Apoptosis and the Heart

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Abstract

The phenomenon of programmed cell death, marked by *de novo* transcription and translation of proteins and often a unique phenotype termed apoptosis, has become a subject of intense investigations in broad areas of biological sciences. PCD/apoptosis has also been demonstrated in the cardiovascular system as part of developmental and physiological conditions as well as pathophysiological/disease processes. The emerging literature already suggests the possible involvement of PCD/apoptosis in many cardiac pathologies which have been recently reviewed in detail [20]. It is clear however that the evidence generated to date, although interesting, must be pursued by more detailed biochemical and molecular investigations of the spatial and temporal relationships of PCD to the underlying cardiapathology and especially to elucide the molecular underpinning of PCD in myocytes.

Finally, preliminary experiment with the potent multiple action neurohormonal antagonist, carvedilol, indicate that it is possible to block ischemia induced apoptosis in cardiac tissue. However, many more studies will be necessary to explore the discrete mechanisms of carvedilol action on PCD/apoptosis.

Key words: apoptosis, heart, PCD, ischemia corvedidol, cardioprotective drugs

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Apoptosis or Programmed Cell Death (PCD) became a major focus of research in cancer, immunology, developmental biology, neurobiology and cardiovascular sciences. The new era of PCD research is still not clearly defined as phenotypic and genomic markers are interchanged frequently. The traditional description for cells that ceased to demonstrate viability "necrosis", is no longer believed to be the primary term synonymous for a non-functional and non-viable cell as the role of PCD/apoptosis steadily unfolds.

Necrosis versus Apoptosis

Necrosis has been associated with conditions that lead to cell membrane damage, osmotic imbalances, ion fluxes, cell swelling and death.. The passive influx of ions and accumulation of Ca^{+2} are the hallmarks mechanism of necrosis. It is generally accepted that expression of new mRNA or proteins is not required for the necrotic process [1]. Necrosis accounts for numerous destructive changes associated with pathological conditions such as hypoxia, energy deprivation and inflammation; a prime example is neuronal death in response to ischemia, a condition that is marked by lack of critical levels of oxygen and glucose which within minutes, lead to severe perturbations of cell function, swelling, lysis and death.

PCD, first coined by Lockshin and Williams [2] to describe the developmentally-regulated death of specific larval muscles following the emergence of the adult moth is now recognized as a primary and wide spread mechanism by which cell viability and tissue remodeling is regulated. PCD is distinct from necrosis by its requirement of new gene expression. PCD originally implied that death of cells occurred within a developmental context in response to a definable physiological stimulus. This early sentiment, however, has now been expanded to include cell death that involves gene-mediated process which is not necessarily associated with the triggering stimulus. Thus, the developmental researchers use PCD to describe specific cell loss that takes place in a spatial and temporal fashion that is predictable, and is induced by physiological conditions of the developing organism. However, most of the non-developmental researchers that study PCD, merely associate PCD to any cell death that is dependent on *de novo* gene expression regardless of the nature (physiological or non-physiological) of the stimulus.

In all cases of PCD, the trigger for the event may be loss of or gain in external signals that lead to activation of the program and *de novo* gene expression. This is illustrated by the ability of RNA or protein synthesis inhibitors to markedly interfere with PCD. Additional support for the

dependence of PCD on specific gene expression is the C. Elegans model where 131 cells die at specific developmental stages; in this model PCD is dependent on a group of genes termed ced (cell death) which if mutated, block the loss of the targeted somatic cells, and these cells persist in the mutant animals [3].

This introduction cannot be complete without attempting to discuss "apoptosis", a term introduced by Kerr et al [4] to describe a common series of morphological changes that accompany the death of cells in a wide variety of tissues. These investigators noted discrete phenotypic changes including "blebbing" of the cell membrane and "shedding" of cytoplasmic "blebs" that can be phagocytosed by other cells. The nuclei of apoptotic cells display electron-dense chromatin along the inner surface of the nuclear membrane and coincident with these morphological changes, the DNA within the nucleus is digested by an endonuclease that cleaves the chromatin between individual nucleosomes. Such digested DNA, when size-fractionated by electrophoresis, produces a "ladder" appearance consisting of multiples of 180 bp of DNA fragments. However, this phenomenon is not universal to all instances of apoptosis. Taken together apoptosis is a morphological description of the phenotypic changes seen in many but not all examples of cell death. Thus, PCD and apoptosis may not identify the same phenomena and therefore, caution should be exercised in the interchangeable use of these terms. Clearly, within the category of PCD, not all dying cells display apoptosis and numerous examples to this fact have been reported in both invertebrate and vertebrate developmental biology research. Finally, both PCD and apoptosis may account for cell death; for example, when cilliary neurons of the developing chick die during neurogenesis, death appears to be non-apoptotic while their death due to target removal elicits the apoptotic pheotype.

Over this background, PCD and apoptosis in normal and disease hearts will be discussed. PCD in cardiac tissue is an emerging topic as only a relatively small number of studies addressed this phenomenon in isolated myocytes and cardiac tissue. Furthermore, most studies so far have focused on the phenotypes more closely related to apoptosis since the identification of genes associated with PCD seems to have met significance technical difficulties.

APOPTOSIS AND THE HEART

In Vitro evidence for PCD/apoptosis in cardiac myocytes

Heart injury following ischemia or ischemia and reperfusion has been a subject of numerous studies over the past 3 decades. However, while necrosis is believed to account for some of the myocytes death, the mechanism(s) of the delayed, or ultimate, cell death has not been clarified. In a recent report by Tanaka et al. [5], cultured neonatal rat myocytes have been exposed to hypoxia (95% N_2 +5% CO_2) and markers of DNA damage assessed along with mRNA of the known apoptosis antigen-Fas. In this system, hypoxia induced DNA fragmentation typical to internucleosomal breakdown has been observed in myocytes only within 12 hrs; this event was also confirmed by nick-end labeling *in situ*. Furthermore, Fas mRNA levels in the myocytes were increased (2 fold) suggesting a role for the Fas-Fas ligand mechanism in hypoxia induced myocyte death. These observations were confirmed and extended by Umansky et al [6] in rat neonatal cardiomyocytes studied by light microscopy, electron microscopy, flow cytofluorometric analysis and DNA electrophoresis. In this study, rat cardiomyocytes were exposed to ischemia alone (by oxygen, serum and glucose deprivation) or "ischemia and reperfusion" (restoration of oxygen, serum and glucose following designated periods of deprivation). Following 8 hrs of ischemia, over 90% of the cells have displayed shape change - small, round appearance, contraction and loss of adherence. All of the adherent and most of the non-adherent cell remained impermeable to propidium iodide but nuclear morphology assessed by Hoechst 33342 showed enhanced chromatin condensation. "Reperfusion" of the cells resulted in over 50% of non-adherence with marked permeability to propidium iodide and apoptotic appearance after staining with Hoechst 33342 dye. However, many cells still reverted to normal morphology after "reperfusion". Thus, nuclear morphology alone cannot be viewed as a reliable marker of apoptosis in this system. Ultrastructural analysis of the "ischemic" cells revealed both apoptotic and necrotic cells and this mixed phenotype was also clearly seen after "reperfusion". Non adherent cells found after "ischemia" (prior to "reperfusion") were largely necrotic whereas following reperfusion, both necrotic and apoptotic cells were observed. Furthermore, in non-adherent cells of the "ischemia" alone period, evidence for internucleosomal DNA degradation was observed but much more so after "reperfusion". Further effort to examine whether *de novo* synthesis proteins are associated with the phenotypic changes of apoptosis, the rat cardiocytes were treated with cycloheximide or puromycin; however, neither agent blocked cell death in the cardiomyocytes exposed to "ischemia" or "ischemia and reperfusion". In fact, both protein synthesis inhibitors induced myocyte death and apoptotic DNA fragmentation. Furthermore, an array of growth factors (PDGF, $TGF\beta_1$, EGF, FGF, IGF) or protease inhibitors (pepstatin, leupeptin, TPCK, TLCK, calpain inhibitors) failed to block "ischemia" induced cell death except of the IGF and calpain inhibitors that blocked apoptosis.

These data generated in an *in vitro* system clearly demonstrate that apoptosis is an important component of cardiomyocyte death induced by "ischemia" and "reperfusion". However, ischemia may not be the only trigger of non-necrotic cardiomyocytes death. In an *in vitro* preparation of rat papillary muscle exposed to stretch by rapid weight overload over several hours, evidence for apoptosis in both myocyte and non-myocytes was noted along with marked expression of the Fas protein. The overstretching procedure also elicited an oxidant stress evidenced by superoxide anion formation; the detrimental

role of the oxidative stress in apoptosis and muscle dysfunction is inferred by the complete abolition of the stretch induced apoptosis and contractile dysfunction by the NO-releasing agent C87-3754 [7].

Evidence for PCD/apoptosis in cardiac tissue *in vivo*

PCD/apoptosis in cardiac development

Myocyte cell death has been documented as early as the primordial cardiac embryogenesis [8]. Recent studies have extended the investigation of PCD/apoptosis into the late gestational and postnatal life in rats [9]. PCD was not evident (based on TUNEL and DNA breaks) in the fetal heart. However postnatally, up to 21 days, DNA strand breaks in myocytes and non-myocytes nuclei were present at all time points (1, 5 and 11 days postnatal). The rate of DNA degradation declined steadily from a high level at day 1 of the postnatal period (incidence highest in the right ventricle) a much lower level at day 5 and day 11. Most notably, bcl-2 mRNA levels (which is translated to an antiapoptotice protein) were high in fetal myocytes but decreased markedly at day 1-5 postnatal but progressively increased again at day 11-21 postnatal. Also, the expression of bcl-2 was lower in the right ventricle (where PCD was at higher rate) than in the left ventricle (where PCD incidence was lower). These data taken together suggest that PCD in myocytes of the perinatally developing heart is inversely related to bcl-2 levels which may be an important control mechanism of cardiac remodeling at the perinatal period. Cardiac apoptosis has recently been found in a 34 week infant. Two weeks before delivery, the atrial and ventricular rates were 144 and 47 beats per minute, respectively. Final diagnosis found that massive apoptosis selectively destroyed the entire right ventricle and the His bundle [21].

PCD and apoptosis in cardiac ischemia

Persistent myocardial ischemia results in cardiac necrosis. While reperfusion is known to be the most effective measure to salvage ischemic myocardium at a certain "time window", it has also been shown that the reperfusion event may carry significant injury to myocytes as reperfusion of the ischemic tissue is associated with a burst of oxygen radicals and acute leukocytic reaction [10]. Since oxygen radicals metabolism may be involved in genes associated with apoptosis [11] it seems plausible to investigate whether cardiac ischemia (30 min) and reperfusion (4 hours) is associated with apoptosis. This hypothesis may have been explored in a rabbit model of cardiac ischemia and reperfusion [12] using transmission electron-microscopy and *in situ* nick-end labeling methods. In this model, apoptosis was clearly detected in the ischemic (but note normal) cardiac tissues; furthermore, permanent ischemia for the same duration did not result in evidence of apoptosis. Also, very brief ischemic insults (5 min) with or without reperfusion failed to elicit apoptosis. Most interesting was the authors findings demonstrating no diminution in the apoptotic response to granulocytopenia suggesting a leukocyte - independent mechanism in the apoptotic response.

The pioneer data reported in the study by Gottlieb et al [12] were recently substantiated in a rat model of cardiac ischemia [13]. PCD was assessed by the TUNEL method and the "Ladder" phenomenon of DNA fragments separated by gel electrophoresis. Moreover, the expression of bcl-2, Bax and Fas proteins in myocytes was examined by immunocytochemistry. In this study, apoptotic cell death comprised the majority of the dying cells (90%) as early as 2 hrs post ischemia and evidence for continued nucleosomal DNA breakdown ("Ladder") could be detected up to 2 days post ischemia. In addition, the expression of Bcl-2 and Fas in myocytes increased dramatically post ischemia. This detailed study strongly suggests that PCD is the major form of myocardial damage produced by cardiac ischemia and that necrosis of myocytes follows apoptosis. The enhanced expression of Fas (a pro-apoptotic protein) may be implicated in the activation of apoptosis which may override the increase in bcl-2 (an Anti-apoptotic protein).

Apoptosis in experimental models of heart failure

Heart failure is marked by progressive deterioration of cardiac function due to ischemic of cardiomyopathic processes. Oxygen stress has been implicated in congestive heart failure in humans. Recently, the possibility of myocyte death in failing heart was studied in a dog model of congestive heart failure induced by multiple intracoronary microembolizations protocol which results in severe reduction of left ventricular ejection fraction [15]. Evidence for cardiocyte apoptosis was based on transmission electron microscopy and *in situ* nick-end labeling of nuclear DNA fragmentation. Clear evidence for apoptosis was found primarily in myocytes adjacent to old infarcts of all dogs with chronic heart failure. However, only 11% of the apoptotic cells were myocytes. This data confirmed for the first time the presence of apoptosis in cardiac tissue, *in vivo* including myocytes of chronic heart failure, thereby supporting an ongoing process of myocardial remodeling process that includes apoptosis.

Apoptosis and the human heart

While research on apoptosis in cardiac physiology and pathophysiology is growing rapidly, little is known on the presence, evolution and mechanisms of apoptosis in the human heart. In one study that attempted to explore the phenomenon in humans, sinus nodes excised from patients with long QT syndrome were examined by light and electron microscopy. The histological abnormalities of the sinus tissue included distinctive focal fibrosis and some degenerating myocytes. Ultrastructural fractures of the degenerated nodal cells were found to be typical of apoptosis. No biochemical confirmation of the structural changes resembling apoptosis or DNA damage was provided. However, narrowed microvessels might have contributed to the pathological process by inducing ischemia thereby leading to apoptosis-like cell degeneration [16].

Cardioprotective drugs that may act by blocking apoptosis

Carvedilol - a multiple action neurohormonal antagonist

Carvedilol is a vasodilating β-adrenoceptor antagonist with potent antioxidant actions. Carvedilol is marketed for the treatment of mild to moderate hypertension and chronic stable angina. Recently, carvedilol phase III clinical trials in patients with congestive heart failure (ischemic and non-ischemic) have been stopped by the FDA (USA) due to marked efficacy of carvedilol in reduction of mortality - 65%. As a member of the β-blockers class and a potent antioxidant, carvedilol has demonstrated potent anti-ischemic and cardioprotective actions in numerous experimental models of ischemic cardiac injury with remarkable consistence across species or experimental paradigms [17]. Moreover, in all studies where carvedilol was compared with equipotent β-blocking regimen of propranolol (a potent non-selective β-blocker) a clear superior cardioprotective action has been demonstrated in carvedilol treated animals. Since ischemia and reperfusion has been shown to elicit apoptotic myocyte death [12] the effect of carvedilol on myocardial apoptosis was investigated in a rabbit model of cardiac ischemia and reperfusion. In this model, ischemia and reperfusion elicits widespread apoptosis in cardiac myocytes as depicted in Figure 1. Carvedilol treatment prior to the ischemic insult significantly reduced the apoptotic myocytes from 14.7 cells per field to 4.5 cells per field ($p < 0.01$). Carvedilol was more potent in blocking ischemia/reperfusion induced apoptosis as compared to an equipotent dose of propranolol. The molecular mechanisms of the anti-apoptotic effect of carvedilol in cardiac ischemia are not fully explored. However, carvedilol's potent antioxidant activity [18] may have contributed to its protective effects as oxygen radical have been shown to induce apoptosis [12]. Furthermore, carvedilol has been shown to suppress the expression of certain genes, such as ICAM-1 [19] and therefore, it is possible that suppression of other genes such as Fas, Bak or Bax, which promote apoptosis, or up-regulation of genes that suppress apoptosis, e.g., Bcl-2, may be regulated by carvedilol. Such studies are now in progress.

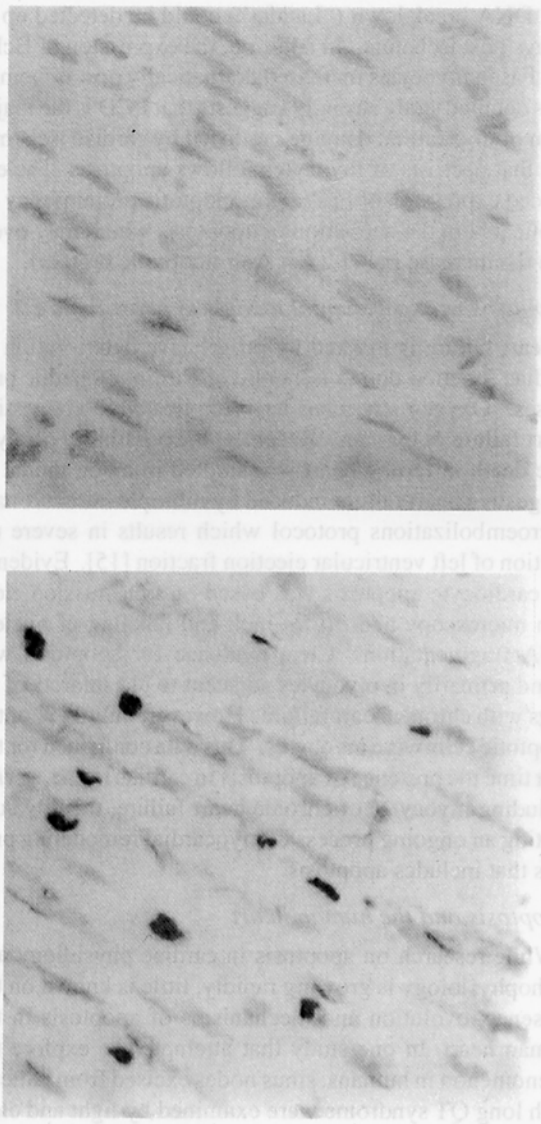

Figure 1 Photomicrographs of rabbit heart tissue after 30 min. of ischemia and 4 hr of reperfusion, incubated with terminal deoxynucleotidyltransferase to add residues of digoxigenin nucleotide catalytically to the 3'-OH end of DNA, and labeled with peroxidase substrate solution. (upper panel) Myocardium without exposure to ischemia. (lower panel) Myocardium exposed to 30 min of ischemia followed by 4 hr of reperfusion.

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