Apoptosis in Heart Failure

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Preservation of homeostasis in mature multicellular organisms and of growth during embryogenesis is achieved through the harmonious collaboration of various active processes, such as cell proliferation, diversification and programmed cell death. The latter is a process which requires energy, specific gene expression and corresponding RNA and protein synthesis.

Apoptosis is a strictly regulated process of programmed cell death.

Cell apoptosis plays an important role in thymus gland remission[1], withdrawal of certain "autoreactive" T-cells[2], control of the rejuvenation rate of certain mature cells, such as lymphocytes, control of aging, as in the regression of prostate, and in the regulation of many other important cell activities, including limiting the immune response.

During embryogenesis of the heart, apoptosis participates in the formation of the cardiac chambers and in the normal growth and development of large vessels, while it also contributes to the postembryonic growth of the conduction system[3].

The excessive or the insufficient function of the apoptotic mechanism are likely to lead to pathological conditions[4]. In the past, it was believed that the apoptotic process is not activated in diversified cells, like the cells of the myocardium and those of the nervous tissue. Recent studies, however, indicate that even in these diversified cells apoptosis takes place in response to stimuli such as ischemia and hypoxia, which under different circumstances would cause necrosis, and that apoptotic cell death plays an important role in myocardial infarction[5], heart failure[6,7] and arteriosclerosis[8].

Apoptosis is evident in the final stages of heart failure[9] of any cause. It still remains unknown however, if it is etiologically linked to the progress of the disease or if it is merely a concomitant finding related to the progressive dysfunction and remodelling of the left ventricle[10].

Morphological characteristics of apoptosis

As already mentioned, there are two types of cell death, apoptosis and necrosis.

Necrotic death is considered a random type of death due to excessive cell injury. Apoptotic death can be induced or programmed. A combined death type does not exist on the cellular level. On tissue level, however, it is possible, in case of increased cytotoxicity or in case of a large and extensive lesion (e.g. ischemia), for groups of necrotic and individual apoptotic cells to coexist.

In histological specimens taken during post-mortem examination of patients who died due to a myocardial infarction, using DNA agarose gel electrophoresis and immunohistochemical and nick end labelling methods, it has been observed that the apoptotic cells were dispersed among the normal myocardial cells in the periphery of the infacted area, while in the central zone, where necrosis of cardiomyocytes was evident, a mixture of necrosed cells and end-labelled apoptotic cells was observed.

Table 1. Morphological and biochemical differences between apoptosis and necrosis.

Apoptosis	Necrosis
Morphological features	
• Elimination of individual cells	• Death of cell groups
• Membrane integrity	• Loss of membrane integrity
• Shrinkage of cells with ultimate formation of apoptotic particles	• Cell swelling and destruction
• Non-inflammatory reaction	• Significant inflammatory response
• Phagocytosis by neighbouring cells and some macrophages	• Phagocytosis by macrophages
• Peripheral arrangement and condensing of chromatin and nuclear fragmentation	• Unclear clusters of chromatin
Biochemical characteristics	
• Caused by normal stimuli	• Caused by abnormal stimuli
• Fully regulated processing with synthesis and activation stages	• Loss of ion homeostasis regulation, does not require macromolecule synthesis
• Needs energy	• Does not need energy
• De novo gene transcription	• No new gene transcription
• Non-random internucleosomal DNA fragmentation	• Random DNA fragmentation

Cell apoptosis is histologically and biochemically different from necrosis[11-13], as shown in Table 1.

Biochemistry of apoptosis

In recent years we have found certain cystein proteases, which display a structure homologous to that of the Ced-3 protein of the nematode Caenorhabditis elegans, which plays a crucial role in programmed cell death[12,13].

These proteases, which belong to the ICE/Ced-3[14] (Interleukin converting enzyme/Cell death-3) family and are called *caspases*[15], are encountered in an inactive form and are proteolytically activated during apoptosis. These enzymes act on various nuclear substrates, such as RNA polymerase, lamines, topoisomerase I etc., which activate a limited proteolysis[16].

The functionally homologous protease granzyme B, responsible for the apoptosis caused by the cytotoxic T-cells, belongs to the same group of proteins.

The DNA fragmentation induced by the activity of endogenous DNAse[17] is also of crucial significance for cell apoptosis. Nuclear DNA is initially fragmented into large segments of 200-300 kb and consequently in smaller segments of 200bp among the nucleosomes.

According to current indications, DNAse, which is normally stored in the endoplasmic reticulum, is released due to an increased inflow of Ca^{2+} through the membranes of the endoplasmic reticulum, which can be caused by a reduction of protein Bcl-2 concentration[18].

Increased [Ca^{2+}] activates other enzymes that are involved in the apoptotic process as well, such as transglutaminase, which contributes to the alteration of apoptotic cell adhesion molecules by revealing immature glucans.

In addition, during apoptosis, a loss of asymmetry of the membrane phosphatides is caused by the presence of phosphatidylserine in the outer surface of the membrane. These membrane alterations help to distinguish the apoptotic cells from the phagocytes in order to achieve their prompt removal (Figure 1).

Methods for detection of apoptosis

Apoptosis is detected in tissue sections[19] with the use of electron microscopy for the identification of apoptotic particles. Nuclear staining with the fluorescent dye Hoechst 22358 allows for the visualisation of nuclear condensation and fragmentation in both cell culture and tissue sections. The most commonly used techniques to detect apoptosis are the following:

1. Apoptotic tissue DNA electrophoresis using agarose gel

This shows a characteristic ladder-like pattern due to the presence of multiple small internucleosomal DNA segments measuring 185b in length[20]. In cases where the DNA is not fully fragmented into internucleosomal segments but in larger segments with lengths of 50-300 kb, apoptosis detection can be achieved through DNA fragmentation using special

Figure 1. Biochemical and physiological changes during apoptosis.

types of electrophoresis (density-gradient and pulsed-field electrophoresis).

The disadvantage of this technique is the inability to determine the apoptotic process in a specific cell type when tissue samples with different cell populations are analysed.

2. In situ labelling of apoptotic cells technique

This allows for the identification of apoptotic cells with a high degree of sensitivity and specificity and is based on the attachment of the 3 ends of DNA internucleosomal fragments to labelled nucleotides with the help of external enzymes.

Two methods of in situ labelling have been developed, depending on the enzyme used:

- The **TUNEL method** (terminal deoxynucleotidyl transferase-mediated d-UTP nick end labelling)[20,21], which uses the enzyme terminal deoxynucleotidyl transferase (TdT DNA) to incorporate labelled nucleotides in the 3′-OH terminal ends of monoclonal or diclonal DNA segments and
- The **ICEL method** (in situ end labelling), which uses the Klenow fragment of E.coli DNA polymerase I to incorporate labelled nucleotides in the 3′-OH terminal ends of diclonal DNA segments with a protruding 5′-end.

The TUNEL method is superior in terms of sensitivity and specificity of detection.

3. Annexin V staining method

This method is based on induced phosphatide asymmetry and on the presence of phosphatidylserine on the outer leaflet of apoptotic cell membrane. Phosphatidylserine is bound to the anticoagulant annexin V[22], properly labelled with a fluorescent dye, allowing for the identification of apoptotic cells.

4. Western blotting for the detection of protein fragments

It is used to verify apoptotic cell death in cell culture models of apoptosis.

The previously described techniques are qualitative techniques and concern the detection of the apoptotic process. An ideal method, however, for the quantification of the apoptosis, would include not only the detection of apoptotic cells but also of the cells that have entered into an irreversible phase and are expected to become apoptotic.

Current research focuses on the signalling pathway of caspases, which are involved in the irreversible apoptotic processes. Activation of caspase-9 is necessary for the activation of caspase-3 in TNF-α induced apoptosis, while caspases -8 and -2 play an almost insignificant role. The activity of caspases is measured using chromogen peptides as substrates.

Factors and conditions responsible for the onset of the phenomenon of cell apoptosis in heart failure

Pathogenic stimuli, capable of leading to apoptosis of myocardial cells, include hypoxia, ischemia, inflammation, cytokines, growth factors and various other toxic factors.

According to one theory, the onset of apoptosis is caused by cell hypoxia[23]. Exposure of myocardial cells (in a culture) to hypoxic conditions has been shown to cause the activation of the compounds Raf-1 and MAPK (mitogen-activated protein kinase).

The MAPK enzyme is an important modulator of the proto-oncogene expression, like the genes *c-myc*[24,25], *c-jun* and *c-fos*, which are involved in the induction of the cell cycle evolution and in the phenomenon of apoptosis[26] (early genes). Scientific data support the view that reperfusion reduces the total extent of cellular apoptosis in ischemic myocardial regions, while it may also accelerate its onset in cells made non-viable due to a reperfusion lesion. It is well known that reperfusion is associated with a direct increase in the production of free radicals and with increased Ca levels in the intracellular space, conditions which are potential inducers of the phenomenon of apoptosis.

Various stimuli, including mechanical stress[27], pressure overload[28] and noradrenalin have been found to increase the rate of myocardial cell apoptosis *in vitro*.

Moreover, it is possible that oxidative stress[29], which is mediated by reactive oxygen species (ROS)[30] as oxygen free radicals, can cause the onset of programmed cell death, playing an important part in the pathogenesis of heart failure[31]. Major ROS include O_2^{-*} and OH^* radicals and non-radicals H_2O_2, HOCl and $ONOO^-$. There is a fragile balance between the formation of these ROS and the endogenous protective mechanisms, which may be inadequate due to disease, genetic defect or malnutrition or may be overcome by various stresses, such as radiation, environmental factors or iron overload, resulting in overproduction of ROS, which leads to damage of the nuclear and mitochondrial DNA (through the upregulation of *p53* gene), of the cell membrane lipids and of the intracellular proteins, which in turn leads to cellular death. In addition, proinflammatory stress-activated cytokines[32], such as TNF-α (tumor necrosis factor-α), IL-1 and IL-6, possibly play a role in the pathogenesis of congestive heart failure, during which there are increased levels of both cytokines and their receptors[33].

Nitric oxide (NO)[34] can have either beneficial or detrimental effects on the myocardium. While in low "normal" concentrations it offers protection against harmful stimuli, such as mechanical stress and noradrenalin, in higher "pathological" concentrations it stimulates the loss of myocytes[35]. It is argued that the induction of the nitrogen monoxide synthase (NOS) enzyme can play an important role in the activation of the apoptotic process in the context of myocardial infarction[36]. NOS induction takes place during the first 48 hours after ligation of the coronary arteries, persists for a period of 14 days and then begins to decrease. Thus, in the dysfunctional myocardium, high NO levels, produced by inducible NOS (iNOS), contribute to progressive myocardial failure by causing apoptosis[37].

Gene regulation of apoptosis

The most complete understanding for the involvement of gene mechanisms in the regulation of cell apoptosis was derived from the study of the nematode C. elegans. The discovery of the death genes *ced-3*[39] and *ced-4*, which participate in apoptosis in these organisms, has led to the discovery of their mammalian counterparts, in the protease family of the interleukin-1-β converting enzyme (ICE). Later it was discovered that the activity of the two aforementioned genes is controlled by another gene, *ced-9*[40]. The sequence analysis of this gene showed that it is homologous to the human oncogene *bcl-2*.

It suffices to cause the expression of ICE to activate the apoptotic mechanism in the mammalian cells. Until now approximately 10 members of this protease family[39] have been discovered and identified. These are called CASPASES (cysteine aspartic acid-specific proteases) because the substrates to which they react are common among them[15].
Caspases are distinguished into two subgroups:

- *Upstream caspases*, which appear to include essential regulatory regions and
- *Downstream caspases*; out of which, the ones sensitive to DEVD oligonucleotides (aspartate-glutamate-valine-aspartate), i.e. caspase-3 and caspase-7, are responsible for the lethal proteolytic breakdown of cellular target proteins, which include nuclear proteins, signalling mechanism proteins as well as cytoskeletal targets.

Caspases are separated by means of proteolytic cleavage from their precursor molecules and each of them separates key enzymes within the cells, such as interleukin 1-β poly (ADP) ribose polymerase (PARP)[41] which repairs DNA molecules that have sustained damage, as well as lamines, molecules that constitute the fibres of the nuclear membrane.

A molecular hierarchy has been suggested for these proteases, in which binding to the first ligand causes the initial proteolysis and activation (caspase-

8), subsequently triggering the proteases that lead to cleavage (caspase-3, -6, -7, -9) of molecules[42], thereby activating the whole mechanism of cell apoptosis.

• Bcl-2 gene family

The *bcl-2* gene protein (MW 25kd) is one of the key inhibitors of apoptosis[43] and it is located in the mitochondrial membrane[44], in the endoplasmic reticulum and the nuclear envelope. Gene transfer experiments have shown that the overexpression of Bcl-2 protein[45] protects many cell types from a large spectrum of apoptotic stimuli and therefore Bcl-2 protein is a modulator of a common final pathway of apoptotic cell death[25,46].

An overexpression of *bcl-2* in the myocardial cells[47] and in the vascular smooth muscle cells prevents *p53*-induced apoptosis.

Except for the *bcl-2* gene, an entire gene group was recently discovered that regulates apoptosis and shows a sequence homologous to that of *bcl-2*. The *bcl-2* genes[48] can be divided into two, functionally competitive, groups[49]:

a) Suppressors of cell death (*bcl-2, bcl-*X_L[50], *MCL-1* and *A1*) and

b) Promoters of cell death (*bax*[51], *bcl-*X_S, *bak*[52], *bad*[53] and *bik*).

All gene proteins of the *bcl-2* family contain well-conserved homologous domains[54], BH1, BH2, BH3 and BH4, which contribute to the formation of heterodimers[55] between pro and anti apoptotic family members or homodimers between molecules of the same protein[56]. Thus, the relative expression ratio of the *bcl-2* family members determines the degree of promotion or prevention of the cell death process[57].

Four principal mechanisms for the *bcl-2* mediated antiapoptotic effect have been proposed:

1. A direct antioxidant effect [58]
2. Inhibition of the release of apoptotic mitochondrial proteins[26]
3. Sequestration and/or modulation of the apoptotic ced-4 protein and its mammalian homologue Apa-1 and
4. Inhibition of a direct cytotoxic effect of the apoptotic regulators *bax* and *bak*.

• Myc, Max, Mad

The *c-myc* oncogene participates in the regulation of apoptosis mainly when combined with cellular proliferation[2,25].

Max and Mad proteins belong to a protein family related to *myc* and are characterised by a homologue region that allows specific binding to a certain DNA sequence as well as to each other.

Myc activity within the cell takes place through the formation of Myc/Max heterodimers[59]. *Myc* can activate cyclin D1 and indirectly cyclin A, which are components of the cyclin-dependent kinases (CDKs) that control the passage to the cell cycle. This is probably a result of Myc/Max direct activation of the CDC25A transcription, which in the absence of growth factors causes apoptosis[55].

Myc overexpression in cell series that have a reduced serum or growth factor concentration or combined with *p53* (which inhibits cell growth), results in interruption of cellular diversification and induction of extensive apoptosis.

• Fas / Apo-1

The Fas protein[60] is a receptor in the cell surface that structurally belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family. It functions as a receptor of the Fas ligand factor (FasL)[61], a 40kD protein that is produced by T-cells. The binding of the Fas protein[62] to the FasL receptor causes apoptosis of the cell that carries the Fas receptor[63].

• P53

p53 is a tumor suppressor gene that encodes a nuclear protein which regulates the transcriptional activation of genes responsible for DNA repairment, cellular division and apoptosis[64]. Expression of high levels of natural-type p53 protein controls cellular proliferation in cases of DNA structural alterations by causing either cell cycle arrest or apoptosis, depending on the size of the induced DNA structural damage and the cell type.

Cellular proliferation arrest is achieved through the overproduction of the, *p53* transcriptionally controlled, p21 protein, which forms compounds with the cyclin kinases and inhibits their action. In addition, *p53* is capable of 3′-5′ DNA extranucleotide activity, a Mg^{2+}-dependent process in the context of DNA repair during transcription, acting as a "proof reading enzyme" for DNA polymerases.

In the case of extensive and non-reparable DNA structural damage, the p53 protein, via an unknown mechanism, leads to cell apoptosis[65] in order to pro-

tect the DNA from accumulating and transmitting mutations to daughter cells [66]. Hypoxia promotes the *p53*-dependent apoptotic process[67]. When cell proliferation occurs as a result of a de-regulated expression of the adenoid oncoprotein EIA or of the *c-myc* oncogene, apoptosis results, except in cases where *bcl-2* or the adenoid homologue *E1B* intervenes. *p53*'s apoptotic effect has been linked to the *p53*-induced expression of Fas, Bax[68,69] and IGF binding protein-3[70].

Molecular mechanisms of apoptosis

• *Caspases' activation mechanisms*

According to recent data, caspases activation may be achieved either within the death receptor complexes of the cell membrane or by means of a mitochondrion-dependent mechanism in the cytoplasm.

1. Death Receptor Pathway

One of the best characterized pathways for the initiation of apoptosis involves the binding of extracellular death signal proteins[71], such as TNF-α, FasL, TRAIL[72] and Apo-3L[73] to their cognate cell surface receptors.

These death receptors contain a distinct cytoplasmic domain (~80 amino acids) that is critical for their apoptotic function, designated as the "death domain".

After binding of their cognate ligands, the death receptors form a homotrimeric complex and, by virtue of death domain-mediated protein-protein interactions, recruit intracellular adaptor proteins to the cell membrane. For example, the pathway that leads from the initial stimulus to the death of the cell, can be represented by TNFR1[74] (TNF-α receptor factor 1). As soon as binding to TNF or Fas is accomplished, some proteins, including TRADD (TNFR-associated death domain protein), TRAF2 (TNF-receptor associated factor 2) and FADD[75] (Fas-associated death domain protein) bind to the endoplasmic section of the receptor and participate in the apoptotic mechanism by activating FLICE[76] (full ICE) and eventually ICE. Consequently, induction of apoptosis by FasL and TNF-α depends on caspase activation[77].

Signalling induced by activation of TNFR1 diverges at the level of TRADD[78]. On the one hand, nuclear translocation of the transcription factor nuclear factor B (NFkB) and activation of c-Jun N-terminal kinase (JNK) are initiated.

On the other hand, TNF-α signalling is linked to the Fas signalling pathway through interaction of TRADD with FADD[75].

2. Stress conditions activator pathways

In contrast to the TNF factor and antiFas, stress conditions activators, which, however, do not cause DNA damage, as well as the ischemia-reperfusion and the ultraviolet C radiation, can cause apoptosis by activating the JNK pathway through a Rac-PAK-MEKK1[80]-SEK-JNK reaction cascade.

Factors that cause DNA damage, such as ionising radiation, ara-C or the drug cisplatin, can activate the JNK pathway, but then the substances involved in the specific reaction cascade are different and follow the ATM-Ab1-MEKK1[80]-SEK-JNK pathway.

The exact mechanism linking the JNK pathway to the apoptosis phenomenon is not yet clear[81].

3. Mitochondrial Pathway

It has also been suggested that mitochondria may play a role in the induction of apoptotic cell death[82].

Cytochrome c, one the mitochondrial respiratory chain proteins, is released into the cytoplasm before the onset of the apoptotic process under conditions of stress and binds to *bax* and *bcl-2*[83,84]. It has been proven that its release can activate proteases through Araf-1, which is a cloned mammalian homologue of the apoptotic factor ced-4 of C elegans[85].

A third protein participates in this pathway, Araf-3, which was recently shown to be identical to caspase-9.

Interaction between caspase-9 and Araf-1 depends on the presence of cytochrome c and dATP or ATP, showing that caspase activation through cytochrome c can be an energy-dependent process[86].

An alternative mechanism has been proposed involving the release of another mitochondrial protein, termed apoptosis-inducing factor (AIF), which has been shown to have proteolytic action. The release of AIF was shown to depend on the opening of the mitochondrial permeability transition pore that results in the breakdown of the proton and electron gradients over the inner mitochondrial membrane.

- ***Apoptosis inhibition mechanisms***

1. Inhibitor of apoptosis protein family

Four proteins have been identified which act as inhibitors of the activity of apoptosis proteins: X-linked IAP, neuronal IAP, c-IAP1, c-IAP2, and survivin[87].

c-IAP1 and c-IAP2[88] were shown to bind to TNF-α receptor associated factor 1 and 2 (TRAF1 and TRAF2) and thus can be recruited to the activated TNFR complex[89].

c-IAP2 was suggested to be involved in TNF-α mediated activation of the NFkB pathway that offers protection against apoptosis.

Direct inhibition of caspase activity was recently shown to be an alternative antiapoptotic mechanism of this class of proteins. More specifically, X-linked IAP, c-IAP1, and c-IAP2 were shown to interact with and inhibit down-stream caspases (caspase-3 and -7), thereby inhibiting apoptosis initiated either by receptor-mediated or by cytochrome c dependent mechanisms.

2. Inhibition of Receptor-Mediated Caspase Activation

As outlined above, initiation of apoptosis by death receptor ligands requires the recruitment of protons to the activated death receptors mediated through death domains and DEDs. Viral proteins were isolated that contain DEDs and promote cell survival when overexpressed in cells. Mammalian homologues of the viral inhibitors termed FLIP[91], I-FLICE, CASH and FLAME-1 show a high degree of homology to caspase-8 and -10, although the protease domain appears to be non-functional. A similar non-functional homologue of caspases-2 and -8 termed apoptosis repressor with caspase recruitment domain (ARC) appears to be selectively expressed in skeletal and cardiac muscle [92].

Most likely, this class of proteins competitively inhibits receptor-induced activation of upstream caspases.

3. Survival factors

Survival factors like interleukin-3 (IL-3), nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1) [93] protect cells from undergoing apoptotic cell death [94]. The cognate receptors belong to the family of protein tyrosine kinase receptors that are implicated in the activation of phosphatidylinositol-3 kinase (PI-3 kinase).

4. Paracrinal factors

According to experimental data, cardiomyocytes were shown to be protected from apoptotic cell death after serum deprivation by cardiotrophin-1 (CT-1).

CT-1, a new member of cytokines IL-6 / LIF (leukaemia inhibitory factor) that activate the down-stream regulation signals through the gp120-dependent pathways, can play an autocrinal role during cardiac development and morphogenesis, promoting survival and proliferation of immature cardiomyocytes and is responsible for adult cardiac hypertrophy[95].

CT-1, however, is at the same time an inhibitor of cardiac myocyte apoptosis via the activation of a MAP kinase-dependent antiapoptotic signalling pathway.

5. Heat-shock proteins

Heat-shock protein hsp27 is an oligomer phosphoprotein that participates in the signalling pathways of programmed cell death, enhancing cellular resistance against the tumor necrosis factor α (TNF-α)[96] by dramatic changes in its cellular position, structural organisation and phosphorylation.

It has been shown that hsp27 inhibits apoptosis. Its anti-apoptotic action is accompanied by a deceleration of cell proliferation[97] and is owed to its ability to cause a decrease of intracellular reactive oxygen species levels induced by TNF-α[98] (Table 2).

Pathophysiological basis of apoptosis in heart failure

To date, it is not clear how the phenomenon of apoptosis leads to ventricular remodelling. Despite the many laboratory and clinical scientific data, which support that the main factor of myocardial cell loss is their necrosis, another possible explanation is the phenomenon of apoptosis, especially in the case of early left ventricular dysfunction that is observed in heart failure.

The mechanisms responsible for the phenomenon of apoptosis remain unknown and we are limited to formulating a number of theories.

According to one theory, cellular hypoxia causes the onset of apoptosis[99,100], while another theory suspects the oxidative mechanism (stress) as the underlying cause[101,102], which induces the up-regulation of the p53 gene and may damage the DNA. Moreover,

Table 2. Apoptosis promoters and inhibitors in the cardiovascular system.

Promoters		Inhibitors
Factors	**Conditions**	
Cytokines:	Hypoxia	Bcl-2 protein
Tumor necrosis factor a (TNF-α)	Reperfusion damage	*bcl-2* group genes, suppressors of cell death: *bcl-X_L, MCL-1, A1*
IL-1, IL-6	Mechanical traction	Nuclear factor kB (NFkB)
Early response genes:	Pressure overload	X-linked IAP proteins, neuronal c-IAP1, c-IAP2, survivin
c-myc, c-jun, c-fos	Oxidative stress	FLIP, I-FLICE, CASH, FLAME-1 proteins
Proteins and peptides	Inflammation	ARC
Noradrenalin	Myocarditis	Survival factors: IL-3, NGF, IGF-1
NO	Transplant rejection	Cardiotrophin-1 (CT-1)
Fas protein	Inducible nitric oxide synthase (iNOS)	Heat-shock protein hsp27
bcl-2 group genes, promoters of cell death: *bax, bcl-$X_S$bak, bad, bik*		

Nakamura[103] claims that the remodelling of the peripheral vascular system can be implicated as a factor of left ventricular dysfunction and all its adverse effects. Studies, both on an experimental level for heart failure, as well as on a clinical level in patient hearts during transplantation have produced findings supporting myocardial cell apoptosis.

Cheng et al[27] studied in dogs the effect of mechanical traction in the induction of apoptosis following a varying degree of stress in the papillary muscle fibres, a condition that simulates the wall stress of chronic heart failure. They found that excessive stress of the papillary muscle fibres results in a significant increase of apoptotic myocardial cells compared to muscle fibres not subjected to increased stress and this was verified by the presence of fragmented DNA.

In another experimental group, an angiotensin-converting enzyme inhibitor (enalapril) and an angiotensin AT1 receptor antagonist (losartan) were administered, showing that apoptosis could be prevented with losartan but not with enalapril, which suggests active drug results in apoptosis that is induced by increased traction forces.

Moreover, Liu and coworkers[104], who also conducted experiments in dogs, induced heart failure after 4 weeks of accelerated ventricular pacing. This kind of heart failure induced programmed cell death at a rhythm greater than that of myocardial cell regeneration. Naturally, these papers should be investigated further to determine whether apoptosis observed in these cases plays a significant role in the pathophysiology of dilated cardiomyopathy or is just a concomitant secondary phenomenon.

Sharov and coworkers[105] induced heart failure in dogs using coronary microembolization. Their findings in the hearts of dogs concurred in favour of apoptosis, especially those found in regions adjacent to old infarcted myocardial tissue.

Finally, the apoptotic mechanism of myocardial cells was observed also in rats, which showed automatic arterial hypertension[106]. It should be noted that rats who had both arterial hypertension and heart failure, displayed a greater degree of myocardial cell apoptosis.

Nevertheless, the existence of the apoptotic phenomenon in experimental animals with heart failure gives researchers the opportunity to interfere in the cell apoptosis process in order to modify the disease progression.

In the clinical setting, Narula and coworkers[9] claim that the progressive deterioration of left ventricular function in patients with end-stage heart failure can be attributed to the phenomenon of apoptosis. They used as research material 7 patient hearts (4 with dilated cardiomyopathy and 3 with ischemic etiology) who had NYHA class IV heart failure and underwent a heart transplantation. Despite the fact that this study supports the view that cell apoptosis is one of the mechanisms that lead to end-stage heart failure, further studies are needed both in patient hearts removed during cardiac transplantation and in specimens from consecutive myocardial biopsies, in order to extract stronger conclusions with respect to the effects of apoptosis and the role it plays in the transition from myocardial hypertrophy to heart failure.

Olivetti et al[107] studied 36 patient hearts with end-stage heart failure, half with ischemic and half

with dilating etiology, which were removed during cardiac transplantation. These researchers found that myocardial cell apoptosis was 232 times increased in these hearts compared to control group hearts that did not have heart failure, while at the same time no correlation was found between the myocardial cells diameter and the degree of apoptosis.

James et al[108] refer to arrhythmogenic right ventricular dysplasia, which is accompanied by a high rate of sudden death and is characterised by a loss of myocardial cells that are progressively replaced by adipose and fibrous tissue.

Mallat et al[109] analysed specimens obtained at autopsy from 8 patients with right ventricular cardiomyopathy caused by arrhythmogenic dysplasia and detected apoptosis in the right ventricular myocardium in 75% of these patients while it was entirely absent in the healthy control group. Apoptosis was usually absent in myocardial regions where there was a mass replacement of cells by adipose and fibrous tissue. It should be noted that it is not yet known whether the accelerated rhythm of cell death through the apoptotic process, which takes place in arrhythmogenic right ventricular dysplasia, is due only to a pre-programmed gene defect or is associated to a certain extent with the damage caused by the consecutive subfatal events of ischemia and reperfusion, ensuing from the multiple ventricular dysrhythmia attacks.

Heart failure has been associated with alterations in the phenotype of myocardial cells, irrespectively of the lesion type, which seem to result from an effect of the neurohormonal system on the myocardial cells[110] on the one hand and of endogenous molecular mechanisms related to apoptosis[111] on the other hand.

Finally, heart failure is characterised by an accentuation of the activity of the sympathetic section of ANS and an increase in the levels of plasma norepinephrine. Overactivation of the adrenergic system can cause myocardial damage, probably due to the direct effect of norepinephrine on the myocardial cells. It has been mentioned that catecholamines induce the apoptotic phenomenon in the myocardium of newborn infants[112]. Both α- and β-adrenergic receptors[113-116] seem to contribute to the death of myocardial cells through the activation of complex biochemical pathways (Figure 2).

It was recently proven that β-receptor stimulation causes the activation of raf-1 and MAP kinase (mitogen activating protein), which has been associated with cellular hypertrophy and survival in myocardial cells. MAP kinase is needed for the inhibition of myocardial cells apoptosis in newborn infants[117].

In low output hemodynamic conditions the renin-angiotensin system is activated, resulting in an increase of serum angiotensin II levels. Two types of angiotensin II receptors have been described, type 1 (AT1) and type 2 (AT2). In neonatal cardiomyocytes, angiotensin II causes hypertrophy and induces several genes that are associated with early responses, such as *c-fos, c-jun and c-myc*[120]. It also increases the apoptotic rhythm by 5 times through the activation of protein kinase C, which is also linked to the increase of Ca in the cytoplasm[121]. In recent studies, blockade of the AT1 receptor inhibited the apoptosis caused by angiotensin[122], while blockade of the AT2 antagonist did not affect the phenomenon of apoptosis. AT2 may induce the apoptotic process by dephosphorylating MAP kinase[123], but its role in the myocardial apoptotic changes in end-stage heart failure is not yet clear.

Plasma levels of inflammatory cytokines, such as TNF-α, are increased in patients with heart failure[74]. TNF-α causes reduced contractility of myocardial cells and a decrease in intracellular Ca levels. In myocardial cells 2 different receptors for TNF-α are encountered: TNFR1 and TNFR2, TNFR1 ligands induce apoptosis. As mentioned previously, the TNF-α factor leads through FADD to the activation of NFkB, which is a potent inhibitor of apoptosis[124]. Therefore, TNF-α seems to have a dual role with respect to myocardial cell apoptosis that depends on the relative activation of various alternative pathways.

The circulation levels of both the atrial natriuretic peptide (ANP) and the brain natriuretic peptide (BNP) are increased in patients with end-stage heart failure. In isolated myocardial cells, normal concentrations of the atrial natriuretic peptide can activate the apoptosis phenomenon[125] through mediation of cGMP mechanisms.

Also, administration of atrial natriuretic peptide decreases the expression of *mcl-1*, a homologue of *bcl-2*, thus limiting the protective effect of the *bcl-2* pathway.

Cellular hypertrophy is characterised by the upregulation of genes and transcriptional factors which promote the development of the cell cycle. Since the myocardial cells are in postmitotic stage, the same stimuli that promote cell growth, instead of leading to cell division, may cause apoptosis. An induced

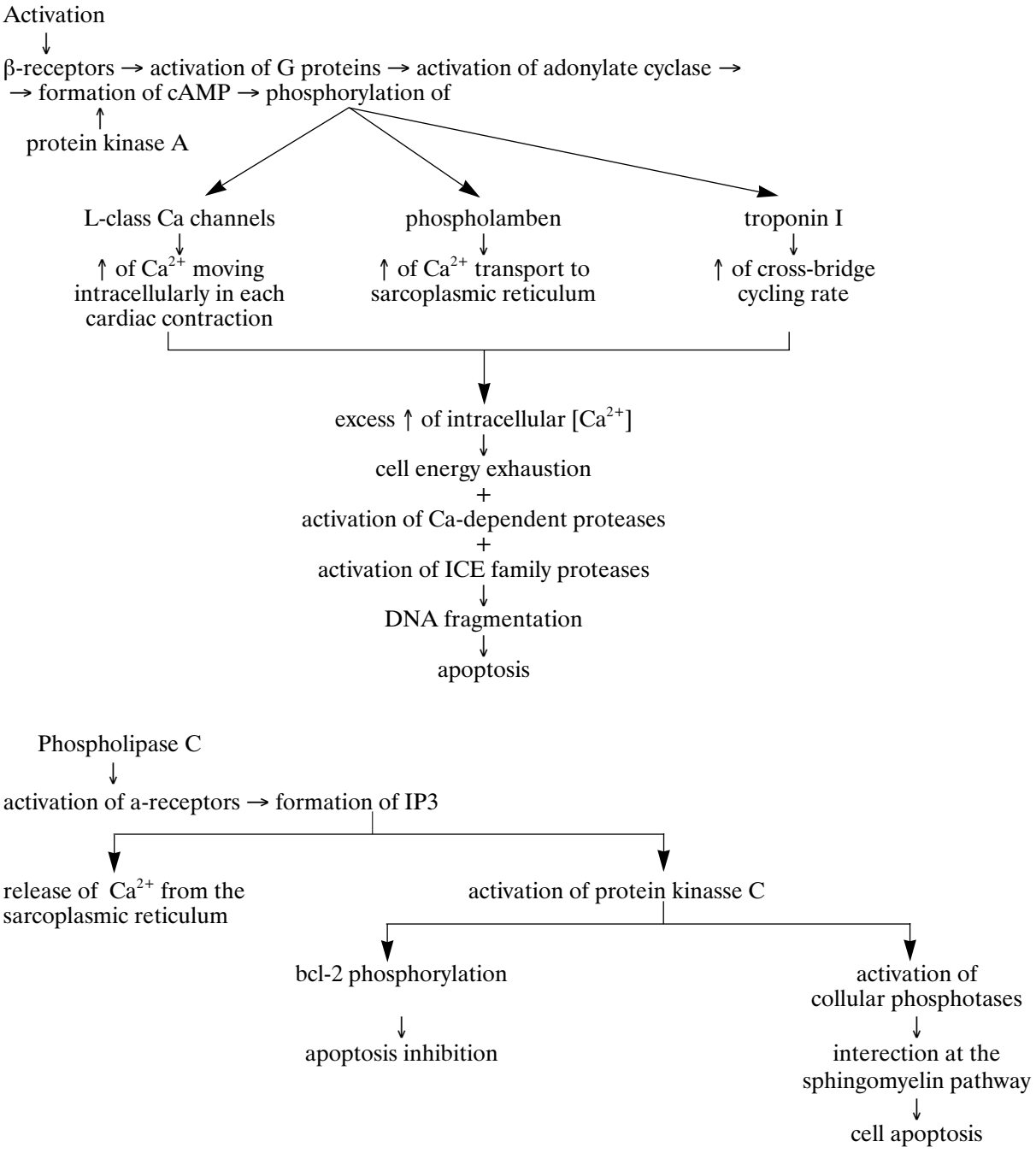

Figure 2. Norepinephrine regulation of myocardial cell apoptosis during the development of heart failure.

overexpression of E2F-1 transcriptional factor in myocardial cells led to diffuse cellular apoptosis[126].

Strategies for the prevention of apoptosis

The creation of experimental heart failure models, where cell apoptosis plays an important role, will allow, through the use of various interventions, to modify the pathways which result in cell death[127]. The in-depth understanding of myocardial cells apoptotic signalling pathways and the development of strategies[128] for their advantageous management may offer new therapeutic possibilities in the field of heart failure. It has been shown that IGF-1[129] prevents, through its receptor, apoptosis of myocardial cells[130] by inhibiting caspases-mediated intracellular pathways of DNA fragmentation. Growth hormone (GH) stimulates the production of IGF-1[131].

hGH reduces Fas-mediated cell death through the overexpression of the anti-apoptotic oncoprotein Bcl-2, which suppresses the activation of cystein protease caspase-3 that affects the cleavage of poly (ADP-ribose) polymerase[132]. It has been found that the inhibitory activity of GH in apoptosis is mediated by the activation of serine-threonine kinase Akt[133], as well as the transcriptional factor NFkB[134].

Also, cardiotrophin-1 (CT-1) inhibits the mechanism of cellular apoptosis.

Up-regulation of apoptosis inhibitors with the overexpression of Bcl-2, through adenovirus transfection, can also constitute a useful form of therapy.

The ability to prevent or even to attenuate the loss of myocardial cells following an acute myocardial infarction and in the early stages of heart failure represents a rational strategy that has as an ultimate objective the prevention of the pathological left ventricular remodeling[135].

Regular exercise can improve the maximum oxygen consumption and reduce the neurohormonal and adrenergic activation in patients with chronic heart failure. Further studies are needed to determine if exercise, combined with ACE inhibitors or b-blockers can revert or prevent left ventricular remodeling[136].

A more complete knowledge of the molecular mechanisms that are responsible for controlling apoptosis in the failing myocardium can instigate a shift of the therapeutic effort towards the myocardium itself. Thus, strategies aiming at preventing further loss of myocardial cells and at promoting their replacement by controlled cellular growth are expected to be scientifically validated.

Cellular apoptotic death through the activation of caspases and proteolytic cleavage of specific target proteins can be prevented by the use of direct inhibitors of caspases or other substances that target some components of the up-regulation of the caspase cascade[138]. Caspase-8 and -3 inhibitors against the TNF-α and Fas-induced cell apoptosis seem to block the activity of procaspase-3 and -9[139].

Also the regulation of NFkB activation probably at the level of caspase activation may play an important role in the TRAIL-induced apoptotic process[140].

IAPs proteins are endogenous suppressors of the final cascade of caspases, the most important of which being XIAP, whose expression control may be an extremely promising molecular target for the regulation of apoptosis through the inhibition of caspase-3[141,142].

The heat-shock proteins (hsp) protect the myocardium from ischemia and reperfusion, by means of endothelial and mechanical recovery, preservation of high-energy phosphorics and reduction of the infarct area. The search of alternative stimuli, due to the nonspecificity and intracellular harmful action of the heat shock, especially in the field of pharmacotherapy and genetic management, can provide vital options that will form an established strategy of myocardial protection[143].

Strategies aiming at crucial components of the apoptotic mechanism originate from the management of viruses. Studies show that infection with the vaccinia virus directly influences the mitochondrion-mediated apoptotic pathway by preventing the permeability transition pore opening[144].

The role of medication, aiming at the modulation of apoptosis, may in the future have important implications in the development of new therapeutic strategies for the heart failure syndrome.

ACE inhibitors allow prevention, delaying or reversal of progressive left ventricular dilatation and improve both its function and long-term survival[145]. It is speculated that this beneficial action of ACE inhibitors (captopril, ramipril, enalapril) is due not only to the inhibition of the production of angiotensin II but also to their ability to promote the activity of bradykinin. The fact that now there are specific inhibitors of angiotensin II AT1 receptors available provides an opportunity to further examine the mechanisms of ACE inhibitors' action.

Interest in the renin-angiotensin II (RAS) system began to develop after we observed that the stimuli leading to myocardial hypertrophy, including angiotensin II, are capable of activating several serine-threonine protein kinases, which, in turn, lead to phosphorylation of nuclear transcriptional factors, such as the *c-myc, c-fos, c-jun* genes, substances that participate in cell transformation, mitotic activity and programmed cell death. The activation of the MAPK protein can be directly induced by angiotensin II. It is possible that the regulation of angiotensin II activity in the process of reactive cell fibrosis development can act as a secondary prevention of the creation of hypoxia conditions at the level of myocardial cells[146]. It is still unknown whether the angiotensin receptor blockers can limit cell apoptosis. It is, however, possible that other drug classes, such as b-blockers[147], can inhibit the phenomenon of apoptosis[148]. Carvedilol, a b-adrenergic blocker with special properties in the peripheral

vessels, as well as its metabolites, is a potent antioxidative factor that can inhibit the formation of free oxygen radicals and thus prevent programmed cell death of myocardial cells[149].

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