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INVITED REVIEW

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The role of apoptosis in myocardial ischemia: a critical appraisal

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Abstract The role of apoptosis in cardiac ischemia is not clarified yet. Own data show that suicidal cell death is apparently not important in global ischemia where it only affects a small number of myocytes (8 %) while the majority of cells, i.e. 92 % die by oncosis. In acute regional ischemia it is most probably not a decisive factor. However, more solid data are needed to justify this statement. Human hibernating myocardium shows an activation of the apoptotic cascade, i.e., apoptosis might contribute to cell loss in this pathophysiological situation of multiple ischemic episodes. Manifold unresolved issues contribute to problems in determining the role of apoptosis in ischemia. These include 1) Uncertainty of the duration of the apoptotic cascade from activation of death receptors at the cellular membrane until DNA fragmentation occurs, 2) The role of the mitochondrial pathway, 3) The mode of removal of myocytes after cell death has occurred, 4) Technical problems such as specificity of the TUNEL method, detection of low abundance proteins such as activated caspases or cytochrome C, statistical considerations. These issues and many others should be clarified before any definite conclusion as to the role of apoptosis in ischemia may be drawn.

Key words Apoptosis – necrosis – cardiac ischemia

Introduction

In 1972, Kerr described the occurrence of apoptosis as a "basic biological phenomenon with wide ranging implications in tissue kinetics" (19). Since then, in a growing number of publications, the importance of apoptosis in a variety of pathophysiological situations has been reported including embryonic development, hematological disorders, and particular emphasis has been placed on the development and regression of cancer cells. Only later, starting in 1994, the role of apoptosis in the cardiovascular system was investigated in situations such as arteriosclerosis, coronary artery restenosis after angioplasty, acute and chronic ischemia, and heart failure (15, 16).

The present short review will report a critical evaluation of the possible role of apoptosis in different situations of ischemia. Cell death in ischemia may occur by two different mechanisms: oncosis or "accidental" cell death, and "suicidal" cell death or apoptosis (Table 1).

Table 1 Cell death in ischemia

	Apoptosis suicidal	Oncosis accidental
Cell volume	shrinkage	edema
Chromatin	condensation	clumping
Mitochondria	intact	damaged
Cell membrane	intact	ruptured
Nucleosomal DNA	cleaved 185 kB	irregular
Inflammation	no	yes

From Majno & Joris, 1995 (25)

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Cell swelling, irregular clumping of chromatin, mitochondrial damage and loss of cell membrane integrity characterize oncosis. A later result is cleavage of the nuclear chromatin into irregular fragments and final disappearance of the injured cell by an inflammatory response. In apoptosis, on the other hand, the cells and nuclei will shrink, the nuclei show dark condensed chromatin and the cellular membrane and mitochondria are apparently unchanged. The nuclear chromatin shows fragmentation into nucleosomal particles of a regular size of about 185 base pairs and the cells will finally disappear by phagocytosis through neighboring cells. Cellular degradation after the occurrence of cell death should be called necrosis independent of the type of cell death, using the correct nomenclature. The term oncosis as a definition of accidental, i.e. ischemic cell death as proposed by Majno and Joris (25) will be used throughout this text. In the following, the role of apoptosis under four different situations will be discussed: acute global ischemia, anisomycin infusion into ischemic myocardium, hibernating myocardium, and acute regional ischemia.

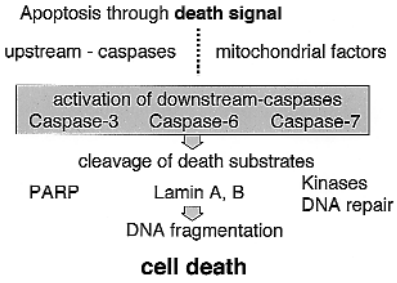

Fig. 1 Simplified version of the major events in the apoptotic cascade.

Experimental results

Acute global ischemia was induced for 90 min at normothermia followed by 6 h of reperfusion in the canine heart. Transmural needle biopsies were removed before and at the end of the ischemic period, and at early (2 h) and late (6 h) stages of reperfusion. Functional recovery and adenine nucleotide contents were measured in addition to morphological analysis using electron microscopy and confocal laser scanning microscopy (14).

The apoptotic cascade consists of many different steps until DNA fragmentation occurs. These include involvement of mitochondrial factors, activation of upstream caspases followed by activation of downstream caspases, cleavage of death substrates and finally, as the very last step, the occurrence of DNA fragmentation (Fig. 1). In the study described here it was evident that activation of mitochondrial factors occurs already during ischemia. Similarly, cleavage of poly(ADP ribose) polymerase (PARP, a DNA repairing enzyme) was present during the ischemic period. At the end of the early reperfusion phase, lamin cleavage was evident. In control and ischemia, 98 % of all myocytes showed an intact lamin content of the nuclear envelope but this sharply decreased to 40 % after 2 h reperfusion. Lamin in its different isoforms, A, B1 and B2, is a main component of the nuclear membrane and its degradation is the prerequisite for the occurrence of DNA fragmentation. At the end of late reperfusion, DNA fragmentation as shown by the TUNEL method was evident in 8 % of all myocytes and in about 8 % of non-myocytes (for extensive data see (14)).

Electron microscopy showed irreversible ischemic injury, i.e. oncotic cell death in the majority of myocytes at the end of ischemia. Only a few interspersed cells were of either normal or slightly injured appearance. From this finding it was concluded that apoptotic and oncotic cell death could occur simultaneously. However, the

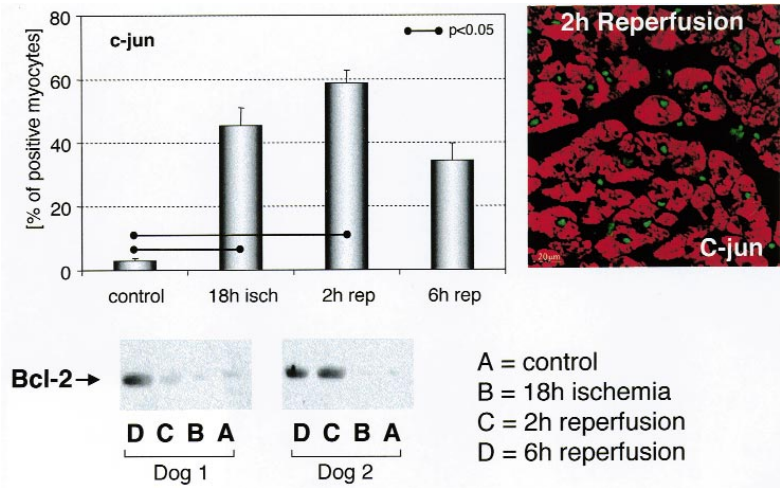

Fig. 2 C-jun appears in cardiomyocyte nuclei during ischemia (graph and immunoconfocal picture) and bcl-2 is upregulated during reperfusion (at the bottom). The confocal micrograph shows myocytes in red by phalloidin and specific labeling in green.

exact mechanism which determines the occurrence of either mode of cell death is still unknown.

Functional recovery and restoration of adenine nucleotide content were minimal further confirming the diagnosis of irreversible ischemic injury.

From this study it was concluded that ischemia initiates the apoptotic cascade but that reperfusion is needed to complete apoptotic cell death by DNA fragmentation.

In a different study, global ischemia was maintained for 18 h at 2 °C, which was followed by reperfusion (24). Here, cellular injury at the end of ischemia was reversible varying from slight to moderate severity and most cardiomyocytes recovered during the reperfusion period, as did cardiac function and the adenine nucleotide pool. However, under this more protective experimental condition, a rate of apoptotic cell death of 7 % of all cardiomyocytes was still observed at late reperfusion preceded by PARP and lamin cleavage similar to the more severe ischemic injury situation described above. Interestingly, while c-jun was absent in control tissue, it appeared during the ischemic period in the nuclei of numerous myocytes and blc-2 expression as determined by Western blot increased markedly during the reperfusion phase (Fig. 2). These findings were interpreted as protective mechanisms counterregulating the susceptibility to apoptotic cell death. C-jun, which has been discussed as either a proapoptotic element or as a survival factor (5, 38), in this experimental situation seems to be more active in the prevention of apoptosis. The interesting conclusion from this experiment is the fact that ischemic cell death but not apoptosis could be prevented by hypothermia.

Anisomycin infusion into ischemic myocardium was studied in pig hearts after ligation of the left anterior descending artery (34). Macroscopically using the TTC staining procedure, anisomycin infusion resulted in salvage of the myocardium in this circumscribed region (Strohm, C., unpublished data). However, numerous cells positive by the TUNEL method (17.5 % of all cardiomyocytes) were scattered throughout the tissue (Fig. 3). This finding is in accordance with other publications describing the ability of anisomycin to provoke apoptosis. This seems to be a specific property of this drug and offers the possibility to use this type of tissue as a positive control to check for the effectiveness (or quality) of methods to detect DNA fragmentation.

Hibernating myocardium was studied in biopsies from human hearts that showed regional hypokinesia or akinesia indicating an adaptation to a chronic perfusion deficit during increased oxygen demand situations or even at rest (9, 12). The characteristic structural alterations consisted of an accumulation of extracellular matrix material, i.e. fibrosis, and an intracellular degeneration. Hypertrophy of myocytes was observed simultaneously with atrophy or cells of normal size, and cellular debris was abundant. The structural changes corresponded to the severity of clinical symptoms, e.g. reduction of regional wall motion, reduction of regional ejection fraction and an increased perfusion deficit. It was postulated that a vicious cycle of tissue damage takes place under this pathophysiological situation in the following way: myocytes are damaged by chronic ischemia. They sequester cellular debris into the extracellular space, which in turn activates fibroblasts and macrophages as evidenced by an increased production of TGF-β_1, a pro-fibrotic growth factor. Myocyte degeneration results finally in cell death and disappearance of the cellular remnants. An elevated synthesis of matrix components such as the different collagens, fibronectin and laminin leads to replacement fibrosis (9). Until now, the question how the myocytes are reduced in number has still not been clarified.

Very small myocardial needle biopsies were obtained from well-defined hibernating regions from patients undergoing coronary bypass surgery. Using Western blot and PCR it was possible to show that the apoptotic cas-

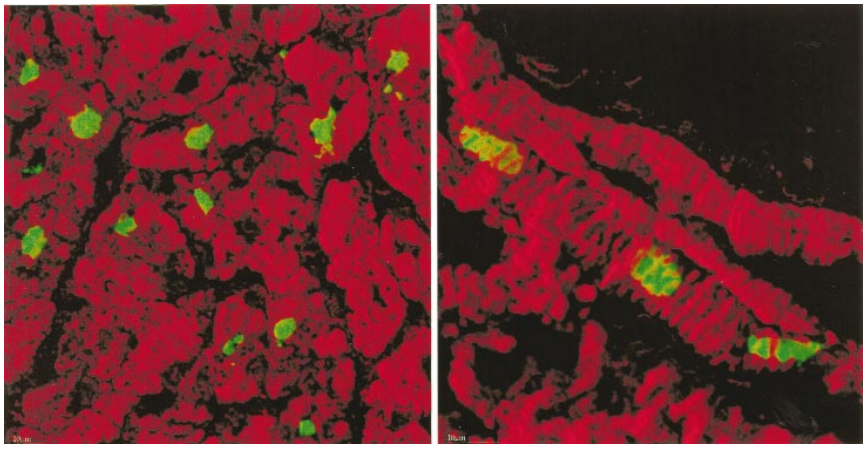

Fig. 3 In salvaged ischemic myocardium from pig heart, numerous TUNEL positive nuclei (green) are present after anisomycin infusion. Red-actin staining of myocytes by phalloidin.

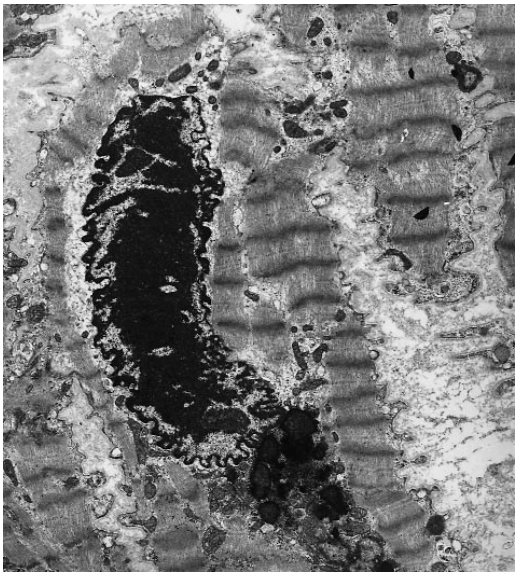

Fig. 4 Electron micrograph showing an apoptotic nucleus with dense chromatin. Reproduced with kind permission from Circulation 1997: 96: 2920–2931 (12).

cade is activated in human hibernating myocardium (10). Caspase 3 was not only elevated on the protein level but its activation was evident by the occurrence of the smaller 17-kDa fragment of the molecule. Similarly, bcl2 levels were reduced and bax was increased. Several myocytes showing DNA fragmentation with the TUNEL method were present, and it was also possible to find several myocytes with the electron microscope that exhibited the condensed nuclear chromatin typical of apoptosis (Fig. 4). Despite the fact that the rate of apoptosis cannot be determined in such small tissue fragments, these data indicate that apoptosis may play a role in eliminating myocytes from hibernating myocardium (11).

Regional ischemia and the role of apoptosis has been studied by our group in canine myocardium after coronary artery ligation (unpublished data). A minimal apoptotic rate of 0.002 % of apoptotic cells was found in the infarcted region indicating that oncotic cell death by far prevails.

To illustrate the confusion with regard to apoptosis in regional ischemia, we present here a summary of data from the literature which shows a great disparity in experimental animals, models, protocols, and methods used to determine apoptosis in its different stages (Table 2). It is evident from this table that the results are so different that a final conclusion as to the role of apoptosis in regional ischemia is still impossible. Differences exist with regard to time point of occurrence (permanent ischemia, limited ischemic interval, during ischemia alone or during postischemic reperfusion), time needed for DNA fragmentation to occur, localization of apoptosis (center of infarct or borderzone), as well as rate of apoptosis.

Table 2 Apoptosis in regional ischemia and reperfusion

Author	Year	Rate of apoptosis	Time	Species
Olivetti (29)	1996	11.6 % border 0.7 % remote	permanent	human
Saraste (32)	1997	0.04 center, 0.9 % border, 0.04 % remote	permanent	human
Kajstura (16)	1996	2.8 – 6.6 x 10^6	2–4.5 h ischemia (ligature)	rat
Cheng (4)	1996	155–700/10^6 border 84/10^6 remote	3 h to 1 – 2 d ischemia	rat
Takemura (37)	1997	absent	30 min isch/2 – 24 h rep	rabbit
Kajstura (17)	1998	76 – 225/10^6	45 min-6 d isch (constriction)	rat
Ohno (27)	1998	oncotic	30 min isch / 2 – 24 h rep	rabbit
Kurrelmeyer (22)	2000	12 – 40 %	3 – 24 h isch	mouse
Okamura (28)	2000	20 %	30 min isch / 6 h rep	rat

isch ischemia; *rep* reperfusion

Unresolved issues

Time course of apoptosis

The time course of the apoptotic cascade and the time interval needed until DNA fragmentation occurs are still unclear. This problem cannot be studied in tissue samples because of their static properties and until now published data are only available from non-cardiac cells such as lymphocytes and cancer cells.

Studies carried out by our group in adult myocytes in culture have recently shown that the breakdown of the mitochondrial pore potential is the first indicator of activation of the apoptotic cascade (36). This occurred 2 h after stimulation of the cells with 0.2 μM H_2O_2. The TUNEL reaction was positive after 14 h indicating that the completion of the apoptotic process is a time-consuming event (Fig. 5). This fact is important for the determination and evaluation of the feasibility of apoptotic rates in tissue in ischemia and under other pathophysiological conditions.

Furthermore, two other problems arise: 1) at what step is the entire apoptotic process still reversible? Upstream caspases? Downstream caspase activation? Or is cleavage of the death substrates, such as PARP and lamin, the decisive step and determines the point of no return? 2) It has been our own observation (K. Suzuki, data not shown here) that upon inhibition of caspase activation using caspase inhibitors, apoptosis is prevented, i.e. DNA fragmentation leading to TUNEL positivity is absent. However, as shown in *in vitro* unfixed cardiomyocytes using the propidium iodide stain the cells switched to oncotic cell death (36). Therefore, the final question is: can apoptosis definitely be inhibited without the occurrence of oncotic cell death? This information may be critical for possible future therapeutic interventions. In cardiomyocytes, these problems are still unanswered and in other cell systems different opinions prevail, i.e. a definite conclusion is still lacking.

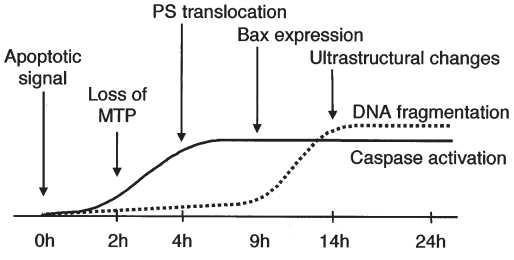

Fig. 5 Time course of the different steps of the apoptotic cascade in adult cardiomyocytes in culture.

The role of mitochondria

The central role of mitochondria in apoptosis was established several years ago (for reviews see (6, 7, 21, 30)). It has become evident that multiple proteins of the huge bcl-2 protein family regulate mitochondrial function. Proapoptotic factors such as bax, bak and bid counteract antiapoptotic components such as bcl-2 and bcl-X_L resulting under physiological circumstances in a balance in favor of protection from cell death and in preservation of the mitochondrial permeability pore complex (PTP). In pathological situations leading to the occurrence of cell death, PTP is disturbed, and cytochrome c, AIF (apoptosis inducing factor) and procaspase 9 are released from the intermembrane mitochondrial space into the cytoplasm where Apaf-1 is activated. This, in turn, will activate caspase 9 and, indirectly, other downstream caspases such as caspase 3, 6, and 7, but also protein kinases, phosphatases and others (Fig. 6) and thus promote DNA fragmentation by activation of endonucleases.

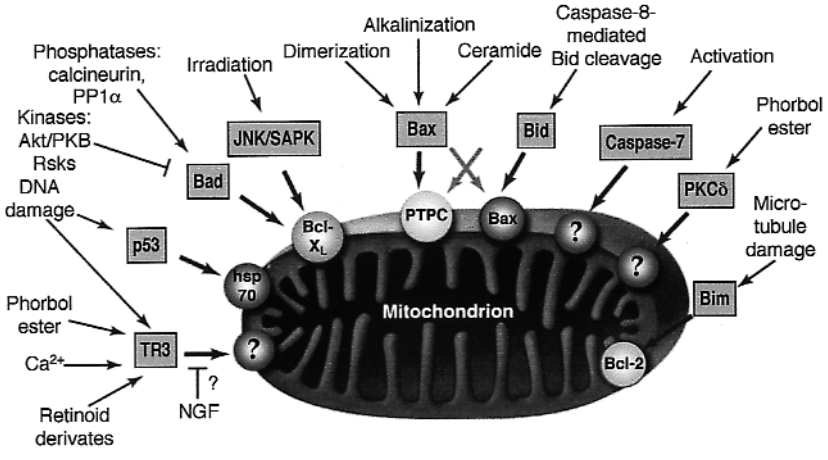

Fig. 6 Numerous factors are involved in the mitochondrial pathway of apoptosis (used with kind permission by Science 2000: 1150–1151 (3).

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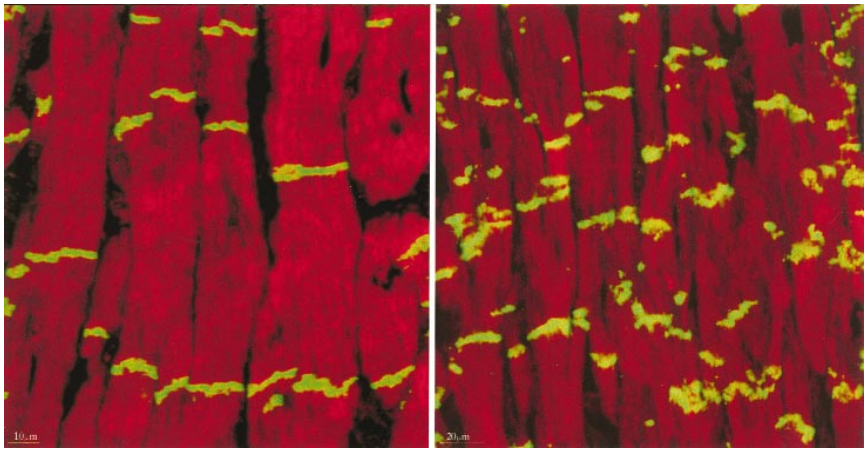

Fig. 7 Confocal micrograph showing the tight connections of intercalated discs between myocytes in normal human myocardium. A: Desmoplakin green. B. Connexin 43 green.

The complex regulatory involvement of the numerous factors influencing mitochondrial function, either localized in, transferred into or released from mitochondria has been studied extensively in isolated cell systems and in cell free preparations but rarely in intact tissue. Only a few groups have tried to establish the basic mechanism of apoptosis in cardiomyocytes (1, 2, 20, 35; for review see (13)).

Most studies on apoptosis have been carried out in either cancer cells or in blood borne cells (Jurkat, HL 60, lymphocytes, thymocytes, etc.) but not in myocytes. Cardiomyocytes are those cells in mammalian organisms that contain the highest mitochondrial volume fraction (24 % in human myocardium, 43 % in mice (33)). Mitochondrial impact on myocyte apoptosis might therefore be extremely strong, or, on the other hand, it might be downregulated by special mechanisms in order to avoid mitochondrial predominance. This question is still open.

In a recent commentary on a publication by Narula (26), J. Reed (31) pointed out that there exists a significant postmitochondrial regulation of apoptosis in heart failure, i.e., release of proapoptotic factors from the mitochondria does not necessarily result in apoptosis. Obviously, many more studies need to be carried out before the exact regulation of apoptosis in cardiomyocytes is established. Only after the mitochondrial pathway in cardiomyocytes has been clearly defined, the role of apoptosis under different pathogenetic situations such as ischemia or heart failure can be determined and evaluated.

Removal of dead cells

A further unknown factor is the question of the mode of removal of dead cells. The myocyte syncytium is established by the intercalated discs that form tight connections on the basis of fascia adherentes, desmosomes and gap junctions (Fig. 7). The only experimental method to dissolve these junctional complexes is Ca depletion. The mechanism active *in vivo* for disconnection of cells from neighboring myocytes has still to be established. Enzymatic autodigestion by proteases of the cells afflicted by apoptosis, or the activation of macrophages are possible mechanisms. The time course of this process, i.e. the time needed for total removal of an apoptotic cell, is unclear as well. It is feasible that myocytes that have undergone complete DNA fragmentation will persist for a longer period of time until they are removed, thus giving a false positive signal when the TUNEL method is used (Fig. 8).

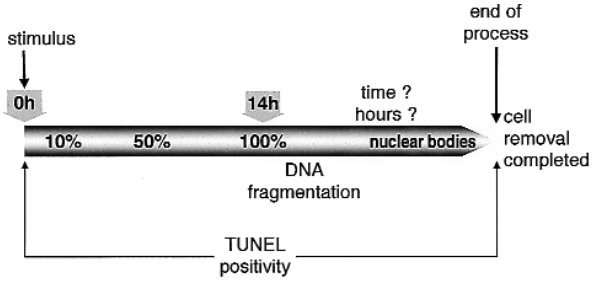

Fig. 8 The duration of TUNEL positivity may be much longer than the time needed for DNA fragmentation to occur. This time interval is still unknown.

Technical considerations

Apart from these more theoretical considerations, practical issues have to be considered. The identification of apoptosis in its different steps encounters technical difficulties, especially when investigating cardiac tissue and not isolated cells. In tissue, either Western or Northern blot techniques can be used to identify activation of caspases, the presence or absence of members of the bcl-2 family, cleavage of substrates or the so-called DNA laddering for the presence of DNA fragmentation. Data obtained by these methods allow for a general estimation of changes but not for an exact localization, i.e. changes

in myocytes can be blurred by alterations in fibroblasts or endothelial or blood borne cells. For this reason, a combination of the methods mentioned above with microscopic evaluation is preferable. Electron microscopy is useful for the determination of the presence of oncosis and, with some luck, for chromatin condensation typical of apoptosis. Fluorescence microscopy or the more sophisticated confocal laser scanning microscopy allow for the localization of bcl-2 proteins, caspases and presence or degradation of substrates, e.g. lamins and PARP. Lack of specificity of antibodies, however, poses problems, as does the cytoplasmic localization of non-abundant proteins such as cytochrome c or the different caspases. Reliability of the TUNEL method for the detection of DNA fragmentation is yet another problem and therefore a careful standardization using positive tissue controls such as intestine needs to be carried out. Since the TUNEL method has been reported to label also oncotic cells as well as those undergoing DNA replication (18, 27), a comparison with results obtained by other methods such as DNA laddering or labeling for DNA strands (ssDNA) is recommended (13). As mentioned above, the duration of TUNEL positivity is another unknown factor that has to be taken into account when evaluating data obtained in tissue. However, the TUNEL method or variations thereof (8, 23) are useful and despite all shortcomings, when applied properly, the best methods available.

When using the different histological methods for the determination of apoptotic rates, statistical problems are apparent:

- Number of experiments or patients to be evaluated
- Random sampling of tissue samples
- Number of tissue sections to be counted
- Reference values for either tissue volume or total number of myocytes or both
- Critical use of microscopy, especially of fluorescent labeling in the confocal microscope.

Summary and conclusion

Apoptosis in ischemia is a multifaceted phenomenon. On the basis of our results we would like to conclude the following:

- Apoptosis is apparently *not* important in global ischemia where it only affects a small number of myocytes (8 %) while the majority of cells, i.e. 92 %, die by oncosis.
- In acute regional ischemia it is most probably not a decisive factor. However, more solid data are needed to justify this statement.
- Human hibernating myocardium (chronic ischemia) shows an activation of the apoptotic cascade, i.e. apoptosis might contribute to cell loss.

It is concluded that the role of apoptosis in *acute* ischemia currently tends to be overestimated. The experimental evidence for the importance of apoptosis in the situation of acute and chronic ischemia is not yet fully established and much more basic work has to be carried out to clarify this issue.

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