cytochrome c release from mitochondria, which was preceded by activation of p38 and JNK kinases. Interestingly, cells where Hsp27 was downregulated were more sensitive to etoposide and p38 and JNK activation as well as cytochrome release from mitochondria was exacerbated (Schepers et al., 2005).

HSP70 AND RESISTANCE OF CELLS TO GENOTOXIC STRESSES

Historically, the effect of Hsps on tumor cell resistance to genotoxic stresses was first studied by radiobiologists in early 80-s when hyperthermia in combination with radiation was introduced in clinic. The main conclusion of these studies was that

thermotolerant cells (i.e. cells with high levels of Hsps) generally do not demonstrate higher radioresistance (see, e.g. (Harston-Eaton et al., 1984)). In early 90-s, however, it was shown that thermotolerant breast tumor cells do demonstrate resistance to some DNA-damaging drugs, e.g., doxorubicine, but not others (e.g. cisplatin, 5-fluorouracil) (Ciocca et al., 1992). Later, besides doxorubicine, resistance to other clinically used anti-cancer drugs such as campothecin, topotecan, etoposide, cisplatin and others was shown (see Table 2). As one can see from the Table 2, protection from various types of DNA-damaging agents (topo I and II inhibitors, crosslinkers, inhibitors of DNA synthesis, UV and gamma-radiation) was found in different normal and transformed tumor cell lines, although in some cells no protection was seen (Table 2). Of note, Hsp70 (also called Hsp72) can protects from almost the same treatments as Hsp27 (cf Table 1). Unfortunately, however, in most of these studies, especially in 90s, high doses of drugs and only short-term viability assay (apoptosis) were used. As we discussed in Introduction, apoptosis is not the main mode of epithelial tumor cell death upon low doses of drugs, so the relevance of most of these studies to clinics is unclear. But for lymphoid cells where protection from drug-induced apoptosis by Hsp70 was also seen, apoptosis usually represent the main mode of their death. Since mechanisms of anti-apoptotic function of Hsps are discussed elsewhere in this book, here we will describe only that is related to genotoxic part of apoptotic pathway.

But first lets consider data regarding effects of Hsp70 on DNA damage itself since decreased damage and/or increased repair obviously should decrease apoptosis and increase overall cell survival. In 2001 R. Bases and collegues found that Hsp70 can associate in vivo and in vitro with HAP-1 endonuclease, a key enzyme in base-excision repair (BER), and stimulates its activity (Kenny et al., 2001). Interestingly, N-terminal ATPase fragment of Hsp70, but not C-terminal substrate-binding fragment was sufficient for activity (Mendez et al., 2003). In recent studies in human leukemic cells R. Bases demonstrated that treatment with siRNA to Hsp70 inhibited repair of abasic sites and sensitized cells to gamma-radiation; however, no evidence of Hsp70 depletion in these cells was presented (Bases, 2005, 2006). In mice with knockout of major inducible Hsp70, Hsp70.1 and Hsp70.3, Hunt et al. found increased levels of spontaneous or radiation-induced chromosomal aberrations and suggested that Hsp70 may be involved in maintaining genomic stability (Hunt et al., 2004). In Hsp70.1/3 knockout fibroblast the authors also found decreased telomerase activity and less inhibition of radiation-induced of DNA-synthesis (apparently indicating impairment of cell cycle checkpoints), but mechanisms of these effect were not elucidated (Hunt et al., 2004). Recent study of Kabakov et al. demonstrated that Hsp70 may directly protect DNA from damage. In MEF expressing Hsp70, radiation-induced DNA damage (assessed by alkaline and neutral comet assays) was significantly reduced (Kabakov et al., 2006). Accordingly, in human lung carcinoma A549, Niu et al (Niu et al., 2006) found that Hsp70 overexpression decreased UVC-induced DNA damage (by alkaline comet assay). Therefore, Hsp70 may be directly involved in protection/repair of DNA, however, further studies are necessary to elucidate the mechanisms.

Table 2. Effects of modulation of Hsp70 levels on response to DNA damaging agents

Treatment	Cell line	Hsp70 level modulation	Effect/assay	References
Campothecin(topo I inhibitor)	U937 human lymphoid	Heat shock	Decrease in apoptosis (morphology, DNA ladder)	(Samali and Cotter, 1996)
	Jurkat human lymphoid	Heat shock, Overexpression	Decrease in apoptosis (Morphology, Tunel)	(Creagh and Cotter, 1999)
	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT, Tunel, DNA Ladder)	(Samali and Cotter, 1996)
Topotecan(topo I inhibitor)	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT)	(Sliutz et al., 1996)
Etoposide(topo II inhibitor)	U937 human lymphoid	Heat shock	Decrease in apoptosis (morphology, DNA ladder)	(Samali and Cotter, 1996)
	MEF	Overexpression	Decrease in apoptosis (MTT)	(Nylandsted et al., 2004)
		ADD70 binding[1]	Increase in apoptosis	(Schmitt et al., 2003)
Doxorubicin (topo II inhibitor)	HL-60	Geldanamycin treatment, overexpression	Decrease in apoptosis (PARP, chromatin condensation, MTT)	(Demidenko et al., 2005)
	MCF-7, MDA-MB231 breast carcinoma	Heat shock	Increase in clonogenic survival	(Ciocca et al., 1992)
	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT assay)	(Karlseder et al., 1996) (Jaattela et al., 1998)
	ME-180 human cervix carcinoma	Antisense	Increase in apoptosis (MTT assay)	(Jaattela et al., 1998)
	HeLa cervix, HCT116 colon carcinoma	siRNA	Decrease in clonogenic survival, increase in senescence	Gabai et al (submitted)
Cisplatin (crosslinker)	MEF, U937, MCF7, HeLa, SW480, HT29, B16	ADD70 binding[1]	Increase in apoptosis (PI, crystal violet)	(Schmitt et al., 2003)
	B16 mouse melanoma, Prob rat colon carcinoma			(Schmitt et al., 2006)

	PC-3 human prostate carcinoma	Antisense	Decrease in clonogenic survival	(Gabai et al., 2005)
Gemcitabine(DNA synthesis)	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT assay)	(Sliutz et al., 1996)
γ–radiation	3T3 mouse fibroblasts	Overexpression	Decrease in apoptosis(Hoehst staining)	(Lee et al., 2001)
	3T3 mouse fibroblasts, RIF mouse fibrosarcoma	Overexpression	Increase in clonogenic survival	(Park et al., 2000b)
	MEF	Overexpression	Increase in clonogenic survival, apoptosis (PARP cleavage, TUNEL assay), necrosis (PI staining)	(Kabakov et al., 2006)
		Hsp70 k/o; overexpression	Increase in clonogenic survival	(Hunt et al., 2004)
	PC-3 human prostate	Antisense	Decrease in clonogenic survival	(Gabai et al., 2005)
	THP-1 human leukemia	siRNA	Decrease in clonogenic survival	(Bases, 2005)
	HCT116 colon carcinoma	siRNA	Decrease in clonogenic survival, increase in senescence	Gabai et al (submitted)
UVB	Wehi murine fibrosarcoma	Overexpression	Decrease in apoptosis (MTT)	(Simon et al., 1995)
UVC	MEF	Overexpression	Decrease in apoptosis	(Steel et al., 2004)
	HCT116	siRNA	Decrease in clonogenic survival	Gabai et al, submitted

[1]ADD70 binding – neutralization of Hsp70 by expression of peptide derived from apoptosis-inducing factor (AIF)

Note: No protection from apoptosis: In MEF: Hsp70-overexpression: etoposide (Steel et al., 2004); γ-radiation (Buzzard et al., 1998) Jurkat, lung carcinoma by HS: γ-radiation (Ekedahl et al., 2003).

No sensitization to apoptosis: In lung carcinoma: by siHsp70: γ-radiation, cisplatin, etoposide (Ekedahl et al., 2003). In PC-3 cells: by antisense - to doxorubicine, UVC, etoposide (Gabai et al., 2005). In colon adenocarcinoma: by antisense - to doxorubicine (Musch et al., 2001).

Two major components are now considered as upstream mediators of DNA-damage-induced apoptosis: p53 and caspase-2 (see ref (Norbury and Zhivotovsky, 2004; Roos and Kaina, 2006) (Zhivotovsky and Orrenius, 2005) for review). Surprisingly, though, there is no data regarding effect of Hsp70 on caspase-2 activity, although Hsp70 apparently inhibits apoptosis upstream mitochondria thus suppressing caspases-9 and 3 (Steel et al., 2004). There are few publications where effect of Hsp70 on p53 was studied. Lee et al found that overexpression of Hsp70 in 3T3 cells reduced accumulation of p53 and its downstream target p21 after gamma-radiation (Lee et al., 2001). Accordingly, overexpression of Hsp70 in MEF also diminished p53 accumulation (Kabakov et al., 2006). We have recently found that modulation of p53 pathway by Hsp70 may be critical for its protective effect. In HCT116 human colon carcinoma cells decrease in Hsp70 expression by siRNA sensitized them to three diverse types of genotoxic agents: UV-radiation, gamma-radiation and doxorubicin (Gabai et al, submitted). Of note, these treatments did not cause apoptosis; instead, they provoke mainly mitotic catastrophe (under UVC radiation), senescence (upon doxorubicine treatment) or combination of both (upon gamma-radiation). Apparently, sensitizing effect of Hsp70 in these cells was dependent on p53, since it disappeared in p53 knockout derivate of this cell line. Using alkaline and neutral comet assays, we assessed DNA damage in Hsp70-depleted cells but did not find significant difference. However, Hsp70 depletion stabilized p53 upon gamma-radiation and doxorubicin and caused higher p21 accumulation (Figure 4A, B). Interestingly, Hsp70 downregulation, similar to Hsp27 downregulation (see above), in HCT116 cells and several other tumor cell lines by itself causes activation of

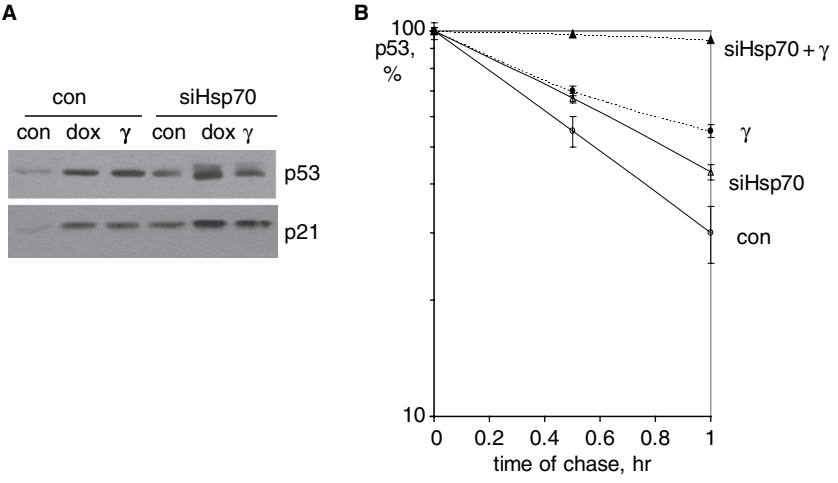

Figure 4. Downregulation of Hsp70 in HCT116 colon carcinoma activates p53/p21 pathway upon genotoxic stresses. **A**. Increase accumulation of p53 and p21 in Hsp70-depleted cells after treatment with doxorubicin (100 nM) or γ-radiation (5 Gy) after 24 h. **B**. Increased stabilization of p53 after radiation of Hsp70-depleted cells. Two hours after γ-radiation (5 Gy) cells were treated with protein synthesis inhibitor emetine (10 μM) for times indicated. Note that Hsp70 depletion itself stabilizes p53 (B) and causes p21 accumulation (A)

p53 pathway and senescence, an effect obviously independent on DNA-damage (Yaglom et al, 2007, see also chapter by M. Sherman in this book). From our and above described data from literature we suggest that Hsp70 may modulate p53 pathway thus protecting cells from apoptosis, senescence, and mitotic catastrophe.

Although p53 is the major effector of genotoxic stresses, and its modulation by Hsp70 may be critical for protection of normal and tumor cells expressing wild-type p53, Hsp70 apparently can protect lymphoid tumor cells which lack functional p53, (e.g., U937, or HL60, see Table 2). As demonstrated in HL-60 cells, Hsp70 expression inhibited etoposide-induced Bax conformation change, its translocation to mitochondria, and downstream events (release of cytochrome c and cytosol, activation of caspase-9 and caspase-3 (Guo et al., 2005). Thus, inhibition of Bax translocation by Hsp70 seems to be common effect for both genotoxic and non-genotoxic stresses (Guo et al., 2005; Ruchalski et al., 2006; Stankiewicz et al., 2005).

Activation of Bax by DNA damage may occur via p53-dependent or p53-independent pathways; in latter case, caspase-2 cleaves Bid thus promoting Bax translocation (Roos and Kaina, 2006). On the other hand, etoposide and UV radiation can cause Bax phosphorylation and translocation via JNK/p38 kinase activation (Kim et al., 2006). Interestingly, both inhibition of Bid cleavage and stress-kinase activity are well-documented effects of Hsp70 for non-genotoxic stimuli such as heat shock, or TNF (e.g., (Gabai, 1997; Gabai et al., 2000; Stankiewicz et al., 2005)). Therefore, it is tempting to speculate that in case of p53-independent apoptosis, Hsp70 exerts its protective effect by inhibiting JNK/p38 and/or Bid cleavage and Bax translocation (Figure 5). However, further research is obviously necessary to clarify this problem.

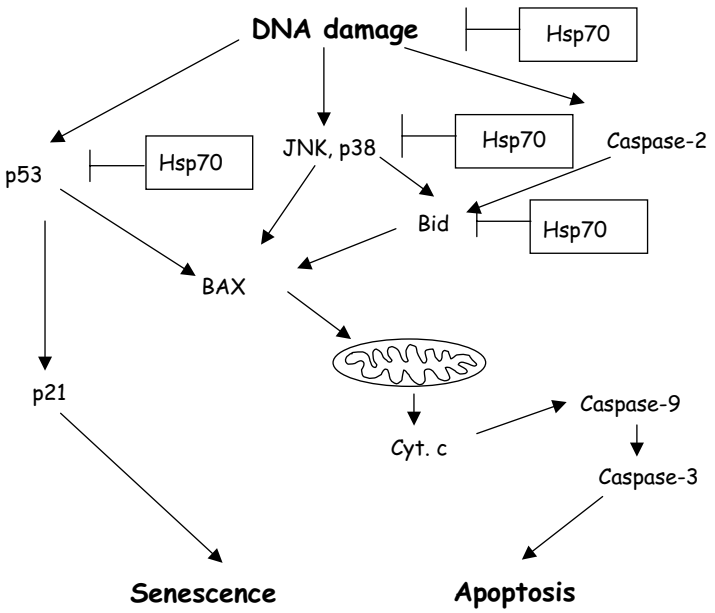

Figure 5. Suggested mechanisms of protection from genotoxic stresses by Hsp70 (see text for details)

HSP90 INHIBITION AND SENSITIZATION OF TUMOR CELLS TO RADIATION AND GENOTOXIC DRUGS

Hsp90 is regarded as a promising target for antineoplastic drug, and currently there are several clinical trials of geldanamicin analogues, a highly specific Hsp90 inhibitors, for treatment of solid tumors. In 2003, several independent groups reported that treatment of various tumor cell lines with geldanamycin or its analogue, 17-AAG, significantly enhance their radiosensitivity as assessed by clonogenic assay (Bisht et al., 2003; Russell et al., 2003) (Machida et al., 2003). Among tumor cell lines where sensitization was seen were gliomas, prostate, lung, colon and cervix carcinomas (Bisht et al., 2003; Russell et al., 2003) (Machida et al., 2003); importantly, there was no radiosensitization by 17-AAG in normal fibroblasts (Russell et al., 2003), but their transformation with E6/E7 oncogenes led to marked sensitization (Bisht et al., 2003). Furthermore, 17-AAG demonstrated significant radiosensitizing effect in vivo (with xenografts in nude mice) (Bisht et al., 2003). There are several possible signaling components affected by Hsp90 inhibition which may be responsible for radiosensitizing effect such as Her-2, Raf-1, ERK and Akt, and treatment with geldanamycin caused degradation of these components. Interestingly, in normal fibroblasts geldanamycin induced same degradation of Her-2, Raf-1 and Akt as in tumor cells, but does not cause radiosensitization (Russell et al., 2003). The authors suggested that tumor cells, in contrast to normal, are more dependent on survival pathways. However, there is another possibility. Along with degradation of components of pro-survival pathways, geldanamycin also activates heat shock response leading to accumulation of Hsp70 and Hsp27, which, as we described above, potentially can protect both normal and tumor cells from DNA-damaging agents, including radiation. Indeed, if tumor lympoid cells were treated first with geldanamycin and then with doxorubicin, they demonstrate resistance to apoptosis, apparently due to Hsp70 accumulation (Demidenko et al., 2005) (Robles et al., 2006), whereas treatment with doxorubicin first and then with geldanamycin had significant synergistic effect (Robles et al., 2006). Since normal cells usually express lower levels of Hsps than carcinoma cells, geldanamycin may induce marked increase of Hsp70/27 in these cells, which may compensate for sensitizing effect of geldanamycin-induced degradation of signaling components. In most carcinoma cells, however, increase in Hsps levels upon geldanamycin treatment may not be significant, and the sensitizing effect prevails. Anyway, for better radio- and chemo-sensitization of tumor cells Hsp90 inhibitors which do not activate heat shock response seems preferable. Of note, both radio-and chemosensitizing effects of geldanamycin were apparently independent on p53 status (Russell et al., 2003) (Robles et al., 2006).

HEAT SHOCK PROTEINS AND CHEMO-RADIORESISTANCE IN CANCER PATIENTS

Intrinsic and acquired chemo- and radioresistance are the major obstacles for effective treatment of cancer. As described above, numerous in vitro studies indicate

that modulation of Hsp27 and Hsp70 levels in various tumor cell cultures affects their sensitivity to radiation and other DNA-damaging agents (Tables 1, 2). The question arises, however, whether expression of Hsps in real human cancers correlates with their chemo- and radiosensitivity. But first lets consider some data indicating that acquired chemoresistance in vitro can be associated with overexpression of Hsps.

This is important since DNA-damaging drugs, in contrast to heat shock, proteasome inhibitors, or geldanamycin, do not usually activate HSF-1 and induce Hsps. Furthermore, several mechanisms of acquired chemoresistance are described; the most common is associated with expression of transporters (MDRs) which facilitates drug efflux. Despite these reservations, however, several studies clearly demonstrate that selection of human tumor cells in vitro with increasing concentrations of chemotherapeutic drugs leads to accumulation of several Hsps. For instance, selection of head and neck carcinoma cell line to cisplatin leads to increased levels of heat shock cognate protein Hsc70 (Johnsson et al., 2000). Accordingly, the same protein was overexpressed in cisplatin-resistant cervix carcinoma (Annalisa Castagna, 2004), or fibrosarcoma with pleotropic resistance (for doxorubicine, etoposide etc) (Davidovich and Roninson, 2000). HCW-2 derivative of HL-60 promyelocytic leukemia cells resistant to daunorubicin, radiation and other treatments demonstrated 5-fold increase in levels of inducible Hsp70 (Salvioli et al., 2003). In melanoma cell line selected for etoposide and cisplatin resistance, overexpression of both Hsp70 and Hsp27 was found (Pranav Sinha, 2003). In radiosensitive subclone of bladder carcinoma, downregulation of Hsp27 was found (Kassem et al., 2002), while radioresistant, but not radiosensitive glioma cells accumulated Hsp70 after radiation (Brondani Da Rocha et al., 2004). Finally, resistance of breast cancer cells to doxorubicine was associated with accumulation of Hsp27 (Liu et al., 2006), while Hsp27-related protein aB-crystalin was overexpressed in melanoma resistant to cisplatin and etoposide (Wittig et al., 2002). Thus, emergence of tumor cells resistant to DNA-damaging drugs can be associated with expression of Hsp70/Hsc70 and/or Hsp27. Although the mechanism by which chemoresistant tumor cells overexpress Hsps has not been elucidated, it may not involve overexpression of HSF-1, at least in some cases. Indeed, expression of HSF-1 did not increase levels of Hsps in PC-3 human prostate carcinoma (Hoang et al., 2000) or U2OS human osteosarcoma cells (Tchenio et al., 2006), while knockout of HSF-1 did not decreased Hsp levels in several tumor cell lines, except HCT116 colon carcinoma (Zaarur, 2006) (Gabai et al, unpublished data). Since promoters of Hsp genes contain many regulatory elements, it is not easy to evaluate which of these elements are responsible for Hsp accumulation during acquired chemoresistance. Among possible candidates is, for instance, CCAAT box located in Hsp70 promoter and activated by p53 analogue, DNp63a; interestingly, wt p53 antagonizes activity of DNp63a and suppress Hsp70 (Wu et al., 2005). Furthermore, topotecan (inhibitor of Topo I, see Table 2) can induce Hsp70 expression in p53 knockout, but not parental HCT116 colon carcinoma, which represents a potential mechanism for Hsp70 activation by genotoxic drugs (Daoud et al., 2003).

There are several clinical studies indicating that Hsp expression in tumors can be associated with resistance to chemo-radiotherapy (see also ref (Ciocca and Calderwood, 2005) for review). In 1993 Ciocca et al found that in breast cancer patients receiving adjuvant chemotherapy, Hsp70 expression was the only independent factor of disease recurrence (Ciocca et al., 1993). In breast cancer patients treated with radiotherapy with or without hyperthermia, expression of Hsp70 after treatment correlated with low probability to attain a complete response (Liu et al., 1996). In locally advanced breast cancer treated with induction chemotherapy, Vargas-Rois observed that high nuclear expression of Hsc70/Hsp70 and total expression of Hsp27 correlated with a shorter disease-free survival (Vargas-Roig et al., 1998). In ovarian tumors resistant to cisplatin or chlorambucil, levels of Hsp27 (by ELISA) were around four-times higher than in sensitive tumors (Langdon et al., 1995). However, in another study, response of ovary carcinomas to chemotherapy did not correlate with levels of Hsp27 (assessed by immunostaining), although patients with Hsp27-negative tumors of stage III-IV had much better progression-free and overall survival (Henriette J.G., Arts, 1999). Local control after radiation in head and neck squamous cell carcinoma was independent on Hsp27 levels (Fortin et al., 2000). However, in patients with esophageal squamous cell carcinoma, better overall survival after radio-and chemotherapy correlates with lower expression of Hsp27 and Hsp70; at the same time, no correlation with modulators of apoptosis, bax and bcl-2, was found (Miyazaki et al., 2005) . Interestingly, better prognosis also correlates with a high expression of p21, demonstrating, obviously, that p53/p21 pathway was activated in the patients with lower expression of Hsp27 and Hsp70. These clinical data are in concordance with in vitro studies indicating that downregulation of Hsp27 or Hsp70 can activate p53/p21 pathway (see above).

CONCLUSION

Available data indicate that both Hsp27 and Hsp70, besides being molecular chaperones, are also implicated in protection of tumor cells from DNA-damaging antineoplastic agents. Most studies demonstrated that artificial modulation of their levels affects cell's sensitivity to radiation and diverse drugs, including inhibitors of topoisomerases I and II, cross-linkers and some others. Besides protection from apoptosis, higher levels of Hsp27 or Hsp70 can also increase clonogenic ability of cells, apparently decreasing senescence and mitotic catastrophe. There are several possible mechanisms of Hsp-mediated protection from DNA damage; they include modulation of p53-dependent pathways (p21, Bax) and p53-independent pathways (stress kinases JNK and p38, ERK, Bid, NF-kB), but their significance needs further elucidation. Intrinsic and acquired resistance to DNA-damaging agents in various tumor cells lines can be associated with overexpression of Hsp27 and/or Hsp70. In patients with certain forms of cancers treated with chemo- or radiotherapy, higher levels of Hsp27 and/or Hsp70 expression correlated with resistance to therapy and worse prognosis.

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