Myocyte Death in the Pathological Heart

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A recent editorial published in Circulation Research by Jutta Schaper and collaborators has raised several challenging questions concerning the role of myocyte death in myocardial diseases. Numerous studies have documented that myocyte death is an important variable in the development of ventricular dysfunction and failure in ischemic and idiopathic dilated cardiomyopathy, long-term systemic hypertension, and during myocardial aging in animals and humans. Loss of myocytes leads to wall restructuring, side-to-side slippage of cells, mural thinning, chamber dilation, fibroblast activation, myocardial scarring, and depressed ventricular function. Collagen accumulation resulting from focal and scattered myocyte death plays a critical role in both ischemic and idiopathic dilated cardiomyopathy, comprising more than 20% of the ventricles in the late stages of the cardiac disease. Contrary to the notion stated in the editorial, coronary blood flow is impaired in both pathological conditions. The importance of cell death as a mechanism of altered hemodynamics has been well demonstrated following myocardial infarction and diffuse cell death associated with limitations in coronary perfusion.

Myocyte death by apoptosis and necrosis documented in our human and animal (for review, see Reference 11) studies has been interpreted as “an epiphenomenon that is not related to the evolution of heart failure...” This conclusion was reached by the authors of the editorial on the basis that cell death in the decompensated heart was not counteracted by an equivalent amount of myocyte proliferation. Questionably, durations of cell apoptosis were indicated, but no attempt was made to emphasize that mitosis lasts between 30 and 45 minutes. This does not imply that cell regeneration compensates for the extent of myocyte loss in the diseased myocardium. Such an imbalance between cell growth and cell death has been a major hypothesis of our laboratory, which has considered inadequate myocyte division as a critical event in the evolution of the pathological heart to terminal failure. Experimentally, inhibition of myocyte apoptosis and necrosis in models of ischemic cardiomyopathy attenuates ventricular dilation, reactive hypertrophy, and diastolic wall stress, demonstrating unequivocally the crucial role of cell death in ventricular remodeling.

Myocyte death is important, but the relevant question is whether it can be recognized and its magnitude actually measured. This technical issue will be addressed first, since according to the editorial, “electron microscopy provides the gold standard for the identification of both apoptosis and necrosis.” We do not share this view; to avoid any possible subjective interpretation of morphological findings, oligonucleotide probes have been developed that allow the recognition of different aspects of DNA damage. Double-strand DNA cleavage with staggered ends can be detected by the terminal deoxynucleotidyl transferase (TdT) assay; double-strand DNA cleavage with single-base overhang can be detected by a polymerase chain reaction (PCR)–generated Taq polymerase probe or hairpin probe with single-base overhang; double-strand DNA cleavage with blunt ends can be detected by a PCR-generated Pfu polymerase probe or hairpin probe with blunt ends; and single-strand DNA cleavage can be detected by the in situ nick translation assay.

The first two recognize different aspects of cell apoptosis, the third depicts cell necrosis, and the last identifies potentially repairable DNA damage. The specificity of these DNA probes has been questioned by electron microscopic findings based on qualitative observations alone or in combination with TdT and Taq labeling of tissue structures. Whether the light, confocal, or electron microscope is used for the detection of apoptosis, we are measuring an event that occurs at a frequency of 0.2% of myocytes or much less. To maintain sampling variability within 10%, 100 positive events have to be found. According to the Poisson statistics, the standard deviation of that estimate, ie, standard error, will be ±√100/100, or 10%. This implies that 50 000 nuclei have to be examined: 1000.002 = 50 000. Moreover, Figure 1 illustrates the proportion between the sampling that can be achieved by the electron microscopic analysis of a tissue section that is 0.2 mm² in area and 500 Å in thickness and the sampling that can be obtained by confocal microscopic analysis of a tissue section that is 150 mm² in area and 5 μm in thickness. The latter equals 50 000. This figure shows the actual proportion between sampling by electron microscopy and confocal microscopy. This difference is 75 000-fold in favor of the confocal microscope.

The efficiency of light, confocal, and electron microscopy reflected by the sampling of 50 000 myocyte nuclei is shown in the Table. The combination of section thickness, number of myocyte nuclear profiles per mm² of myocardium, and the area of the section permit the calculation that the number of myocyte nuclei in one section equals 75 000 for light and confocal microscopy and 40 for electron microscopy. Therefore, the number of sections required for the examination of 50 000 myocyte nuclei is less than one by light and confocal microscopy and 1250 by electron microscopy. The corresponding time/effort is 1 hour and 2 hours for light and...
confocal microscopy. Electron microscopy necessitates 156 days, 8 hours per day. No lunch break is allowed. This value is very conservative, given that 1 hour per grid has been allotted.

Let us now consider the accuracy of the analysis of a nucleus when light microscopy, confocal microscopy, and electron microscopy are used. The average minor diameter of a myocyte nucleus is 4 μm. Both light and confocal microscopy will allow the evaluation of the entire nucleus. The advantage of confocal microscopy is the possibility of optically sectioning the nuclear profile, eliminating the limit in resolution dictated by section thickness.2,5,19,20 This problem cannot be resolved by light microscopy. By electron microscopy, only 1.3% of the minor diameter of the nucleus will be examined. If the major length of a myocyte nucleus, which is nearly 14 μm in dimension, is oriented perfectly perpendicularly to the plane of sectioning, 0.4% of this parameter will be determined. Under the optimal condition of nuclear orientation, in which a mid-section of the nucleus, along its major axis, is longitudinally oriented to the plane of sectioning, 1.3% of the 175 μm³ of nuclear volume will be available for evaluation. However, most frequently, only a minor fraction, such as 30% of the section, contains a portion of a nucleus. This implies that only 0.2% of the nuclear volume is detectable (Figure 2).

It is rather difficult to accept that interpretation of nuclear morphology has been made when the percentage of structure examined varies from a minimum of 0.2% to a maximum of 1.3%. Additionally, the number of TdT-labeled particles is counted on the basis of this magnitude of sampling. We are told that the number of nuclear profiles measured ranges from 50 to 300. Under the best circumstances, ie, 1.3%×300 nuclei, not even four complete nuclei were sampled. Moreover, the number of gold particles reflecting positive TdT labeling has been measured in a semiquantitative fashion by dividing them in two groups: “slightly labeled” and “much more than slightly labeled.”21 After 30 minutes of ischemia, 6% and 11% of TUNEL-positive nuclei were detected light microscopically at 2 and 4 hours of reperfusion. Corresponding electron microscopic results yielded 41% and 83%.27 This 7-fold difference is unacceptable on the basis of magnitude of sampling, section thickness, and resolution.

The authors of the editorial have questioned the electron microscopic part of our study, claiming misinterpretation of findings.1 We strongly object to their evaluation of Figure 11A. This is apoptosis, not a prominent nucleolus. Compact dark appearance of nucleolar material does not occur in prominent nucleoli after fixation by glutaraldehyde and osmium tetroxide. However, to avoid subjective evaluation of morphological features, specific molecular probes have to be used. In our experience, electron microscopy of immersion-fixed rabbit myocardium, under controlled conditions and after acute and chronic pressure overload, results in poor preservation of myocytes, particularly of the mitochondria and sarcoplasmic reticulum compartments.30,31 This situation is worse in human hearts.28 In the work from Fujiwara’s laboratory,26,27 hearts were not arrested in diastole, making impossible the analysis of contracted myofibrillar structures.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Diagram illustrating the amount of myocardial sampling by electron microscopy (EM) and confocal microscopy (CM). See text for details.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Magnitude of sampling that can be obtained by electron microscopy from a myocyte nucleus when the minor and major diameters and the volume of the nucleus sectioned on different planes are considered. See text for details.
and mitochondrial profiles. It is rather sad that the same problems apply to the studies of the authors of the editorial.32–35 Electron microscopy in our study3 was considered of so little value that no numbers were given in a statistical fashion. This was our decision, because the evaluation of nuclei by electron microscopy has no logic and is against any principle of morphometry. Nuclear counts in the liver, lung, skeletal muscle, heart, and blood vessels introduced by Weibel, Loud, and Gundersen were all performed in semithin sections at the light microscopic level (for reviews, see References 36 through 41). A few times, we have counted the numerical density of nuclear profiles in electron micrographs to identify the value of the penetration factor p; this parameter indicates how much of a structure has to be included in a tissue section in order to be visible.29 Evaluation of the morphological characteristics of the myocyte cytoplasm after immersion fixation of the nondiastolic arrested heart does not permit one to distinguish real findings from artifacts. Assessment of nuclear morphology by electron microscopy constitutes an extravagant, unreliable methodology.

The question was raised whether 10% of all DNA fragments or 100% DNA fragmentation has to occur to obtain a positive signal for apoptosis.1 It is not necessary to destroy a large portion of the genome to kill a cell; one unrepaired DNA double-strand break is sufficient to kill a cell and reflects apoptosis.42,43 Additionally, vinculin defines costameres on the plasma membrane, and loss of sarcomelial lining and myofibrillar structures does not leave costameres intact. Therefore, vinculin is a reliable marker for the identification of the integrity or discontinuity of the surface membrane.

A final critical question raised is why a cell undergoes apoptosis or necrosis. This issue has been discussed in almost every review we have published in the last 4 years and is currently being investigated in every laboratory involved in this field. Recently, we have advanced the possibility that preexisting single-strand DNA cleavage may predispose cells to activate their suicide program. Single-strand DNA breaks, which are potentially repairable, can be detected by a reaction catalyzed by the Klenow fragment of DNA polymerase I in the presence of biotinylated deoxyuridine triphosphate (dUTP), deoxyadenosine triphosphate (dTTP), deoxythymidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP). This assay, which is template dependent, results in the incorporation of biotinylated dUTP at 3′ recessed single-strand DNA breaks. The newly generated DNA strand is visualized by extravidin labeled by fluorescein that binds to biotinylated dUTP.44 By this approach, it was possible to document that human myocytes undergoing double-strand DNA cleavage possessed single-strand DNA breaks. This work is in progress and is consistent with the importance of preexisting DNA damage for the activation of the endogenous cell death pathway (J. Kajstura, A. Leri, C.A. Beltromi, P. Anversa, unpublished observation, 2000).

In conclusion, confocal microscopy, combined with the use of probes capable of identifying specific forms of DNA damage, constitutes the most efficient and reliable method for the quantitative assessment of various types of acute cell death in the heart. Electron microscopy is the least efficient, is highly subjective, and is the most inappropriate methodol-

ogy for the quantitative evaluation of ongoing cell death. It is an old technique that failed to detect myocyte apoptosis in the past. A new problem requires a new approach.

References


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