INTERACTION BETWEEN CELL DEATH AND CELL PROLIFERATION IN CANCER

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Apoptosis is molecularly regulated and genetically programmed cell death. A range of environmental, physical or chemical stresses can induce it. It is characterised by a sequence of precisely regulated events that culminate in self-destruction of a cell. Apoptosis is a common phenomenon in developmental processes and in normal physiological conditions when unnecessary cells have to be eliminated. Apoptosis is also the predominant form of cell death triggered by cytotoxic drugs in tumour cells. There are many biochemical and genetic parallels between cell death pathways in different animal species.

Methods of cellular survival under the stressful environmental conditions are also genetically programmed and mediated by the activity of physiological defence mechanisms. That is another, even more conserved and evolutionarily ancient cellular response. This response is mediated by the heat shock proteins (Hsp).

Hsp is a highly conserved family of proteins that play a major role in cytoprotection. However, apoptosis that is induced to eliminate unwanted, damaged or old cells may be understood as another way of protecting tissues, from the great changes in the environment. Consequently, there are many functional interactions between these two, mechanistically opposing, mechanisms that regulate cellular decision to live or to die. Recent studies have established that the survival-promoting effects of Hsp can be partially attributed to the suppression of apoptosis. Therefore there is a great potential in pharmacological applications of Hsp inhibitors that may help inducing apoptosis when that may be beneficial, as in various tumours.

2.1 Heat shock proteins

The eukaryotic stress response is highly conserved and involves the induction of Hsp. Cellular protection against harmful insults relies on transient increase in Hsp production. Many vital functions of the cell, such as maintenance of cell cycle and proliferation are under regulatory control of Hsp. In mammalian cells, the stress response involves the induction of 5 major classes of Hsp families, the small Hsp 27, Hsp 60, Hsp70, Hsp90, and Hsp104. Hsp synthesis is tightly regulated at the transcriptional level by heat shock factors, HSFI and HSF2. In resting cells, HSFI is a monomer but active HSFI exists as a trimer and binds to the heat shock elements, the consensus sequence at DNA.

Hsp function as molecular chaperones in regulating cellular homeostasis and promoting cell survival. The main function of Hsp is helping in folding of nascent proteins, refolding of denatured proteins, inter-organellar transport of proteins and prevention of illegitimate aggregations. Cells failing to respond to stress are sensitive to induced cell death via apoptosis.

| FAMILY | CHAPERONS | LOCATION | FUNCTION |
|--------|-----------|----------|----------|
| Hsp27  | Hsp27, α2-crystallin | cytosol | stabilisation against aggregation |
| Hsp60  | αB-crystallin | mitochondria | prevents aggregation of denatured proteins |
| Hsp70  | Hsp70 | cytosol | folding of nascent proteins |
| Hsp90  | Hsp90α | mitochondrial | interorganellar transport |
| GRP78  | | ER | refolding of denatured proteins |
| Hsp90α | | cytosol | chaperonin-mediated folding of nascent proteins |
| Hsp90α | | | conformational maturation of steroid hormone receptors |
| Grp78  | | | signal transducing kinases |

Table 1. Main families of mammalian Hsps

2.2 Cellular senescence, apoptosis, and necrosis: chaperon overload as a potential regulator

Cells typically die either by apoptosis or necrosis. During necrosis, cell membrane loses its integrity and cell content is released causing an inflammatory response. In apoptosis, however, cell content remains "well-packed" in the apoptotic bodies and inflammation does not develop. Nevertheless, these two forms of cell death share some common features. Both processes could be:

• caused by the same pathophysiological conditions,
• prevented by antiapoptotic mechanisms and
• transformed from one form to another.

There is another cellular state that is seen in some cell types - a nondividing-senescent-state. These cells exhibit only a limited number of replications in cell culture. Morphological and functional properties of a cell change until it reaches a senescent-
state. These cells are unable to undergo apoptosis and are shifted to necrosis upon DNA damage.

The Hsp play an extremely complex role in the regulation of apoptosis. The principal role is maintaining the physiological homeostasis needed for the cell survival. However, by chaperoning the active structure of key apoptotic signalling proteins the Hsp may directly promote apoptosis and act as chaperones of the death.

On the other hand, the protein folding capacities of Hsp may be exhausted due to massive stress, during ageing, or in chronic diseases. In these conditions protein misfolding and aggregation are prevailing. Various levels of chaperone overload may have an important contribution to the signals directing the cell to senescence, necrosis or apoptosis.

### 2.3 Major elements in the mechanism of apoptosis

Apoptosis is an energy-dependent, ubiquitous and genetically controlled physiological process.

- It is morphologically well characterised with nuclear condensation, cell shrinkage, and membrane blebbing.
- The physiological changes involve fragmentation of nuclear DNA into 80-200 oligo-nucleosomal fragments. The DNA fragments are produced by the specific caspase-activated endonucleases. This highly regulated process develops as the response to some initial stimulus followed by a specific cascade of events. Apoptosis proceeds in three phases:
  - The initiation - signalling phase, which involves the activation of surface death receptors, or the mitochondrial pathway;
  - The signal transduction - preparation phase where activation of initiator caspases and certain kinases/phosphatases takes place and
  - The execution - death phase involving activation of effector caspases (Table 2).

| PHASE / CATEGORY | MECHANISM |
|------------------|-----------|
| Initiator caspase | caspase-8, -9, -10, -12 |
| Physiological induction | Bax, Bcl2, JUNOSAGE, interactions |
| Initiator caspase | caspase-8, -9, -10, -12 |
| Physiological induction | Bax, Bcl2, JUNOSAGE, interactions |
| Death receptors | TNFα, Fas (CD95), FADD |
| Protein synthesis | growth factors, cytokines, proinflammatory |
| Preparation phase | |
| Execution phase | |
| Effective caspases | caspase-3, -6, and -8 |
| Physiological feature | membrane blebbing, apoptotic body formation, DNA fragmentation |

Table 2. The most important members of the apoptotic machinery

### 2.4 Sites of initial signalling events

#### 2.4.1 Plasma membrane

Activation of the TNF and the Fas receptor, the so-called death receptors on the plasma membrane activates factors that promote cell death. The superfamily of TNF receptors is implicated in the inflammatory and immune response. Death receptors contain an intra-cytoplasmic domain called death domain. Through this domain receptors interact with the cytosolic proteins and propagate the death signal by activating caspases. They are the final executioners in a stereotyped cascade leading to cell death.

In the late execution phase, apoptosis is characterised by marked changes in cell morphology, including membrane blebbing, loss of the membrane phospholipid asymmetry and exposure of phosphatidylserine on the surface. The phosphatidylserine can be recognised by the immune system. Hsp can help translocate phosphatidylserine to the cell surface making cells more vulnerable to immune lysis.

The role of Hsp70 is pleiotropic to cellular life and in some cases over-expression of Hsp70 is protecting from apoptosis, but in other cases it may promote the cell death. Hsp90 is helping in propagation of the apoptotic signal from plasma membrane.

#### 2.4.2 Cytosol

In the cytosol stress kinases are important elements of 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 ERK is associated with mitogenic stimulus, whereas the JNK and p38 kinases are stress responsive.

The small Hsp27 is activated by p38-activated phosphorylation. The phosphorylated dimers of Hsp27 interact with Daxx, a protein that contains the death domain. Association with Hsp27 prevents Daxx from interaction with another serine/threonine kinase. That is the way of inhibiting the Fas-mediated apoptosis.

Hsp70 has a general inhibitory role in stress kinase pathways. Hsp72 also interacts with peptide binding domain of JNK and is necessary for JNK down-regulation.

#### 2.4.3 Nucleus

The biochemical signature of apoptosis is DNA damage and nucleosomal fragmentation of DNA that is resulting from activation of specific endonucleases. These enzymes cleave the chromatin to shorter, oligo-nucleosomal DNA fragments.

Hsp play a major role in protecting the cells from DNA damage induced by various agents. Members of the Hsp27 and Hsp70 families have a protective role for the DNA integrity against oxidative stress. Nuclear Hsp72 suppresses the appearance of apoptosis after DNA damage.

The specific form of DNA damage occurs with telomere shortening. At the critical length of the telomere regions, around 7kb, cells go to the state of senescence, which may further proceed to apoptosis. Telomere regions are maintained by enzyme telomerase and Hsp90 is necessary for the enzyme activity.

#### 2.4.4 Mitochondria and reactive oxygen species

The mitochondrion appears to be the central coordinator of apoptotic events. Many proapoptotic and signal transduction pathways converge on the mitochondrion to induce the membrane permeabilisation. Rupture of the outer membrane and formation of the mega-channel, permeability transition pore (PTP) is the starting event. The adenine nucleotide translocator present in the inner mitochondrial membrane and the voltage-dependent anion channel at the outer membrane are the major components of PTP. These proteins are responsible for the lethal changes in mitochondrial...
membrane potential and release of certain molecules from intermembrane space to cytosol. The reaction is controlled by Bcl-2 and Bcl-2 related proteins. The PTP formation is connected with the Bax protein and physical disruption of the outer membrane. Changes in membrane permeability lead to matrix swelling and finally to leakage of cytochrome c, and this is a starting signal for the execution phase of apoptosis.

The second mitochondrial protein involved in apoptosis is activator of caspases (Smac/DIABOL0). This protein inhibits inhibitor of apoptosis (IAP) that blocks processing of effector caspases -3 and -9. The release of cytochrome c from mitochondria drives the assembly of the high molecular weight caspase-activating complex - apoptosome. The apoptosome contains oligomerised Apaf-1, which in the presence of dATP and caspase-9 helps auto-activating cleavage of caspase -3, an executioner of apoptosis.

Hsp27 may decrease caspase activity by binding to cytochrome c and down-regulate mitochondria pathway of caspase dependent cell death. Hsp70 and Hsp90 suppress apoptosis by directly associating with Apaf-1 and blocking formation of apoptosome.

There is another role of mitochondria in the development of apoptosis. Mitochondria are primary site of reactive oxygen species (ROS) formation. ROS have a major role in mediation of cellular damage. ROS can be generated in the electron transport chain, xanthine and other flavoprotein oxidases, auto-oxidation of catecholamines, thiols, intracellular xenobiotics, haemoglobin and NAD(P)H oxidase. In normal cell there is a balance between pro- and anti-oxidant pathways. Upon stress stimuli an imbalance in redox system develops that leads to accumulation of ROS. ROS may induce damage to cell by oxidizing the membrane lipids, proteins and DNA. The overproduction of ROS is associated with many forms of apoptosis and necrosis. ROS-induced apoptosis is associated with up-regulation of Fas death receptor. Anti-apoptotic protein Bcl-2 prevents generation of ROS.

Small Hsp27 and Hsp70 appear to be protective agents against oxidative stimuli, by elevating reduced glutathione level, or stimulating glucose-6-phosphate dehydrogenase activity, or inhibiting lipid peroxidation.

Nitric oxide (NO) is an important signalling molecule regulating a number of diverse physiological processes and is produced by nitric oxide synthases (NOS). There are three types of NOS in the cell: neuronal, endothelial and inducible. NO inhibits apoptosis, through up-regulation of survival kinases, and inhibition of caspase-3.

2.4.5 Endoplasmic reticulum

The ER plays important function in intracellular calcium homeostasis. Conditions leading to alteration of ER intraluminal oxidative status can also induce stress. Participation of ER in induction and progression of apoptosis involve the disturbed Ca++ signalling and accumulation of unfolded proteins. Glucose regulated proteins (Grp) belong to the Hsp70 family and could be induced by ER stress. After translocation across ER membrane they act as apoptotic regulators by protecting the host cell against stress-induced death.

2.5 Effector molecules

2.5.1 Caspases

Caspases represent the family of proteases that hydrolyse proteins at aspartate residues. There are 14 types of caspases that are classified into 3 major groups, which are:

- initiator
- inflammatory
- effector caspases

The activation of caspases is organised through an apoptotic cascade pathway. The TNF-induced apoptosis involves activation of initiator caspases -8 and -10. The mitochondrial pathway involves initiator caspase -9 and effector caspases -3, -6.

Hsp27 inhibits mitochondrion - dependent caspase activation. The small Hsp α- and β- crystallines, inhibit both mitochondrial and death receptor pathways. Hsp-70 binds to caspase-3 and inhibits its activity.

2.5.2 Nucleases

There are various endonucleases expressed in the cell. The deoxyribonuclease (DNase) implicated in apoptosis is an Mg++ endonuclease called caspase-activated DNase (CAD). Hsc70, with its cofactor Hsp40 is involved in folding of CAD.

2.5.3 Transglutaminases

Tissue transglutaminase (TGase) is a member of a family of enzymes that catalyse protein cross-linking by transamidation. Transamidation has an important role in packing the cells in the tissue. At the late phase of apoptosis this protein cross-linking is important for preventing the massive inflammatory processes. TGase binds and hydrolyses ATP and GTP. The enzyme is inhibited by NO and is activated by increased intracellular Ca++ concentration. TGase expression is inversely correlated with the expression of...
antiapoptotic protein Bcl-2, and inhibition of TGases confers protection against apoptosis.

2.6 Heat shock proteins and caspase independent apoptosis

It has been shown that the signalling pathways are interrelated and that caspase-dependent pathways may interconverge with caspase-independent pathways in induction of apoptosis. The therapeutic use of Hsp modulation in anti-cancer protocols points to the importance of caspase-independent apoptotic pathways, which are predominant pathways of apoptosis in tumour cells. A number of enzymes and lipid molecules participate in the development of caspase-independent apoptosis.

Serine proteases are participating in amplification of apoptosis. Granzyme, which is a serine protease, is an activator of caspasines, and activator of cytochrome c release. The surface-expressed Hsp70 mediates the apoptosis of tumour cells by binding of granzymes. Cathepsins are a class of proteolytic enzymes involving 3 major groups: cysteine proteases, aspartyl proteases and serine proteases (B, C, L, H, K, S, and O). The enzymes are of lysosomal origin and are involved in peptide formation and protein degradation. They are involved in autophagy-associated apoptosis and oxidative stress-induced apoptosis. In tumour cells cathepsin-B is the most important mediator of cell death.

The Hsp70/Hsp90 chaperone plays an important role in lysosomal proteolytic pathways. Hsc70 is involved in uptake of cytosolic proteins into the lysosomal lumen.

2.6.1 Calpains

Calpains are calcium-dependent proteases involved in cytoskeletal reorganisation and muscle protein degradation. The enzymes are heterodimers composed of small regulatory and large catalytic subunit. Calpains and caspasines often act in a synergistic way in promotion of apoptosis.

2.6.2 Ceramide induced apoptosis

Ceramide is a lipid mediator in induction of apoptosis. It activates stress activated protein kinase signalling pathway. Hsp70 protects cells from ceramide-induced apoptosis.

2.6.3 Apoptosis inducing factor

Apoptosis inducing factor (AIF) is a mediator of caspase-independent apoptosis. AIF translocates from mitochondria to both cytosol and nucleus. Bcl-2 and Hsp70 can inhibit AIF translocation.

2.6.4 Anoikis

Anoikis is a type of cell death where cells fail to find substratum and connection with other cells or extracellular matrix. The lack of integrin-mediated interactions with extracellular matrix induces apoptosis. It mainly occurs at epithelial cells and it assumes proper compartmental positioning in specialized structures. Failure of anoikis contributes substantially to tumour progression and facilitates metastasis. It is possible that cytoskeletal alterations and cell-matrix detachment could release death receptors leading to death domain induced apoptosis.

The phosphorylated form of Hsp27 helps the stability of integrin. It was shown that Hsp27 inhibit metastatic potential in melanoma cells.

2.6.5 Heat shock proteins and antiapoptotic mediators

Hsp are involved in negative regulation of pro-apoptotic pathways, but also in activation of anti-apoptotic mediators. Hsp 70 acts in helping Bcl-2 activation.

Apoptosis itself inhibits Hsp synthesis by down-regulating the respective transcription factor HSF-1. In that manner apoptosis stops one of the important surviving signals.

2.6.6 Molecular mechanisms of Hsp action

Hsp act as molecular chaperones preventing protein aggregation and promoting protein folding. Hsp function as oligomers and often form chaperone complexes with each other. The biological role of Hsp is mediated by their ability to interact with protein or polypeptide substrates. The peptide binding activity of Hsp 70 is mediated through interactions between its C-terminal peptide binding domain and hydrophobic residues exposed in unfolded substrates. Association of Hsp70 with its target peptides is further regulated by the activity of its N-terminal ATPase domain (Figure 2.)

Hsp may function in “passive mode” when they 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 ATP-dependent degradation of unfolded or misfolded proteins. The passive mode is typical during stress when cellular ATP level is low. The active mode prevails when cells have recovered and the ATP level is increased again. Many proteins, such as protein kinases and nuclear hormone receptors, require the continuous help of Hsp chaperone complex to keep them in activation competent state. However, Hsp have no priority or selection between substrates and hence the chaperone function is extended to pro-apoptotic factors, too. Hsp 60 promotes apoptosis by helping in the maturation of procaspase 3.

Figure 2. Molecular organisation and structure of Hsp70

2.6.7 Heat shock proteins and cellular homeostasis

Hsp have essentially a dual function in the cell. They are eliminating misfolded and damaged proteins produced by stress and other
insults. However, they also play a critical role in the maintenance of cellular homeostasis by continuously chaperoning of a number of cellular proteins.

- Redox homeostasis

Hsp act as antioxidants, and haem oxygenase is one of the Hsp members that are responsible for the production of antioxidants biliverdin and bilirubin. The redox state of the cell influences Hsp synthesis and a decreased glutathione level may lead to direct activation of HSF-1. On the contrary, strong oxidative agents block activation of HSF-1, and its binding to DNA. It has been shown that mild changes of redox homeostasis lead to activation of HSF-1. However, large changes cause HSF-1 inhibition.

- Cell organisation

Hsp help in stabilising the cytoskeleton and cytoarchitecture by direct interactions with cytoskeletal proteins. Inhibition of major cytoplasmic Hsp, Hsp90 leads to increased cellular lysis and disruption of cytoplasmic organisation. Small Hsp protect actin filaments and help cell survival in apoptosis.

2.7 Heat shock proteins as pharmacological targets in apoptosis modulation

2.7.1 Heat shock protein inhibition - an efficient way to induce apoptosis of tumour cells

Apoptosis in tumour cells

From the various mouse models and cultured cells it becomes evident that acquired resistance to apoptosis is hallmark of most, if not all, types of cancers. Although tumour cells are resistant to apoptosis, they are not completely devoid of death. Cell death in tumour cells is mostly associated with cellular senescence and mostly involves caspase-independent routes of apoptosis or necrosis. A tumour cell may escape from caspase-mediated apoptosis either by over expressing antiapoptotic proteins or by severe mutations in proapoptotic factors. The antiapoptotic Bcl-2 is known to be over-expressed in many tumours. In Hodgkin’s lymphoma, mutations of Fas receptor were found and caspase-8 is frequently mutated in neuroblastoma. Tumour cells also have ways to escape caspase-dependent apoptosis, by expressing survivin, an inhibitor of apoptosis. The survivin expression is associated with poor prognosis. Mutations in the tumour-suppressor p53 gene are one of the major mechanisms of the tumour escape from apoptosis. Hsp regulates the function of p53.

2.7.2 Heat shock proteins in tumour cells

It was found that members of the Hsp family, such as Hsp70, Hsp27, and Hsp90 are over-expressed in several tumour cells. It has been shown that Hsp90 can inhibit apoptosis by direct physical interaction with apoptotic molecules. There are numerous examples of Hsp involvement in tumorigenicity; Hsp27 is over-expressed in colon carcinoma cells, Hsp90 in prostate cancer and Hsp70 in breast tumours where it is found to be necessary for the progression. Hsp bind to caspases inhibiting their activation, but they are also efficient in blocking caspase independent apoptosis. These characteristics make inhibition of Hsp an efficient tool in inducing a cell-specific apoptosis. Depletion of Hsp70 and Hsp90 in tumour cells induces their apoptosis.

Aging and various degenerative diseases induce accumulation of damaged/misfolded proteins that are produced by the oxidative stress and proteotoxic insults. At the same time the essential chaperone functions of Hsp are also impaired. Increased demands of chaperone function may exceed the available chaperone capacity leading to imbalance of cellular homeostasis.

On the other hand tumours undergo facilitated evolution due to increased proliferation. Conventional antitumour therapies (chemotherapy, radiotherapy, hyperthermia) all induce Hsp in surviving cells. The over-expression of Hsp may help the accumulation of mutations in tumours, which can help their further progression to more aggressive types of malignant cells. Use of Hsp inhibitors may affect this process and release some of mutations that have been rescued by Hsp before.

2.7.3 Enforced apoptosis of tumour cells

Inhibitors of Hsp can suspend the Hsp-dependent block of both caspase-mediated and caspase-independent apoptosis of tumour cells. It is well known that Hsp are not selective in their chaperoning function. They assist in the folding of a variety of cellular proteins. Consequently, Hsp inhibitors will target a number of different molecules. That makes inhibition of Hsp potentially very effective in induction of tumour cells apoptosis.

Although there are efficient inhibitors of Hsp60 and Hsp70, targeting of Hsp90 represents a central attraction in Hsp-related tumour inhibition. Inhibition of Hsp90 induces apoptosis in various tumour cells and also leads to a defect in number of proliferative signals. The most important Hsp90 inhibitor is geldanamycin and its derivatives. Hsp90 inhibition leads to dissociation of various Hsp90 client proteins from chaperone complex and to their consecutive degradation by the proteasome. Some drugs interact with Hsp90, like cisplatin, taxol and the antibacterial, novobiocin, and influence its function.

It appears that applying low doses of Hsp90 inhibitors together with conventional chemotherapeutic represents an effective way to target various cancers. Cytoprotective effects of Hsp come from the inhibition of stress-induced apoptosis. Rescue from apoptosis may also be helpful in anticancer protocols, where bystander non-malignant cells are also damaged by the therapy.

At some point Hsp inhibitors may act as Hsp inducers. Hsp synthesis is regulated at transcriptional level by HSF-1. Hsp90 and Hsp70 have been shown to bind to HSF-1 and keep it repressed in the absence of stress. During stress, misfolded proteins occupy both chaperones, which results in dissociation, nuclear translocation and activation of HSF-1. Pharmacological Hsp inhibition may therefore paradoxically lead to an increase in their amount.

Increased Hsp may lead to tumour cell sensitisation against immune attacks, providing a simultaneous protection of bystander cells in various cancer therapies, such as chemotherapy, radiotherapy, hyperthermia etc. Tumour cells may express Hsp on their surface, which leads to their enhanced recognition by the natural killer cells of the native immune system, and a specific anti-tumour immunity may develop. Extracellular Hsp, released as a result of cell death and taken up by antigen-presenting cells...
through Hsp receptors, are involved in the cross presentation of chaperoned peptides.

Proteasome inhibitors up-regulate Hsp synthesis by increasing amounts of misfolded proteins that compete for Hsp with HSF-1. The level of various Hsp, as well as the amount of Hsp, which are not occupied by damaged, misfolded proteins, can be critical in cytoprotection and cell survival.

2.7.4 Therapeutic use of heat shock protein up regulation

A number of clinical applications can be derived from the general cytoprotective / antiapoptotic role of Hsp. It could be applied in cardioprotection, in cellular defence against stroke and in various neurodegenerative diseases, as well as, for the improvement of efficacy in tissue transplantation. Hsp induction eases the deleterious consequences of chronic diseases, such as diabetes, and conditions like Alzheimer’s, Parkinson’s or prion disease, where the accumulation of misfolded proteins is the major cause of neurodegeneration. These conditions may gain beneficial effects from the Hsp over expression.

Cell life and proliferation, as well as, cell death involves regulation through the dynamic conformational changes of a number of apoptotic molecules, involving various oligomerisation and autoactivation steps. These suggest an extensive need for molecular chaperones. Hsp are capable of assisting in all these processes. Their proapoptotic role is usually balanced, and very often overwhelm by their participation in cytoprotection. Therefore, finely tuned balance of Hsp function is a key point for regulation of cell death, or survival, and also for making switch between two forms of cell death, apoptosis and necrosis.

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