Review

Apoptosis in chronic hibernating myocardium: sleeping to death?

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Abstract

Is the ‘smart heart’ smart enough? Since the introduction of the term ‘hibernating myocardium’, this has been referred to as the ‘smart heart’, however more recently several publications have suggested that cell death accompanies the hibernation process, so that revascularisation of patients with hibernating myocardium should be performed without delay. Other data, however, point to cellular dedifferentiation instead of cellular degeneration, which means that cardiac hibernation is an adaptive mechanism capable of preserving the myocardial viability for a prolonged period. In an attempt to find an answer to the above-mentioned question, this review summarises and discusses the findings in this field, also giving attention to possible explanations for the discrepant findings. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Since it became generally accepted that apoptosis can occur in terminally differentiated cells, such as cardiomyocytes, many researchers have focused on the occurrence of apoptosis in acute pathophysiological conditions, i.e. in ischaemia/reperfusion or acute myocardial infarction. Many data have come to light that suggest the importance of this type of cell death in these situations [1–5]. Considerable attention has also been paid to the question of whether or not apoptosis is an important factor in the progression from compensated hypertrophy to heart failure [6–9]. However, not much is known about the role of cell death in intermediate adaptive processes like hibernation.

2. Hallmarks of hibernating myocardium

The term ‘hibernating myocardium’ describes an important clinical situation that characterises dysfunctional but viable myocardium as a result of an oxygen shortage due to a chronic or repetitive underperfusion accompanied by a limited coronary flow reserve. It has been suggested that the underperfused myocardium can retain its viability by down-regulating its function, thereby regaining the balance between the need for and the availability of oxygen. After revascularisation, for example after coronary artery bypass grafting (CABG), the cardiac function of patients recovers, but in the setting of chronic hibernating myocardium this can take a few months to 1 year [10]. Although dysfunctional, hibernating myocardium partially retains the ability to respond to inotropic challenges. Myocardial function improves upon low-level dobutamine infusion, but deteriorates with a high-level infusion. The high glucose tracer analogue (fluorodeoxyglucose, FDG) signal, which can be obtained with positron emission tomodraphy (PET) in hibernating segments, suggests that glucose uptake is stimulated in those areas, although direct evidence is missing [11–13]. In patients who show a delayed functional recovery after reperfusion, the hibernating myocardium undergoes structural remodelling. These
structural changes are characterised by cellular and extracellular features. Important subcellular alterations are: redistribution of nuclear heterochromatin; depletion of sarcomeres; accumulation of glycogen (which might be related to the suggested glucose uptake stimulation [11]); occurrence of aberrantly shaped but healthy mitochondria; and degradation of structured sarcoplasmic reticulum, giving rise to cardiomyocytes with structural hallmarks of foetal heart cells, i.e. a dedifferentiated phenotype [14]. Indeed, the structural alterations are accompanied by altered expression levels of several proteins, confirming the dedifferentiation hypothesis [15]. It remains unknown whether this dedifferentiation is reversible, however the recovery of function after reperfusion is suggestive of a normalisation of the subcellular structure of the cardiomyocytes. During dedifferentiation the cells do not become atrophic, but there is an increase in the amount of extracellular space [16,17]. This observation, and the knowledge that functional recovery after revascularisation is often incomplete, gives rise to the question of whether or not cell death occurs. Ultrastructural alterations in cardiomyocytes, suggestive of cellular apoptosis-like degeneration, have been detected in biopsy tissue from a limited number of patients [18–20]. Therefore, the following questions arose: does degeneration occur in chronic hibernating myocardium (CHM) and, if so, does cellular dedifferentiation eventually result in degeneration of the cardiomyocytes?

3. Is apoptosis in CHM clinically important?

In a chronic situation, even low percentages of cell death could be of long-term clinical importance [21]. If cell death were present, even a very low rate would result in a considerable percentage of cell loss after a long period. In that case, one would not expect functional recovery after revascularisation, which can be seen in the majority of patients [22–24]. On the other hand, the basis of incomplete recovery emphasises the possible involvement of progressive cell death. If the latter does in fact play a role, revascularisation without delay is indicated, although some data (discussed below) suggest that dedifferentiated cardiomyocytes are more resistant to oxygen shortage than normally structured cells in remote areas.

4. The search for apoptosis in chronic hibernating myocardium in humans

Until now, there has been only very limited research with a view to quantitating apoptosis in CHM. The techniques used to achieve this were limited to in situ DNA nick end-labelling (TUNEL, discussed below) [19,20,25], evaluation of some apoptosis-related proteins (P53 and Bcl-2) [25], ultrastructural analysis [18–20,25] and, recently, the evaluation of caspase mRNA content [26]. Across research groups, different findings were reported [18–20,25]. In some publications it is suggested that cardiomyocyte degeneration and cell death play an important role in CHM, although no data are given on the frequency of apoptotic cell death [18,19]. Recently, however, Angelini et al. found an exceptionally high mean apoptotic rate of 8.9% (next to a very high mean percentage of fibrosis: 37.7%) in their patient population with CHM, when using TUNEL [20]. If these percentages truly represent apoptosis in CHM, recovery after revascularisation would be expected to occur not at all or only to a very moderate extent. These data are in sharp contrast with our own findings, which suggest that apoptosis is absent or is present only to a very limited extent in CHM [25]. In all the biopsies from patients with coronary artery disease (CAD) whom we have investigated with electron microscopy so far (n = 530), we have found only three cardiomyocytes with structural changes suggestive of apoptosis (Borgers, unpublished data). Moreover, the hitherto published electron microscopic (EM) figures of apoptotic cardiomyocytes in CHM clearly lack obvious structural dedifferentiation characteristics [18,19]. If apoptosis is indeed a major feature in the presented patient populations, the possibility remains that dedifferentiated and degenerated cardiomyocytes are two different cell populations (i.e., apoptosis is not per se inherent to structural hibernation).

In a recent publication the TUNEL method was used, combined with EM evaluation, and validation of the expression of the pro-apoptotic P53 and anti-apoptotic Bcl-2 proteins in CHM biopsies, but no evidence of apoptotic cardiomyocytes was found, although the internal (TUNEL-positive interstitial cells) and external (TUNEL-positive cells in spleen) controls were positive. There was no evidence of up-regulation of P53 or Bcl-2 which is, when combined with the TUNEL data, indicative of the absence of an apoptotic threat [25]. Recent findings of Elsässer and Schaper [26], however, suggest that this threat could be present in a subset of their patient population since they found evidence for an upregulation of caspase-3 mRNA, but caspase protein content or caspase activity was not assessed.

5. The search for apoptosis in animal models for hibernation/cardiomyocyte dedifferentiation

For the moment, only a few animal models in which cardiomyocyte dedifferentiation occurs are known. The only validated animal model consists of experimentally induced chronic atrial fibrillation in the goat [27]. Recently, a sheep model with chronic coronary stenosis was presented, in which the functional (dysfunctional but stress
responsive myocardium) and the structural hallmarks (such as cardiomyocyte dedifferentiation) of CHM were present [28]. Cardiomyocyte dedifferentiation can also be seen in a sheep model of patchy necrosis, in cells bordering microinfarcted areas [29,30]. In a pig model of subacute hibernation, structural aspects of cardiomyocyte dedifferentiation were present, sometimes accompanied by patchy necrosis [31,32]. In some of these models, apoptosis detection was performed, but there was a tremendous variation in the results, which can possibly be attributed to differences across animal models or the methods used [25,32].

Using the goat model described above, TUNEL analysis, combined with EM and the evaluation of Bcl-2 and P53 expression levels, was performed to investigate whether cardiomyocyte dedifferentiation is accompanied by cardiomyocyte apoptosis. However, as in human CHM biopsies, evidence of neither apoptosis nor apoptotic threat was found. Hence it was suggested that cardiomyocyte dedifferentiation, to an extent similar to that noted in CHM, probably does not lead to apoptosis [25]. The question of cell death has also been raised in connection with a pig model for hibernation devised by Chen et al. [32], in which LAD coronary artery stenosis was applied for 24 h to 4 weeks. This model, however, seemed to be confounded by patchy necrosis in the majority of the animals, and extensive cardiomyocyte apoptosis (up to 9.8±4.6%) was found mainly in areas bordering the patchy fibrotic areas. The recovery of contractile function after revascularisation, inherent to true hibernation, was not established in this study. Since hibernation was relatively short in the protocol used in this model, the results might indicate that necrotic cell death may indeed be a prerequisite for subsequent apoptotic events occurring in the infarct border zones.

On the other hand, in another recently proposed model with patchy necrosis induced by intracoronary injection of microbeads in the sheep [29], no evidence of cardiomyocyte apoptosis with the TUNEL method 6 weeks after embolisation could be found (Dispersyn, unpublished data). Failure to find apoptotic cardiomyocytes in infarcted border zones in this model, in contrast with the findings in the pig model of Chen et al., could be attributed to the assessment of apoptosis at different time points after infarction. Nevertheless, in the sheep model, extensive apoptosis of interstitial cells was detected in the fibrotic areas, consistent with the described disappearance of infiltrated and proliferated interstitial cells [33].

In order to further elucidate the prevalence of apoptosis in CHM, a thorough investigation into apoptosis (preferably using a broad spectrum of detection methods) has to be performed in the sheep model with chronic coronary stenosis, the model that most closely mimics CHM. So far, EM evaluation clearly revealed cardiomyocyte dedifferentiation, but failed to show degenerative events [28].

6. Methodological pitfalls and limitations of study material

If we want to find an answer to the question of the occurrence and importance of apoptosis in CHM, we first have to focus on several methodological aspects.

6.1. Detecting apoptosis by DNA nick end-labelling

Of the various methods of detecting apoptosis, only a few have been applied so far to search for apoptosis in CHM [18–20,25]. In general, the terminal transferase (TdT) mediated dUTP nick end-labelling (TUNEL) method is used most frequently. This method has as its main advantage the fact that it allows the in situ detection and identification of cells with DNA fragmentation, and therefore possibly apoptotic cells [34]. However, there is debate about the specificity of this method and its sensitivity can easily be modified [35–38]. Nevertheless, if the method is used properly, it can provide the investigator with valuable information, making possible the quantification of different cell types in which DNA fragmentation occurs. Another commonly used method to evaluate the occurrence of apoptosis is the detection of DNA laddering with a view to assessing DNA fragmentation [39,40]. However, in an experimental cell system it was proved that this method is useful only if more than 2% of the cells are apoptotic [40,41], a percentage that is most likely not reached in CHM. In working with the TUNEL method, one can avoid false-positive results by adequately including controlled reactions that pertain to the time and temperature of incubation, proper choice of internal positive and negative control tissue, etc. [5,35,36]. Even under well-controlled conditions, when using the TUNEL method, one should investigate a large quantity of tissue in order to obtain an idea of the rate of apoptosis.

There remains the possibility that cells undergoing apoptosis can escape TUNEL positivity, thereby giving rise to an underestimation of the percentage of apoptosis. It is not excluded that a portion of the apoptotic cells are removed by phagocytosis in a stage before DNA fragmentation (hence TUNEL positivity) occurs [42–44]. In other words, it is possible that only when the amount of apoptotic cells is greater than the temporally and locally available phagocytic capacity, can the typical DNA fragmentation be detected. To further clarify this matter, other techniques capable of detecting early apoptosis-related features (such as the detection of phosphatidylserine flip/flop by the annexin-V method [45], preferentially at the EM level [46,47]) should be used in parallel with the TUNEL method and/or with the more specific Taq polymerase based DNA in situ ligation assay [48]. Investigating caspase activation may be a valid alternative, although there is evidence emerging that caspases are not only
activated in apoptotic cell death, but also in some cases of necrotic cell death [49].

Another puzzling observation, however, is the virtual absence of phagocyted fragments in phagocytic cells in CHM. The previously published figures showing engulfment of apoptotic cardiomyocytes by phagocytes in other cardiac diseases cannot be interpreted unambiguously given that it is difficult to disclose the origin of the phagocyted fragment or even the nature of the phagocyte [7,50–52].

6.2. Ultrastructural verification of apoptotic events

EM must still be considered the gold standard as far as the identification of apoptotic cells is concerned. However, only limited studies seem to make use of this technique. Until now no previous reports show ultrastructural evidence of cardiomyocyte apoptosis in ischaemia/reperfusion studies or in the setting of dilated cardiomyopathy. When EM is used, many structural changes have been proposed as pre-apoptotic changes. For example, margination and condensation of chromatin to various extents have been described as pre-apoptotic [7,52,53]. The reasons for this denotation are entirely unclear since, to our knowledge, there is no way to unambiguously distinguish these changes from similar changes occurring in pre-necrotic or irreversible necrotic cells. Only end-stage chromatin condensation (half-moon appearance) is a typical hallmark of an apoptotic nucleus [37,40], which, however, is only seldom observed in cardiomyocytes. This assumption is further substantiated by Ohno et al., who found, using EM, TUNEL positivity in the majority of clearly necrotic cells [18]. To make the issue even more complex, Kanoh et al. recently demonstrated that a large number of TUNEL-positive cardiomyocytes had ultrastructural features of neither apoptosis nor necrosis, but were instead considered ‘living’ cells [38]. It should be emphasised that only by using various appropriately applied techniques (reviewed in Refs. [54,55]), such as TUNEL, DNA laddering assays, detection of apoptosis-related proteins and caspases activation assays in combination with ultrastructural analysis, can one obtain a good appreciation of the occurrence and extent of apoptosis.

6.3. Patient selection

It is likely that in a proportion of the patient population under study, other concomitant pathologies are present, which may be the cause of the observed degenerative events. As a result, it is difficult to dissociate the effect of this pathology on the long-term survival of chronic hibernating cardiomyocytes. Consequently, the use of different patient selection criteria in different research centres may, at least in part, be responsible for the discrepant findings [18–20,25]. Moreover, the severity and duration of coronary events in the patient populations may differ geographically (e.g., waiting list for CABG), so that these parameters may be an important contributing factor [56,57].

6.4. Biopsy tissue selection

Per definition, chronic hibernating myocardium is viable myocardium and therefore has to be free from non-viable infarctions [10]. In the clinical setting, however, infarcted areas are present most of the time and it is known that, in the infarcted border zones, cell degeneration and apoptosis do occur [2,5]. This makes the interpretation of the finding of apoptotic phenomena difficult since one cannot rule out a causal relationship between hibernation and infarction on the one hand, and apoptosis on the other. Therefore, it cannot be stressed enough that if we want to answer the question of whether cellular hibernation (dedifferentiation) finally results in degeneration, possibly through apoptosis, strict selection criteria have to be established so that tissue that truly consists of hibernating myocardium (viable and able to recover function) is chosen. Differences in the selection criteria adopted for patients and tissue most probably form the basis of the discrepant results described in recent literature [18–20,25]. The selection of patients and biopsy areas can be performed with several non-invasive imaging techniques able to resolve tissue viability, for example PET [58–60]. However, histologic examination of the biopsies thereafter should exclude those areas with infarctions that could not be detected by these techniques, because of their limited sensitivity and specificity. Thus, besides functional and biochemical selection criteria similar to those used in clinical studies [23,24,59,60] (hypofunctional, but stress-responsive and actively metabolising myocardium), histological selection criteria (dedifferentiation hallmarks with exclusion of infarcted areas) should be used. Moreover, the same criteria should be used when validating animal models of CHM [12].

6.5. Biopsy tissue availability

A major drawback in the search for apoptosis in CHM is the limited amount of tissue that can be investigated. For obvious ethical reasons, per patient only one or at most two needle biopsies of about 15 mg can be taken. These small biopsies contain only a limited number of cardiomyocytes (around 180,000 cardiomyocyte nuclei) [61]; hence it can be readily understood how an occasional apoptotic cell could be overlooked, especially if only part of the biopsy is used for apoptosis detection, which is most often the case. Logically, it is very difficult to interpret negative results as to the relevance of apoptosis in this pathological condition. It is clear that a validated animal model of chronic hibernating myocardium is needed in
which the typical functional and structural hallmarks of CHM are present, in order to have access to unlimited amounts of tissue for research.

6.6. Tissue processing

Probably one of the most important technical factors in the detection of apoptosis by the TUNEL method is that the tissue samples should be processed (fixed or frozen) as soon as they have been biopsied [62]. Since nuclear DNA fragmentation due to autolysis occurs soon after the biopsies have been taken, and since, further, the sensitivity of the TUNEL method, which cannot distinguish between nucleosomal cleavage and non-specific DNA fragmentation [37,63], can easily be increased, this can be a major source of false-positive results. A possible argument for this is the observation that many of the published pictures of TUNEL-positive cardiomyocytes show a concentrated staining at the nucleus [7,64]. Tissue treated with DNase before TUNEL is performed contains similar stained nuclei without the occurrence of nuclear fragments [65]. However, in many tissues in which apoptosis is known to occur physiologically (programmed cell death), TUNEL positivity is seen as a more scattered appearance, probably due to nuclear fragments, with ‘leaking’ of the staining into the cytosol, which is consistent with the known loss of nuclear membrane integrity during the apoptotic process [36,47,66]. It was concluded from this study that RNA synthesis and splicing could be a source of false-positive results. In hearts with dilated cardiomyopathy, TUNEL performed at the EM level showed that all TUNEL-positive cells were living cells without any features of apoptosis or necrosis. Moreover, all the TUNEL-positive cells were PCNA (proliferating cell nuclear antigen; required for both DNA replication and repair) positive and Ki-67 (a replication associated antigen) negative, suggesting that the TUNEL-positive cells suffered from DNA damage and had increased levels of DNA repair [38]. That the TUNEL method in combination with PCNA detection is a powerful tool for searching for DNA damage has already been suggested by Hegyi et al. [35] and Coates et al. [66], who deliberately used this technique (combined with P53 immunolabelling) to search for sites of enhanced DNA damage. The fact that no evidence for an upregulated PCNA expression and TUNEL positivity could be found in the dedifferentiated cardiomyocytes from CHM or chronic fibrillating atria suggests that, not only apoptosis, but also extensive DNA damage, is probably absent in these settings [25].

Another, albeit highly speculative, hypothesis is that, under certain circumstances, a multinucleated cardiomyocyte can lose one of its nuclei, possibly involving DNA fragmentation, without resulting in cell death, and that this event would give TUNEL-positive results. An increase in the number of myocyte nuclei is a process known to occur in hypertrophic surviving cardiomyocytes after myocardial infarction [67]. It is not known whether the reverse of this process is possible, but it can be speculated that cardiomyocytes might survive with fewer nuclei.

7. Possible triggers of apoptosis in CHM

The important question of which factors can trigger cardiomyocyte apoptosis in CHM is far from answered. First of all, little is known about potential triggers for cardiomyocyte apoptosis; in addition, little research has been done in this field in CHM. Even the actual triggers for the typical functional and structural changes in CHM are unknown. It might be possible that the hypothesised triggers for cardiomyocyte dedifferentiation — possibly to a different extent — may also cause apoptosis. Since chronic hibernating myocardium was originally defined as chronically underperfused [10], one could assume that the ischaemia leads to cellular hypoxia. It is known from in vitro experiments that hypoxia can trigger the death of cardiomyocytes through apoptosis [68,69], but it is highly unlikely that chronic hibernating cardiomyocytes are truly hypoxic. It is generally believed that by down-regulating their function, cardiomyocytes adapt to the lowered oxygen availability and thereby restore the oxygen supply/demand ratio [10]. More direct evidence that cardiomyocytes from CHM are not hypoxic has come from the finding that high-energy phosphate content is unaltered in CHM [70]. Moreover, cytochemical analysis provided evidence that those cells are not calcium overloaded and do not present uncoupling of their oxidative phosphorylation, as shown by a normal activity of cytochrome c oxidase and the absence of mitochondrial NADH oxidase and proton translocating ATPase [71,72]. Nevertheless, it has been suggested that hibernating myocardium can be the result of repetitive stunning because not the basal coronary flow, but the flow reserve, would be diminished [13,73]. Therefore, it cannot be excluded that repetitive periods of hypoxia occur which could have been missed in the above-mentioned study since biopsies are taken in the most favourable circumstances, i.e. under anaesthesia demand ischaemia is unlikely to occur.

Another possible trigger for apoptosis with reference to
CHM could be cardiomyocyte stretching. Because of the hypocontractility of the hibernating segment, it is clear that hibernating cardiomyocytes undergo a different mechanical load than normal cardiomyocytes in remote segments. There are data from in vitro, ex vivo and in vivo studies to the effect that stretching of cardiomyocytes can trigger apoptosis of those cells [74–76]. However, the in vivo observation that apoptosis at the border zone of an infarct is the result of local mechanical stretch may be particularly difficult to interpret due to the concurrence of passive stretch and oxygen shortage. If stretch were sufficient to trigger apoptosis, the infarcted area would enlarge gradually, so that perhaps both stretch and oxygen shortage are needed to induce apoptosis. Restoration of oxygen supply would then be sufficient to stop the enlargement of the infarct. But on the basis of the available evidence, it cannot be ruled out that delayed cardiomyocyte death is a direct consequence of ischaemic injury without the involvement of stretch. On the other hand, some data suggest that physical stretch causes the cardiomyocytes to be more susceptible to other apoptosis-triggering conditions, thereby supporting the hypothesis that stretch is a significant player in structural remodelling. Stretch is known to induce cardiomyocytes to increase (i) angiotensin II secretion, (ii) the amount of AT$_1$ receptors and (iii) the synthesis of P53, all changes which can be counteracted by IGF-1, a factor known to be able to inhibit apoptosis in some circumstances [77–79]. Although it is conceivable that stretch is present in hibernating myocardium, the fact that P53 up-regulation was not observed in CHM suggests that severe stretch is not present in this situation [25].

8. Are chronic hibernating cardiomyocytes preconditioned to resist apoptosis?

There is indirect evidence that dedifferentiated cardiomyocytes from CHM tolerate ischaemia better than non-dedifferentiated cardiomyocytes. Indeed, when CHM biopsies are not immediately fixed, ultrastructural abnormalities — such as loss of intramatricial granules in the mitochondria and subsequent mitochondrial swelling — suggestive of acute ischaemia can be observed in normally structured cardiomyocytes without dedifferentiation features, but not in those that are clearly dedifferentiated [72]. This may indicate that dedifferentiated cardiomyocytes are better protected against ischaemia. It could be hypothesised that endogenous protective mechanisms, such as an increased expression of certain heat shock proteins, are up-regulated in CHM, although direct evidence of such up-regulation is missing. Nevertheless, it is known that ischaemic preconditioning induces the efficient translation of stress proteins [80]. Several of these chaperones are subsequently translocated to the nucleus, possibly to protect against degradation of DNA that has become more susceptible to this due to a transformation of the chromatin organisation into a nuclease-sensitive conformation (as is the case in apoptosis) [81,82]. Heat shock proteins, such as Hsp70, Hsp27 and αB-crystallin, are known to protect against ischaemic cardiac damage. Unlike with ischaemic preconditioning — which also attenuates apoptotic cell death induced by ischaemia/reperfusion [83] — in a pig model of short-term hibernation, mRNA expression of Hsp70 (and several other apoptosis-modulating proteins) was not altered during coronary stenosis, nor during subsequent stunning [84]. Whether this also applies to CHM remains to be investigated, but in cases of chronic heart failure (DCM and ICM), protein expression levels of Hsp72, Hsp27 and Hsp90 were not significantly changed, whereas the levels of chaperonin Hsp60 nearly doubled [85].

An important question is whether degenerative changes occur at a later stage of chronic hibernating myocardium; in other words: how long can dedifferentiated cardiomyocytes survive? As suggested earlier, it is possible that, in patients with a longer history of CHM, apoptosis does in fact become a common feature. Whether dedifferentiation eventually results in apoptosis is a key question, which is very difficult to answer since validation of chronic hibernation models has only just started.

9. Conclusion

In view of the limited and discrepant findings hitherto reported, there is no evidence that fulminant cardiomyocyte apoptosis takes place in CHM in humans, or that cardiomyocyte dedifferentiation leads to apoptosis. Moreover, because of the above-described methodological limitations and ethical constraints to obtaining adequate study material, interpretation of human data should be done with great care with respect to clinical relevance. Only validated animal models which fulfill all criteria of CHM will be able to fill this lacuna.

References


