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Review

Apoptosis in relevant clinical situations: contribution of apoptosis in myocardial infarction

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Abstract

Myocardial infarction is associated with increased TUNEL-positivity in cardiac resident and infiltrated cells. Apoptosis of proliferated interstitial myofibroblasts and infiltrated inflammatory cells may have a role in terminating tissue repair processes after infarction. Lateral and endocardial border zones of infarction within the risk area have frequent appearance of TUNEL-positive cardiomyocytes. Although the typical ultrastructural morphology of apoptosis has rarely been detected in ischaemic cardiomyocytes, there are many reports in which the TUNEL method was used for assessment of cardiomyocyte apoptosis. It has become evident that TUNEL-positivity reflects a wide range of cellular conditions; viable cells undergoing DNA repair, apoptosis, and necrosis. Therefore, it is controversial whether TUNEL-positive cardiomyocytes in infarcted myocardium are all apoptotic. Methods which will be more specific for identifying apoptosis are required for future study. TUNEL-positivity can be attenuated by anti-apoptotic interventions such as inhibition of caspases, mitochondrial protection, free radical scavenging, and some conventional pharmacotherapies. However, it remains to be determined whether anti-apoptotic interventions result in satisfactory reduction of infarct size. The injurious impact of myocardial ischaemia comes from a mixture of pro-apoptotic and necrosis-promoting signals, and the target of both signals is mitochondria. Through a common pathway they may cause permeability transition. Interventions which act only at the post-mitochondrial stage of apoptosis may fail to reduce infarct size, whereas those acting at pre-mitochondrial and mitochondrial stages may reduce infarct size. Progress in investigating the basic mechanisms of apoptosis and recognition of the modes of cardiomyocyte death will contribute to advances in cardioprotective therapy in myocardial infarction.

Keywords: Apoptosis; Infarction; Ischemia

1. Introduction

For a long time necrosis has been thought to be the mode of myocardial cell death. Apoptosis as a pathogenetic factor in heart disease was introduced relatively late compared to other fields of medicine [1]. Apoptosis research since 1994, in relation to myocardial ischaemia and infarction, is summarized in Table 1 [2–40]. Since the first report documented reperfusion-induced apoptosis in rabbit cardiomyocytes [2], many reports focused on confirming the appearance of apoptosis in ischaemic cardiomyocytes and on its distribution in vivo in animals [7–9] and humans [4,10,11,15,17]. Assessment of apoptosis was usually performed using a combination of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) [41] by light microscopy and genomic deoxyribonucleic acid (DNA) ladder detection. A variety of proapoptotic pathways (intracellular cation changes [12], cytokines [13], ceramide [21]) were defined. Molecular genetic analyses were used in apoptosis research on hearts, and the roles of intracellular pathways (mitogen-activated protein kinases (MAPKs) [14], c-Jun *N*-terminal kinases (JNKs) [33]) and the proapoptotic gene, p53 [16] were clarified. In 1997–1999,

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Table 1
Current apoptosis research in myocardial ischaemia (or hypoxia) and infarction[a]

Years	Species/reperfusion	Aim			Documents	Assessment for apoptosis
		P	M	I		
1994	Rabbit/(+)	*			Appearance of apoptosis in reperfused myocardium [2]	ISEL and DNA ladder
	Rat/in vitro		*		Induction of Fas and apoptosis triggered by hypoxia [3]	TUNEL and DNA ladder
1995	Human/?	*			Presence of apoptosis in infarcted (<a few days) hearts [4]	TUNEL and DNA ladder
	Rat/(+)			*	Attenuation of infarction by pretreating with IGF-1 [5]	TUNEL
	[Cell line	*			A novel assay for apoptosis using annexin V [6]	Immunocytochemistry]
1996	Rat/(−)	*			Increased apoptosis 1–2 days after coronary occlusion [7]	TUNEL, ladder, antimyosin
	Rat/(−)	*			Increased apoptosis early after infarction [8]	TUNEL and DNA ladder
	Rat/(+/−)	*			Acceleration of apoptosis by reperfusion [9]	TUNEL and DNA ladder
	Human/?	*			Apoptosis in the border zones of infarction (<10 days) [10]	TUNEL and DNA ladder
	Human/(+/−)		*		bcl-2 and bax expression in myocardial infarction [11]	Not performed
	Rabbit/in vitro		*		Apoptosis triggered by acidosis [12]	TUNEL
	Rat/in vitro		*		TNF-α-induced apoptosis [13]	ISEL, ladder, comet assay
1997	Rat/(+)		*		p38MAPKs activation by ischaemia–reperfusion [14]	DNA ladder
	Human/(+)	*			Apoptosis in human acute myocardial infarction [15]	TUNEL and DNA ladder
	Rat/(−)		*		p53 expression as one of mediators of apoptosis [16]	TUNEL and DNA ladder
	Human/?	*			Apoptosis early after acute infarction [17]	TUNEL and DNA ladder
	Rat/in vitro		*		bcl-2 prevents p53-induced apoptosis [18]	TUNEL and DNA ladder
	Mouse/(−)		*		p53-independent apoptosis in hypoxic myocardium [19]	TUNEL and DNA ladder
	Rat/(+)		*		Apoptosis inhibition in the ischaemic preconditioning [20]	TUNEL and ELISA
	Rat/in vitro		*		Ceramide-induced apoptosis [21]	TUNEL and ELISA
	Mouse/(−)			*	Infarct attenuation by transgenic IGF-1 overexpression [22]	TUNEL, ladder, antimyosin
	Rat/(+)			*	Infarct attenuation by transfection of NF-kB decoy [23]	Not performed
	Rabbit/(+)			*	Infarct attenuation by PARS inhibition [24]	Not performed
	Rat/in vitro			*	Inhibition of apoptosis by an extract from soy flour [25]	TUNEL and DNA ladder
	Rat/(+)			*	Farct attenuation by cyclosporin A [26]	Not performed
	[Cell line		*		Prevention of cytochrome *c* release from mitochondria by bcl-2 [27]	TUNEL and DNA ladder]
1998	[Cell line		*		Cloning of caspase-activated DNase (CAD) and inhibitor of CAD [28]	Ladder and caspase assay]
	[Cell line		*		Apoptosis without DNA fragmentation by an inhibitor of CAD [29]	Ladder and caspase assay]
	Rabbit/(+/−)		*		Apoptosis as a maskerade of oncosis in myocardial infarction [30]	Electron microscopic TUNEL
	Rabbit/(+)	*			Apoptosis of non-cardiomyocytes in myocardial infarction [31]	TUNEL and DNA ladder
	Rat/(+)		*		Co-localization of caspase-3 and apoptotic change in ischaemic cells [32]	TUNEL and DNA ladder
	Rat/(−)		*		JNK activation in apoptotic cells remote from infarction [33]	TUNEL and DNA ladder
	Rat/(−)		*		Redistribution of plasma membrane phospholipids on apoptosis [34]	TUNEL and DNA ladder
	Rat/(+)			*	Apoptosis prevention by ZVAD-fmk [35]	TUNEL and DNA ladder
	Rabbit/(+)			*	Apoptosis prevention by carvedilol [36]	TUNEL and DNA ladder
	Rat/(+)			*	Apoptosis attenuation by anti-complement therapy [37]	TUNEL and DNA ladder
1999	Rabbit/(+)			*	Apoptosis and infarct size reduction by p38 MAPK inhibitor [38]	TUNEL and DNA ladder
	Rat/(+)			*	Apoptosis and infarct size reduction by NHE inhibitor [39]	TUNEL and DNA ladder
	Rat/(+)		*		Differential regulation of apoptosis by bcl-2, AP-1, and NF-kB [40]	TUNEL and DNA ladder

[a] Aim: P, phenomenon; M, mechanism; I, intervention. ISEL, in situ end-labeling; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling. Several breakthrough reports on noncardiomyocytes are noted in [. . .].

attempts to attenuate ischaemic myocardial injury through modulating apoptosis were reported. The tools used for this therapeutic approach were insulin-like growth factor-1 (IGF-1) [22], nuclear factor-*k*B (NF-*k*B) [23], poly-[adenosine diphosphate (ADP)-ribose]-synthetase (PARS) [24], caspase (cysteine aspartic acid protease) [35,42–44], oxidative stresses [36], complement [37], p38MAPK [38], Ca overload [39], and anti-apoptotic proteins [45–47]. However, the possibility that a majority of the so called 'apoptotic cell death' of cardiomyocytes may be a phantom of oncotic cell death was postulated from an experimental study using an electron microscopic TUNEL method [30].

In the present review, we discuss the current perspective of the relevance of apoptosis in myocardial cell death related to myocardial infarction, and the possibility of therapeutic anti-apoptotic interventions in this field.

2. Modes of myocardial cell death; apoptosis and non-apoptosis

Necrosis has been contrasted to apoptosis in the modes of cell death. However, necrosis is recognized as the sum of degenerative changes that follow any type of cell death, including apoptosis [48]. Apoptosis in its original meaning is characterized by the morphological changes listed in Table 2. Early in necrosis, mitochondria and the entire cytoplasm become swollen, and this is followed by plasma

Table 2
Apoptosis vs. oncosis (necrosis)

	Apoptosis	Oncosis (necrosis)
Plasma membrane	Intact until late in the process	Destroyed early
Morphologic features	Chromatin condensation (pyknosis)	Swelling of entire cytoplasm (oncosis)
	Nuclear fragmentation (karyorhexis)	Mitochondrial swelling
	No swelling of mitochondria until late	
	Cell shrinkage	
Biochemical features	Double-strand beaks of DNA with mono- or oligomers of 180–200 base pairs (endonucleolysis)	Nonspecific degradation of DNA
	Protein degradation by specific proteases	Protein degradation by nonspecific proteases
	Surface exposure of phosphatidylserine before membrane disintegration	
Fate	Heterophagic elimination and little inflammation	Leakage of contents and secondary inflammation
Biologic meaning	Physiological and pathological, tightly regulated	Accidental and unregulated

membrane disintegration and leakage of cell contents, leading to inflammation. In apoptosis, chromatin condensation (pyknosis) and margination occur [49]. The nucleus becomes fragmented (karyorhexis) and the cell shrinks, typically giving the appearance of a so called apoptotic body. Such apoptotic cells are eliminated by heterophagy. There is little or no swelling of mitochondria or other organelles until late in the process. According to this classic meaning of apoptosis, most of cardiomyocyte death due to ischaemic injury which has been termed apoptotic may in fact be necrotic cell death. Following the morphologic definitions of apoptosis, its biochemical characteristics [adenosine triphosphate (ATP)-requiring step-wise DNA fragmentation culminating in the formation of mono- and/or oligomers of 180–200 base pairs by endonucleases] were elucidated. However, the simple use of TUNEL-positivity and DNA ladder detection as a determination of apoptosis was apt to result in misunderstandings of the modes of cardiomyocyte death. TUNEL detects single-strand DNA breaks as well as double-strand DNA breaks with free 3′-OH termini. Therefore, TUNEL may not be specific for apoptosis and some necrotic cells may be TUNEL-positive. A DNA ladder may also not be specific for cardiomyocyte apoptosis in vivo since non-cardiomyocytes, whose number exceeds cardiomyocytes in myocardial tissue, may contaminate tissue samples. Therefore, the need for assessing plasma membrane integrity by electron microscopy has been emphasized by Ohno et al. [30]. Electron microscopy combined with TUNEL revealed that TUNEL-positive cardiomyocytes had membrane damage, and they speculated that TUNEL-positivity may be secondary to irreversible membrane damage. In their concept, myocardial cell death consists of oncosis, apoptosis, and necrosis (the final feature of oncosis and apoptosis). The term ‘oncosis,’ is applied to the classic pattern of cell death with swelling [49].

In another way Anversa et al. [50] classified myocardial cell death into three forms; necrosis (corresponding to oncosis), apoptosis, and necrosis–apoptosis (a final form of cell death as mentioned above). They tried to distinguish between these three forms by using histochemical techniques and confocal microscopy. In the helical twist of DNA, endonucleases produce two strands with stagger ends and blunt ends [51]. Stagger ends consist of double-strand breaks in the internucleosomal DNA with one- (due to Ca-dependent DNase I) or two-base 3′ overhangs or longer overhangs (due to pH-dependent DNase II) involving four bases. In contrast, other endonucleases and exonucleases produce cleavage of internucleosomal and nucleosomal DNA, which results in blunt ends of the digested fragments [50,51]. Anversa et al. assumed that the first and second types of double-strand breaks may be produced by apoptosis-related DNases while the third form (blunt ends) may be produced by necrosis-related DNases. They postulated that a *Taq* polymerase-generated probe, a TdT reaction [52], and a *Pfu* polymerase-generated probe identified products with single-base 3′ overhangs, single- or longer-base 3′ overhangs, and blunt-ends of DNA fragments, respectively and that these three probes may contribute to distinguishing between apoptosis and non-apoptosis.

Since cardiomyocytes rendered ischaemic sometimes have features of both irreversible structural changes and TUNEL-positivity, Ohno et al. [30] speculated that some final step in the apoptotic process may be activated during oncosis and that this activation has no relevance to the extent of infarction in vivo. Although these speculations raise the question as to whether the potential benefits of anti-apoptotic interventions are a reality or an illusion, a significant number of reports on anti-apoptotic interventions claim at least partial involvement of apoptosis in diseased states (Table 3).

In cultured neonatal cardiomyocytes, we compared TUNEL-positivity to simultaneously stained propidium iodide (PI)-positivity, a marker of irreversible membrane damage, by confocal microscopy [53]. When cardiomyocytes were kept hypoxic, PI-positivity increased with a short time delay after the onset of hypoxia. TUNEL-positivity also increased with time, but never exceeded the rate of PI-positivity. Specifically, TUNEL-positive cells

Table 3
Anti-apoptotic interventions[a]

Species	Protocol	Anti-apoptotic intervention	Effects
(1) Rat/in vivo	30-min ischaemia (I) and 6-h reperfusion (RP)	Pretreatment with a caspase-3 or caspase-1 inhibitor	Apoptosis reduced but infarct size not reduced [42]
(2) Mouse/in vivo	Permanent coronary occlusion	Pretreatment with a broad caspase inhibitor (ZVAD-fmk)	Apoptosis reduced [43]
(3) Rat/in vitro	48-h hypoxia (H)	Pretreatment with ZVAD-fmk	Apoptosis reduced [44]
(4) Rat/in vivo	30-min I and 24-h RP	Pretreatment with ZVAD-fmk	Apoptosis and infarct size modestly reduced [35]
(5) Rat/Langendorff	30-min I and 30-min RP	Human bcl-2 eDNA transfection by HVJ	Apoptosis inhibited, CK leakage reduced, post-ischaemic function improved [45]
(6) Rat/in vitro	4-h H and 2–4-h reoxygenation	Human bcl-2 eDNA transfection by HVJ	Myocyte survival improved, LDH leakage reduced [46]
(7) Mouse/in vivo	30-min I and 2-h RP	Transgenic bcl-xl overexpression	Apoptosis reduced but infarct size not reduced [47]
(8) Rabbit/in vivo	45-min I and 24-h RP	Pretreatment with a PARS inhibitor	Infarct size reduced, function improved [24]
(9) Rat/in vivo	20-min I and 24-h RP	Pretreatment with IGF-1	Apoptosis reduced, CK leakage reduced [5]
(10) Mouse/in vivo	Permanent coronary occlusion	Transgenic IGF-l overexpression	Apoptosis and necrosis reduced Remodeling attenuated [22]
(11) Rat/in vivo	Permanent coronary occlusion	Transfection of NF-*k*B decoy	Infarction reduced [23]
(12) Neonatal rat/ in culture	8-h H and 16-h reoxygenation	Exposure to the anti-apoptotic factor purified from soy flour	Apoptosis reduced, cardiomyocyte death prevented [25]
(13) Rat/ Langendorff	30-min I and RP	Pretreatment with cyclosporin A, an inhibitor of MPT	Function improved, ATP/ADP ratios increased, [26] apoptosis assay not performed
(14) Rat/in vivo	30-min I and 120-min RP	Use of anti-SAPKs property of carvedilol	Apoptosis and infarct size reduced [36]
(15) Rat/in vivo	30-min I and 60-min RP	Pretreatment with p38 MAPK inhibitor	Apoptosis and necrosis reduced, cardiac function better recovered [38]
(16) Rat/in vivo	225-min I or 45-min I and 180-min RP	Oral pretreatment with NHE inhibitor to reduce Ca overload	Mortality and arrhythmia reduced, apoptosis reduced, bcl-2/bax ratio increased [39]

[a] I, ischaemia; RP, reperfusion; H, hypoxia; CK, creatine kinase; HVJ, hemagglutinating virus of Japan; PARS, poly(ADP-ribose)synthetase; IGF-1, insulin-like growth factor-1; MPT, mitochondrial permeability transition; MAPK, mitogen-activated protein kinase; SAPKs, stress-activated protein kinases; NHE, Na-H exchanger.

were always positive for PI in our in vitro system. In addition, in our system preincubation with a broad caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(OMe)-CH_2F (ZVAD-fmk), resulted in a 60% reduction of PI-positive cells. Thus, our study indicated that TUNEL-positivity could be induced secondary to plasma membrane damage and also that broad caspase inhibition prevented plasma membrane disruption and cardiomyocyte death, although the mechanisms involved remain to be determined.

In contrast to oncosis with internucleosomal DNA fragmentation, evidence for apoptosis without DNA fragmentation was recently reported [29]. A caspase-activated deoxyribonuclease named CAD (caspase-activated DNase), which breaks at internucleosomal DNA sites, was identified and cloned, and an inhibitor of CAD named ICAD [28] was also identified. ICAD, by binding to CAD, inhibited DNA fragmentation induced by caspase activation, but those cells were killed as a result of caspase activation rather than escaping from suicide [29]. These results suggest that caspase activation is able to kill cells without DNA fragmentation, that DNA fragmentation may not be the hallmark of apoptotic cell death and that 'backward signaling' from activated caspases to determinants of cell death, such as mitochondrial permeability transition pores, may have a role in caspase activation-induced cell death. Thus, it is difficult to draw a clear line between apoptotic cell death and non-apoptotic cell death by the appearance of fragmented DNA in the modes of elimination of ischaemic cardiomyocytes.

3. Appearance of apoptosis in myocardial infarction

3.1. Frequency of TUNEL-positivity

Although TUNEL-positivity is not the best hallmark of apoptosis in cardiomyocytes, since there is no other way to semiquantitatively assess apoptosis in myocardial tissue, it is used in this section. In acute myocardial infarction (within 10 days after onset, no description of whether or not coronary arteries were reperfused) in humans, 12% of cardiomyocytes were TUNEL-positive in the border zone of the infarction, and less than 1% in areas remote from the infarcted zone [10]. However, experimental studies have shown that the appearance of TUNEL-positivity depends on the time after the onset of infarction that the analysis is made, and also on the presence or absence of reperfusion [2,7–9]. TUNEL-positivity increases during the first 6 h to 2 days after permanent coronary occlusion [8] and then decreases over 4 weeks [7], and reperfusion increases TUNEL-positivity. Under experimental conditions, 11–15% of cardiomyocytes rendered ischaemic and reperfused were TUNEL-positive [30,35]. The absolute value of TUNEL-positivity changes according to experimental de-

sign, e.g. ischaemic duration, species, sensitivity of TUNEL in each lab. Again it also needs to be considered that TUNEL-positive cardiomyocytes include necrotic cells.

3.2. Process of infarct-related apoptosis

The possible mechanisms involved in caspase activation and the appearance of apoptosis in myocardial infarction are illustrated in Fig. 1. The major intermediate regulator of caspase activation is a mitochondrial pathway. At present two apoptogenic factors are known; a ~50 kDa apoptosis-inducing factor (AIF) and cytochrome *c*. AIF which acts by activating caspases does not require cytochrome *c* to exert its apoptotic action. Cytochrome *c* itself is not apoptogenic but holocytochrome *c*, which lacks a heme group, is released reversibly or irreversibly from mitochondria injured by numerous factors including reactive oxygen species and Ca overload. Cytochrome *c* binds to apoptotic protease-activating factor-1 (Apaf-1). Since Apaf-1 has an ATP binding site, this activates the caspase cascade in an ATP-dependent manner. The requirement of ATP in the promotion of apoptosis may derive from the involvement of Apaf-1. Caspase activation via release of cytochrome *c* from mitochondria is induced by oxidative stress to the cytoplasm or mitochondria and possibly by unidentified signals from activated death factor receptors at the plasma membrane, such as Fas and tumor necrosis factor (TNF)-receptors. In the presence of deoxy-ATP, Apaf-1 activates caspase-9, which is an upstream enzyme in the caspase cascade, and then caspase-3 is activated. Caspase-3 induces CAD activation, which leads to DNA fragmentation, and it also cleaves cytoskeletal proteins such as actin, fodrin and lamin, leading to alteration of the cytoskeleton. Another activator of the caspase cascade is the tumor suppressor gene p53. In many organs and tissues, p53 is activated in response to DNA damage which can also be induced by ischaemia. Although the process of caspase activation by p53 has not been elucidated in detail, p53 induces transcription of many genes with p53 binding sites. p53 suppresses expression of B cell lymphoma/leukemia-2 (bcl-2) related proteins, resulting in a pro-apoptotic direction in the cell. It also induces PIGs (p53-induced genes) which amplify the activation of reactive oxygen species. However, contrasting results were obtained in a gene-targeting study, in which even in p53-

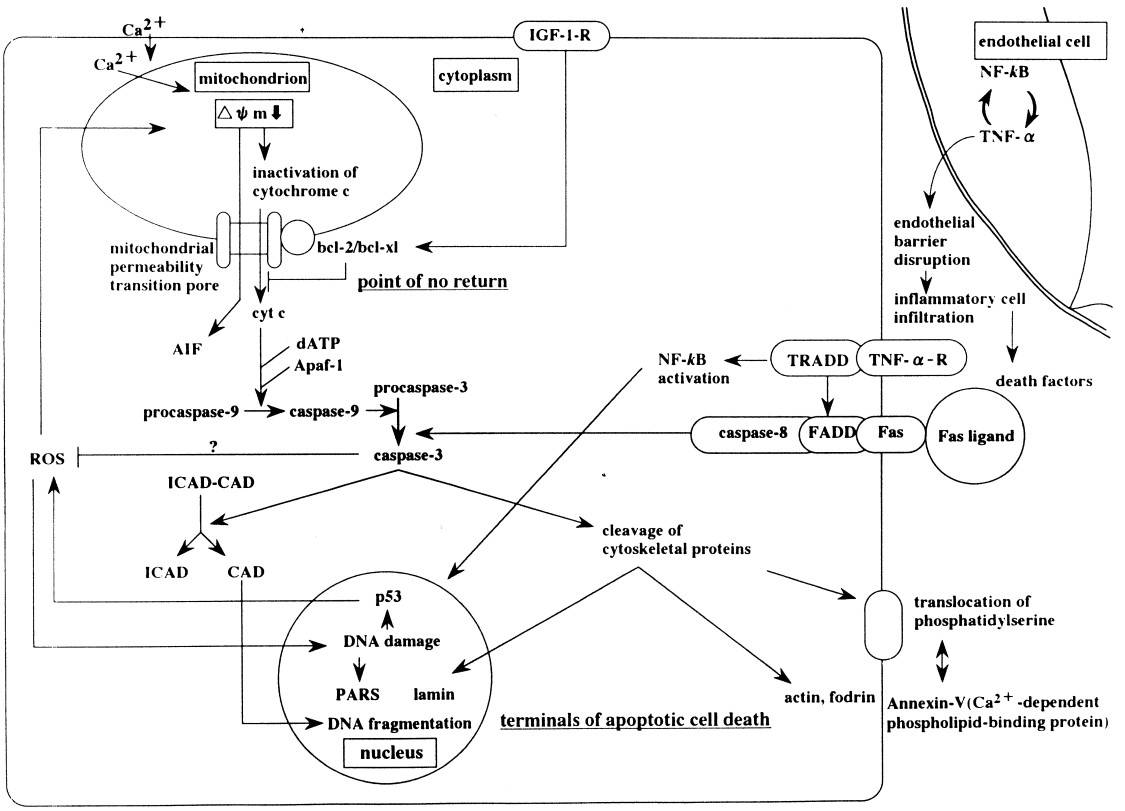

Fig. 1. Putative apoptotic signal pathways in ischaemic cardiomyocytes. Putative apoptotic pathways in the ischaemic cardiomyocytes are illustrated. Pro-apoptotic signals induce mitochondrial permeability transition leading to release of AIF (apoptosis-inducing factor) and cytochrome *c* into cytoplasm. These apoptogenic factors activate caspases as does the Fas–Fas ligand pathway. AIF activates caspase-3 but it has not been determined whether it also activates caspase-9. Activated caspase-3 cleaves the ICAD (inhibitor of CAD), and free CAD (caspase-activated DNase) cleaves DNA. Ischaemic impacts on the myocardium are not exclusive in the apoptotic signal pathway and both pro-apoptotic and necrosis-promoting signals are crossed in the ischaemic myocardium. ROS, reactive oxygen species; $\Delta\psi m$, mitochondrial membrane potential; (⊣) indicates inhibition.

knockout mice, the appearance of ischaemia-induced apoptosis was not affected [19]. To fully determine whether cardiomyocyte apoptosis is independent of p53-related gene expression, the roles of functional homologues of p53, such as p73 need to be investigated in p53-knockout mice [54].

3.3. Apoptosis in the myocardium remote from the infarcted zone

TUNEL-positive cardiomyocytes appear quite a bit less in noninfarcted myocardium remote from the risk areas. In human, after recent myocardial infarction, specimens of myocardium remote from infarcted areas had 0.7% of TUNEL-positive cardiomyocytes in contrast to 0% in control hearts [10]. Also in human hearts, bax, a pro-apoptotic protein, was overexpressed in 13% of patients with acute infarction but in 83% of patients with old infarction [11]. The pathophysiologic significance of such cardiomyocyte apoptosis in noninfarcted myocardium remote from infarcted zones remains to be determined, including the possibility of its relation to ventricular remodeling after infarction.

3.4. Apoptosis of noncardiomyocytes

TUNEL-positivity in myocardial infarction is not limited to ischaemic cardiomyocytes. Abundant numbers of non-cardiomyocytes such as coronary endothelial cells, interstitial macrophages, myofibroblasts and infiltrated neutrophils undergo apoptosis [31]. The pathophysiologic significance of the elimination of these cells by apoptosis has not been fully determined but conceptually their elimination may have a purpose.

Apoptosis of ischaemic coronary endothelial cells may help to quickly reseal the intima of coronary vasculatures by new endothelium since apoptosis shortens the time required for the elimination of injured cells. Leukocytes and macrophages which accumulate extensively in infarcted myocardium to rapidly scavenge injured tissue have cytotoxic factors such as inflammatory cytokines and proteases. When the scavenging process is completed, the infiltrated cells need to be eliminated from infarcted zones promptly so that surviving cardiomyocytes are not killed. If the inflammatory cells die of necrosis, toxic components may be released and may cause excessive inflammation. Therefore, elimination of these cells by apoptosis appears to be meaningful.

Massive appearance and subsequent decrease of myofibroblasts after infarction were reported [31]. The myofibroblasts probably synthesize collagen for replacement by fibrosis. If proliferating myofibroblasts remained for a long time after infarction, increased collagen may inappropriately increase the stiffness of infarcted hearts triggering post-infarction heart failure.

It is not known what kinds of changes may occur after attenuation of apoptosis in such noncardiomyocytes. This is important from a therapeutic point of view because non-specific anti-apoptotic interventions will alter their fate.

4. Therapeutic strategies

4.1. Therapeutic strategies and limitations

To examine the proposition that anti-apoptotic interventions may have a cardioprotective effect in myocardial infarction, the following issues must be considered. First, cardioprotective agents must act at the pre-mitochondrial or mitochondrial stage of apoptosis. Interventions acting only at a post-mitochondrial stage may reduce apoptosis but may not reduce infarct size (Fig. 2). With respect to interventions at the pre-mitochondrial and mitochondrial stages, it is sometimes hard to clearly differentiate whether the therapeutic tools are specific for apoptosis or specific for necrosis. Second, there is the possibility that anti-apoptotic interventions reduce DNA fragmentation in necrotic cell death as well as in apoptotic cell death, leading to a discrepancy between the therapeutic effects on reduction of DNA fragmentation and infarct size. Third, the use of TUNEL-positivity and DNA ladder formation as markers of therapeutic effect may be misleading with regard to the therapeutic efficacy of an agent and the interpretation of the results. For instance, combining the techniques of TUNEL, a *Taq* polymerase-generated probe and nuclear replication markers revealed that TUNEL-positive cardiomyocytes in patients with dilated cardiomyopathy were viable cells in the process of DNA repair rather than apoptotic cells [55]. Such new techniques will be required for future apoptosis research. Fourth, it is currently hard to completely determine the presence or absence of anti-apoptotic properties in some of the conventional cardioprotective therapies previously reported (Fig. 2). This may in part be due to the fact that apoptosis was not considered in therapeutic attempts reported before cardiac apoptosis research was introduced in 1994.

Although interpretation of the results of currently reported interventions is not straightforward (Table 3), representative interventions are listed in the following sections.

4.2. Caspases

A gene targeting approach for caspases began recently. Overexpression of caspase-3 in transgenic mice induced contractile dysfunction and nuclear damage [56]. However, so far there are no reports on the analysis of myocardium in caspase knockout animals.

Effects of ZVAD-fmk as a caspase inhibitor are broad and irreversible, and it reduced TUNEL-positivity and infarct size modestly [35]. There are several things to be

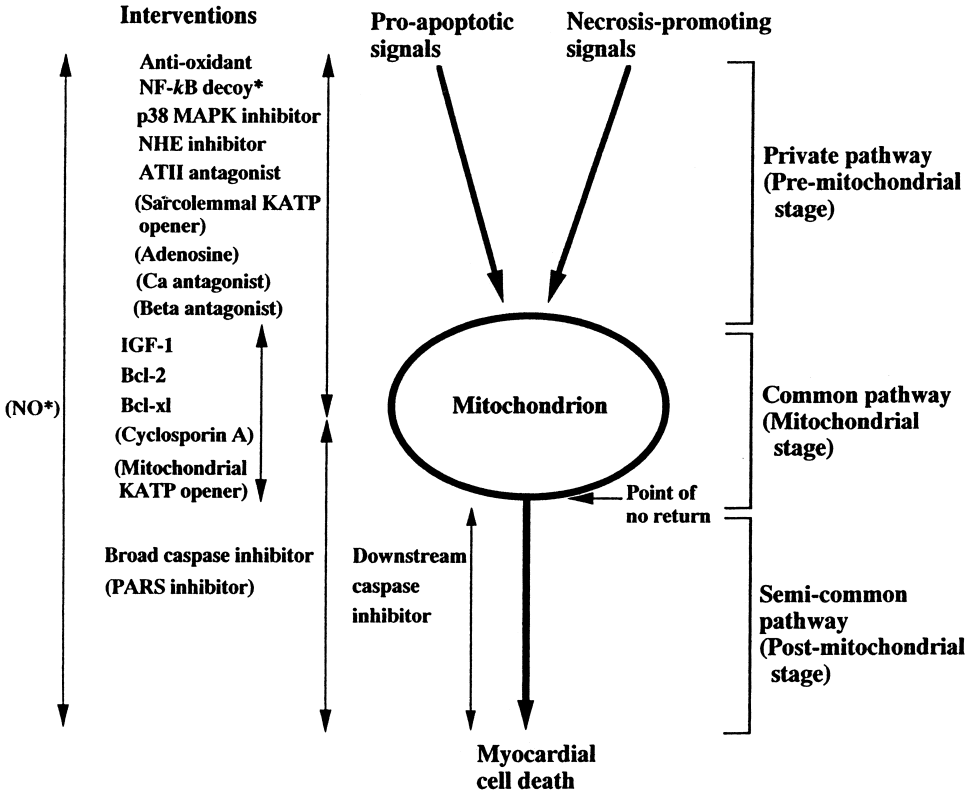

Fig. 2. Anti-apoptotic and anti-necrotic interventions. Sites of action of representative anti-apoptotic and anti-necrotic interventions are illustrated. Interventions work at the pre-mitochondrial (private pathway), the mitochondrial (common pathway), and/or at the post-mitochondrial (semi-common pathway) stages. Arrows indicate the range of functioning sites. The interventions with parenthesis are reported to be anti-necrotic, but their anti-apoptotic properties are not established. * NO and NF-*k*B have in general both anti-apoptotic and pro-apoptotic actions.

considered concerning the action of ZVAD-fmk. First, ZVAD-fmk is a pseudopeptide for intracellular caspases and its effects are dose-dependent [57]. Second, there is evidence with noncardiac cell lines that ZVAD-fmk accelerates endogenous generation of reactive oxygen species leading to cytotoxic action in association with caspase inhibition [58]. The authors speculated that caspase may have a role in inhibiting reactive oxygen species generation in their cell lines and that inhibition of caspase resulted in secondary cytotoxicity. It remains to be determined whether this harmful mechanism also works in ischaemic cardiomyocytes. Third, broad caspase inhibition reduces apoptosis in infiltrating inflammatory cells in ischaemic myocardium. Neutrophils which escape apoptosis and acquire a prolonged survival may sustain free radical generating activities as documented in an in vitro system [59]. Therefore, there is a significant limitation to the use of ZVAD-fmk alone to greatly attenuate infarct size, and whether it offers long-term cardioprotection against ischaemic injury remains obscure.

In contrast to broad caspase inhibition, specific inhibitors of downstream caspases, acetyl-Val-Glu-Ile-Asp(Ac-YVAD)-CHO and acetyl-Asp-Glu-Val-Asp (Ac-DEVD)-CHO, a reversible caspase-1 and -3 inhibitor, respectively, attenuated TUNEL-positivity but did not reduce infarct size [42]. This indicates that intervention only at a post-mitochondrial stage fails to protect ischaemic myocardium. At present there is little which could explain the discrepancies in the infarct limiting effects in studies utilizing different subtypes of caspase inhibitors. One possible explanation is that ZVAD-fmk may have additional characteristics which other caspase inhibitors do not possess. ZVAD-fmk does not attenuate cytochrome *c* release from mitochondria [60], but it may abolish all activities of AIF, an unstable apoptogenic protein released from mitochondria [61]. Also, ZVAD-fmk may be able to inhibit reactive oxygen species-induced cardiomyocyte apoptosis by a mechanism not involving the cytochrome *c*–caspase-3 pathway [62]. In addition, it is possible that ZVAD-fmk may inhibit Fas-mediated apoptosis through its broad caspase inhibition, although it remains to be determined whether a Fas-mediated apoptotic pathway is involved in ischaemic myocardial injury.

4.3. IGF-1

In neuronal tissue, IGF-1 inhibits apoptosis through IGF-1 receptor activation-dependent inhibition of caspase activation. IGF-1 administration prior to ischaemia reduced myocardial enzyme release and myocardial inflammation

in rats [5,22]. In cultured cardiomyocytes, IGF-1 attenuated TUNEL-positivity, enhanced cell survival, and inhibited caspase-3 activation [63]. The mechanisms of attenuating myocardial injury are not fully understood, but in primary cultured cardiomyocytes IGF-1 modulates the bcl-2 family. IGF-1 increases the level of anti-apoptotic bcl-2 and decreases that of pro-apoptotic bax, thereby, attenuating the mitochondrial pathway of apoptosis [63] (Fig. 1).

4.4. PARS

Ischaemia–reperfusion is associated with caspase-3-induced cleavage of PARS, the chromatin-bound DNA repair enzyme which catalyzes attachment of ADP ribose units from nicotinamide adenine dinucleotide (NAD) to nuclear proteins following DNA damage. Excessive activation of PARS depletes the reduced form of NAD (NADPH) and finally leads to necrosis since maintenance of energy equivalents is essential for completion of apoptosis. It is thought that in part, this is why PARS is cleaved early in apoptosis. Once cleaved PARS cannot consume NAD and energy equivalents, and thus energy will not be impaired so that apoptosis can proceed to completion. NAD is necessary for mitochondrial respiration, and depletion of NADPH causes to a fall in the intracellular level of ATP leading to failure in the completion of apoptosis and the occurrence of necrosis. This mechanism of cell death is termed the 'PARS Suicide Hypothesis.' Pharmaceutical inhibition of PARS synthase activity just before reperfusion was reported to reduce infarct size after reperfusion and improve ventricular function in isolated rabbit hearts [24]. The mechanisms by which ischaemic myocardial cell death is attenuated by PARS inhibition have not been fully determined. They may involve the inhibition of oxidative stress-induced PARS activation and the resultant maintenance of NAD and ATP, and also the inhibition of PARS-induced acceleration of neutrophil infiltration.

4.5. NF-kB

NF-*k*B is a nuclear transcription factor present in many cell types. Activation of NF-*k*B has both anti-apoptotic and pro-apoptotic features depending on the cell type. An anti-apoptotic function for NF-*k*B was reported in studies in which fibroblasts derived from NF-*k*B knockout mice had increased sensitivity to the cytotoxic effect of TNF-α [64]. However, NF-*k*B also acts as a pro-apoptotic factor. It acts as an important signal transducer of proinflammatory cytokines such as interleukin (IL)-2, -6 and TNF-α and of reactive oxygen species, adhesion molecules, Fas ligands, and nitric oxide (NO) synthase. NF-*k*B is activated by TNF-α or TNF receptor-associated death domain protein (TRADD) and by reactive oxygen species (Fig. 1). NF-*k*B exists as a cytoplasmic complex bound to its inhibitory protein I*k*B. Phosphorylation of I*k*B by oxidative stresses induces dissociation of I*k*B from the complex, and the activated NF-*k*B translocates from the cytosol to the nucleus promoting inflammation. Therefore, suppression of the effects of NF-*k*B may attenuate apoptosis in the heart. Transfection of myocardium with decoy-binding sites for NF-*k*B causes inhibition of NF-*k*B effects, and significantly reduced infarcted area [23]. This effect may have been obtained by suppressing numerous actions of NF-*k*B, not all of them mediated by apoptosis inhibition.

4.6. Bcl-2 family

Bcl-2 is a mitochondria-anchored anti-apoptotic protein originally identified as a proto-oncogene. The bcl-2-related family is composed of 16 proteins through the three bcl-2 homology domains. Among them, at least four proteins (bcl-2, bcl-xl, bcl-w, mcl-1) have anti-apoptotic properties. The anti-apoptotic bcl-2 proteins exert cardioprotection by multiple mechanisms such as a direct anti-oxidant effect, inhibition of pro-apoptotic bcl-2 proteins, mitochondrial membrane stabilization which maintains mitochondrial transmembrane potential and subsequent inhibition of mitochondrial swelling and release of cytochrome *c* and/or AIF into the cytoplasm. Ischaemic preconditioning with reduced apoptosis was associated with upregulation of bcl-2 in rats [65]. Transfection of human bcl-2 into the heart with a virus vector inhibited TUNEL-positivity, reduced creatine kinase level, and improved cardiac function after ischaemia-reperfusion in vivo and in vitro [45,46]. However, there is one report showing that TUNEL-positivity after ischaemia–reperfusion was attenuated but infarct size was not reduced in transgenic mice overexpressing bcl-xl [47]. At present only few reports exist on the effect of overexpression of anti-apoptotic bcl-2 proteins in myocardial ischaemia, details of reports have not been published and results are controversial. At least it is assumed that even anti-apoptotic bcl-2 proteins may not offer cell survival despite effective inhibition of apoptotic events (probably through membrane stabilization and inhibition of cytochrome *c* release into cytoplasm), if the cell have already suffered from permanent damage of mitochondrial respiration.

4.7. Mitochondrial permeability transition

Cyclosporin A is an inhibitor of mitochondrial permeability transition. Isolated hearts pretreated with cyclosporin A had a greater recovery of post-ischaemic cardiac function and ATP/ADP ratios and less mitochondrial damage after reperfusion [26]. It was also reported that cyclosporin A stimulates endothelin receptor-mediated NO release [66]. As described below NO reduces oxidative stresses and inhibits caspase activation. Protection by cyclosporin A is concentration-dependent but its effect is diminished under the severe Ca overload or adenine

nucleotide depletion which occurs upstream of the mitochondrial permeability transition.

4.8. Mitogen-activated protein kinase

p38 MAPK, a member of the MAPK family which is activated during cellular stresses, is one of the signal transducers for apoptosis. Whether SB203580, an inhibitor of p38 MAPK, might reduce infarct size was investigated [38]. Administration of SB203580 before ischaemia and after reperfusion reduced TUNEL-positivity of cardiomyocytes and infarct size, and improved cardiac function. The reduction of TUNEL-positivity was closely related to the reduction of infarct size. Nevertheless, it is possible that inhibition of p38 MAPK may reduce necrosis and necrosis associated TUNEL-positivity through undefined mechanisms. However, the close relation between reduction of TUNEL-positivity and infarct size reduction by this intervention suggests at least partial involvement of apoptosis cascades in this mechanism.

4.9. Ca overload

The processes leading to myocardial cell death are mediated by a variety of Ca-sensitive signal transduction events [67]. Ca influx activates Ca regulated proteases such as calpains in relation to the induction of apoptosis. However, it should be noted that Ca overload associated with ischaemia–reperfusion is not a specific trigger for apoptosis since it is also known to be important for the induction of accidental cell death.

4.9.1. Ca antagonist

It is controversial whether Ca antagonists really reduce myocardial infarct size. This probably comes from that inhibition of Ca influx through the L-type Ca channel cannot prevent Ca overload induced by ischaemia–reperfusion. There are no published reports on the effects of Ca antagonists on myocardial apoptosis associated with infarction.

4.9.2. Sodium–hydrogen exchanger inhibitor

The level of sodium–hydrogen exchanger (NHE) mRNA in ischaemic myocardium is elevated in the animal model of coronary occlusion and is increased by reperfusion. When an NHE inhibitor was administered before the onset of ischaemia, it reduced the mortality and TUNEL-positive cardiomyocytes, and increased the ratio of bcl-2 to bax in the ischaemic area [39]. NHE inhibitors protect ischaemic myocardium by attenuating Na overload-triggered Ca overload of cells and inflammatory cell infiltration into ischaemic myocardium, which are both anti-necrotic and anti-apoptotic.

4.9.3. ATP-dependent potassium channel opener

Sarcolemmal ATP-dependent potassium channel (KATP) openers attenuate Ca overload by shortening the action potential duration. KATPs are also present in the inner membrane of mitochondria where they regulate mitochondrial volumes and energetics. The mechanisms involved in mitochondrial protection by KATP opening remain obscure. Pretreatment of myocardium with diazoxide, a mitochondrial KATP opener, mimicked the cardioprotection obtained by preconditioning [68]. Since infarct size reduction by ischaemic preconditioning usually exceeds the amount of TUNEL-positive cardiomyocytes (up to 15% of risk areas), the powerful cardioprotection by preconditioning cannot be explained solely by apoptosis inhibition. Although there are no reports on the relation between the cardioprotective action of KATP openers and apoptosis, it is highly possible that mitochondrial KATP opening attenuates permeability transition.

4.10. Nitric oxide

NO donors are basic pharmaceuticals used in myocardial infarction. NO has pro-apoptotic and anti-apoptotic properties through cyclic GMP (cGMP)-dependent and cGMP-independent mechanisms [69]. The cGMP-independent action of NO may in part mediate the potent oxidant peroxynitrite. The anti-apoptotic actions of NO may involve at least two mechanisms. First, an increase in intracellular cGMP by NO decreases intracellular Ca concentration which is one of the key signals of apoptosis. Second, NO can S-nitrosylate thiols. If cardioprotective thiols such as glutathione are depleted, caspases could be targets of S-nitrosylation by NO, resulting in caspase inactivation. There are many reports on the relation between NO and apoptosis in many cell types. However, there has been no direct proof of the relation between NO and cardiomyocyte apoptosis in association with myocardial infarction.

4.11. Other conventional pharmaceuticals

4.11.1. β-Antagonist

There is no clear evidence that β-antagonists in general modulate apoptosis. Anti-apoptotic properties have only been reported for carvedilol. Carvedilol is a β-antagonist with additional pharmacological activities and its ability to reduce infarct size has been reported [36]. Since its anti-ischaemic effect exceeds that of propranolol, a property in addition to β-antagonism has been emphasized. Although many of the reports noted significant effects with pretreatment, post-treatment with this agent also attenuates infarct size. Therefore, further studies will be required to elucidate the effect of this agent in a clinical setting of myocardial infarction. Stress-activated protein kinases (SAPKs) are one of the apoptotic signal transducers. When administered before reperfusion, carvedilol attenuated ischaemia–reperfusion-induced Fas expression in cardiomyocytes and activation of SAPKs in myocardium at risk, but such

effects were not observed with propranolol, a conventional β-antagonist [36]. These results suggest that the anti-ischaemic property of carvedilol may be due to its specific anti-apoptotic action which propranolol does not possess.

4.11.2. Angiotensin II antagonists

There is no evidence that angiotensin II (ATII) antagonism attenuates cardiomyocyte apoptosis in acute myocardial infarction. However, involvement of ATII in post-infarction apoptosis in zones remote from risk areas has been suggested [70]. In the surviving portion of the infarcted hearts, stretch-induced ATII release through the paracrine system occurs. ATII has two specific (AT_1 and AT_2) receptors in target organs. It was reported that stretch-induced endogenous ATII release triggered cardiomyocyte apoptosis in vitro [71]. It is known that ATII activates pro-apoptotic p53 transcription through AT_1 receptor activation. This mechanism of inducing apoptosis may contribute to post-infarction remodeling. However, the extensive stretch in vitro which induces cardiomyocyte apoptosis may not occur in physiological conditions [72]. In addition, there are contradictory in vitro data that AT_1 receptor-mediated signal was anti-apoptotic [73]. Therefore, the role of endogenous ATII in its receptor-mediated cardiomyocyte apoptosis in overloaded hearts in vivo remains to be determined.

4.11.3. Adenosine

Adenosine A_1 and A_3 receptors in cardiomyocytes independently protect them from ischaemic stresses. A_2 receptors in coronary vasculatures have a role in maintaining coronary circulation. In the AMISTAD trial adenosine was proven to reduce myocardial infarct size after thrombolytic therapy in humans [74]. As one of the mechanisms of cardioprotection by adenosine, the protein kinase C-KATP pathway has been suggested. There are no reports on the relation between the cardioprotective actions of adenosine and attenuation of apoptosis in ischaemic cardiomyocytes.

5. Conclusions

There is controversy whether the biologic form of cell death in so called ‘cardiomyocyte apoptosis’ is ‘apoptotic’ or ‘oncotic.’ This issue is especially important in ischaemic heart disease because acute ischaemia is one of the most injurious stimuli to metabolically active hearts, and such stimuli sometimes exceed the range of simply pro-apoptotic signals. Therefore, the role of apoptosis in myocardial infarction and the future of anti-apoptotic interventions as therapeutic tools remain obscure. However, it should be remembered that apoptosis is one of the early causes [75] rather than a terminal event that is associated with the end stage of disease, and there are reports of beneficial effects of anti-apoptotic interventions. Therapeutic approaches to apoptosis in ischaemic heart disease are in their infancy and quite long from becoming practical. Through extensive apoptosis research, however, progress is being made in understanding the mode of ischaemic cardiomyocyte death, and this may finally lead to new therapies for the reduction of infarct size.

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