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Cellular Stress Responses

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1. Introduction

Cells encounter many internal and external stimuli, some of which may induce stresses, when they are a part of a normal tissue or when they grow in a culture. These stresses trigger responses which may change cellular responses to subsequent environmental signals or even cause cell death. Exposure to stress over time may cause the accumulation of damage to DNA, proteins and lipids. If not repaired, this will enhance susceptibility to aging associated illnesses, like neurodegenerative diseases, diabetes, heart diseases, etc., and to cancer. The cellular stress responses must be taken into account when the cells are used in cell therapies and in regenerative medicine.

The cellular stress response is a reaction to changes or fluctuations of extracellular conditions, which damage the structure and function of macromolecules (Kültz, 2003). Depending on the severity and duration of stress encountered, cells either re-establish cellular homeostasis to the former state or adopt an altered state in the new environment. Therefore, different stressors and different intensities of stresses trigger different cellular responses: (1) induce cell repair mechanisms; these use considerable amounts of available resources and often result in recovery of normal cells, (2) induce cell responses that result in temporary adaptation to some stressors, (3) induce autophagy or (4) trigger cell death. Some important mechanisms of cell adaptation to stress and their inducers will be described in this chapter.

1.1 Cell repair mechanisms

1.1.1 Altered gene expression

Stressors can damage intracellular macromolecules, including proteins, DNA, RNAs and lipids. The stress responses are mediated through induction of molecular chaperones (Buchberger et al., 2010), clearance of damaged macromolecules (Kroemer et al., 2010), growth arrest and changed gene expression patterns (Spriggs et al., 2010), etc. First we shall describe some examples of altered transcription.

1.1.1.1 Modulation of transcription upon stress

The changes in gene expression are often mediated by micro ribonucleic acids (miRNAs, reviewed in Leung and Sharp, 2010), which are short noncoding RNAs of about 22 nucleotides. There were about 950 miRNA discovered by April 2010, which could target about 60% of mammalian mRNAs. The stress responses modify the synthesis of miRNAs and the activities of miRNA-protein complexes, consequently the expression of their mRNA

targets. miRNAs bind to mRNAs and either accelerate the degradation or inhibit the translation of mRNA, i.e. modulate the stability and/or translational potential of their targets.

The transcription factor, transformation-related protein 53 (p53, chapter 1.1.3.2) regulates the expression of miRNA at the levels of transcription and processing. p53 was found to induce the transcription of the primary transcripts miR-34a, miR-34b and miR-34c upon DNA damage. These repress some target genes to promote growth arrest and apoptosis. The expression of p53 itself must be precisely regulated. A miRNA (miR-125b) was found, which keeps the expression of p53 low in humans. The repression of miR-125b is reversed upon DNA damage with the activation of p53 through a protein kinase cascade (Leung and Sharp, 2010).

The level of target gene repression depends on the relative concentrations of target genes and miRNAs. For example, MICA and MICB are extracellular ligands of an immune activating receptor (NKG2D) of natural killer cells (NK) and T cells. MICA and MICB are induced by several types of cells under stress, like heat shock, viral infection, oxidative stress and DNA-damage. Their translation is inhibited by miRNAs in normal cells, however, upon stress the transcription of MICA and MICB is upregulated, while the levels of appropriate inhibiting miRNAs remain unchanged. It seems that the levels of mRNA exceed the quantity that can be inhibited by miRNA; consequently MICA and MICB are expressed. Their binding to the receptor NKG2D, which is expressed on natural killer cells and T cells helps to eliminate the virus-infected cells. Interactions in the cells are more complex, as the level of miRNA-mediated repression depends also on the expression amount of other mRNAs of the transcriptome, which are targeted by the same miRNA. The outcome of miRNA repression depends also on interactions with other stress proteins that can modulate the activity of miRNA protein complexes, e.g. by inhibiting the access to target mRNA.

The subcellular location of miRNA can change as a consequence of stress. Most of miRNA are diffused in the cytoplasm; they have to associate with the member of Argonaute protein family for activity. After a maturation process at Argonaute protein, mature miRNA guide the Argonaute-containing complexes to target sites in mRNAs that are partially complementary to the miRNA sequence, and induce repression of gene expression at the level of mRNA stability or translation. Upon stress some of miRNAs, mRNAs and Argonaute are in stress granules.

Sometimes, miRNA can time the stress response. Timing is important in acute stress responses, such as during inflammation. Then nuclear factor-κB (NF-κB, chapter 1.1.3.3) upregulates the transcription of miRNAs along with other inflammatory responsive genes through a cascade of reactions in macrophages. RNAs are synthesized as pre-RNAs; all pre-RNAs, including miRNA, are synthesized in about 2 hours. The processed mature miRNA peak about 24 hours later; therefore, the action of miRNA is delayed.

1.1.1.2 Translational regulation of gene expression during stress

Nutrient stress, temperature shock, DNA damage and hypoxia can lead to changes in gene expression patterns caused by shutdown and reprogramming of protein synthesis through selective recruitment of ribosomes to mRNAs (Spriggs et al., 2010). This is regulated by elements in 5′ and 3′ untranslated regions of mRNAs, like internal ribosome entry segments, upstream open reading frames and miRNA target sites.

In eukaryotes, the initiation of translation is inhibited often by phosphorylation of the eukaryotic initiation factor 2 (eIF2), which is a part of the so called ternary complex. The

ternary complex is composed of eIF2, initiator tRNA (tRNAi) and GTP and is loaded onto a small ribosome subunit, which binds mRNA and recognizes the start of translation, codon AUG. This triggers the hydrolysis of GTP, uncoupling of tRNAi from eIF2, release of initiation factors and start of translation (Spriggs et al., 2010). In mammalian cells, four stress-related kinases phosphorylate eIF2, which lead to reduction in initiation codon recognition. A second mechanism for nonspecifically reducing levels of protein synthesis is by preventing recruitment of the translational machinery to the mRNA. This is done by interfering with m7G cap recognition, which is a modified base at the extreme 5′ end of the mRNA. mRNAs for stress response proteins evade global repression of translation by several mechanisms. For example, a cap-dependent recruitment of mRNA may be bypassed, by the internal ribosome entry sites (IRES). IRES were originally detected in viruses and are parts of RNA, which facilitate binding of mRNAs to 40S ribosomal subunits (Spriggs et al., 2010). This involves cofactors IRES trans-acting factors (ITAF). Changes in the abundance or activity of ITAFs influences the degree of IRES mediated translation; this is often used during stress conditions.

Heat shock factors (HSF) are inducible transcriptional regulators of genes encoding stress proteins, like molecular chaperones and others. HSF1 is the most important regulator of expression of heat shock proteins (Hsp, chapter 1.1.2.1) in vertebrates (Akerfelt et al., 2010). Hsp are molecular chaperones and among others assist proteins in folding or prevent and reverse protein missfolding and aggregation. HSF1 is converted from monomer to trimer in response to temperature shock and oxidative stress. Monomeric HSF1 is a phosphorylated protein and is interacting with Hsp90. On stress, HSF1 dissociates from Hsp90, which enables its trimerization and binding to heat shock elements of Hsp genes. To enable a versatile regulation, these processes are more complex. HSF1 interacts with different HSP at different phases of its activation cycle. Trimeric HSF1 is inactive when bound to multimeric chaperon complex composed of Hsp90, co-chaperone p23 and immunophilin FK506-binding protein 5 (FKBP52, also FKBP4, Akerfelt et al., 2010). Elevated levels of both, Hsp90 and Hsp70, prevent trimer formation of HSF1. Activated HSF1 can bind to Hsp70 and Hsp40. There seem to be activation-attenuation cycles of HSF1, during which there are extensive posttranslational modifications of HSF1, including acetylation, phosphorylation and sumoylation. HSF1 is phosphorylated also under non-stress conditions, while phosphorylation-mediated sumoylation of a Lys residue of the regulatory domain occurs on exposure to heat shock. SUMO stands for small ubiquitin-related modifier. This are small proteins that are covalently attached to and detached from other proteins to modify their function. While stress-induced sumoylation is rapid after the heat shock, acetylation is delayed, as it is regulated by the balance of acetylation by p300-CBP (CREB-binding protein) and deacetylation by nicotinamide adenine dinucleotide dependent histone deacetylase sirtuin 1, SIRT1. Increased expression of SIRT1 was reported to enhance and prolong the DNA-binding activity of HSF1 at the promoter of HSP70.1. Therefore SIRT1 maintains HSF1 in a state which enables DNA binding, while the acetylated form can not. Sirtuins are a family of NAD^+ (nicotinamide adenine dinucleotide) dependent histone deacetylases, which influence gene transcription, metabolism, DNA repair and organism life span (Majmundar et al., 2010). Sirtuins are sensors of the cellular redox state, as they respond to the changes of ratios of oxidized/reduced forms of NAD^+.

Other HSF, like HSF2 also bind to the promoters of HSP genes. Upon stress-induced transcription of HSP genes, both, HSF1 and HSF2 accumulate into nuclear stress bodies.

Nuclear stress bodies are thought to participate in rapid, transient, and global reprogramming of gene expression through different types of mechanisms including chromatin remodeling and trapping of transcription and splicing factors (Biamonti & Vourc'h, 2010).

1.1.2 Protein quality control and repair

Proteins have numerous functions in cells: enzymatic, transport, structural, for molecular recognition, signal transduction, etc. They are often damaged as a consequence of stress; even their normal biogenesis is an error-prone process and may lead to stress. For example, truncated polypeptides that result from incomplete translation, misfolded intermediates, and unassembled subunits of protein complexes have exposed hydrophobic regions, which may facilitate aggregation (Buchberger et al., 2010). Failure to clear aggregated proteins leads to cell stress common to many disorders, especially to neurodegenerative diseases. The environmental stress triggers can induce nonnative posttraslational modifications and damage proteins in other ways and consequently induce cell stress as well.

Some degree of protein damage occur normally in every cell, however, the extent of protein damage increases by adverse intrinsic and environmental conditions, like unbalanced protein synthesis, oxidative stress, metabolic stress, some environmental toxins and pollutants, elevated temperature, high-energy radiation, etc. To cope with considerable extent of protein damage, the damaged proteins are either repaired by molecular chaperones or degraded by the ubiquitin proteasome system or autophagy (Buchberger et al., 2010). Ubiquitin is a protein of 76 kDa, which marks the proteins for degradation by the protease, 26S proteasome. The attachment of ubiquitin (ubiquination or ubiquitinylation) is an ATP-consuming process by the cascade of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligase (E3). E2 and E3 enzymes cooperate with molecular chaperones. Polyubiquinated substrates are transferred to the proteasome for degradation. Aggregated proteins, which are degraded by selective autophagy, are also ubiquinated.

Some types of protein damage are prevalent in specific organelles of eukaryotic cells (Buchberger et al., 2010). The errors in glycosylation, disulphide bond formation and membrane insertion are more frequent in the endoplasmic reticulum (ER) and secretory pathway, while these proteins are generally thermodynamically more stable, therefore less sensitive to heat stress compared to cytosolic proteins.

1.1.2.1 Stress repair and stress response

The stress repair mechanisms enable cells to survive the stress induced damage. Chaperones are a family of proteins that assist the non-covalent folding or unfolding of proteins and the assembly or disassembly of multimeric macromolecular structures, but do not occur in these structures during their normal biological functions when these are in correctly folded state. There are different classes (families) of chaperones, which serve different functions, including folding of newly made proteins, the repair of the damage caused by misfolding, membrane transport, keeping the protein precursor in translocation-competent state, assistance in protein degradation, etc. Chaperons are efficient quality control components for proteins. Many chaperons were discovered as their expression was elevated after the heat shock, thus they were named heat shock proteins (Hsp). Chaperons of Hsp70 family interact with short extended peptide stretches of hydrophobic and basic amino acid residues

of unfolded, natively folded or aggregated proteins (Buchberger et al., 2010). The binding of Hsp70 prevents aggregation of these proteins and can even induce conformational changes. The rounds of Hsp70 binding and release to protein substrates can promote the disaggregation of proteins. This is enhanced by cooperation with Hsp100 family chaperones. The cells have several Hsp40 chaperones (J domain containing chaperones), which provide substrate specificity of Hsp70, stabilize Hsp70-substrate interactions and trigger ATP hydrolysis by Hsp70. Nucleotide exchange factors, like Hsp110 are also required for ADP release and rebuilding of ATP at Hsp70.

Chaperon Hsp90 is thought to bind and stabilize partially folded but inactive conformations of its substrates; some of the substrates may be recognized in extended conformations. It hydrolyzes ATP like Hsp70; it interacts with a large number of co-chaperones. Its substrates are proteins involved in signal transduction, like protein kinases and transcription factors (Buchberger et al., 2010). Chaperones Hsp90 and Hsp70 can cooperate; then they are physically coupled by co-chaperone Hop.

An Hsp60 family member in eukaryotic cytosol is TRiC (TCP-1 Ring Complex or chaperonin containing TCP-1; CCT). Like other Hsp60 family members, TRiC is a barrel shaped protein complex that encapsulates substrates into protected folding environment. Its substrats are subunits of oligomers, with beta sheet secondary structures (Buchberger et al., 2010) and possibly late folding intermediates or misfolded proteins. There are also other small heat shock protein family members in the cytosol. ATP-independent small heat shock proteins (sHsp) bind misfolded proteins to prevent their aggregation and may loosen aggregates by coagreggation therefore facilitating subsequent re-folding by Hsp70. Hsp70 and Hsp40 present aggregated proteins to Hsp100. The solubilized protein can re-enter chaperone-mediated folding cycles or degradation by ubiquitin-proteasome system; this is implied by the presence of ubiquinated proteins within aggregates, colocalization of 26S proteasome, and the increased aggregate formation and delayed removal of aggregates upon inhibition of proteasome. However, ubiquitin proteasome system is not the only pathway for aggregate removal, especially as 26S proteasome can become inhibited by the aggregate formation. Irreversibly aggregated proteins in aggresomes are degraded primarily by selective autophagy. This autophagy requires ubiquitin-binding proteins; perhaps ubiquination is important for this degradation as well.

Stressed cells attempt to repair or degrade acutely damaged proteins and may undergo adaptive responses to reduce protein damage by decreasing global protein translation and increasing the molecular chaperones and proteins of proteolytic system. The well known adaptive responses in eukaryotic cells are the heat shock response (HSR) and unfolded protein response, which is the response to an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (UPR, Buchberger et al., 2010). Expression of genes, which are upregulated by HSR, are under the control of HSF1 (chapter 1.1.1.2, Akerfelt et al., 2010). Under non stress conditions, the monomeric HSF1 is in the cytosol bound by Hsp90, perhaps also Hsp70/40. These chaperones are tugged away under heat stress, which enables trimerization of HSF1, the activation of HSF1 trimer and expression of HSR target genes.

The ability to respond to ER stress is critical for cell survival, as chronic or unresolved ER stress can lead to apoptosis. Increases of protein synthesis or protein missfolding rates that exceed the capacity of chaperones, changes in calcium concentration in the ER lumen, oxidative stress and disturbances of the redox balance in the ER lumen contribute to development of ER stress (Tabas & Ron, 2011). In eukaryotic cells, the ER stress is sensed by

three upstream signaling proteins that start cascades of corrective reactions. The activities of these three pathways are collectively called unfolded protein response. All ER stress response pathways are activated when there is imbalance of unfolded proteins and chaperones. The evolutionary oldest pathway is triggered by activation of IRE1 (inositol-requiring protein-1). In mammalian cells there are two isoforms: IRE1α in all cells, and IRE1β in gastrointestinal and respiratory tracts. IRE1 is likely activated by dissociation of BiP and binding of unfolded proteins. ER stress induces the dimerization of the IRE1 luminal domains and positions the cytosolic domains close for trans-autophosphorylation of the kinase activation loop. Phosphorylated IRE1 is the specific endonuclease which cleaves the mRNA of X-box binding protein 1 (XBP-1), which is then translated into a transcription factor, which induces the expression of many genes involved in UPR. The second pathway of UPR is initiated by activation of kinase PERK (protein kinase RNA (PKR)-like ER kinase). Similarly to IRE1, PERK is autophosphorylated and undergo homomultimerization upon stress. Then it phosphorylates the α-subunit of the translation initiation factor eIF2 (eukaryotic translation initiation factor-2), which results in attenuation of global translation initiation. Translation of the gene encoding the transcription factor ATF4 (activating transcription factor-4) is favored by limiting amounts of eIF2. It enables the expression of CHOP (C/EBP-homologous protein, GADD153, gene name Ddit3), which through interaction with other transcriptional regulators induces and suppresses numerous genes, which repair the ER stress. CHOP induces also the transcription of GADD34 (growth arrest and DNA damage-inducible protein-34), which dephosphorylates phosphorylated eIF2α and restores global protein translation.

A third pathway is mediated through transcriptional factor ATF6 (activating transcription factor-6, Buchberger et al., 2010). In unstressed cells ATF6 is bound to BiP in the ER. Upon ER stress, BiP dissociates to allow transport of ATF6 to Golgi, where it is processed and its fragment is released to cytosol for the subsequent activation of genes in the nucleus. The three pathways can be activated by any type of ER stress; there is different timing of activation (Tabas & Ron, 2011). IRE1, ATF6 and PERK pathways are sequentially activated upon the prolonged ER stress. Prolonged IRE1 and CHOP activation can trigger apoptosis.

Molecular chaperones seem to have also other biological functions, which are not connected with protein folding (Henderson, 2010). Such proteins are called moonlighting proteins. Based on the extreme sequence conservation between the chaperone members within families, the researchers have long assumed that they have the same functions. However, it appears that they may have different moonlighting activities despite of close sequence similarities. Some molecular chaperones act as receptors in the plasma membrane and have signaling functions in the extracellular fluid. The latter may be important in stress response. Circulating molecular chaperones may be a danger signal, as stress is a danger to all organisms and needs to be integrated into the homeostatic regulation.

1.1.3 Signaling pathways

1.1.3.1 mTOR pathway

Protein mTOR (mammalian target of rapamycin, also FK506 binding protein 12-rapamycin associated protein 1; FRAP1) is a serine/threonine protein kinase, which regulates growth by maintaining the balance between anabolic processes, like macromolecular synthesis, and catabolic processes, such as autophagy. It is involved in cell cycle progression, DNA

recombination, and DNA damage detection. mTOR functions in regulatory pathways that control ribosome biogenesis and cell growth when nutrient concentrations change (particularly the levels of essential amino acids), like in a hypoxic environment of solid tumors (OMIM, 2011).
mTOR is the catalytic subunit of two complexes, mTORC1 and mTORC2 (Sengupta et al., 2010). mTORC1 is a homodimer, composed of mTOR and regulatory-associated protein of mTOR (raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor). mTORC2 is composed of Rictor (RPTOR-independent companion of mTOR), mLST8, Deptor, mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1, also PRR5). Rictor is required for mTORC2 catalytic activity; it is proposed to recruit substrates to mTORC2.
The upstream signals that regulate the activity of mTORC1 are growth factors, amino acids, glucose, oxygen levels (Sengupta et al., 2010). The downstream actions of mTORC1 include protein synthesis, autophagy and many metabolic pathways. mTORC1 is a critical mediator of the cellular response to many types of stress, such as DNA damage, drops in the level of energy, oxygen, amino acids and glucose. It is therefore involved in many stress responses in physiological and pathophysiological states, possibly in aiding the resistance of tumor cells to conventional therapy.

1.1.3.2 p53

Transformation-related protein 53 (p53) is a transcription factor, which responds to many types of cellular stress, such as DNA damage, hypoxia and oncogene activation. It regulates target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair or changes in metabolism (Speidel, 2010). In addition to its functions as a transcription factor in the nuclei, cytoplasmic p53 has transcription independent activities. Both, transcription dependent and transcription independent activities of p53 may lead to apoptosis. There are accounts when transcription independent mechanisms are essential for the full apoptotic response (Speidel, 2010). p53 is also a BH3 domain protein and can interact with some members of BCL2 family (chapter 1.4.1.3). The apoptotic stimuli will induce transcription dependent and transcription independent activities of p53 in most cases; the two complement each other. A few connections of the two signaling pathways have been identified by demonstrating the roles of p53 transcription targets Puma, Mdm2, IGFBP1 in transcription-independent apoptosis.
p53 is kept inactive in unstressed cells. The concentration and activity of p53 are regulated mostly on the posttranslational level. The half life of p53 protein in normal, unstressed cells, is about 6 to 20 minutes, while it can be hours under stress. Post-translational modifications are selectively activated by different stress signals. p53 is then modified by phosphorylation, methylation, monoubiquination, sumoylation, neddylation and glycosylation (Ak & Levine, 2010). p53 is polyubiquinated under non-stressed conditions by mouse double minute 2 homolog (MDM-2, E3 ubiquitin ligase, chapter 1.1.2), which speeds up its degradation on the proteasome. On the other hand, MDM-2 is transcriptionally regulated by p53, so there is an autoregulatory loop. MDM-2 functions as a heterodimer with MDM4. There is another autoregulatory loop between MDM-2 and MDM-4, as MDM-2 ubiquinates MDM-4. Further, MDM-2/MDM-4 and MDM-2/p53 complexes are regulated by protein kinases CHK-1/2 and ATM (Ak & Levine, 2010). DNA damage activates the ATM kinase (ataxia-telangiectasia mutated gene), which inhibits MDM-2 and activates p53. The members of calcium

calmodulin kinase superfamily, CHK-1/2 kinases, phosphorylate MDM-2, which dissociates from MDM-4 resulting in increased levels of p53. The failure to polyubiquinate p53 results in its high intracellular levels. Ubiquitin proteases, HAUSP (herpesvirus-associated ubiquitin-specific protease, also ubiquitin-specific protease 7; USP7) and USP42 remove ubiquitin from p53 and stabilize it. Stress signal results in higher levels of p53, which leads to transcription of selected genes and cell cycle arrest, apoptosis or the cells loose the ability to divide (are senescent). Different stress signals result in different post-translational modifications of p53 and transcription of different sets of genes.

p53 regulates the expression of miRNA (chapter 1.1.1.1) at the levels of transcription and processing. Most p53 mutations found in cancers are in a domain required for miRNA processing and transcription (Leung & Sharp, 2010). p53 enhances the processing of a population of pre-miRNA in cancer cells. Therefore loss of p53 function in transcription and processing might contribute to tumor progression.

The interruption of ribosomal biogenesis during the cell replication cycle results in an increase in free ribosomal proteins; some of them bind to MDM-2 and inhibit its polyubiquination, which stabilizes and activates p53 (Ak & Levine, 2010). Mutations in some tumor suppressor genes, e.g. retinoblastoma protein, free the transcription factor E2F-1, which transcribes ARF tumor suppressor gene. ARF is transcribed from an alternative reading frame of the cyclin-dependent kinase inhibitor 2A gene (CDKN2A). ARF protein binds and inhibits MDM-2 and raises levels of p53 within the cells. Similarly, the mutations that activate oncogenes Ras and myc increase ARF levels. In this way ARF acts as tumor suppressor by initiating p53-dependent responses. Activity of p53 is ubiquitously lost in human cancer either by mutation of the p53 gene itself or by loss of cell signaling upstream or downstream of p53 (Toledo & Wahl, 2006).

p53 responds to the intrinsic stress. There is another transcription factor, nuclear factor-κB complex (NF-κB), which responds to extrinsic stress (chapter 1.1.3.3, K10). p53 and NF-κB cannot function at the same time in the same cell. On activation of one, the other is inactivated.

1.1.3.3 NF-κB

NF-κB has been detected in many cell types, which express cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins in health and in various disease states. It is activated by different stimuli, like cytokines, free radicals, ultraviolet irradiation, and bacterial or viral products. Inappropriate activation of NF-κB leeds to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and AIDS, while its persistent inhibition may result in apoptosis, inappropriate immune cell development, and delayed cell growth (Chen et al., 1999).

NF-kB is a complex; a heterodimer from the protein of Rel family (p65, Rel A, Rel B, c-Rel) and from the p50/p52 set of proteins (Ak & Levine, 2010). NF-κB is in the cytosol associated with the inhibitor IκB in the absence of stress signals. IκB is phosphorylated upon stress by IκB kinase (IKK), then ubiquinated and degraded by a proteasome. NF-κB shifts to the nucleus resulting in transcription of genes with NF-κB response elements. The activation of these genes results in cellular replication, inflammatory responses mediated by tumor necrosis factor, and cell survival signals. NF-κB transcribes the gene of IκB-α, which is one of its negative regulators. The result is the autoregulatory loop of the transcribed IκB-α with NF-κB, which alternatively activates and shuts down the transcription of IκB-α.

1.2 Adaptation to stress

Acclimation and physiological adjustments that lead to tolerance to a certain degree of stress is a well known and proved concept, which enables the organisms to survive in different environmental conditions. Some experimental proofs are emerging that the same concept is true at the cellular level; i.e. that the cells can adapt to environmental stress to a certain degree and this makes them more resilient to environmental stresses. In 2010 our group has shown that a mild stress can inhibit triggering of apoptosis through the intrinsic pathway (chapter 1.4.1.1) in the primary liver cells (hepatocytes; Nipic et al., 2010). This is the first evidence to our knowledge of apoptotic pathway shut down caused as a consequence of mild stress. The state of the cells after encountering the mild stress, was named preapoptotic cell stress response. This state is temporary, since the cells revert to a normal state in a few days in the absence of further stressors (Nipic et al., 2010, Banic et al., 2011). Despite the inactivation of apoptosis triggering by caspase-9 through the intrinsic pathway, apoptosis can be triggered when the inducer is strong enough, however, the apoptosis executing enzymes caspase-3 and -7 are activated to a lesser degree than when the cells are in the normal state. This seems like a mechanism of an adaptive response in the stressed cells.

Adaptations to sublethal stress, which result in greater stress tolerance, were observed also in mouse blastocysts, where applying hydrostatic pressure improved their survival after freezing and in suboptimal culture conditions (Pribenszky et al., 2005). Similar results were obtained also in bovine blastocysts (Pribenszky & Vajta, 2011). Also, signals from DNA damage can facilitate osmotic stress adaptation (Kültz, 2005).

The phenomenon that a sublethal stress induces a resistance to mild stress, was observed from bacteria to multicellular organisms and humans. Some cells have to tolerate large changes in the environment because of their position in the organism. The cells in papilla of mammalian kidney have to tolerate varying degrees of hyperosmotic stress during urine concentration; the degree of hyperosmolarity depends also on the organism's hydration state. These cells are adapted to ever changing environment. The proteomic approach was used to compare the expression in hyperosmotic renal papilla and an adjacent iso-osmotic region, the cortex (Gabert & Kültz, 2011). Of 1877 proteins that were common to both regions, there were 212 comparably overexpressed in the cortex and 80 proteins in the papilla. In response to tonicity changes in papilla, protein expression altered significantly, mainly that of metabolic enzymes, molecular chaperones, proteins involved in redox balance, transport and transcription. During antidiuresis 15 different proteins changed significantly, while 18 different proteins changed significantly during diuresis relatively to normally hydrated controls. Proteins significantly altered by diuretic state are structure proteins (actin, tubulin), signaling (Rho GDP dissociation inhibitor, abhydrolase domain-containing protein 14B), chaperones (Hsp beta-1, αB crystallin, T complex protein-1) and those with anti-oxidant functions (α-enolase, GAPDH, LDH). Therefore, many genes, which are commonly overexpressed in stress, have been identified to be induced as a result to environmental changes in the kidney papilla. The possible pathways of adaptation remain to be determined.

Discovering the mechanisms of adaptation to stress may prove tremendously important, also because it is conceivable that the cells, during the process of malignant transformation and the resulting tumor cells, undergo adaptations, which enable them to resist the signals that would trigger cell death in their normal state.

1.3 Autophagy

Autophagy is an intracellular lysosomal (vacuolar) degradation process characterized by the formation of double-membrane vesicles, autophagosomes, which sequester cytoplasm. It is involved in growth, survival, development and death of cells. Autophagy is a term used for several processes: (1) macroautophagy, (2) microautophagy and (3) chaperone-mediated autophagy (Funderburk et al., 2010). (1) In macroautophagy, the cytoplasmic material is engulfed by a double-membrane, which fuses subsequently to the lysosome. Sequestered material is indiscriminately removed. (2) Lysosomes engulf a portion of cytosol in microautophagy. (3) Chaperone-mediated autophagy is a way for removal of selected proteins. These proteins can be modified, e.g. ubiquinated. As macroautophagy is the most prevalent form, it is often referred to as autophagy; this term shall be used in such manner below. Autophagy starts by formation of an isolation membrane (phagophore) around the portion of cytosol. The membrane elongates and seals on itself to form a double membrane vacuoles autophagosomes. These then fuse with lysosomes where the entrapped components are degraded.

Autophagy is conserved among eukaryotic cells and occurs at a basal rate in most cells. It is a mechanism for quality control to eliminate protein aggregates and damaged organelles, like mitochondria (Scarlatti et al., 2009). During cell stress, autophagy is a process through which the cells can reuse the resources. For example, the starvation-induced autophagy helps to recycle the amino acids for protein synthesis and produce the substrates for oxidative phosphorylation when the supplies of nutrients are limited. Degradation of whole regions of cytoplasm therefore generates free amino acids, which can be metabolized to meet energy demand during periods of stress. Autophagy may be the last attempt to rescue the cells from dying. As the intracellular processes are complex and intertwined, autophagy may have a role in some cases of cell death, too. While this was shown during the development of *Drosophilla* and *Dictyostelium discoideum*, there is no evidence for such process in mammals so far. However, autophagy can be involved in cell death in cultured mammalian cells and can occur upstream of, alongside to or during the final stages of apoptosis. It is often challenging to determine the role of autophagy in cell death, as observing autophagic structures is not sufficient to demonstrate the involvement of autophagy in cell death.

Apoptosis and autophagy may be co-regulated in the same directions, as the anti-apoptotic Bcl-2 and Bcl-xL proteins negatively regulate autophagy by binding to Beclin 1 (mammalian Atg6, see below), and pro-apoptotic BH3-only proteins may reverse this effect by displacing these interactions. Apoptosis can also suppress autophagy (Luo & Rubinsztein, 2010). The interplay between autophagy and apoptosis is currently a fast developing field of research.

The signaling of autophagy is mainly through serine/threonine kinase mTOR (chapter 1.1.3.1). Amino acid deprivation inhibits mTORC1, which leads to induction of autophagy (Sengupta et al., 2010). The autophagic process liberates amino acids, which reactivate mTORC1 and the replacement of lysosomes consumed during autophagy. Another main regulator of autophagy is a protein Beclin 1 (Bcl-2-interacting protein, reviewed in Scarlatti et al., 2009, Funderburk et al., 2010). Beclin 1 was discovered first as the interacting partner of the anti-apoptotic protein Bcl-2 through its BH3 domain (chapter 1.4.1.3). Subsequent studies have indicated that the endogenous Bcl-2 regulates Beclin 1. Also, it was found out that Beclin 1 interacts with many different proteins; however, the physiological roles of many of these interactions are not fully understood. In mammals, the core autophagy complex is thought to form by binding of Beclin 1 to the class III phosphatidylinositol 3-kinase VPS34 and protein VPS15 (Funderburk et al., 2010). This is based also on the

similarity with the autophagy complex of its yeast counterparts Atg6 and Vps34. The association of Beclin 1 with additional sets of proteins implies that Beclin 1 may be a connection point between autophagy, endocytic and cell death pathways. Elucidating its diverse roles may uncover its involvement in heart disease, pathogen infection, development and neurodegeneration.

1.4 Cell death

There are many pathways for a cell to die; the best known are necrosis, apoptosis and in some circumstances autophagy.

1.4.1 Apoptosis

The term apoptosis was first used by Kerr and coworkers (1972) to describe a cell death with several of typical morphological manifestations (Kroemer et al., 2009): rounding up of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, little or no ultrastructural modifications of organelles, plasma membrane blebbing, maintenance of plasma membrane integrity until the final stages of the process, phagocytosis of remains of the cells. There is no inflammation in tissue as the consequence of apoptosis (Savill & Fadok, 2000; Kurosaka et al., 2003).

There are biochemical and functional heterogeneities, as apoptosis is triggered through different biochemical pathways. The cysteine proteases caspases are central to triggering apoptosis (caspase-dependent triggering), although the apoptosis triggering can be caspase-independent, too. These and some of the main apoptosis regulators shall be described in the following chapters.

1.4.1.1 Caspases

The term caspases (cysteine-dependent aspartate-specific protease) describes a family of proteolytic enzymes with cystein in the active site, which cleave the substrates after the aspartate residue (Denault & Salvesen, 2002). Caspases -1, - 4, -5, -11 and -12 are important in development of cytokines, like interleukines 1ß and 18. The caspases -2, -3, -6, -7, -8, -9 and -10 are important in apoptosis signaling. In normal cells, they are inactive zymogens (procaspases), which are activated through dimerization and proteolytic cleavage upon the apoptotic stimuli (Denault & Salvesen, 2002). Caspases can trigger and execute apoptosis through cascades of reactions. Caspases -2, -8, -9 and -10 are initiating caspases, i.e. activate the executioner caspases, -3, -6 and -7. These activate other proteins, whose action result in apoptosis morphology.

There are several molecular pathways of triggering apoptosis; however the central two involve proteases caspases. These are (1) the extrinsic pathway, which originates from the cell surface, and involves activation of caspase-8 and (2) the intrinsic or mitochondrial pathway originating through the activation of caspase-9 (Salvesen & Dixit, 1997). The two pathways converge to activate caspase-3, which is the best understood executioner caspase. The extrinsic pathway is important in immune responses. The intrinsic or mitochondrial pathway can be activated by many stress stimuli, including DNA damage or extensive perturbation of mitochondrial membrane potential. It is also activated through caspase-3; it then enhances apoptosis.

Although not all caspases are involved in the regulation of apoptosis, the overexpression of any one of them culminates in cell death (Norberg et al., 2010). Knockout of any of caspases results in higher cell numbers.

1.4.1.2 Caspase – independent pathways

Many apoptosis regulators are associated with mitochondrial membranes and are released into the cytosol upon apoptotic stimuli. One of the better known pathways of caspase-independent triggering of apoptosis involves apoptosis-inducing factor (AIF, Norberg et al., 2010). It is anchored with its N-terminal into the mitochondrial inner membrane. AIF is triggered by increased calcium or early lysosomal permeabilization; these are frequent in cell death signalling after ischaemia/reperfusion injury and treatment with cytotoxic drugs. AIF can be cleaved by calpain I, which has a mitochondrial localization signal and needs μM amounts of Ca^{2+} for activation. Then cleaved AIF is released from mitochondria upon the mitochondrial permeabilization. It is transported into the nucleus, where it contributes to a large scale DNA fragmentation and chromatin condensation. Several studies detected that antioxidants can inhibit AIF-induced cell death and intracellular ROS levels may regulate AIF cleavage and release.

AIF has to be cleaved from the membrane in order to be released from mitochondria. Unless it is cleaved before the permeabilization of the mitochondrial outer membrane, it would be reasonable to expect that other soluble mitochondrial proteins, e.g. cytochrome c, would be released first and would activate apoptosis through caspase-dependent mechanisms (Norberg et al., 2010). The elimination of AIF does not protect the cells from apoptosis induced by most drugs.

1.4.1.3 Apoptosis regulators - BCL2 family proteins

The proteins of BCL2 family (B cell lymphoma-2) are important regulators of apoptosis; some of them are pro-apoptotic, others are anti-apoptotic. All family members share characteristic BCL2 homology domains (BH). The anti-apoptotic members, like BCL-2 and BCL-xl (BCL-2-related gene, long isoform) have four BH domains (BH1-BH4). Some pro-apoptotic BCL2 members have three (effector domains), others one BH domains (BH3-only proteins). BAX (BCL-2-associated x protein) and BAK (BCL-2 antagonist killer 1) have three BH domains (BH1-BH3), while e.g. BID (BCL-2-interacting domain death agonist), BIM (BCL-2-interacting mediator of cell death), Puma (p53-upregulated modulator of apoptosis) and Noxa have a single BH domain (BH3). Anti-apoptotic BCL2 proteins control the integrity of the mitochondrial outer membrane in mammals by inhibiting pro-apoptotic members (Chipuk et al., 2010). Upon activation, the effector pro-apoptotic proteins homo-oligomerize into pores in the outer mitochondrial membrane and promote its permeabilization. This releases apoptosis regulators, among others cytochrome c, which binds to APAF-1 (apoptotic protease activating factor-1) and procaspase-9 in the cytosol to oligomerize and form a complex named apoptosome. This activates procaspase-9, therefore turns on the intrinsic apoptotic pathway (chapter 1.4.1.1).

The BH3-only proteins are activated in response to cellular stress. Some of them (at least BID and BIM) are called direct activators, as they can promote the oligomerization of BAX and BAK. For example, the stress activated BID may be sequestered by an anti-apoptotic member, which prevents apoptosis. In the case of the further stress, other BH3-only protein can replace the BID, so it may activate BAX or BAK. Cellular stress can cause also transcriptional regulation of the BCL-2 family. The newly synthesized BH3-only proteins can interact with anti-apoptotic proteins and lower the threshold for BAK and BAX activation. For example, if BCL-2 is associated with PUMA, any future induction of BIM is not inhibited and results in the permeabilization of mitochondrial outer membrane.

Therefore the progression of stress to apoptosis is determined through complex interactions between the BCL-2 family proteins.

1.5 Cellular stress responses in regenerative medicine

As it was described above, the cellular responses to stress result in reparation of damage, cell death or in adaptation to mild stress that prevents excessive apoptosis. The processes of cellular adaptation to stress, i.e. the acquired resilience of cells to apoptosis by mild stress may be of value in regenerative medicine. There are indications that modulation of stress mechanisms is useful to improve the outcome of transplantations. For example, cold ischaemia pretreatment correlates with increased regeneration of epithelial cells immediately after the transplantation of kidney allografts (Naesens, 2011). Also, the adaptations to increased hydrostatic pressure improve the survival of murine and bovine blastocysts after freezing or in suboptimal culture conditions (Pribenszky & Vajta, 2011, chapter 1.2). On the other hand, it was observed that deterioration of mechanisms of cellular adaptation to stress result in lesser survival of kidney grafts. Old donor age decreases the chances of successful kidney transplantation (Naesens, 2011). Namely, many processes associated with aging are general pathways involved in tissue damage and stress responses; examples are the changes in mitochondrial physiology, increased susceptibility to apoptosis, impaired regeneration and repair, replicative senescence (when the cells become senescent as the result of breaks in DNA), etc.

There are examples of pre-treatments of cells to manipulate stress response pathways that minimise cellular damage and improve the transplantation outcome. Preconditioning of model cells of retinal pigment epithelium with non-lethal oxidative stress protects these cells from cell death induced by oxidative-stress (Sharma et al., 2009). The cell line ARPE-19 was used to mimick the conditions of oxidative stress, which is encountered by retinal cells transplanted for repairing the age-related macular degeneration.

The application of moderate shear stress on liver tissue slices was better than no shear or high shear stress, with the conclusion, that perioperative flow management is needed to regulate shear stress, i.e. to avoid the excessive shear stress on liver tissue upon a massive liver resection (Torii et al., 2005). Interestingly, the absence of shear stress also resulted in destruction of sinusoidal structures, which supports the need for constant perfusion of donor liver.

Targeting specific proteins involved in stress response was shown to increase the stress resistence of grafts in several cases. Increased synthesis of heme oxygenase-1, which is known to increase the cellular resistance against oxidative injury, improved liver graft viability in Lewis rats (Uchida et al., 2003). Other stress proteins, like heat shock proteins, are protective in models of transplantation; however, there is a need to develop strategies for their upregulation in clinical practice. Overexpression of heat shock protein 90-binding agent geldanamycin and some of its analogs protected renal cells from oxidative stress and reduced kidney ischaemia-reperfusion injury in a mouse model (Harrison et al., 2008). Rat mesenchymal stem cells engineered to overexpress Hsp20 were resistant to oxidative stress; this increased their survival after transplantation into infarcted heart by about twofold (Wang et al., 2009). A further example of targeting the stress responses is the protection of β cells by inhibition of iNOS (Hynes et al., 2011). Transplanted β cells fail because of specific autoimmune reactions and also due to non-specific inflammatory reactions. Proinflamatory cytokines, like interleukin 1β, can induce β cell destruction in a nitric oxide-dependent manner, as it stimulates inducible nitric oxide synthase iNOS, which produces NO cytotoxic

to β cells. Lentiviral-based strategy was used to inhibit iNOS expression through short hairpin interfering RNA; this improved protection of β cells (Hynes et al., 2011).
Pharmacological preconditioning may improve the survival of grafted cells as well. For example, trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine, TMZ) is a widely used anti-ischaemic drug for treating angina in cardiac patients. The stem cells were preconditioned with TMZ and used in an *in vivo* rat model of myocardial infarction (Wisel et al., 2009). A significant increase in the recovery of myocardial function and up-regulation of Akt and BCL-2 levels were observed in hearts transplanted with TMZ-preconditioned cells. Similarly, the treatment of ventromesencephalic grafts with the p53 inhibitor, pifithrin-α enhanced the survival of dopamine cell transplants and augmented behavioral recovery in Parkinsonian rats (Chou et al., 2011).
Clearly, using cells' ability to adapt to stress conditions or manipulating the stress response mechanisms to improve the cellular adaptation to stress betters the survival of cells and the transplantation outcome in experimental models. The challenge remaining is the finetuning of experimental techniques to suit the needs of regenerative medicine.

1.6 From triggers to consequences of stress responses

Any deviation from the ideal environment could be a stressor for cells, i.e. the stress is caused by too much or too little of an agent, stimulus or other environmental condition. Heat or cold, modification of pH, hyper- or hypo- osmolarity, the increased concentrations of reactive oxygen species, etc., all result in cellular stress.
Stressors can trigger two types of cellular responses, from within cells and by the immune system. The stress responses of non-immune cells, are described sometimes as an intrinsic stress, while the stress responses of the immune system are called extrinsic stress (Ak & Levine, 2010). The latter functions by signaling with cytokines and clonal selection of cells. The transcription of many of the genes that participate in immune response is regulated by NF-κB (chapter 1.1.3.3), which is a growth and division-promoting factor and can thus turn into an oncogene. The intrinsic stresses can be DNA damage, hypoxia, low levels of glucose and amino acids, interference with mitochondrial and ribosomal biogenesis, the action of some toxins, etc. The protein p53 responds to such stressors (chapter 1.1.3.2). p53 also regulates many genes that prevent DNA damage or help in the DNA repair. Hypoxia, glucose levels and mitochondrial and ribosomal biogenesis are regulated by the interactions of the p53 pathway genes with the insulin-like growth factor 1 (IGF-1)/mTOR pathways and the regulation of the endosomal compartment by p53-induced genes (Sengupta et al., 2010). The activation of p53 or NF-κB including pathways are mutually exclusive within the cells. The activation of p53 results in slowing glycolysis and restoring oxidative phosphorylation, while the activation of NF-κB pathway activates cell division and utilizes large amounts of glucose and predominant use of glycolysis (Ak & Levine, 2010).
Encountering the stressors is the normal consequence of living in a fluctuating environment, therefore, the cells have developed mechanisms to ameliorate the stress or to adapt to it. This is achieved through the repair of damage, adaptation, reuse of resources and a limited cell death. As living with stressors is unavoidable in the life of organisms and cells, does the stress matter? The cells have to divert at least some of their resources from other pathways, to deal with stressors, as it is described throughout this chapter. Our cells are well adapted to a mild stress for a short time, however, there are potentially serious consequences of the long term stress.

One of the recently established hallmarks of cancer is the presence of stress phenotypes (Leung & Sharp, 2010). The expression of microRNA is often aberrant in cancer; also, microRNA expression patterns often correlate with clinically relevant tumor characteristics. Several microRNAs regulate genes that control proliferation, apoptosis, differentiation, tumor invasion or tumor metastases, and are therefore directly involved in cancer initiation and progression. MicroRNAs are also important in regulating the development of immune cells and in modulating innate and adaptive immune responses (Stern-Ginossar et al., 2008).

The connection of prolonged stress to the development or deterioration of various pathologies is clearly seen in the consequences of aggregated proteins accumulation, which is a hallmark of many neurodegenerative diseases (Petrozzi et al., 2007). Cell stress and stress proteins have a profound effect in triggering/developing the cardiovascular diseases, too. It was first observed in 1970's that the patients infected with *Mycobacterium tuberculosis* or *M. leprae* have antibodies to an antigen, which was later identified as Hsp60 (chapter 1.1.2.1). It is now confirmed that Hsp60 and other chaperones, like Hsp10, Hsp70 and Hsp90 family members are strong imunogens and imunomodulators in experimental models of arthritis, diabetes and atherosclerosis (Shamaei-Tousi et al., 2007). The reason for this may be that many chaperones are potent activators of immune cells and may act as adjuvants and as immunogens, possibly as the latter in the case of Mycobacterial infection. Atherogenesis may be driven by crossreactive immunity to bacterial Hsp60 proteins. Namely, the host Hsp60 are expressed on the stressed endothelial cells. Endogenous chaperones may protect ischaemic myocardium. Hsp70 members inhibit some caspase-dependent and independent apoptosis. Hsp27 was shown also to inhibit cytochrome c-dependent activation of apoptosis and to stablize cytoskeletal structures. The major cytoskeletal lesions of myocardium may occur during ischemic injury, therefore, the stabilization of these structures could be important for cell survival (Haigs & Yankner, 2010).

Accumulation of damaged macromolecular structures over time was long recognized to lead to aging. The rate of aging and the appearance of age-related signs are modulated by stress responses (Haigs & Yankner, 2010). Central to it are protein and DNA damage repair mechanisms and mitochondrial respiratory metabolism. These are controlled by insulin/IGF-1, mTOR, sirtuin and AMPK (AMP-activated protein kinase) pathways. The coordinated action of these pathways are central to maintaining homeostasis in normal and stress conditions through the regulation of nutrient sensing and stress response pathways. There are many studies, which link the decline in the effectiveness and integration of stress responses to ageing and the development of age-related diseases. Modulation of mitochondrial and metabolic functions and mobilization of macromolecular maintenance and repair leads to life extension in modeling organisms.

Cellular stress can at least contribute to, or even trigger, many diseases and malignant transformations and has an important role in aging. To ameliorate or repair these processes the cells are being used in cell therapies and regenerative medicine. For maximal therapeutic success it is important to use the cells in the best condition possible or those adapted to stress as described in chapter 1.5. Discovering the detailed mechanisms of stress responses, some of which were described in this chapter, will improve the assessment of the condition of transplanted cells and to better their handling and transplant survival rates.

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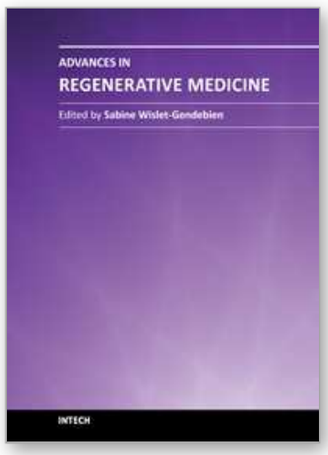

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Even if the origins of regenerative medicine can be found in Greek mythology, as attested by the story of Prometheus, the Greek god whose immortal liver was feasted on day after day by Zeus' eagle; many challenges persist in order to successfully regenerate lost cells, tissues or organs and rebuild all connections and functions. In this book, we will cover a few aspects of regenerative medicine highlighting major advances and remaining challenges in cellular therapy and tissue/organ engineering.

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