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Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy A comprehensive review

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Abstract

Heat shock proteins (Hsp) form the most ancient defense system in all living organisms on earth. These proteins act as molecular chaperones by helping in the refolding of misfolded proteins and assisting in their elimination if they become irreversibly damaged. Hsp interact with a number of cellular systems and form efficient cytoprotective mechanisms. However, in some cases, wherein it is better if the cell dies, there is no reason for any further defense. Programmed cell death is a widely conserved general phenomenon helping in many processes involving the reconstruction of multicellular organisms, as well as in the elimination of old or damaged cells. Here, we review some novel elements of the apoptotic process, such as its interrelationship with cellular senescence and necrosis, as well as bacterial apoptosis. We also give a survey of the most important elements of the apoptotic machinery and show the various modes of how Hsp interact with the apoptotic events in detail. We review caspase-independent apoptotic pathways and anoikis as well. Finally, we show the emerging variety of pharmacological interventions inhibiting or, just conversely, inducing Hsp and review the emergence of Hsp as novel therapeutic targets in anticancer protocols.

Keywords: Apoptosis; Cancer; Heat shock proteins; Molecular chaperones; Necrosis; Tumor suppression

Abbreviations: 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; AIF, apoptosis-inducing factor; Ask1, apoptosis signal-regulating kinase-1; BAD, Bcl-2-associated death protein; BAG, Bcl-2-associated athanogene protein; Bax, B-cell lymphoma-2 protein (Bcl-2)-associated X protein; Bcl-2, B-cell lymphoma-2 protein; CAD, caspase-activated deoxyribonuclease; DNase, deoxyribonuclease; ER, endoplasmic reticulum; FADD, Fas-associated death domain protein; Grp, glucose-regulated protein; Hip, Hsc70-interacting protein; Hop, Hsp70-Hsp90 organizing protein; HSF, heat shock factor; Hsp, heat shock protein; IAP, inhibitors of apoptosis proteins; JNK, c-Jun N-terminal kinase; MMPT, matrix metalloprotease; Mn-SOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; NOS, nitric oxide synthase; p23, 23-kDa co-chaperone of Hsp90; PARP, poly-ADP ribose polymerase; PCD, programmed cell death; PI-3-kinase, phosphatidyl-inositol-3-kinase; PS, phosphatidylserine; PTP, permeability transition pore; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; Smac/DIABLO, second mitochondria-derived activator of caspases; TNF, tumor necrosis factor.

Contents

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1. Introduction

Programmed cell death (PCD) is a common phenomenon in developmental processes or in normal physiological conditions, where the old or damaged cells have to be eliminated. Two distinct forms of cell death, apoptosis and necrosis, have been characterized. Apoptosis is induced by an array of extra- or intracellular stimuli. Organisms are equipped with their own physiological defense to cope with environmental stress and are under the control of genetic machinery in order to prevent or induce cell death depending upon the severity of the stress. Heat shock proteins (Hsp) are highly conserved and play a major role in cytoprotection. Apoptosis resistance is associated with the high expression of Hsp, hence, the present discussion is focused on the functions of Hsp in apoptosis regulation. We also review the pharmacological applications using Hsp inhibitors to induce apoptosis in various tumor models.

1.1. Heat shock proteins: molecular chaperones

The eukaryotic stress response is highly conserved and involves the induction of Hsp. Hsp are detected in all living cells. Both in vivo and in vitro studies have shown that various stressors transiently increase the production of Hsp as protection against harmful insults. Hsp induction was first identified in the salivary glands of *Drosophila melanogaster* upon application of heat shock (Ritossa, 1962). The unique nature of Hsp synthesis was correlated with the acquisition of thermotolerance and cytoprotection. Later, the interest on Hsp tremendously expanded as more and more functions of normal resting cells involving Hsp were uncovered. The multifunctional roles of Hsp in cells show that

Hsp is one of the major regulatory proteins in the cell, and vital functions of cell, such as the maintenance of the cell cycle, are associated with Hsp. The role of Hsp in cell proliferation was reviewed (Pechan, 1991; Helmbrecht et al., 2000).

In mammalian cells, the stress response involves the induction of 5 major classes of Hsp families, namely, the small Hsp exemplified by Hsp27, Hsp60, Hsp70, Hsp90, and Hsp104 (Table 1; Lindquist & Craig, 1988; Craig et al., 1994; Morimoto et al., 1994; Scharf et al., 1998). Hsp synthesis is tightly regulated at the transcriptional level by heat shock factors (HSF). Although various HSF were reported, HSF-1 was shown to be the main regulator of the short-term induction of Hsp. In resting cells, HSF-1 is a monomer; however, active HSF-1 exists as a trimer and binds to the heat shock elements (the consensus DNA sequence for heat shock factor binding; Morimoto et al., 1992). In addition, some members of the HSF family help the long-term induction of Hsp or exhibit important roles in the regulation of gene expression and developmental processes (Morimoto, 1998).

Hsp function as molecular chaperones in regulating cellular homeostasis and promoting cell survival (Hartl, 1996; Bukau & Horwich, 1998). Various studies demonstrate that Hsp-induced cytoprotection can be attributed partly to the suppression of apoptosis (Samali & Orrenius, 1998), but the precise mechanism of these effects remains to be elucidated. As an additional evidence showing the tight connection between cytoprotection and resistance to apoptosis, cells failing to respond to stress are sensitive to induced cell death via apoptosis (Sreedhar et al., 1999).

Table 1
Major heat shock proteins

Hsp	Co-chaperones or isoforms	Expression	Localization
Hsp27	various isoforms	constitutive/ inducible	cytoplasm/nucleus
Hsp60	Hsp60/Hsp10-cyt	constitutive/ inducible	cytoplasm
	Hsp60/Hsp10-mito	constitutive	mitochondria
Hsp70	Hsc70	constitutive/ inducible	cytoplasm/nucleus
	Hsp70.1	constitutive/ inducible	cytoplasm
	Hsp70.2	constitutive	cytoplasm
	Hsp70.3	constitutive	cytoplasm
	Hsp70	constitutive/ inducible	mitochondria
	Grp75	constitutive	ER/cytoplasm
	Grp78	constitutive/ inducible	ER/cytoplasm/ nucleus
	Hsp105	constitutive	cytoplasm/nucleus
Hsp90	Hsp90-α	constitutive/ inducible	cytoplasm/nucleus
	Hsp90-β	constitutive/ inducible	cytoplasm/nucleus
	Hsp90-N	constitutive/ inducible	cytoplasm/nucleus
	Hsp75/TRAP-1	constitutive/ inducible	mitochondria

Only the major Hsp families were included. Hsp27 collectively refers to various members of the relatively diverse family of the small Hsp. The most important member of the fifth major Hsp family, Hsp104, is expressed only in yeast, and therefore, it is not included in the table.

1.2. Cellular senescence, apoptosis, and necrosis: chaperone overload as a potential regulator

Cells typically die either by apoptosis or necrosis. These two forms of cell death are probably much closer to each other than previously thought (Proskuryakov et al., 2003). Both necrosis (where the cell membrane looses its integrity and the cell content is released causing an inflammatory response) and apoptosis (where the cell content remains "well-packed" in the apoptotic bodies and inflammation does not occur) (1) can be caused by the same pathophysiological exposures; (2) can be prevented by antiapoptotic mechanisms; and (3) can be transformed from one form to the other.

Several cell types exhibit only a limited number of replications in cell culture. Morphological and functional properties change until the cell reaches a nondividing—senescent—state (Hayflick & Moorhead, 1961; Smith & Pereira-Smith, 1996). Senescent fibroblasts are resistant to PCD (Wang, 1995), are unable to undergo p53-dependent apoptosis, and are shifted to necrosis upon DNA damage (Seluanov et al., 2001). However, apoptosis resistance is not a general feature of senescent cells, which may also be apoptosis-prone depending on the cell type and apoptotic stimuli (Zhang et al., 2002). Senescent fibroblasts promote carcinogenesis of neighboring cells by secreting tumorigenic factors (Krtolica et al., 2001). Therefore, the accumulation of senescent cells may contribute to the age-dependent dramatic increase of cancer incidences.

As we discussed in Section 1.1, Hsp-induced cytoprotection can rescue cells from apoptosis. However, the protein folding capacity of Hsp may be exhausted due to a massive stress resulting in protein misfolding and aggregation, as well as during aging, where oxidized proteins accumulate, or in any chronic disease. This chaperone overload leads to gross disturbances in cellular life (Csermely, 2001a), and increasing severity of the malfunction in protein folding assistance may lead to cell senescence, apoptosis, or necrosis, respectively. Thus, Hsp may not only constitute the most ancient defense mechanism of our cells, but also behave as direct sensors of their functional competence. Various levels of chaperone overload may have an important contribution to the signals directing the cell to senescence, apoptosis, or necrosis (Soti et al., 2003a).

1.3. Evolution of apoptosis: bacteria and eukaryotes

Apoptosis is a universal phenomenon. In principle, this "altruistic" form of cell death ensuring the survival of the

whole organism by sacrificing some of its cells was considered to help multicellular organisms. However, bacteria, yeast, and unicellular protozoa also develop various forms of PCD, which is required in certain developmental processes (such as lysis of the mother cell in sporulation) or helps the survival of the whole colony upon environmental stress, like nutrient starving (Lewis, 2000). The protective role of Hsp is acting against apoptosis here as well: their induction effectively suppressed the autolysis of *Escherichia coli* (Powell & Young, 1991).

Phylogenetic analysis reveals a specific affinity of several eukaryotic proteins involved in apoptosis (such as metacaspases or the OMI/Htra2 protease), with homologues from α-proteobacteria, suggesting a mitochondrial origin of the respective genes. Exploration of the bacterial homologues of many apoptotic proteins, such as caspases, shows a greater diversity than seen in eukaryotes inferring a horizontal gene transfer of the respective genes from bacteria to eukaryotes. Considering these results, a double acquisition of most proteins involved in apoptotic machinery can be hypothesized as having the first event with the domestication of the promitochondrial endosymbiont and the second at the stage of the primitive multicellular eukaryote (Koonin & Aravind, 2002).

1.4. Major elements of the mechanism of apoptosis

Apoptosis (Greek word meaning *falling off*) is an energy-dependent, ubiquitous physiological process genetically controlled by the expression of evolutionarily conserved genes, which either mediate or suppress the process of cell death. Apoptosis is well characterized by distinct morphological and physiological changes. The morphological changes include nuclear condensation, cell shrinkage, and membrane blebbing, whereas the physiological changes are fragmentation of nuclear DNA due to activation of specific endonucleases cleaving nuclear DNA into 80–200 oligonucleosomal fragments and the activation of caspases, resulting in partially digested proteolytic protein products (Table 2; Kerr et al., 1972; Evan & Littlewood, 1998; Lawen, 2003; Shivapurkar et al., 2003). Apoptosis is a highly regulated process, and it mainly responds to the initial stimulus followed by a cascade of events, hence, it can be divided into 3 phases: (1) the initiation phase (or *signaling phase*), which involves the activation of surface death receptors (mainly the tumor necrosis factor [TNF] family members), the mitochondrial pathway or the initiation of apoptosis by other stimuli (e.g., those affecting the endoplasmic reticulum [ER]); (2) the signal transduction phase (or *preparation phase*), where activation of initiator caspases and certain kinases/phosphatases takes place; followed by (3) the execution phase (or *death phase*), which involves the activation of effector caspases (Thornberry & Lazebnik, 1998). Therapeutic approaches to modulate apoptosis target the cross-roads of major signaling pathways (Dickson, 1998). Hsp are ideal targets, since they both contribute to the pathways themselves and act as chaperones for key molecules in apoptosis.

Table 2
Most important members of the apoptotic machinery

A. Signaling phase	
Death receptors	TNF-α, Fas (Apo1/CD95), FADD
Physiological inducers	ROS, Ca^{2+}, JNK/SAPK activation
Protease activators	granzymes, calpains, cathepsins, proteasome
B. Preparation phase	
Initiator caspases	caspase-8, caspase-9, caspase-10, caspase-12
Physiological inducers	Bax, ROS, MMPT, cytochrome *c*, apoptosome
Nucleases	AIF, endonuclease G, PARP
C. Execution phase	
Effector caspases	caspase-3, -6, and -7
Physiological changes	membrane blebbing, apoptotic body formation, DNA fragmentation

Only the key molecules of the apoptotic machinery were included. For the full names of the abbreviations, see the list of abbreviations.

2. Heat shock proteins and caspase-dependent apoptosis

Hsp have an extremely complex role in the regulation of apoptosis (Fig. 1). At first glance, due to their cytoprotective role, they inhibit the apoptotic response. We will give many exciting examples of the molecular mechanisms of how Hsp inhibit key steps in the apoptotic cascade and how Hsp fight to maintain the physiological homeostasis of the cell, which is an important requirement for cell survival (Wei et al., 1994; Punyiczki & Fesus, 1998; Samali & Orrenius, 1998; Jolly & Morimoto, 2000). However, there are many elements of the apoptotic machinery, where Hsp either serve as chaperones of a key signaling protein or directly promote apoptosis. These signaling elements, where Hsp "chaperone the death," will be also reviewed.

2.1. Sites of initial signaling events

2.1.1. Plasma membrane

The major players of the initiation phase of the apoptotic events at the plasma membrane are the death receptors, like the TNF or the Fas (Apo-1/CD95) receptor families. Binding of the appropriate ligands to TNF receptors activates various TNF receptor-associated factors, leading to the formation of an early complex, which is responsible for the activation of nuclear factor-κB (NF-κB) and a consequent cell survival. The second complex contains caspase-8 and leads to the mobilization of the apoptotic machinery. In case NF-κB activation has been achieved, the second complex will recruit the caspase-8 inhibitor FLIP protein and the apoptosis is blocked (Rothe et al., 1994, 1995; Rathmell & Thompson, 1999; Arch & Thompson, 1998; Ashkenazi & Dixit, 1998; Micheau & Tschopp, 2003).

Due to the pleiotropic role of Hsp70 to cellular life, its contribution to cell surface receptor-mediated apoptosis is

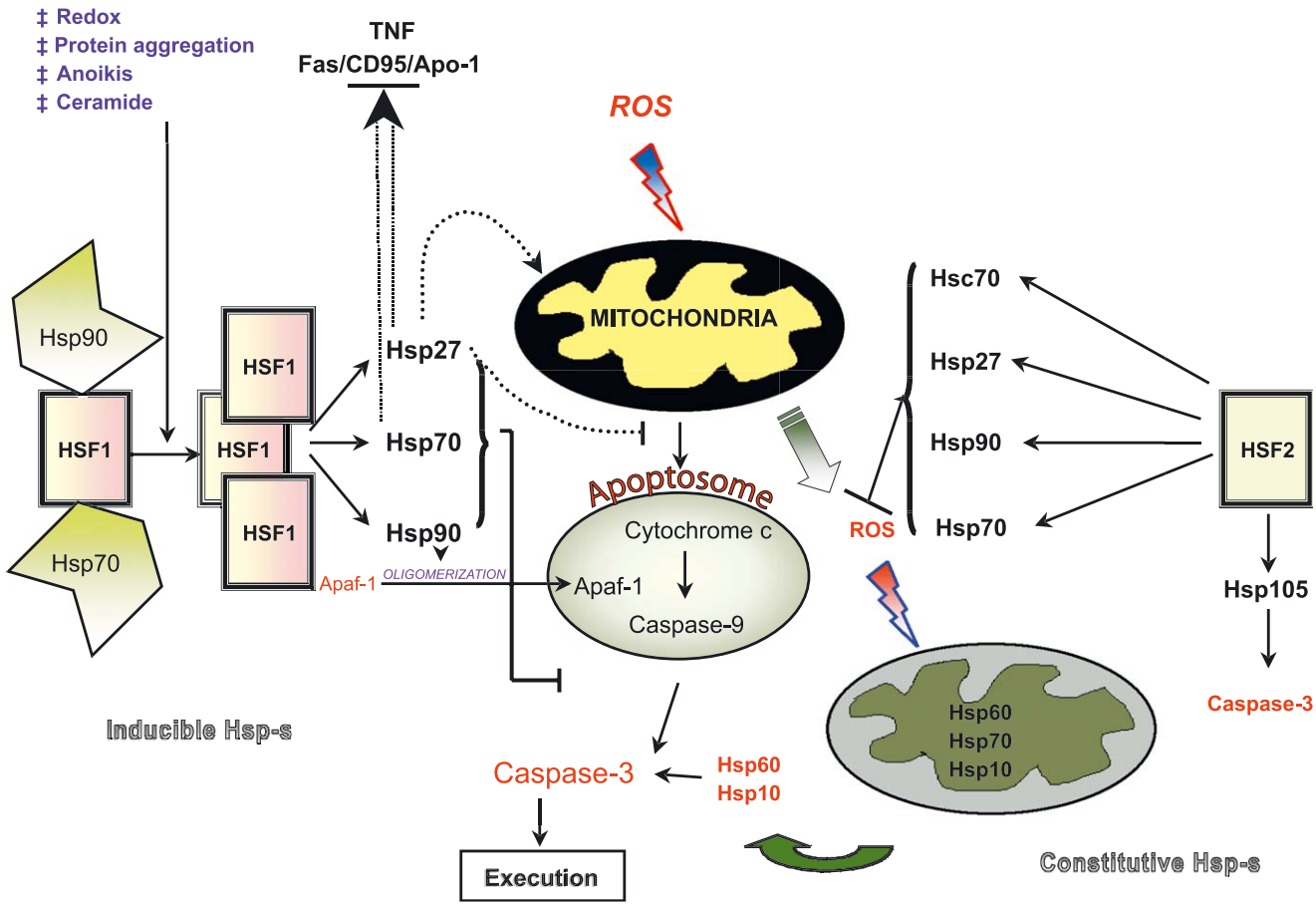

Fig. 1. Hsp in the regulation of the major events in apoptosis. Various forms of stress induce Hsp and may also trigger the induction of apoptosis. Members of 3 of the major Hsp families (Hsp27, Hsp70, and Hsp90) inhibit apoptosis by inhibiting caspase-3. Hsp are also interfering with oxidative stress. Hsp90 is inhibiting the oligomerization of the Apaf-1 complex, while Hsp27 and Hsp70 inhibit the signaling pathway from surface receptors, such as the TNF-α or Fas receptors. On the other hand, release of mitochondrial Hsp60/Hsp10 helps the activation of caspase-3 and Hsp90 is required for TNF-α signaling.

difficult to assess in gross terms. Hsp70 overexpression reduced Fas-induced apoptosis (Schett et al., 1999). Contrary to this, vector-mediated Hsp70 overexpression failed to protect Jurkat cells from apoptosis, compared with heat up-regulated Hsp70 (Mosser et al., 1997). In addition, overexpression of Hsp70 showed enhancement of Fas-mediated apoptosis in Jurkat cells (Liossis et al., 1997). Besides showing the action of Hsp70 at various points preventing or promoting apoptosis, these results may also reflect the varying initial levels of Hsp70, as well as other chaperones in the different systems, which may have set the Hsp70 requirement for cytoprotection at different levels.

We know more about the specific role of Hsp90 in the membrane-associated initial events. In gross terms, inhibition of Hsp90 by antisense oligonucleotides abolished the heat shock-induced protection of neuroblastoma cells from apoptosis, which could not be achieved using antisense Hsp70 or Hsp27 (Lee et al., 2001). Similarly, inhibition of Hsp90 resulted in apoptosis, contributing to the recruitment of death domain proteins to their respective receptors (vanden Berghe et al., 2003). However, Hsp90 is an important factor in helping the propagation of the apoptotic signal from the plasma membrane (Galea-Lauri et al., 1996). Hsp90 is necessary for the activity of the death domain kinase and the receptor interacting protein, which sensitize cells to TNF-induced cell death (Lewis et al., 2000; Chen et al., 2002a). Additionally, an isoform of Hsp90, Hsp75/TRAP-1, is interacting with the TNF receptor and probably promoting TNF signaling (Song et al., 1995).

In the late execution phase, apoptosis is characterized by marked changes in cell morphology, which include membrane blebbing and exposure of phosphatidylserine (PS) on the membrane (Martin et al., 1995). The acto-myosin system has been proposed to be the source of contractile force that drives bleb formation (Coleman et al., 2001). Most of the Hsp are associated with the acto-myosin complex either directly or through actin-binding proteins (Clark & Brown, 1986; Koyasu et al., 1986; Margulis & Welsh, 1991; Kellermayer & Csermely, 1995). In support of the role of Hsp in bleb formation, the blebs were delimited by an Hsp27-containing F-actin ring, suggesting F-actin reorganization, where Hsp27 acts as a cap-binding protein (Huot et al., 1998).

Another late apoptotic event, the exposure of PS on the outer layer of the plasma membrane, is due to the loss of

plasma membrane phospholipid asymmetry (Martin et al., 1995). The role of Hsp in PS externalization is not known but external PS can be recognized by the immune system where Grp94, a homologue of Hsp90, helps the recognition (Schild & Rammensee, 2000).

In several cases, Hsp translocate to the cell surface after the initiation of apoptosis, making the cells more vulnerable to immune lysis (Feng et al., 2002; Sapozhnikov et al., 2002). There is a correlation between surface expression of Fas and Hsp70 protein during heat-induced apoptosis in rat histiocytoma cells (Sreedhar et al., 2000).

2.1.2. Cytosol

In the cytosol, stress kinases are important elements of the signal transduction pathway in inducing and or modulating the apoptotic response. Among the mitogen-activated protein kinases, the activation of the signal-regulated protein kinase (ERK1/2) is associated with a mitogenic stimulus, whereas the c-Jun N-terminal kinase (JNK) and the p38 kinases are stress responsive.

The small Hsp, Hsp27, is phosphorylated in vivo by a protein kinase termed MAPKAP kinase-2, which is activated by p38 kinase-induced phosphorylation. Charette et al. (2000) demonstrated that the phosphorylated dimers of Hsp27 interact with Daxx, a protein that contains a death domain, specifically binds to Fas, and through the activation of the JNK kinase, enhances Fas-mediated apoptosis. Binding of the phosphorylated form of Hsp27 to Daxx prevents Daxx interaction with the apoptosis signal-regulating kinase-1 (Ask1), a serine/threonine kinase, thereby inhibiting the Fas-mediated apoptotic pathway.

Hsp70 has a rather general inhibitory role in stress kinase pathways. Hsp72 acts as a direct inhibitor of Ask1: its physical interaction with the kinase was demonstrated in in vitro binding assays using Ni+-NTA-agarose beads. Hsp72 antisense oligonucleotides prevent the inhibitory effects of Hsp72 in H_2O_2-induced Ask1 activation and consequent apoptosis of NIH3T3 cells (Park et al., 2002). Hsp72 also interacts directly with the peptide-binding domain of JNK (Park et al., 2001; Gabai et al., 2002). Using the antisense RNA approach, it was found that accumulation of Hsp72 is necessary for JNK down-regulation. However, mutant Hsp70 was able to inhibit JNK activation but not apoptosis, suggesting that the chaperone activity of Hsp70 is required for the inhibition of apoptosis but not for JNK inhibition (Mosser et al., 2000). Hsp70-induced JNK inhibition regulates the Bid-dependent apoptotic pathway (Gabai et al., 2002). Constitutive expression of another member of the Hsp70 family, Hsp105a, also protected cells from stress-induced apoptosis through JNK inhibition in neuronal PC12 cells (Hatayama et al., 2001), although the molecular mechanism behind this phenomenon is not known, and overexpression of the same protein enhanced oxidative stress-mediated apoptosis in mouse embryonal F9 cells (Yamagishi et al., 2002). Similar to the inhibitory effect of Hsp72 on the JNK kinase, addition of purified recombinant Hsp72 to a crude cell lysate reduced p38 kinase activation, while depletion of the whole family of Hsp70 proteins with a monoclonal antibody enhanced p38 activation (Gabai et al., 1997, 2000).

The role of Hsp90 in stress kinase regulation is not known in such details like that of Hsp27 or Hsp70. However, Hsp90 is necessary for the folding and activation competence of a large number of kinases (Pratt & Toft, 2003). Dissociation of the Hsp90-Raf1 complex results in apoptosis in mast cells (Cissel & Beaven, 2000) and in B-lymphocytes (Piatelli et al., 2002), where the disruption of the mitogen-activated protein kinase cascade is accompanied with the activation of JNK in the dexamethasone-induced mast cell apoptosis model (Cissel & Beaven, 2000). The down-regulation of Raf can also be induced by the sequestration of its activator, B-cell lymphoma-2 protein (Bcl-2)-associated athanogene protein-1 (BAG-1), by Hsp70 after various forms of stress (Song et al., 2001).

2.1.3. Nucleus

Nucleosomal fragmentation of DNA is a biochemical signature of apoptosis resulting from the activation of specific endonuclease activation resulting in the cleavage of the chromatin to shorter DNA fragments called oligonucleosomal DNA fragments (Compton, 1992). However, DNA damage may also be an early signaling event of the apoptotic cascade as well. In a few cell types, the early phase of apoptosis is associated with the appearance of high molecular weight DNA fragments suggesting a large-scale reorganization of chromatin. Formation of high molecular weight DNA fragments in cerebellar granule neurons accompanies both caspase-dependent and -independent types of cell death, indicative of multiple mechanisms in the regulation of excision of DNA loop domains during neuronal cell death (Bezvenyuk et al., 2000). As another early nucleus-dependent event, histone H1.2, has been recently shown to translocate to the cytoplasm in response to DNA damage and promote the release of cytochrome *c* by activating the Bcl-2 protein, Bak (Konishi et al., 2003).

Hsp play a major role in protecting the cells from DNA damage induced by various damaging agents (Samali & Orrenius, 1998). Most Hsp translocate to the nucleus after stress, and members of Hsp27 and Hsp70 families have a protective role against oxidative stress as we will describe in Sections 2.1.4 and 6.3.1. These features pose Hsp as elements of an efficient mechanism to protect the integrity of DNA. Indeed, overexpression of Hsp25 was shown to reduce oxidative DNA damage after TNF-α treatment (Park et al., 1998). One of the major hydrophilic by-products of lipid peroxidation, *trans*-4-hydroxy-2-nonenal, binds to DNA, resulting in the formation of exocyclic guanosine adducts and acts as a mutagen (Hu et al., 2002). Appearance of Hsp72 in cell nucleus was seen in dimethylarsinic acid-treated human alveolar L-132 cells, where nuclear Hsp72 suppresses the appearance of apoptosis after DNA damage

(Kato et al., 1999). A smaller fraction of Hsp90 also translocates to the nucleus upon stress (Csermely et al., 1995), and Hsp90 tightly interacts with histones (Schnaider et al., 1999), inducing a condensed state of the chromatin (Csermely et al., 1994). However, a role of Hsp90 in the protection of DNA against oxidative or other forms of damage has not been shown yet.

A rather specific form of DNA damage occurs with telomere shortening. At a critical length of the telomere regions at the end of the chromosomes around 7 kb, cells go to the state of cellular senescence, which may further proceed to apoptosis. Telomere regions are synthesized/maintained by the telomerase enzyme. Hsp90 directly interacts with telomerase and is necessary to achieve its enzyme activity (Holt et al., 1999). Consequently, Hsp90 was reported to increase telomerase activity in prostate carcinomas (Akalin et al., 2001). Moreover, Hsp90 is an essential component for the activation of the Akt kinase, which is activates the telomerase and thus acts against apoptosis (Haendeler et al., 2003).

2.1.4. Mitochondria and reactive oxygen species

Although initial studies suggested that organelles like mitochondria, ER, and lysosomes do not play a major role during apoptosis (Kerr et al., 1972), later, it was found that mitochondria are the central coordinators of apoptotic events (Ferri & Kroemer, 2001). Many proapoptotic and signal transduction pathways converge on the mitochondria to induce mitochondrial membrane permeabilization (Fig. 2). There are several competing models to explain the rupture of the outer mitochondrial membrane as a result of the opening of the mega-channel, called the permeability transition pore (PTP). The adenine nucleotide translocator present in the inner mitochondrial membrane and the voltage-dependent anion channel in the outer membrane are the major components of the PTP and are responsible for the lethal change in mitochondrial membrane potential (Martinou et al., 2000). The PTP induces the mitochondrial translocation and multimerization of the proapoptotic protein, B-cell lymphoma-2 protein (Bcl-2)-associated X protein (Bax). Bax, in turn, helps the

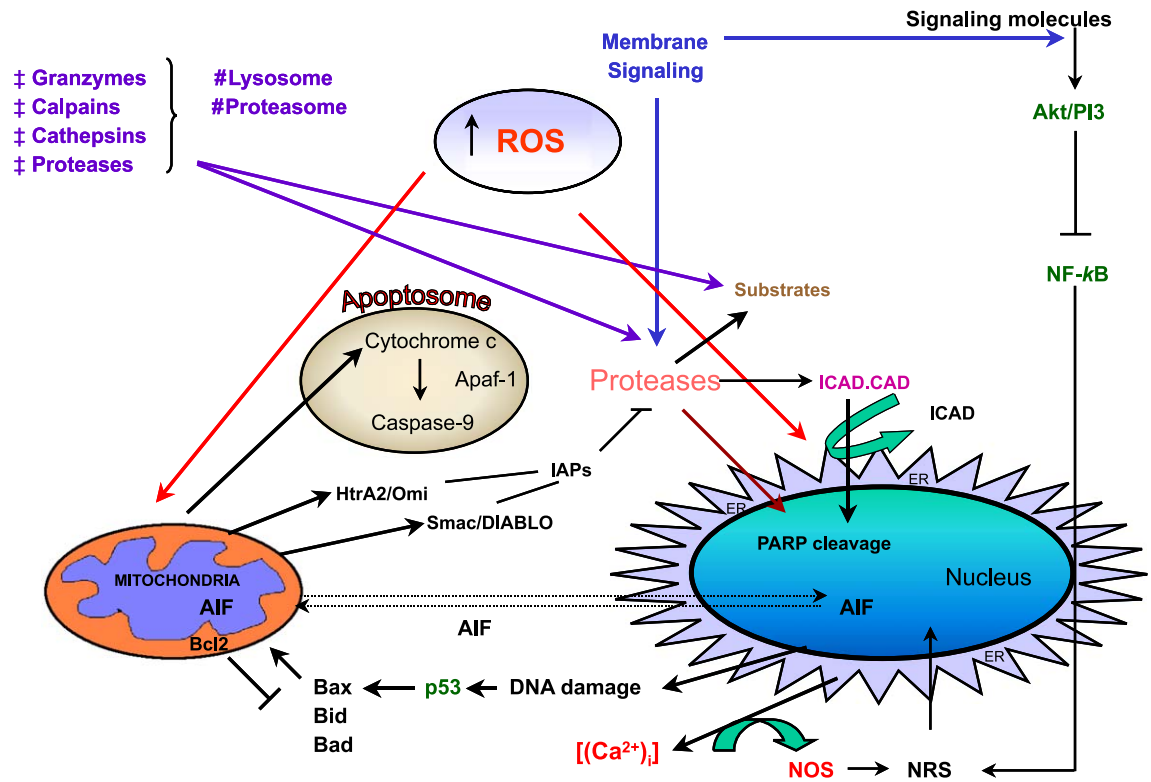

Fig. 2. Major pathways of mitochondrial apoptosis. Many proapoptotic and signal transduction pathways converge on mitochondria to induce mitochondrial membrane permeabilization. The PTP induces the mitochondrial translocation and multimerization of the proapoptotic protein, Bax. Bax, in turn, helps the permeabilization of the inner mitochondrial membrane resulting in the leakage of cytochrome *c* and other mitochondrial inter-membrane proteins that give a "go" signal for the execution phase of apoptosis. The second mitochondria-derived activator of caspases (Smac/DIABLO) is a mitochondrial protein that inhibits the IAP (such as XIAP, c-IAP1, and c-IAP2) after its release to the cytosol. IAP are known to block the processing of the effector caspases, caspase-3 and -9. Apart from Smac/DIABLO, there is another mitochondrial protein, HtrA2/Omi, that also inhibits IAP. The release of cytochrome *c* from mitochondria drives the assembly of the high molecular weight caspase-activating complex called apoptosome. The apoptosome contains oligomerized Apaf-1, which in presence of dATP and caspase-9, recruits and helps the autoactivating cleavage of caspase-3, an executioner of apoptosis. On the figure, several elements of ROS-induced apoptosis are also included, such as the formation of the highly toxic nitrogen reactive species (NRS). Finally, in the executioner phase of apoptosis, endonucleases, such as CAD, are activated.

permeabilization of the inner mitochondrial membrane resulting in the leakage of cytochrome *c* and other mitochondrial intermembrane proteins that give a "go" signal for the execution phase of apoptosis (De Giorgi et al., 2002).

The second mitochondria-derived activator of caspases (Smac/DIABLO) is a mitochondrial protein that inhibits the "inhibitors of apoptosis proteins" (IAP, such as XIAP, c-IAP1, and c-IAP2) after its release to the cytosol. IAP are known to block the processing of the effector caspases, caspase-3 and -9 (Shibata et al., 2002). Apart from Smac/DIABLO, there is another mitochondrial protein, HtrA2/Omi, that also inhibits IAP (van Loo et al., 2002).

The release of cytochrome *c* from the mitochondria drives the assembly of the high molecular weight caspase activating complex called apoptosome. The apoptosome contains oligomerized Apaf-1, which, in the presence of dATP and caspase-9, recruits and helps the autoactivating cleavage of caspase-3, an executioner of apoptosis (Acehan et al., 2002).

Immunodepletion of Hsp27 from cytochrome *c*-activated cytosol resulted in a decreased caspase activity. In parallel experiments, Hsp27 was co-precipitated with both cytochrome *c* and procaspase-3. These data suggest that Hsp27 sequesters both cytochrome *c* and procaspase-3, and thus prevents the correct formation/function of the apoptosome complex (Concannon et al., 2001). Indeed, Hsp27 binds to cytochrome *c* released from the mitochondria to the cytosol and prevents cytochrome *c*-mediated interaction of Apaf-1 with procaspase-9, thus interfering specifically with the mitochondrial pathway of caspase-dependent cell death (Garrido et al., 1999; Bruey et al., 2000). Later studies showed that Hsp27 is also localized in the mitochondria and promotes the retention of cytochrome *c* (Paul et al., 2002). Similarly, Hsp27 inhibits the release of another proapoptotic molecule, Smac/DIABLO, as well (Chauhan et al., 2003). Interestingly, Hsp27 was shown to form a complex with cell death-inhibiting RNA, which is a noncoding RNA competing with the mRNA of several antiapoptotic proteins, such as that of Bcl-2 for ribonucleoprotein-mediated degradation (Shchors et al., 2002). Altogether, these observations show that Hsp27 plays a major role in protecting mitochondria during activation of apoptosis (Samali et al., 2001).

Mitochondrial Hsp60 and its co-chaperone, Hsp10, are associated with procaspase-3 in Jurkat cells. In the staurosporin-induced apoptosis model, Hsp60 and Hsp10 release the active caspase-3. Similar effects were demonstrated in cell free systems, suggesting a role for Hsp60 and Hsp10 in regulating apoptosis in presence of cytochrome *c* and dATP (Samali et al., 1999; Xanthoudakis et al., 1999). In agreement with the previous findings, antisense oligonucleotide-induced decrease in mitochondrial Hsp60 induced the release of cytochrome *c*. On the contrary, cytoplasmic Hsp60 was found to sequester several antiapoptotic molecules, such as Bax or Bak (Kirchhoff et al., 2002).

In vitro experiments with purified recombinant Hsp70 showed its inhibitory role for cytochrome *c*/dATP-mediated caspase activation but not for the oligomerization of Apaf-1. Hsp70 suppresses apoptosis by directly associating with Apaf-1 and blocking the assembly of a functional apoptosome (Beere et al., 2000). In contrary, overexpression of Hsp105a, a Hsp70 homologue, is associated with the induction of apoptosis involving cytochrome *c* release, caspase-3 activation, and poly-ADP ribose polymerase (PARP) cleavage in mouse embryonal F9 cells (Yamagishi et al., 2002).

Hsp90 directly binds to Apaf-1 to prevent the formation of apoptosome complex (Pandey et al., 2000). In addition, the reactive cysteines present on Hsp90 were able to reduce cytochrome *c*, suggesting a role for Hsp90 in modulating the redox status in resting and apoptotic cells (Nardai et al., 2000). Hsp90 is known to help the vascular endothelial growth factor (VEGF)-induced expression of the antiapoptotic Bcl-2 (Dias et al., 2002); however, the exact mechanism of this action is unknown.

It seems that we are just starting to uncover the role of Hsp in mitochondrial apoptotic events. Data on their involvement in the formation of the PTP, as well as in the action of several pro- or antiapoptotic proteins, such as HtrA2/Omi or the IAP, are spuriously lacking. Discovery of the plenitude of the possible regulatory functions of Hsp in these processes is an exciting task of future research efforts.

Mitochondria are primary sites of reactive oxygen species (ROS) formation. It has been shown in many model systems that the cellular redox homeostasis plays an essential role in cell survival and cellular signaling. ROS or free radicals were reported to have a major role in the mediation of cellular damage (Thannickal & Fanburg, 2000). Although the origin of ROS remains to be determined, mitochondria are thought to be the major source of ROS in vivo (Boveris & Chance, 1973). However, ROS can be generated by a variety of mechanisms, like the mitochondrial and microsomal electron transport chain, xanthine oxidase and other flavoprotein oxidases, auto-oxidation of hydroquinones, catecholamines, and thiols, intracellular xenobiotic mechanisms, NADP(H)-oxidase, as well as by the auto-oxidation of hemoglobin. In a normal cell, there is always a balance between pro- and antioxidant pathways. Upon stress stimuli, an imbalance of the redox milieu develops and leads to the accumulation of ROS. ROS may induce some specific effects, serving as messengers of cellular damage and can also cause a general damage in the cell by oxidizing the membrane lipids, proteins, and DNA. The overproduction of ROS is associated with many forms of apoptosis and necrosis (Buttke & Sandstrom, 1995; Zamzami et al., 1995; Suzuki et al., 1997).

ROS-induced apoptosis was shown to be associated with the up-regulation of the Fas death receptor (Bauer et al., 1998). As an interesting cross-talk between different pathways, the antiapoptotic protein Bcl-2 also prevents the mitochondrial generation of ROS after the opening of the PTP (Gottlieb et al., 2000).

There is a correlation between ROS generation and the induction of Hsp (Schoeniger et al., 1994; Gorman et al., 1999). Showing a general protective role of Hsp against ROS, heat shock transcription factor-1 deficiency increases mitochondrial oxidative damage in mouse hearts (Yuan et al., 2002a).

Small Hsp, such as Hsp27, emerged as novel and important factors to protect against oxidative stimuli, blocking an important initiation factor of apoptotic processes (Arrigo, 2001). Indeed, small Hsp elevate reduced glutathione levels by promoting an increase in glucose-6-phosphate dehydrogenase activity and by a somewhat smaller activation of glutathione reductase and glutathione transferase (Preville et al., 1999; Arrigo, 2001). As another example of their action, the cytotoxicity induced by TNF-α and inflammatory cytokines via ROS production is inhibited by overexpression of Hsp27 or other small Hsp, such as α-crystalline, in L929 fibroblasts through the overexpression of Bcl-2, an antiapoptotic protein, which helps in maintaining the mitochondrial integrity (Park et al., 1998).

Members of the Hsp70 family play a similar role in oxidative protection like the small Hsp. Hyperoxia-mediated lipid peroxidation was attenuated in A549 human lung adenocarcinoma cells by overexpressing Hsp70. Increased expression of Hsp70 did not detectably alter a number of major antioxidant enzymes, such as manganese superoxide dismutase (Mn-SOD), catalase, and glutathione peroxidase, suggesting a specific protective role for Hsp70 against hyperoxia (Wong et al., 1998). Hsp70 was shown to be cardioprotective by conferring oxidative protection after heat shock preconditioning (Su et al., 1999). There is also a correlation between Mn-SOD activity and Hsp72-mediated cardioprotection, although Hsp70 does not bind to Mn-SOD directly (Suzuki et al., 2002). Rat histiocytic cells expressing Hsp70 were shown to be resistant to heat-induced apoptosis partially through the inhibition of ROS induction. In this system, hydrogen peroxide-induced Hsc70 expression along with Hsp70 suggested a role for Hsc70 in protection against ROS (Sreedhar et al., 2002a). Hsp72 reduces the formation of 8-hydroxy-2′-deoxyguanosine- and *trans*-4-hydroxy-2-nonenal-modified proteins in ischemia-reperfused liver of rats (Yamagami et al., 2002). The role for Hsc70 as an antioxidant has also been shown in reoxygenation injury in the intestinal cell line Caco-2 (Gebhardt et al., 1999) and in monocytes (Chong et al., 1998).

Nitric oxide (NO) is an important signaling molecule regulating a number of diverse physiological processes and is produced by a group of enzymes called nitric oxide synthases (NOS), namely, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) (Christopherson & Bredt, 1997; Mayer & Hemmens, 1997). NO inhibits apoptosis acting through up-regulation of survival kinases, like Akt (Dimmeler et al., 1998), and in an in vitro model by a direct inhibition of caspase-3 via *S*-nitrosylation (Rossig et al., 1999). Indeed, NOS is interacting with caspase-3 in an NO-dependent manner (Matsumoto et al., 2003). On the other hand, NO is combined with superoxides forming nitrogen peroxides, which are efficient initiators of the apoptotic response. Hsp70 attenuates the NO-induced apoptosis in RAW264.7 macrophages by maintaining the mitochondrial integrity (Klein & Brune, 2002), but protection by Hsp70 also involves the up-regulation of intracellular glutathione (GSH) (Calabrese et al., 2002; Sreedhar et al., 2002a).

Hsp90 has been shown to have a regulatory role in eNOS- and nNOS-mediated NO production; hence, inhibition of Hsp90 helps the induction of apoptosis by diminished NO production and by increased NOS-dependent superoxide production in certain cellular systems (Garcia-Cardena et al., 1998; Bender et al., 1999; Pritchard et al., 2001; Billecke et al., 2002). Hsp90 was also shown to inhibit superoxides generated by nNOS but not from xanthine oxidase (Song et al., 2002). As a more direct role in oxidative stress, Hsp90 protects cells from iron overload-induced oxidative stress (Fukuda et al., 1996).

2.1.5. Endoplasmic reticulum

The ER plays a critical role in protein biosynthesis and maintenance of intracellular calcium homeostasis. A variety of conditions, including disruption of intracellular homeostasis and alteration of ER intraluminal oxidative environment, can induce ER stress and lead to apoptosis (Chen & Gao, 2002). The participation of the ER in apoptosis initiation and progression is assumed to involve at least two mechanisms, the unfolded protein response and disturbed Ca^{2+} signaling (Kaufman, 1999; Patil & Walter, 2001). Although the complete understanding of the contribution of ER stress to the development of apoptosis is missing, recent studies suggest the involvement of the ER proteases, caspase-7 and -12, in this process (Nakagawa et al., 2000). The unfolded protein response also induces the CHOP/GADD153 transcription factor, which also promotes ER-induced apoptosis (Kaufman, 1999).

Glucose-regulated proteins (Grp) are a class of ER proteins composed of several members, which are induced by ER stress, like depletion of ER intraluminal Ca^{2+} or disturbances of protein glycosylation. Grp78 is involved in polypeptide translocation across the ER membranes and acts as an apoptotic regulator by protecting the host cell against ER stress-induced cell death. However, the mechanism of this protection is obscure. From a cell free system, it was found that Grp78 inhibits ER-induced apoptosis through direct binding and inhibition of the proapoptotic caspase-7 and -12 (Rao et al., 2002; Xie et al., 2002; Reddy et al., 2003). Recently, Grp78 induction was found to be associated with NF-κB activation (Chen & Gao, 2002); further, the Grp78-induced inhibition of Ca^{2+} disturbances and oxidative ER stress-induced apoptosis was extensively studied. Other important chaperone of the ER, calreticulin, and protein disulfide isomerase exert similar protective role like Grp78 (Liu et al., 1997a; Ko et al., 2002).

Recently, a rather exciting interplay between the ER and mitochondria has been uncovered during the apoptotic process. This interaction involves the junctions between the two organelle and opens the possibility that pro- and antiapoptotic molecules, like Bcl-2, regulate calcium fluxes through this junction (Berridge, 2002). As an additional hint for the specific interaction of these two organelles, two proapoptotic mitochondrial molecules, Bax and Bak, were shown to be localized to the ER and promote caspase-12-dependent apoptosis (Zong et al., 2003). It is a completely unexplored question if ER chaperones are involved in the regulation of the ER/mitochondrial junctions.

2.2. Effector molecules

2.2.1. Caspases

The family of proteases known as caspases specifically cleave proteins at aspartate residues. Caspases exists as inactive zymogens (procaspases). There are about 14 types of caspases reported in the literature (Ahmad et al., 1998; Thornberry & Lazebnik, 1998) and are classified into 3 major groups, which are initiator, inflammatory, and effector caspases (Nicholson & Thornberry, 1997). The activation of caspases is organized as a cascade in various apoptotic pathways. Thus, TNF-induced apoptosis involves the activation of the initiator caspases-8 (FLICE) and -10, which further activate the effector caspases-3, -6, and -7. The mitochondrial apoptosome-mediated pathways involve the activation of the initiator caspase-9, which further activates the same set of effector caspases, caspase-3, -6, and -7.

Hsp27 helps in retaining the mitochondrial integrity and inhibits mitochondrion-dependent caspase activation, but its direct involvement in caspase inhibition was not shown (Samali et al., 2001). However, immunoprecipitation experiments suggest that Hsp27 binds to procaspase-3 and inhibits its processing (Concannon et al., 2001). Interestingly, methylglyoxal modification of Hsp27 inhibits its interaction with procaspase-3 (Sakamoto et al., 2002). The small Hsp, α- and β-crystallines, inhibit both mitochondrial and death receptor pathways and are thought to be involved in the inhibition of autocatalytic maturation of caspase-3 (Kamradt et al., 2002).

Hsp70 binds to caspase-3 through its pseudosubstrate, the carboxy terminal EEVD sequence, and inhibits caspase-3 activity. However, the availability of the EEVD sequence depends on Hsp70 conformation and is dependent on ATP binding to Hsp70. Later, it was shown that Hsp70-mediated caspase inhibition is a result of reduced processing of procaspase-3 but not due to inhibition of the activity of the processed enzyme (Mosser et al., 1997).

2.2.2. Nucleases

From the functional point of view, the deoxyribonuclease (DNase) implicated in apoptosis may be classified in the groups of Ca^{2+}/Mg^{2+} endonucleases, Mg^{2+} endonucleases, acid endonucleases, cation-independent endonucleases, and Zn^{2+}-sensitive endonucleases (Ribeiro & Carson, 1993; Counis & Torriglia, 2000). However, Zn^{2+}-sensitive endonucleases were reported only in in vitro reconstitution of intact HeLa S3 nuclei and in apoptotic U937 cytosolic extracts (Kimura et al., 1998). Recently, a new Ca^{2+}/Mg^{2+} endonuclease was identified and called as the NUC70 cytoplasmic endonuclease. The activity of NUC70 is inhibited by caspase inhibitors suggesting that this nuclease is a cytoplasmic target for effector caspases (Urbano et al., 1998). An additional set of reports showed a Ca^{2+}/Mg^{2+} endonuclease activity associated with recombinant cyclophilins A, B, and C (Montague et al., 1994, 1997).

The major nuclease responsible for apoptosis-induced DNA fragmentation is a Mg^{2+} endonuclease called as caspase-activated DNase (CAD) or DNA fragmentation factor (DEF40). In proliferating cells, CAD exists in a complex of its inhibitor ICAD/DEF45, and the caspase-3-induced cleavage of ICAD leads to the release of the CAD endonuclease activity (Liu et al., 1997b; Enari et al., 1998). Hsc70, with its cofactor Hsp40, is involved in the folding of CAD. CAD is released from ribosomes as a heterocomplex with ICAD, which also assists in the folding of CAD during its synthesis (Sakahira & Nagata, 2002).

2.2.3. Transglutaminases

Tissue transglutaminase (TGase) is a member of the transglutaminase family catalyzing protein cross-linking by transamidation. There are several classes of enzymatically active TGases identified as having an important role in packing the cell at the late phase of apoptosis to prevent a massive inflammatory process (Lorand & Graham, 2003). Apart from transamidation, TGases also bind and hydrolyze ATP and guanosine triphosphate (GTP) (Tucholski & Johnson, 2002). The intracellular TGase activity is inhibited by GTP and NO and is enhanced by increases in intracellular Ca^{2+} level (Fesus, 1998).

TGase expression is inversely correlated with the expression of the antiapoptotic protein Bcl-2, and inhibition of the enzyme confers protection against apoptosis (Oliverio et al., 1999). Indeed, initially it was thought that TGase is only proapoptotic, as it sensitized SK-N-BE and 3T3 cells for apoptosis-inducing hyperpolarization of mitochondria followed by increased production of ROS. Furthermore, TGase overexpressing cells were sensitive to staurosporin-induced apoptosis (Piacentini et al., 2002). However, recent evidence suggests that TGase may also act as an apoptotic inhibitor through the cell cycle-regulating retinoblastoma protein (Antonyak et al., 2001; Boehm et al., 2002; Tucholski & Johnson, 2002).

Although the role of Hsp in TGase action has not been explored yet, it is of considerable interest that the ER chaperone protein disulfide isomerases, like Erp57, were shown to possess TGase activity (Chandrashekar et al., 1998; Natsuka et al., 2001).

3. Heat shock proteins and caspase-independent apoptosis

In this section, we will review the emerging alternative pathways of apoptosis, which are not centered around caspase activation. We would like to apologize for this dissection, which will turn to be rather artificial in some cases. Obviously, the signaling pathways are interrelated and, therefore, "caspase-independent" pathways may sometimes converge with caspase-dependent ones. In Section 3.6, we will briefly summarize anoikis, where currently no caspase-independent pathways are known. However, the therapeutic use of Hsp modulation in anticancer protocols makes it especially important to review their effects on caspase-independent apoptotic pathways, which are many times the major pathways of apoptosis in tumor cells (Nylandsted et al., 2000).

3.1. Serine proteases

Granzymes are a family of serine proteases often associated with perforin in activated T-lymphocytes and natural killer cells. A number of granzymes, granzyme A–G, have been isolated and cloned from mouse cytotoxic lymphocytes and natural killer cells; however, in humans, only a few of them were identified. Granzyme B has been found in the nucleus, as well as in cytoplasmic granules of killer thymocytes, and is involved in target cell apoptosis during lymphocyte-mediated cytotoxicity (Trapani, 2001; Pardo et al., 2002; Raja et al., 2003).

Granzyme cascade cleaves caspases in vivo, resulting in a massive amplification of caspase-dependent apoptotic pathways (van de Craen et al., 1997; Barry et al., 2000). Although these proteases can directly activate caspases, predominantly they induce the activation of the Bid protein and the consequent mitochondrial membrane changes and cytochrome *c* release (Wang et al., 2001). Furthermore, granzyme-mediated apoptosis requires Bid cleavage not involving caspase activation (Sutton et al., 2000), suggesting that granzyme-mediated apoptosis can be caspase-independent. Granzyme A is a typical mediator of caspase-independent apoptotic pathways, where it directly activates the ER DNase, GAAD, and promotes single strand DNA nicks and consequent apoptosis (Fan et al., 2003). Similarly, the involvement of granzyme C during caspase- and mitochondrial depolarization-independent cell death was reported (Johnson et al., 2003). In contrast, a recent report shows that granzyme B requires procaspase-3 for apoptosis initiation, where caspase-3 mutant cells failed to suffer granzyme B-dependent apoptosis (Metkar et al., 2003).

Very little is known about the role of Hsp in granzyme-mediated cell death pathways. It has been shown that Hsp27 does not interfere with granzyme B-induced activation of caspases (Bruey et al., 2000). However, Hsp27 co-precipitates with granzyme A from cytoplasmic lysates, although it is not a substrate for granzyme A (Beresford et al., 1998). Surface-expressed Hsp70 mediates the apoptosis of tumor cells by binding and uptake of granzyme B (Gross et al., 2003).

3.2. Cathepsins

Cathepsins are a class of proteolytic enzymes containing several types of proteases. The 3 major classes of cathepsins are the cysteine proteases, comprising cathepsins B, C, L, H, K, S, and O, followed by the aspartyl proteases, comprising cathepsins D, E, and F, and finally, a serine protease, cathepsin G. Being of lysosomal origin, these enzymes play a major role in peptide formation and protein degradation (Buhling et al., 2002; Takuma et al., 2003). Cathepsins are often involved in various forms of autophagy-associated apoptosis (Uchiyama, 2001). Oxidative stress-induced, caspase-independent apoptosis often involves the activation of cathepsin D (Kagedal et al., 2001; Takuma et al., 2003). Cathepsin B is released from lysosomes in liver cells after TNF-α treatment or in p53-induced apoptosis and contributes to cell death (Werneburg et al., 2002; Yuan et al., 2002b). In several tumor cells, cathepsin B is the most important mediator of cell death (Foghsgaard et al., 2002). Its activation can be prevented by the activation of the NF-κB pathway (Liu et al., 2003). Cathepsins emerge as important players of caspase-independent apoptosis, but in several cases, their activation also converges to caspase-dependent pathways (Mathiasen & Jaattela, 2002; Turk et al., 2002).

Although little is known about the role of Hsp in the regulation of these proteases compared with caspases, Hsp73 and Hsp90 were found accumulated in lysosomes of proximal tubular epithelial cells in rat kidneys in acute gentamicin nephropathy, suggesting a role of these proteins in protein degradation (Komatsuda et al., 1999; Agarraberes & Dice, 2001). The whole Hsp70/Hsp90 chaperone complex was found in lysosomal membranes, suggesting a major role for these chaperones and additional co-chaperones in proteolytic pathways (Agarraberes & Dice, 2001). Hsc70 was also found within the lumen of lysosome and the effective uptake of cytosolic proteins by these organelles was shown to depend on the availability of Hsc70 (Terlecky et al., 1992; Cuervo et al., 1997).

3.3. Calpains

Calpains are calcium-dependent proteases thought to play an important role in cytoskeletal reorganization and muscle protein degradation. Calpains exist as heterodimers comprised of a small regulatory subunit and one of the 3 large catalytic subunits: calpain-1, -2, or -3 (Goll et al., 2003). Calpains and caspases often synergize in the apoptotic process, especially in neuronal cells. However, in various cancer cells, such as in ovarian and breast cancer (Bao et al., 2002; Mathiasen et al., 2002), or in cisplatin-mediated apoptosis of melanoma cells (Mandic et al., 2002), calpain-mediated, but caspase-independent, apoptosis is the major

pathway of cell death. In the latter model, calpain cleaves Bid independently of caspase activation and thus triggers a consecutive apoptosis (Mandic et al., 2002).

Grp94 was shown to protect human neuroblastoma cells from hypoxia/reoxygenation-induced apoptosis involving calpains (Bando et al., 2003). Grp94 was also shown to be cleaved by calpain in etoposide-induced apoptosis (Reddy et al., 1999). It is interesting to note that cisplatin, which may interact with Grp94 (Itoh et al., 1999b; Soti et al., 2002), induces the activation of calpain in the apoptotic process (Mandic et al., 2003).

3.4. Ceramide-induced apoptosis

Ceramide has been recognized as a lipid mediator in the induction of apoptosis. Multiple, diverse apoptotic inducers are known to increase ceramide concentration in apoptotic cells (van Blitterswijk et al., 2003). Ceramide-induced apoptosis can be inhibited by Bcl-2 (Ruvolo et al., 2002). In agreement with this, mitochondria were shown to sense the sphingolipid signals (Tomassini & Testi, 2002). Fas induces ceramide generation during the initiation phase of apoptosis, which, in turn, may contribute to death receptor clustering (Rufini & Testi, 2000; van Blitterswijk et al., 2003). Ceramide was also shown to activate apoptosis by activating multiple pathways, which are both caspase-9 and -3 dependent (Movsesyan et al., 2002). Ceramide has multiple targets in the cell, including various kinases and phosphatases, leading to the activation of certain transcriptional factors (Alesse et al., 1998; Pettus et al., 2002). Its role and regulation was suggested to be dependent on intracellular glutathione (GSH) levels (Lavrentiadou et al., 2001). Besides caspase-dependent routes of ceramide-induced apoptosis (Movsesyan et al., 2002; Ruvolo et al., 2002), cathepsin-(De Stefanis et al., 2002) and calpain-dependent pathways (Poppe et al., 2002) were also shown to participate in ceramide-induced cell death. Indeed, ceramide has been shown to interact with cathepsin D directly and promote the autocatalytic activation of the enzyme (Pettus et al., 2002).

Although ceramide inhibits the heat shock response in some cells, especially suppressing the induction of Hsp70 (Kondo et al., 2000), its major action was proved to be the induction Hsp, such as small Hsp (Chang et al., 1995). Ceramide also induces stress-activated protein kinase (SAPK) signaling (Westwick et al., 1995). It was hypothesized that Hsp70 protect cells from ceramide-induced apoptosis, where increased ceramide levels are associated with the activation of SAPK/JNK kinases and induced Hsp70 synthesis (Verheij et al., 1996). In agreement with this assumption, a later study showed the suppressive effect of Hsp70 on ceramide-induced cell death (Ahn et al., 1999).

3.5. Apoptosis-inducing factor

The apoptosis-inducing factor (AIF) is a recently identified mediator of caspase-independent apoptosis. AIF translocates from the mitochondria to both the cytosol and the nucleus. Nuclear AIF induces peripheral chromatin condensation and high molecular weight DNA fragmentation to about 50-kb-long DNA fragments. Overexpression of one of the major antiapoptotic proteins, Bcl-2, inhibited AIF translocation (Cande et al., 2002a, 2002b). Hsp70 and Hsc70 (but not Hsp27) directly inhibited AIF translocation both in vitro and in intact cells. In agreement with this, the depletion of Hsp70 using antisense oligonucleotides sensitized cells to AIF-mediated apoptosis (Ravagnan et al., 2001).

3.6. Anoikis

Anoikis (Greek word meaning *state of homelessness*) is defined as a type of cell death where cells fail to find their substratum and the lack of the integrin-mediated extracellular matrix interaction induces apoptosis (Frisch & Screaton, 2001; Grossmann, 2002). Anoikis mainly occurs in epithelial cells, functioning to prevent the shedding and relocalization of these cells. It also assures proper developmental positioning of epithelial cells in specialized structures, such as the luminal structures of the mammary gland. Failure of anoikis contributes substantially to human tumor progression and facilitates metastasis (Frisch & Screaton, 2001). Anoikis utilizes mostly the Fas pathway, leading to caspase-dependent apoptosis. However, besides the general mechanism, there are some anoikis-specific elements, like the direct recruitment of caspase-8 to unligated integrin receptors (Stupack et al., 2001). An additional specific pathway involves FADD (Fas-associated death domain protein), which is primarily a nuclear protein, but its localization may be regulated by cell adhesion. Signaling pathways from integrins to FADD are currently being studied (Frisch, 1999). It is possible that cytoskeletal alterations, which accompany cell-matrix detachment, could release death receptors from a sequestered state, leading to death domain-induced apoptosis.

We have rather indirect evidence for the participation of Hsp in anoikis. The phosphorylated form of Hsp27 was shown to help the stability of integrin in platelets together with Hsp70 and Hsp90 (Polanowska-Grabowska & Gear, 2000). As a possible consequence of its role in anoikis regulation, overexpression of Hsp27 was shown to inhibit the invasive and metastatic potential of melanoma cells (Aldrian et al., 2002), where anchorage-dependent cell growth is related not only to the Hsp27 protein content, but also to its phosphorylation status by p38 kinases and its F-actin association. Hsp60 activates $\alpha 3\beta 1$-integrin, which is involved in the adhesion of metastatic breast cancer cells to lymph nodes and osteoblasts (Barazi et al., 2002).

BAG, a co-chaperone of Hsp70/Hsc70, binds and sequesters the death domain protein, FADD, leading to the inhibition of anoikis. Conversely, the inhibition of the chaperone activity of BAG leads to anoikis (Frisch, 1999). In agreement with a key role of BAG in anoikis regulation,

most of the metastatic tumors show a high expression of BAG (Cutress et al., 2002).

Currently, we do not know caspase-independent pathways of anoikis. However, the down-regulation of caspase-induced apoptosis in various tumors indicates that their discovery, and the characterization of the involvement of Hsp in caspase-independent anoikis regulation, is probably only a question of time.

4. Heat shock proteins and antiapoptotic mediators

Hsp are involved not only in the regulation of the various proapoptotic pathways, but also in the maintenance and activation of antiapoptotic mediators (Fig. 3). To better understand the pleiotropic role of Hsp, we chose to give a separate summary of their role in the regulation of anti-apoptotic pathways in the following section.

4.1. Heat shock proteins and B-cell lymphoma-2 protein pathway

The Bcl-2 family of proteins consists of more than 20 members, which form various homo- and heterodimers with each other, either promoting or inhibiting apoptosis (Gross et al., 1999; Cory & Adams, 2002). The relative expression of these pro- and antiapoptotic proteins is thought to decide the fate of cell during apoptosis by regulating mitochondrial membrane integrity (Cecconi, 1999; Gross et al., 1999).

As we will describe in detail in Section 6.2, BAG is a nucleotide exchange factor for Hsp70 (Höhfeld, 1998). The BAG proteins make a direct link between Hsp and Bcl-2 helping Bcl-2 activation. The role of other Hsp in Bcl-2 regulation has not been elucidated yet, except the role of Hsp90 in up-regulating Bcl-2 proteins after VEGF addition (Dias et al., 2002).

4.2. Heat shock proteins and phosphatidyl-inositol-3-kinase/Akt pathway

The other major antiapoptotic pathway, the phosphatidyl-inositol-3-kinase (PI-3-kinase)/Akt pathway, mainly responds to growth factor withdrawal. The Akt kinase is activated via the PI-3-kinase, and the activation stalls apoptosis (Downward, 1998). Once activated, Akt phosphorylates the proapoptotic factors, like Bcl-2-associated death protein (BAD), enabling them to bind to the 14-3-3 protein, which sequesters and inhibits them (Henshall et al., 2002). Akt also phosphorylates eNOS at Ser-1179, enhancing the production of NO as another mechanism to inhibit apoptosis as described in Section 2.1.4 (Cirino et al., 2003). Endothelial cell survival pathways are known to involve Akt, and it remains to be seen whether NO plays a role in this process.

Hsp27 associates with Akt and protects its kinase activity from heat stress and serum deprivation in PC12 embryonal carcinoma cells (Mearow et al., 2002). In neutrophils, Hsp27 association is necessary for Akt activation. Akt-induced phosphorylation of Hsp27 results in its dissociation from Akt and enhanced neutrophil apoptosis (Rane et al., 2003). Hsp90 is a necessary chaperone for the Akt kinase by interacting both the Akt-activator 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt itself (Sato et al., 2000; Basso et al., 2002; Fujita et al., 2002). In

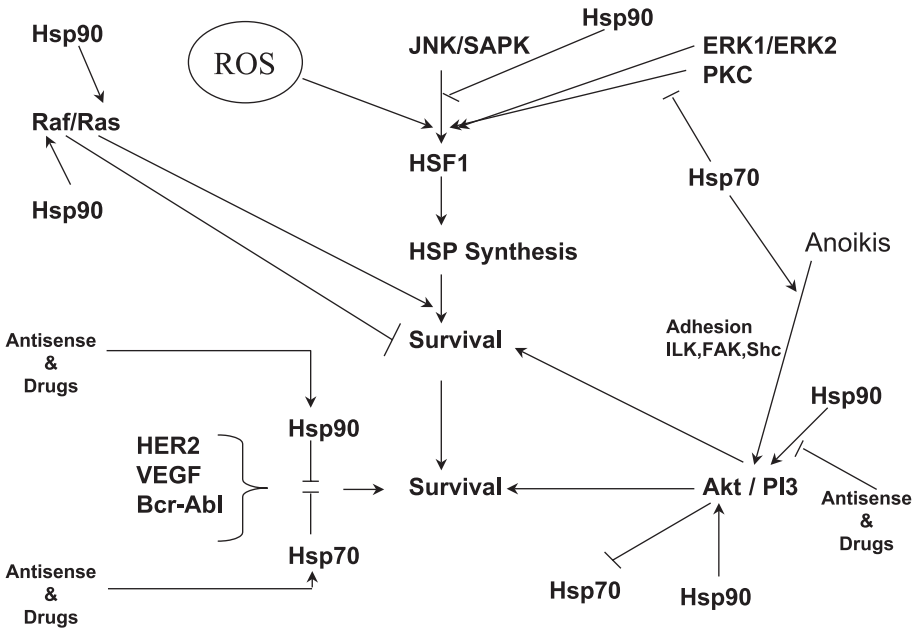

Fig. 3. Interactions of Hsp with the signaling network regulating cell survival and apoptosis. On the figure, a comprehensive summary is given about the network of Hsp interactions with various apoptosis-related signaling pathways, such as the activation of stress kinases, the Akt survival pathway, as well as various interactions with Hsp synthesis itself.

connection with this, Hsp90 serves as a molecular scaffold to promote the Akt-induced phosphorylation and activation of eNOS (Fontana et al., 2002). Interestingly, the ER chaperone, calreticulin, suppressed the Akt kinase activity during cardiac differentiation (Kageyama et al., 2002).

5. Modulation of modulators: effect of apoptosis on heat shock protein induction

The preceding sections gave numerous examples of how Hsp regulate apoptosis. However, key pro- and antiapoptotic processes also regulate Hsp synthesis. In this section, we will briefly summarize the (unfortunately rather limited) knowledge on the regulation of Hsp synthesis by the apoptotic process. In principle, apoptosis inhibits Hsp synthesis by down-regulating the respective transcription factor, HSF-1. Thus, activation of Fas inhibited heat-induced activation of HSF-1 and the up-regulation of Hsp70 (Schett et al., 1999). In agreement with this, HSF-1 was shown to undergo apoptosis-induced proteolysis by a caspase-like protease (Zhang et al., 1999). Similarly, glycogen synthase kinase-3, an apoptosis-promoting kinase, inhibits the activation of HSF-1. The PI-3-kinase-dependent survival pathway suspends this action by blocking glycogen synthase kinase-3 (Bijur & Jope, 2000). Thus, the apoptotic pathway itself stops one of the survival signals, Hsp synthesis. It remains to be seen if this mechanism is generally operating in tumor cells or if it is one of those pathways that becomes damaged in order to promote tumor survival.

6. Molecular mechanism of heat shock protein action

Hsp act as molecular chaperones preventing protein aggregation and promoting protein folding. Hsp almost never act alone: they tend to oligomerize, as well as to form chaperone complexes with each other. All major chaperone classes have their co-chaperones, which are smaller chaperone proteins usually regulating the folding cycle of the chaperone complex. Hsp have other general roles as well. Due to their pleiotropic action on the cell, they profoundly influence cellular homeostasis including the redox balance and cell organization. In Section 6.1, we briefly summarize the contribution of these general roles of Hsp to the regulation of the apoptotic process.

6.1. Heat shock proteins as molecular chaperones of apoptosis

It is a rather interesting question whether the divergent roles of Hsp in apoptosis regulation, summarized in the preceding sections, require a bona fide chaperone function of these proteins. When working in "passive mode," chaperones may behave as ATP-independent "holders" of damaged proteins, sequestering them and preventing their fatal aggregation. In ATP-dependent "active mode," chaperones are working as "folders" helping in the folding, transport, and/or ATP-dependent degradation of unfolded or misfolded proteins. While the passive mode is typical during stress when the cellular ATP level is low, the active mode prevails when the cell has recovered and the ATP level is increased again (Ellis, 1993; Hartl, 1996; Bukau & Horwich, 1998; Saibil, 2000; Hartl & Hartl, 2002). A number of proteins, such protein kinases and nuclear hormone receptors, require the continuous help of the Hsp90 chaperone complex to keep their activation-competent state (Pratt, 1997; Csermely et al., 1998; Richter & Buchner, 2001). It is also an interesting question, whether the particular Hsp modulates the apoptotic process as a passive or active chaperone or by a specific interaction, which is not equivalent with any of the general chaperone functions mentioned.

Unfortunately, the exact mechanism of apoptosis-related Hsp function has been explored in relatively few cases in detail. The chaperone function of Hsp70 was clearly established in the regulation of the proapoptotic p53 protein both in its wild type and mutant forms (Zylicz et al., 2001). Similarly, Hsp70 and its homologue, Hsp105, are required to suppress poly-Glu-induced protein aggregation and the consecutive apoptosis (Kobayashi et al., 2000; Ishihara et al., 2003).

It is important to note that Hsp have no priority or selection between their substrates as molecular chaperones, and hence their chaperone functions are extended to proapoptotic factors too. For example, Hsp60 promotes apoptosis by helping in the maturation of procaspase-3 (Samali et al., 1999; Xanthoudakis et al., 1999) and several Hsp are needed for the death-receptor induced signaling pathways.

6.2. Role of co-chaperones

Molecular chaperones almost always need co-chaperones for the regulation of the binding and release of substrate proteins. The major co-chaperones include BAG-1, CHIP (C-terminal Hsp90 interacting protein), Hsp40, Hip (Hsc70 interacting protein), Hop (Hsp70-Hsp90 organizing protein), and p23 (a 23-kDa co-chaperone of Hsp90) (Frydman & Höhfeld, 1997; Buchner, 1999; Caplan, 1999; Morimoto, 2002).

BAG-1 was first identified as an antiapoptotic protein because of its binding to Bcl-2 and promoting cell survival (Takayama et al., 1995). Later, the specific binding of BAG-1 and its homologues to Hsc70/Hsp70 was demonstrated (Höhfeld & Jentsch, 1997; Takayama et al., 1997; Nollen et al., 2000; Thress et al., 2001). The amino terminus of BAG-1 is involved in Bcl-2 interaction (Takayama et al., 1995) and the carboxy terminus is required for other protein interactions including Hsp70 binding (Stuart et al., 1998; Doong et al., 2002). Interestingly, the Hsp90 co-chaperone, Hip, also binds to Hsp70 and competes for BAG-1 binding. While Hip stabilizes Hsp70 in its peptide binding form,

BAG-1 helps to release the peptides from Hsp70 (Höhfeld, 1998). BAG-1 overexpression suppresses activation of caspases and apoptosis (Höhfeld, 1998; Chen et al., 2002b). As mentioned in Section 4.1, BAG-1 binds to Bcl-2, which is the key element of its antiapoptotic action (Takayama et al., 1995). BAG-1 also binds and sequesters the death domain protein, FADD, leading to the inhibition of anoikis (Frisch, 1999). In agreement with the key role of BAG in anoikis regulation, most of the metastatic tumors show high expression of BAG (Cutress et al., 2002). In addition, nuclear localization of BAG-1 during radiation therapy was found to be associated with poor prognosis (Yamauchi et al., 2001), which may be related to the inhibitory role of BAG-1 on the DNA damage-inducible protein, GADD34-related apoptotic cascade (Hung et al., 2003).

CHIP interacts with Hsp70/Hsp90 and inhibits their chaperone activity. CHIP is also implicated in ubiquitin-mediated protein degradation (Höhfeld et al., 1995; Ballinger et al., 1999; Cardozo et al., 2003). A recent study revealed that Hsp90-specific inhibitors led to the CHIP-dependent degradation of ErbB2, suggesting that CHIP may connect the Hsp90 chaperone machinery to the proteasome (Zhou et al., 2003). The role of CHIP in apoptosis has not been elucidated yet.

Hsp40 denotes a rather large chaperone family having a common domain, the J domain. Hsp40 isoforms are primarily involved in the regulation of Hsp70 chaperone activity (Cheetam et al., 1994, 1996). Hsp40 binds unfolded proteins and transfers them to Hsp70. After the transfer is completed, Hsp40 leaves the Hsp70 complex, and helps the hydrolysis of Hsp70-bound ATP (Bukau & Horwich, 1998; Hartl & Hartl, 2002). The J domain is possessed by several oncogenes (Stubdal et al., 1997), which may also modify Hsp70 function. Hsp40 helped to refold chemically denatured CAD; however, Hsp40 could not restore the DNase activity of CAD (Sakahira & Nagata, 2002). In association with either Hsc70 or Hsp70, Hsp40 protected macrophages from NO-mediated apoptosis (Gotoh et al., 2001). The Hsp70/Hsp40 system was shown to be effective in reducing the cellular aggregates in polyglutamine expansion-related nuerodegenerative disease (Kobayashi et al., 2000), thus, preventing the cause of massive neuronal apoptosis.

Hip regulates the Hsc70 chaperone by inhibiting its ATPase activity (Höhfeld et al., 1995). Hop is an adaptor of the Hsp70 and Hsp90 chaperones and also acts as a receptor for extracellular prion proteins (Lässle et al., 1997; Zanata et al., 2002). Although the specific role of Hop in apoptosis is not studied, it acts as a part of the chaperone complex of Hsp70/Hsp90 (Frydman & Höhfeld, 1997).

p23 is a co-chaperone of Hsp90 that stabilizes Hsp90/substrate complexes (Johnson et al., 1996). Both p23 and Hsp90 bind to double-stranded RNA-activated protein kinase (PKR), a member of the eukaryotic elongation factor-2α kinase family having an established role in tumor suppression and apoptosis. Dissociation of either of these two molecules, Hsp90 or p23, induces apoptosis (Donze et al., 2001). p23 also helps Hsp90 to chaperone the Bcr-Abl dysregulated protein kinase, where disruption of the chaperone complex leads to apoptosis (Shiotsu et al., 2000).

As a part of the Hsp90 chaperone complex, $p50^{cdc37}$ is involved in stabilizing a number of intrinsically unstable protein kinases, including Cdk4, Cdk6, Raf, and the Src family kinases. $p50^{cdc37}$ is an oncogene in mice (Stepanova et al., 2000a) and its overexpression is associated with many tumor types (Stepanova et al., 2000b). Loss of $p50^{cdc37}$ leads to apoptosis of human prostate epithelial cells (Schwarze et al., 2003). In conjunction with this, $p50^{cdc37}$ is required for the inactivation of the NF-κB survival pathway by TNF-α (Chen et al., 2002a).

6.3. Heat shock proteins and cellular homeostasis

Hsp not only function to refold or eliminate misfolded/denatured proteins, but they also play a critical role in many aspects of cellular life as continuously expressed chaperones of a number of cellular proteins (Pratt, 1997; Bukau & Horwich, 1998; Hartl & Hartl, 2002; Pratt & Toft, 2003). The general role of Hsp in the maintenance of cellular homeostasis prompted us to include a section that summarizes their role in apoptotic regulation from this angle. Some of the basic facts we list here may have been mentioned before, but we think it is worthwhile to repeat them here and show in a different context.

6.3.1. Redox homeostasis

Oxidative stress is an important signal for the apoptotic process. Moreover, protection against changes in the cellular redox homeostasis gives an efficient tool to regulate various apoptotic pathways. HSF-1 knockout mice showed an extreme sensitivity for oxidative stress (Yuan et al., 2002a) Indeed, as we summarized in Section 2.1.4, Hsp act as antioxidants in maintaining the cellular redox homeostasis. Heme oxygenase is the Hsp responsible for the production of the antioxidants biliverdin and bilirubin, while Hsp70 was shown to acquire both enhanced peptide binding ability and peptide complex stability under oxidative conditions (Callahan et al., 2002; Papp et al., 2003).

The redox state of the cell also influences Hsp synthesis. Thus, a decrease in reduced glutathione level may lead to a direct activation of HSF-1. On the contrary, strong oxidative agents inhibit the trimerization of HSF-1 blocking its DNA-binding ability. A more reduced cellular environment helps chaperone induction, while millimolar concentrations of a reducing agent impair the activation of HSF-1. As a summary of these results, mild changes of redox homeostasis lead to the activation of HSF-1, however, large changes in redox homeostasis cause HSF-1 inhibition (Jacquier-Sarlin & Polla, 1996; Kato et al., 1997; Papp et al., 2003).

6.3.2. Cell organization

The eukaryotic cytoskeleton constitutes 3 major components: microfilaments, intermediate filaments, and micro-

tubules. Extensive research on this field showed the importance of Hsp in stabilizing the cytoskeleton by direct interactions with cytoskeletal proteins (Clark & Brown, 1986; Koyasu et al., 1986; Margulis & Welsh, 1991; Gao et al., 1992). It was suggested that Hsp play a more intricate role in the organization of the cytoplasm, serving as highly dynamic, low-affinity extensions of the cytoskeleton, sequestering a plethora of proteins, and resembling the early (but partially invalid) concept of the ''microtrabecular lattice'' (Schliwa et al., 1981; Csermely et al., 1998; Csermely, 2001b). Indeed, recent work established that inhibition of the major cytoplasmic Hsp, Hsp90, by specific inhibitors, as well as by anti-Hsp90 ribozyme, leads to increased cellular lysis under various conditions, which is in agreement with the disruption of cytoplasmic organization as a result of the disruption of Hsp complexes (Pato et al., 2001; Sreedhar et al., 2003a).

Gross disturbances of cytoskeleton lead to apoptosis. As a general role, the small Hsp are well known to protect the actin filaments and help cell survival in apoptosis (Geum et al., 2002; Paul et al., 2002). Anti-Hsp27 antibodies induce neuronal apoptosis by disrupting actin filaments (Tezel & Wax, 2000). The Hsp70 homologue, Hsp105, protects microtubules and displays an antiapoptotic function (Saito et al., 2003). These examples give a further emphasis on the role of Hsp in the stabilization of the cytoskeleton and cytoarchitecture and thus in prevention of the apoptotic process.

7. Heat shock proteins as pharmacological targets in apoptosis modulation

The preceding sections gave numerous examples on how Hsp can regulate the apoptotic process. This makes any pharmacological intervention, which regulates the synthesis or activity of Hsp, an exciting tool to modulate apoptosis in pathological conditions. Here, we summarize our knowledge of the role of Hsp in tumor cell survival and apoptosis. We also highlight, why Hsp inhibition does not lead to the death of the whole organism and vice versa, why Hsp activation is not causing tumor cell proliferation.

7.1. Heat shock protein inhibition as an efficient way to induce the apoptosis of tumor cells

7.1.1. Apoptosis in tumor cells

When Bcl-2 was discovered as an oncogene involved in the promotion of cell survival, it was thought that antiapoptotic genetic lesions are necessary for tumors to arise (Vaux et al., 1988). Indeed, from various mouse models and cultured cells, it is evident that acquired resistance to apoptosis is a hallmark of most, if not all, types of cancers. Although tumor cells are resistant to apoptosis, they are not completely devoid of death. Cell death in tumor cells is mostly associated with cellular senescence and mostly involves caspase-independent routes of apoptosis or necrosis.

A tumor cell may escape from caspase-mediated apoptosis either by overexpressing antiapoptotic proteins or by debilitating mutations in proapoptotic factors. For example, the antiapoptotic Bcl-2 is known to be overexpressed in many tumors (Cory & Adams, 2002). In case of Hodgkin lymphoma, mutations of the Fas receptor were found (Gronbaek et al., 1998). Caspase-8 is also frequently mutated in neuroblastoma, a childhood tumor of the peripheral nervous system (Grotzer et al., 2000).

Besides changes in caspase-dependent apoptotic pathways, tumor cells also undergo changes to prevent caspase-independent apoptosis. For example, the IAP family member, survivin, is also highly expressed in many tumors. The survivin expression is associated with poor prognosis (Yamamoto et al., 2002).

Mutation of the tumor suppressor p53 gene is one of the major mechanisms of the tumor cell escape from apoptosis. The function of p53 is regulated by Hsp (Lam & Calderwood, 1992; Sepehrnia et al., 1996; Zylicz et al., 2001). Moderate expression of functional p53 in the presence of basal Hsp synthesis protects cells from heat-induced apoptosis (Sreedhar et al., 2002b). On the other hand, overexpression of Hsp with mutated p53 failed to give protection (Sreedhar et al., 2002a).

7.1.2. Heat shock proteins in tumor cells

Hsp, such as Hsp70, Hsp27, and Hsp90, which can inhibit apoptosis by direct physical interaction with apoptotic molecules, are also overexpressed in several tumor cells (Soti & Csermely, 1998; Jolly & Morimoto, 2000). For example, Hsp27 enhances the tumorigenicity of colon carcinoma cells (Garrido et al., 1998), Hsp70 is highly expressed in human breast tumors and is needed for their survival (Nylandsted et al., 2000), and Hsp90 was reported in prostate carcinomas (Akalin et al., 2001). Similarly, Hsp bind to procaspases, inhibiting their activation (Beere & Green, 2001). As we have summarized in Section 2, Hsp also block several caspase-independent pathways of apoptosis, which makes their inhibition an efficient tool in inducing a relatively tumor-cell-specific apoptosis. Depletion of Hsp70 from tumor cells by various methods induces their apoptosis (Wei et al., 1995; Nylandsted et al., 2000, 2002). Inhibition of Hsp90 in tumor cells results in the dimerization-induced activation of death receptors, suggesting that Hsp keep death proteins in an apoptosis-resistant state by a direct association (Hulkko et al., 2000).

Aging and various diseases, such as neurodegenerative diseases, induce the accumulation of damaged/misfolded proteins due to oxidative stress and/or proteotoxic insults. Moreover, chaperone function (Nardai et al., 2002), Hsp induction, and the degradation of damaged proteins are all impaired under these conditions (Soti & Csermely, 2000, 2002). In such cases, the increased demand of chaperone function may exceed the available chaperone capacity, which

results in an unbalance of cellular homeostasis termed *chaperone overload* (Csermely, 2001a). Recently, Hsp90 and other Hsp were shown to buffer the phenotypic appearance of hidden mutations in *Drosophila*, *Arabidopsis*, and *E. coli* (Rutherford & Lindquist, 1998; Fares et al., 2002; Queitsch et al., 2002) involving both a possible repair of mutant proteins and epigenetic changes at the level of the chromatin structure (Sollars et al., 2003). Tumors undergo facilitated evolution due to the increased proliferation and selection pressure. Conventional antitumor therapies (chemotherapy, radiotheraphy, hyperthermia, etc.) all induce Hsp in surviving cells. The overexpression of Hsp may help the accumulation of hidden mutations in tumors, which can help their further progression to more aggressive types of malignant/metastatic cells (Caporale, 1999; Csermely, 2001a). Indeed, the induction of Hsp70 by hyperthermia and anticancer drugs was reviewed and was shown to be more effective in chemoresistant tumors (Brozovic et al., 2001). Use of Hsp inhibitors may affect this balance and release some of the hidden mutations harbored by Hsp before.

7.1.3. Enforced apoptosis of tumor cells

Inhibitors of Hsp can suspend the Hsp-dependent block of both caspase-mediated and -independent apoptosis of tumor cells. Moreover, due to the Hsp-induced simultaneous stabilization of various proteins, Hsp inhibitors (as opposed to, e.g., protein kinase inhibitors) target not only a specific molecule, but a number of molecules, which makes them potentially more effective in the induction of tumor cell apoptosis (Huang & Ingber, 2000; Sreedhar et al., 2003b).

Although we have various Hsp inhibitors for Hsp60 (mizobirine, Itoh et al., 1999a) and for Hsp70 (deoxyspergualine, Nadler et al., 1992, 1994; sulfoglycolipids, Whetstone & Lingwood, 2003; the anticancer drug, MKT-077, Wadhwa et al., 2000), so far, it were the members of the 90-kDa Hsp family that gave the possibility of developing specific Hsp inhibitors, which are effective in clinical trials (Table 3) because of the specific ATP-binding site on the N-terminal domain of the 90-kDa Hsp, the Bergerat fold (Bergerat et al., 1997; Stebbins et al., 1997). Targeting of Hsp90 became a central attraction in Hsp-related tumor inhibition, giving a new pharmacological target in cancer therapy. The most important Hsp90 inhibitors are geldanamycin (Whitesell et al., 1994), its less toxic analogue, 17-allylamino-17-demethoxy-geldanamycin (17-AAG; Schulte & Neckers, 1998), radicicol, and its more stable oxime derivatives (Soga et al., 1998; Agatsuma et al., 2002), which have a higher affinity for Hsp90 than geldanamycin (Roe et al., 1999; Schulte et al., 1999). Recently, new geldanamycin analogues (Hargreaves et al., 2003) and a third class of inhibitors, the purine scaffold inhibitors, were developed (Chiosis et al., 2002), and there are ongoing efforts to synthesize even more Hsp90-interacting drug candidates (Table 3).

A recent report showed an important element of tumor specificity of Hsp90 inhibitors (Kamal et al., 2003). When Hsp90 becomes activated, it forms a large complex with various co-chaperones in tumor cells; on the contrary, it is found in a latent, uncomplexed state in normal cells. The geldanamycin-derivative, 17-AAG, binds to the tumor-specific, complexed form of Hsp90, with a 100-fold higher affinity than the latent form in nontransformed cells (Kamal et al., 2003). This difference also raises the possibility that active Hsp90 behaves as a tumor-selective catalyst in converting geldanamycin derivatives to their active conformation (Neckers & Lee, 2003).

Hsp90 inhibition leads to the dissociation of various Hsp90 client proteins from the chaperone complex and to their consecutive degradation by the proteasome (Schulte et al., 1997; An et al., 2000; Blagosklonny, 2002; Solit et al., 2002). Inhibition of Hsp90 induces apoptosis of various tumor cells (Hostein et al., 2001; vanden Berghe et al., 2003). Hsp90 inhibition also leads to a defect in a number of proliferative signals including the Akt-dependent survival pathway (Munster et al., 2001, 2002; Basso et al., 2002). Moreover, inhibition of Hsp90 was shown to be successful in reducing chemoresistant tumors with poor prognosis (Munster et al., 2001, 2002).

Importantly, there are additional drugs that interact with Hsp90, such as the widely used chemotherapeutic agents cisplatin (Itoh et al., 1999b), taxol (Byrd et al., 1999), as well as the antibactericid novobiocin (Marcu et al., 2000). Cisplatin was recently shown to bind to a novel nucleotide binding site of Hsp90 at its C-terminus (Soti et al., 2002), while novobiocin binds to several domains of Hsp90 (Marcu et al., 2000; Soti et al., 2002). The C-terminal nucleotide binding pocket has a unique nucleotide binding specificity

Table 3
Pharmacological modifiers of Hsp action

Drug	Affected Hsp
A. Hsp inhibitors	
Geldanamycin and 17-AAG	Hsp90
Radicicol	Hsp90
Cisplatin	Hsp90
Novobiocin	Hsp90
Deoxyspergualine	Hsp70, Hsp90
MKT-077	Hsp70 (mot2)
Mizobirin	Hsp60
B. Hsp inducers	
Amphetamine	all Hsp
Carbenoxolone	all Hsp
Geldanamycin and 17-AAG	all Hsp
Proteasome inhibitors	all Hsp
Stannous chloride	all Hsp
p38 kinase inhibitors	Hsp27
Geranyl-geranyl-acetone	Hsp70
C. Hsp co-inducers	
Aspirin	all Hsp
Bimoclomol	all Hsp

Among the Hsp inhibitors, only geldanamycin, its less toxic derivative, 17-AAG, and radicicol are the only relatively specific inhibitors of Hsp90. Aspirin and bimoclomol do not induce Hsp themselves, but only help the induction process provoked by any by environmental stress.

(Soti et al., 2003b), as well as a differential effect on Hsp90 client proteins (Soti et al., 2002), which gives hope that selective inhibitors against this segment of Hsp90 can be developed, showing novel properties in various anticancer protocols.

Hsp inhibitors, thus, may block a number vital pathways for cell proliferation, such as important checkpoint kinases of the cell cycle (Pratt, 1997; Csermely et al., 1998; Pratt & Toft, 2003), and promote apoptosis. However, recent data revealed that they may also sensitize tumor cells against various attacks by helping their lysis under hypoxia, complement attack, or mild detergent treatment (Sreedhar et al., 2003a).

7.1.4. Heat shock protein inhibitors as heat shock protein inducers

Hsp synthesis is tightly regulated at a transcriptional level, where the transcription factor HSF-1 plays a major role. Several cytoplasmic chaperones, such as Hsp90 and Hsp70, have been shown to bind to HSF-1 and keep it repressed in the absence of stress. During stress, both chaperones become occupied by misfolded proteins, which results in the dissociation, nuclear translocation, and activation of HSF-1. The dissociation of HSF-1 from the promoter regions of Hsp genes also requires the action of several molecular chaperones including Hsp90 (Abravaya et al., 1992; Ali et al., 1998; Zou et al., 1998; Bharadwaj et al., 1999; Kim & Li, 1999; Kim et al., 1999a). Hence, Hsp90 inhibitors may cause the transcriptional activation of HSF-1 by disrupting Hsp/HSF-1 complexes. Indeed, geldanamycin and its analogue, 17-AAG, were shown to activate HSF-1 (Kim et al., 1999b; Bagatell et al., 2000), and another geldanamycin analogue, herbimycin A, attenuates heat stress-induced apoptosis in rat hepatocytes parallel with Hsp70 induction (Sachidhanandam et al., 2003). Thus, the inhibition of Hsp paradoxically leads to an increase in their overall amount, which should be taken into account as a potential disadvantage when clinical applications of these drugs are designed. Indeed, both geldanamycin and 17-AAG were shown to antagonize the action of cisplatin in human colon adenocarcinoma cells (Vasilevskaya et al., 2003).

7.1.5. Ongoing clinical trials with heat shock protein inhibitors

Given the pleiotropic roles of Hsp, it not surprising that only the most specific Hsp inhibitors, those for the 90-kDa Hsp, have reached the level of clinical trials so far. Although the very first Hsp90 inhibitor, geldanamycin, showed clear antitumor effects, it encountered difficulties in clinical trials due to its high hepatotoxicity in some of the human tumor models (Supko et al., 1995). Thus, a search for new classes of Hsp90 inhibitors with lower toxicity began and an analogue, 17-AAG, was successfully developed. 17-AAG possesses all the Hsp90-related characteristics of geldanamycin (Schulte & Neckers, 1998; Kelland et al., 1999), with lower toxicity (Brunton et al., 1998; Chiosis et al., 2003; Workman, 2003), and could enter Phase I clinical trials (Neckers et al., 1999; Neckers, 2002). Both geldanamycin and 17-AAG can be metabolized by reduced nicotine adenine dinucleotide (NADH) quinone oxidoreductase 1 (DT-diaphorase), which is known to potentiate antitumor activity by stabilizing the tumor suppressor p53. Quinone oxidoreductase 1 may be a major factor in conferring on 17-AAG, as well as its parent compound geldanamycin the advantage that they specifically accumulate in tumor cells (Chiosis et al., 2003; Workman, 2003), which helps to explain why Hsp90 inhibitors are generally not so toxic to patients as one would expect from the pleiotropic roles of Hsp90 inhibited by them.

Combination therapies, applying low doses of Hsp90 inhibitors together with conventional chemotherapeutic agents, seem to be an effective way to target various cancers. For example, in the case of Bcr-Abl-expressing leukemias, a low dose of geldanamycin is sufficient to sensitize these cells to apoptosis in the presence of ineffective concentrations of doxorubicin, through caspase activation (Blagosklonny et al., 2001). In another example, 17-AAG, in combination with taxol, showed enhanced cytotoxic effects on taxol-resistant ErbB2 overexpressing breast cancer cells (Munster et al., 2001; Sausville, 2001). As an alternative strategy, the synthesis of geldanamycin hybrids, such as the adduct with steroids (Kuduk et al., 1999, 2000) as well as with inhibitors of the PI-3-kinase-related survival pathway (Chiosis et al., 2001), conferred further selectivity and efficiency for these drugs besides their tumor-specific accumulation.

7.2. Therapeutic use of heat shock protein up-regulation

A number of clinical applications can be derived from the general cytoprotective/antiapoptotic role of Hsp, like cardioprotection, cellular defense against stroke, and various neurodegenerative diseases, as well as an efficient use of Hsp inducers to help tissue transplantation. Therefore, the induction of Hsp has a number of benefits and a wide range of potential clinical applications. However, antiapoptotic action may help tumor cell survival. Fortunately (as we summarized in Sections 3 and 7.1.1), apoptosis differs to a large extent from the usual caspase-dependent pathway in tumor cells. Additionally, Hsp induction may sensitize tumor cells for immune attack, providing a simultaneous protection of bystander cells in various cancer therapies, such as chemotherapy, radiotherapy, hyperthermia, and other protocols. We will summarize these elements of therapeutic use of Hsp induction in the following sections and compare the existing methods for inducing Hsp at the end of the section.

7.2.1. Sensitization of cancer cells for immune attack

Increased Hsp may lead to tumor cell sensitization against immune attacks by two mechanisms: tumor cells may express Hsp on their surface, which leads to their enhanced recognition by the natural killer cells of the native immune system (Multhoff et al., 1997; Multhoff, 2002), and a specific antitumor immunity that may be developed by

Hsp-related antitumor vaccination (Chu et al., 2000; Srivastava, 2002; Baker-LePain et al., 2003).

Hyperthermia has been used for a long time as an adjuvant of other various cancer protocols (Crile, 1963). Besides the proapoptotic effect of high temperatures and the hyperthermia-induced extra bioavailability of chemotherapeutic drugs, the induction of Hsp may also lead to their expression on the tumor cell surface. Various Hsp, such as Hsp70, can often be found on the surface of tumor cells (Ferrarini et al., 1992; Multhoff et al., 1995; Multhoff & Hightower, 1996; Berger et al., 1997). Interestingly, extracellular Hsp90-α had a stimulatory effect on the growth of some lymphoid cell lines (Kuroita et al., 1992) and Grp78 was identified as a potential intercellular signal-transducing protein between pancreatic cancer cells (Furutani et al., 1998). Tumor cell surface Hsp may be derived from the hosting cell itself and may also come from neighboring tumor cells undergoing a necrotic process and releasing Hsp as "danger signals." Extracellular Hsp bind to the surface Hsp receptors (such as the scavenger receptor CD91, CD36, and the Toll-like receptors 2 and 4) and act as natural adjuvants activating the innate immune system, leading to cytokine release, up-regulation of MHC II complexes in antigen-presenting cells, as well as increased dendritic cell maturation (Multhoff et al., 1997; Binder et al., 2000; Jolly & Morimoto, 2000; Moser et al., 2002; Multhoff, 2002; Srivastava, 2002; Baker-LePain et al., 2003).

At the end of the 1980s, Hsp70, Hsp90, and Grp94 (termed gp96) were identified as tumor-specific antigens expressed on the surface of various tumor cells (Srivastava et al., 1986; Ullrich et al., 1986; Konno et al., 1989). Differences in protein structure of various tumor-derived, surface-expressed Hsp were minor. However, their immunogenicity showed major differences. This apparent discrepancy led Pramod Srivastava to suggest that the Hsp-related immunogenicity resides in a great variety of peptides, which are noncovalently associated to and "presented" by the Hsp (Srivastava & Heike, 1991; Srivastava & Maki, 1991; Srivastava et al., 1994). Indeed, Hsp in the cytoplasm and in the ER may play a significant role in transporting, trimming, and presenting antigenic peptides to the MHC-I molecules (Spee & Neefjes, 1997; Binder et al., 2001; Chen & Androlewicz, 2001; Menoret et al., 2001). Extracellular Hsp, released as a result of cell death and taken up by antigen-presenting cells through Hsp receptors (Basu et al., 2001; Binder et al., 2001; Li et al., 2002), are involved in the cross-presentation of chaperoned peptides on MHC molecules of antigen-presenting cells. Hsp may also serve as adjuvants of the immune response potentiating the effects of other antigen-presenting mechanisms (Baker-LePain et al., 2003). Hsp induction may help these processes and may overcome the limitations of aging- (Wick & Grubeck-Loebenstein, 1997; Pawelec et al., 2002) and chaperone overload-induced (Csermely, 2001a) immunosuppression.

Peptide-chaperone complexes can be efficiently formed as covalent adducts and can be used to promote the immune response against viral infections and various forms of cancer, which is a technology platform applied by StressGen (Chu et al., 2000). As a related, but not immune attack-based, approach to use Hsp-related gene induction as an anticancer therapy, suicide genes with heat shock promoters greatly enhanced the efficiency of hyperthermia in breast cancer xenografts (Braiden et al., 2000).

7.2.2. Protection of cells from apoptosis

From the now classical observations of Currie et al. (1988), we know that Hsp have a significant role in cardioprotection. Similarly, Hsp induction helps the survival of neurons after stroke (Yenari et al., 1998; Hoehn et al., 2001), as well as improves the efficiency of tissue transplantation (Perdrizet et al., 1993). Hsp induction eases the deleterious consequences of chronic diseases, such as diabetes (Nanasi & Jednakovits, 2001; Kurthy et al., 2002). Conditions, like Alzheimer's, Parkinson's, Huntington's, or prion disease, where the accumulation of misfolded proteins is the major cause of neurodegeneration (Warrick et al., 1999; Carmichael et al., 2000; Sittler et al., 2001), as well as conditions such as trauma, where neuroregeneration becomes necessary (Kalmar et al., 2002), also gain beneficial effects from Hsp overexpression.

Unfortunately, in many of the above pharmacological experiments, the exact mechanism is not known. However, we believe it is not a misleading statement that most of the above cytoprotective effects of Hsp come from the inhibition of stress-induced apoptosis. Rescue from apoptosis may also be helpful in anticancer protocols, where by-stander, nonmalignant cells are also damaged by the therapy. As an example, the Hsp90 inhibitors, radicicol and geldanamycin, prevent the neurotoxic effects of anticancer drugs (Sano, 2001). This, together with the additional cytotoxic effects of Hsp inhibitors in tumor cells, makes combination therapy of Hsp inhibitors and conventional anticancer drugs a promising approach. Obviously, many additional studies have to be done to show the extent and reason of the differential effects of Hsp inhibitors, which, in parallel, may kill the cell by inducing an apoptotic process and save the cell by inducing Hsp.

7.2.3. Methods of heat shock protein activation

Obviously, heat shock is the archetype of the induction of Hsp (Ritossa, 1962). However, whole body or partial hyperthermia may not be feasible in many circumstances. As we mentioned in Section 7.1.4, an interesting and important side effect of Hsp inhibitors is their Hsp-inductive character, which is due to the disruption of the Hsp/HSF-1 complexes, as well as the disruption of chaperone-induced HSF-1 release from the Hsp gene promoter regions (Kim et al., 1999b; Bagatell et al., 2000). However, there are many additional protocols to induce Hsp synthesis.

As a very "traditional" method, amphetamine-induced lipolysis elevates body temperature, which, in turn, leads to Hsp synthesis and to improved post-ischemic heart recov-

ery (Maulik et al., 1995). Proteasome inhibitors up-regulate Hsp synthesis by increasing the amount of misfolded proteins that compete for Hsp with HSF-1 (Meriin et al., 1998; Kim et al., 1999a; Ashok et al., 2001). Some of the protein kinase inhibitors were also shown to induce Hsp27 induction (Kawamura et al., 1999). Stannous chloride has been shown as a nontoxic, efficient inducer of Hsp, thus, improving the success rate of tissue transplantations (House et al., 2001). Geranyl-geranyl acetone, a nontoxic Hsp inducer, has been shown to suppress ethanol- and hydrogen peroxide-induced apoptosis of rat hepatocytes (Ikeyama et al., 2001). Similarly, the antiulcer drug, carbenoxolone, has also been shown to be an inducer of Hsp70 (Nagayama et al., 2001).

Most of the above methods introduce a certain level of stress to the cells and thus provoke Hsp synthesis. However, in most of the diseases, it seems to be more efficient if the administered drug does not induce Hsp but just helps the natural Hsp induction provoked by the natural stimuli on the cell. This help in Hsp induction was termed as chaperone co-induction by Vigh et al. (1997). The best-known chaperone co-inducer is aspirin, which enhances Hsp synthesis (Jurivich et al., 1992; Ghavami et al., 2002). Another family of drug candidates exemplified by bimoclomol helps the induction of Hsp synthesis by perturbing various membrane structures and helping the release of putative lipid-signaling molecules, as well as by the prolongation of the binding of HSF-1 to the heat shock element on the DNA (Vigh et al., 1997; Hargitai et al., 2003; Török et al., 2003). These drug candidates (acting like "smart drugs" by a selective interaction only with cells that are in danger) may provide an important novel therapy in a number of acute and chronic diseases.

8. Conclusions and perspectives

The involvement of Hsp in a multitude of intracellular actions places them as central coordinators in deciding the fate of cell. The level of various Hsp (the Hsp pattern), as well as the amount of Hsp, which are not occupied by damaged, misfolded proteins, can be critical in cytoprotection and cell survival. We need much more comparative investigations on the induction of various Hsp, as well as on their occupancy, to get a full picture of the optimal levels of these proteins. However, from the number of successful clinical studies, one point is already clear: Hsp can be used as novel molecular targets for pharmacological and therapeutic interventions both to prevent and to cause apoptosis.

8.1. Pleiotropic role of heat shock proteins in apoptosis

The dynamic conformational changes of apoptotic molecules involving various oligomerization and autoactivation steps after the death signals either from the death receptor(s) or from intracellular stress suggest an extensive need for chaperones. The highly dynamic interactions between various members of the apoptotic cascade, like receptor dimerization, procaspase/caspase recruitment to the receptor complexes, dATP/cytochrome *c*/Apaf-1/caspase-9 complex formation, etc., are in most cases influenced by molecular chaperones, the Hsp. Being central regulators of assembly, transport, and folding of other proteins, Hsp play a major role in apoptotic signaling events. However, their proapoptotic role is balanced and usually overcome by the well-known Hsp-induced cytoprotection. This finely tuned balance is not only a key point in regulating cell death or survival but also serves as a switch between the two forms of cell death, apoptosis and necrosis.

8.2. Exciting areas of further research

Among the multitude of exciting ideas for further experiments, the following areas may be listed as important fields where we feel there is a significant gap in the current knowledge on the interactions of Hsp and apoptosis:

- the interaction of Hsp with various components of death receptor complexes certainly deserves more attention in light of recent reports on the membrane- and raft-association of various Hsp;
- it would be exciting to know if Hsp90 is also involved in protecting the DNA against oxidative damage;
- we are just at the start to uncover the role of Hsp in mitochondrial apoptotic events. Data on their involvement in the formation of the PTP, as well as in the action of several pro- or antiapoptotic proteins, such as HtrA2/Omi or the IAP are especially lacking;
- whether Hsp are involved in the regulation of the recently discovered ER/mitochondrial junctions is a completely unexplored area;
- our knowledge of the interactions of Hsp with effector nucleases is very limited;
- the involvement of Hsp in caspase-independent forms of apoptosis, as well as in anoikis, is a rapidly developing area of research;
- we are just beginning to explore the role of various co-chaperones in the regulation of apoptosis;
- the disturbingly complex effects of Hsp inhibitors, as well as Hsp inducers, on caspase-dependent and -independent forms of apoptosis give a beautiful possibility for well-designed experiments analyzing the dose dependence of the applied drugs and the interrelationships of Hsp patterns, chaperone function, chaperone occupancy with drug-induced or -prevented apoptosis;
- the Hsp-related links among cellular senescence, apoptosis, and necrosis are just about to be uncovered;
- last, but not least, changes of the apoptotic process in aging organisms deserve much more attention, especially in light of the profound changes of Hsp during the aging process.

We hope that the review both helped the reader to organize the knowledge on Hsp and apoptosis and gave some hints about the excitement and happiness we have working in this field.

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