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REVIEW

Apoptosis in cardiovascular disease

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Cardiologists are well acquainted with cell death due to ischaemic necrosis in the myocardium. Loss of the integrity of the cell membrane leads to leakage of intracellular dehydrogenases and kinases, as well as structural proteins such as troponin T, into the interstitial spaces and ultimately into the plasma. Fluid and ions flood into the cell causing the cytoplasmic swelling which is observed by microscopy in early necrosis. Mitochondrial swelling and disruption occur early in necrosis while nuclear fragmentation occurs late in the process. The necrotic cells invoke an acute inflammatory response and complement is bound; repair by fibrosis follows.

Apoptosis is a mechanism of cell death less familiar to cardiologists which has very different characteristics. In apoptosis the cell is induced actively to participate in its own destruction by synthesising enzymes which degrade and fragment the DNA strands within the nucleus. The process was recognised initially as a part of normal morphogenesis—for example, apoptosis is necessary to create interdigital spaces to form the hand in the human embryo, and it is the way tadpoles lose their tails. Apoptosis can be regarded as a neat and tidy way to remove tissue without creating a damaging inflammatory process. The condensed remnants of the apopotic cell are incorporated into adjacent cells; these may be either the specialised tissue cells (hepatocytes or myocytes) or endothelial cells that do not normally have a phagocytic function.[1 2] Ingestion of apoptotic cells by macrophages also occurs following expression of thrombospondin on the surface of the cell[2 3] and involves the vitronectin receptor of macrophages. The ingestion process does not invoke an acute inflammatory response.

Features of apoptosis and methods of recognition

The essential process in apoptosis is the production of endonucleases which begin to cut the DNA into short 180 base pair fragments or multiples of this length. If a tissue in which apoptosis is occurring is homogenised and the DNA extracted it can be run on agarose gels and the multiple DNA fragments can be recognised as a characteristic ladder pattern with multiple bands at regular short intervals. This method is widely used and is sensitive in detecting apoptosis; however, in a tissue where several cell types are present it gives no indication of which cell type is undergoing apoptosis.

The nuclei in apoptosis when examined by electron microscopy have characteristic ultrastructural features with chromatin condensing into peripheral crescentic masses followed by the nucleus collapsing into itself to form a small dense body. The cytoplasm of the cell also condenses but mitochondria remain intact until the cell is just a small dense body, a process graphically described as "baling". Electron microscopy is tedious and time consuming if large areas of tissue are to be studied. Simple staining of the DNA by dyes such as propideum iodide does allow the nuclear condensation to be recognised by light microscopy but the method works best in single cell layers such as those grown in culture dishes rather than in whole tissues. The technical difficulties in demonstrating apoptosis in tissues resulted in the process being largely ignored, although it was described in 1980.

A major revolution in technology occurred in 1992 with the introduction of the TUNEL technique,[4] now widely available as commercial kits which allow any laboratory with the capacity to cut histological sections ostensibly to identify apoptotic cells in human tissues. Apoptotic indexes (the proportion of any particular cell type marking as positive in human tissues) are being reported in a steadily increasing number of scientific papers. Six papers on apoptosis in human vascular tissue appeared in 1993, 30 in 1995.

The TUNEL technique depends on the preferential binding of terminal deoxynucleotidyl transferase (TdT) to the exposed 3'hydoxyl ends of DNA at the sites of strand breaks, created in the apoptotic process. TdT is then identified by adding deoxyuridine triphosphate (dUTP) labelled with biotin, a process known as nick end labelling. A range of methods is used in the different commercial kits available to produce a positive signal from the biotin including immunoflourescence and peroxidase.

The TUNEL method is not without its technical challenges. The optimum reagent concentrations vary from tissue to tissue and it is all too easy to obtain apoptotic indexes that are too high to be credible. False negatives are equally easy to obtain. The specificity of a positively marked nucleus for certain cell death may not be absolute. The technique may in fact be oversensitive and detect a degree of strand breaking that indicates remedial DNA damage. One conclusion to be drawn is that more than one method of showing apoptosis must be used in any particular study of human tissue. A TUNEL index in isolation is almost impossible to interpret. Despite these cautions the wealth of papers now appearing indicates that apoptosis

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is present in the atherosclerotic plaque and that it occurs in cardiac myocytes.

Triggers for apoptosis

Inherent in the physiological role of apoptosis is the concept that a cell which has become surplus to requirements should autodestruct.[5] Specific triggers for the induction of apoptosis are known. The p53 tumour suppresser protein acts on cells with DNA damage to invoke apoptosis rather than allow survival of a cell with aberrant genomic material. Loss of p53 is an important factor in the development of malignant tumours. A cell surface antigen Fas, also known as APO-1, acts as a receptor for a ligand (FasL) which induces apoptosis. FasL is as yet poorly defined but occurs on cytotoxic lymphocytes. The Fas receptor has a 70 amino acid sequence that is homologous with the tumour necrosis factor (TNF)α receptor 1. This sequence is necessary for the induction of apoptosis and has become known as the "death domain". TNFα itself will enhance apoptosis acting on this domain in the TNF 1 receptor. The Fas-FasL mechanism is important in eliminating unwanted immunocompetent cells reacting to self and may be part of the mechanism by which autoimmunity is avoided.[6] In mice, mutations in the genes controlling Fas and FasL lead to autoimmune disease.[7]

Perforin and granzymes are normally regarded as substances released from cytotoxic lymphocytes that destroy target cells by forming holes in the external cell membrane in a process akin to necrosis. Perforin and granzymes can however also induce apoptosis after entering into the target cells.[8 9]

The best known factors controlling apoptosis are a multigene family of Bcl-2-like proteins with homologous structure, some of which such as Bcl-2 itself inhibit apoptosis[10 11] and others such as Bax and Bak promote it. A determining factor for a cell continuing to exist without going into mitosis or apoptosis may be the ratio of different components of the extended Bcl-2 family that are being produced. One potent trigger of apoptosis in cell lines that are capable of division in adult life is withdrawal of growth factors. The general rule for a cell is divide or die. For cell lines that do not divide in adult life such as neurones and myocytes apoptosis must be under tight suppression. Once apoptosis is initiated via a surface receptor a cascade of enzymes are induced within the cell that act as the effector mechanism. The demonstration of one or more of these enzymes within cells using immunohistochemistry is a valuable adjunct in confirming apoptosis is occurring. These enzymes include the ICE (interleukin converting enzyme) family of cysteine proteases.

Apoptosis in human cardiovascular disease

ATHEROSCLEROSIS

In the evolution of a plaque from a fatty streak to a raised fibrolipid plaque, a crucial step is the formation of a core of acellular lipid. This core is traditionally regarded as being formed by the necrosis of lipid filled foam cells of macrophage origin. The mechanism responsible for necrosis was far from clear but combinations of hypoxia and cytotoxic lipid peroxides formed within the cell cytoplasm were popular explanations. It is now clear that to some extent macrophage death is via apoptosis.[12 13] At the margins of the core, macrophages when examined by electron microscopy show ultrastructural features of both necrosis and apoptosis, and in these areas of the plaque TUNEL positive macrophages are taken up by other viable macrophages. ICE expression confirms apoptosis is occurring.[14] The trigger for apoptosis could be a reduction of macrophage colony stimulating factor (M-CSF), the normal growth factor for this cell line, or be caused by TNFα which is produced abundantly by activated macrophages within plaques. Fas expression has not been studied.

In plaques which become vulnerable—subject to a high risk of disruption—the balance of collagen synthesis and degradation is a major factor in determining the amount of collagen in the plaque cap. Increasing numbers of lipid filled macrophages are associated with a fall in the number of smooth muscle cells that synthesise the collagen.[15] There is now evidence that this decline is mediated by apoptosis,[16] possibly triggered by the proximity to macrophages.[17 18] Activated macrophages produce TNFα and interleukin-1 β, which may be the effector mechanism.[19]

THE MYOCARDIUM

Myocytes like neurones have no potential for regeneration by mitosis and last the life of the individual. One unanswered question is whether there is an age related loss of myocytes by apoptosis in the absence of any vascular or myocardial disease. In both experimental models and human myocardial disease there is now abundant evidence that myocyte apoptosis can be induced. What is totally unresolved is whether this process is primary or secondary and its importance in leading to progressive myocardial dysfunction.

In animal models ischaemia, reperfusion, and myocardial infarction lead to apoptosis in myocytes.[20–22] The distribution of TUNEL positive myocytes suggests that at the margins of areas of infarction caused by necrosis, apoptosis may be an additional mechanism of cell death. In one of the models using ischaemia produced by microspheres injected into the coronary artery of the dog, apoptosis was regarded as a factor in the development of cardiac failure in long term studies.[22] There is some evidence from human studies that infarcted myocardium shows DNA laddering on agarose gel electrophoresis indicating apoptosis concomitant with necrosis.[23] Other stimuli identified as producing myocytes staining as TUNEL positive in animal models include rapid ventricular pacing,[24] mechanical stretch,[25] pressure overload hypertrophy,[26] ventricular hypertrophy due to hypertension,[24] and cardiac rejection where apoptosis occurs concurrently with nitric oxide synthetase induction.[27]

The clear implication from this wide range of models is that the induction of apoptosis is a

common final pathway of a large number of insults and myocyte loss may be an important mechanism in end stage disease. Based on this experimental model, four important studies have been made of human myocardial disease.[28–31]

In the conduction system, James[28] performed simple observational studies indicating that loss of conduction myocytes may be due to apoptosis. The studies illustrated TUNEL positive cells in conditions in which progressive loss of conduction tissue occurs. The illustrations in some cases showed very high apoptotic indexes. The work lacked other markers to confirm the observations.

In a study of hearts obtained at autopsy from subjects dying of coronary artery thrombosis in which reperfusion of the occluded artery had been achieved in life, apoptotic myocytes were found particularly in the border zone between viable myocardium and the infarcted tissue.[29] The study was small and carried out on necropsy material, which further magnified the complexities of the TUNEL method but it confirmed observations made in experimental models.

Narula and colleagues[30] undertook a study of seven hearts from subjects with advanced cardiac failure (three with ischaemic disease and four with idiopathic dilated cardiomyopathy), obtaining the material at the time of cardiac transplantation. Apoptotic myocytes were observed in five cases with indexes ranging from 5–35%. Of the five positive cases, four were from the patients with idiopathic dilated cardiomyopathy. Apoptotic myocytes were not evenly distributed throughout the myocardium but occurred in focal areas. Confirmation of apoptosis was obtained by DNA extraction and demonstration of laddering on gels in four hearts. The workers concluded that apoptosis was occurring, that it contributed to continuing myocyte loss and, therefore, possibly to heart failure and, as a preliminary observation, idiopathic dilated cardiomyopathy showed a greater degree of apoptosis than ischaemic disease. Nevertheless questions remain and the work can only be considered preliminary. The recorded apoptotic indexes were high; the patient with the highest apoptotic index (35·5%) had the highest ejection fraction and cardiac index, which seems discordant with apoptosis causing continuing myocardial dysfunction. Apoptosis is an event that can be recognised only for about 24 hours before the cell is removed. Such high indexes cannot therefore be a reflection of the state of the whole myocardium and are not true reflections of a rate of myocyte loss unless it is postulated that the patients were in extremis. Other work showed that in tissue extracts of hearts with dilated cardiomyopathy there was at least a sixfold increase in deoxyribonuclease 1 (DNAse 1) compared with normal myocardium.[31] No other myocardial disease states were examined.

Work by Mallat *et al*[32] concentrated on arrhythmogenic right ventricular dysplasia using eight necropsy specimens. In six of eight hearts with right ventricular dysplasia TUNEL positive myocytes were observed. TUNEL positive myocytes were not evenly distributed, but in local areas up to 28% of myocytes were labelled. These areas tended to be away from the areas of myocardium which were already replaced by adipose tissue. Expression of CPP32, an ICE related product, within myocytes was demonstrated. The use of necropsy material introduced another variable into what is already a technique marked by its difficulties; however, four control specimens showed no positivity by TUNEL in myocytes.

Summary

The current state of knowledge concerning the role of apoptosis in the myocardium is therefore far from complete. The high indexes reported are puzzling; if they did reflect the rate of myocyte loss in the absence of any regenerative capacity ventricular function would be expected to fall rapidly. It may be better at the present time to regard positive TUNEL marking as an indicator of myocyte damage with DNA fragmentation which might be repairable. The rate at which TUNEL positive cells are removed in the myocardium is unknown. If the lag time for removal of apoptotic cells is days or even longer the high indexes are more plausible. In patients with end stage heart failure, irrespective of its cause, apoptosis is likely to be a non-specific mechanism of slow myocyte loss. The case for apoptosis having a primary role is more convincing in arrhythmogenic right ventricular dysplasia. Loss of myocytes by apoptosis would not be expected to induce inflammation and reparative fibrosis, and would be a logical mechanism for the bland replacement of myocytes by adipose tissue, which is so typical of right ventricular dysplasia.

The position and importance of apoptosis in human cardiac disease is only just beginning to be clarified—a further flood of papers in this area will follow. Cardiologists will need to interpret the results with caution and beware of the difficult technology involved. It is, however, an important subject—apoptosis could conceivably be blocked therapeutically.

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IMAGES IN CARDIOLOGY

Tricuspid valve endocarditis

A 65 year old woman with chronic renal insufficiency who had been on venous haemodialysis for six years presented with a history of weight loss, persistent fever, chest pain, and cough lasting for several months as well as recent onset of heart failure. A loud pansystolic heart murmur was detected. Tricuspid valve endocarditis with gross tricuspid insufficiency was diagnosed by means of echocardiography. Despite broad spectrum antibiotic treatment and supportive therapy she developed septic shock and fatal multiorgan failure.

Necropsy confirmed tricuspid valve endocarditis, consisting of two bulky soft vegetations with smooth glistening surface, measuring 4 and 3·5 cm, attached to the anterior valve leaflet, and numerous smaller vegetations on the other leaflets (A).

Histological examination of the vegetations showed huge fungal masses, consisting of septate filaments with a 45° branching characteristic of *Aspergillus* spp (B, Grocott silver stain, × 250). The lungs displayed numerous infarcts related to arterial emboli, which contained aspergillus colonies.

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