Significance of Myocytes With Positive DNA In Situ Nick End-Labeling (TUNEL) in Hearts With Dilated Cardiomyopathy

To the Editor:

Recently, Kanoh et al.1 seriously questioned the reliability of the DNA in situ nick end-labeling (TUNEL) assay as a method of detecting cardiomyocyte apoptosis (CA) in dilated cardiomyopathy. Our experience with both failing2 and infarcted3 human hearts is remarkably different. We agree that TUNEL positivity is not 100% specific to what is morphologically defined as apoptosis. However, if one accepts double-strand DNA breaks as one of the hallmarks of apoptotic cell death (which Kanoh et al must even question), our experience is that the detection of this preferred substrate by the TUNEL assay results in reasonable (0.1% to 1.0%) and reproducible (r = 0.88) estimates of CA frequency in relevant samples. DNA ladders are demonstrable in the TUNEL-positive areas when the amount of positive cells exceeds ~0.04%.

We think that the key reason for the findings by Kanoh et al is their lack of appropriate standardization of the TUNEL assay. As we have pointed out previously,2–5 one must go beyond the manufacturer’s instructions to avoid erroneously false-positive and false-negative results. This can be done by using adjacent tissue sections treated with DNase I as a positive control of apoptosis and by interrupting the staining reaction on the appearance of positive signal in these sections. This procedure confirms the optimal sensitivity of the assay and normalizes it for differences in tissue permeability.2–4 Using this approach, TUNEL positivity is never zero; rather, it is in the range of 0.003% to 0.01% in normal myocardium. Furthermore, the very high numbers of labeled cells in the positive samples of Kanoh et al (7.9% and 5.8% using the electron microscopic immunogold assay and light microscopic TUNEL staining, respectively) point to problems in standardization (or the presence of artefacts) when methods based on DNA fragmentation are used.

We and others have repeatedly observed features of apoptotic morphology, such as condensed instead of hypertrophied nuclei, in TUNEL-positive cardiomyocytes.2–4 Why Kanoh et al failed to find morphological evidence of CA could be due to the very small number of cells studied per tissue sample (10% of the ~500 cells that would be required to find 1 truly apoptotic cell).2–3 Because only 15% (n = 6) of the biopsies were positive, how many of the 20 patients actually contributed to their positive data?

The value of the TUNEL assay lies in its excellent signal-to-noise ratio and, hence, suitability for the quantification of very low amounts of positive cells. Although potentially more specific tests, such as the Taq polymerase assay, should be rigorously tested for the quantification of CA, we think that the TUNEL assay is currently the method of choice for this purpose, provided that it is allowed to perform at its best.

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The Significance of Expression of Proliferating Cell Nuclear Antigen in the Cardiovascular System: Mitosis or DNA Repair?

To the Editor:

We read the article by Kanoh et al.1 with great interest. They showed that the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick-end labeling (TUNEL) technique is not specific for apoptosis, which was pointed out previously.2–3 Although not specific, TUNEL is a selective method1 and, within the limits imposed by its high sensitivity, the TUNEL technique stains apoptotic nuclei.1,2–3 Kanoh et al found that a number of TUNEL-positive nuclei expressed proliferating cell nuclear antigen (PCNA) in myocytes from hearts with dilated cardiomyopathy.1 A few years ago, we found that TUNEL-positive nuclei can express PCNA at the edge of the lipid core of human advanced atherosclerotic lesions.2 This region, the periphery of the acellular core of atheroma, is chiefly occupied by lipid-laden macrophage foam cells. We have come to the same conclusion as Kanoh et al: the coexpression of PCNA and TUNEL (PCNA being the accessory protein of DNA polymerase δ4) indicates DNA repair synthesis. We also suggested that this repair may presage apoptosis in human atherosclerotic lesions.2 Indeed, the presence of TUNEL-positive PCNA-negative nuclei (or nuclear fragments) in the lesions2 may suggest late stages of apoptosis.

These observations underline the most likely possibility: the expression of PCNA and certain other markers of DNA synthesis, which are frequently used to estimate the fraction of proliferative cells, may also show DNA repair synthesis.1,2,5 The positivity of nick-end labeling techniques, including TUNEL, proves extensive DNA damage and large numbers of DNA breaks exist in these nuclei.

Taken together, the expression of PCNA in TUNEL-positive nuclei suggests that the positivity of some markers of DNA synthesis in certain (probably terminally differentiated) cells, such as macrophages in atherosclerotic lesions and myocytes in the heart, is due to DNA repair rather than mitosis. This view is supported by our finding that PCNA expression without cytological evidence of mitosis was seen in some human macrophages incubated in the presence of oxidized LDL (unpublished data). Any results obtained using markers of DNA synthesis, therefore, should clearly be interpreted with caution.

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Response

Dr. Hegyi and Skepper came to the same conclusion that we did: the coexpression of proliferating cell nuclear antigen (PCNA) and DNA in situ nick-end labeling (TUNEL) suggests severe DNA repair in macrophages and cardiac myocytes.1,2 In addition, they suggested that this DNA repair may presage apoptosis in human atherosclerotic lesions. In our study on dilated cardiomyopathy (DCM), each myocyte with positive TUNEL (indicating the presence of both single- and double-stranded DNA fragments) was not ultrastructurally apoptotic or necrotic, but living and PCNA-positive. These cells were negative for the Taq polymerase-based in situ ligation assay, which detects double-stranded DNA fragments with single-base, 3′ overhangs and is specific for apoptotic mechanisms. Therefore, we do not think that TUNEL- and PCNA-positive myocytes themselves are in the process of apoptosis. However, we assume that these myocytes with severe DNA repair may proceed to apoptosis in the future, because we think that myocyte loss in DCM may be due to apoptosis.

TUNEL is useful for screening apoptotic cells, but it is not specific for apoptosis.2,3 DNA ladders indicating the presence of double-stranded DNA fragmentation in the tissue do not differentiate which myocytes or interstitial cells are apoptotic in cardiac tissues, especially in the infarcted myocardium, which has rich infiltrating interstitial cells.4 The apoptotic morphology of myocytes at the light microscopic level is not reliable, except for the finding of apoptotic bodies, because bizarre-shaped nuclei, which may look like apoptotic nuclei by light microscopy (but are not apoptotic by electron microscopy), are observed in hypertrophied and/or failing hearts.2 Therefore, we think that both electron microscopic analysis (including electron microscopic TUNEL [EM-TUNEL]) and the Taq polymerase-based in situ ligation assay are necessary for the precise detection of apoptotic cells in vivo, as was shown in our study.

Saraste et al. questioned the percentage of positive TUNEL myocytes in our study. However, this percentage was 0.1% to 1.0% in the autopsied and explanted hearts from the 10 patients with DCM and ischemic cardiomyopathy in their study5 and 1% in the 40 specimens from the 20 patients with DCM (7.9% in the 6 TUNEL-positive specimens) in our study.1 Considering that the tissue size of an endomyocardial biopsy specimen is very small compared with that of tissue sections obtained from autopsied and explanted hearts and that the distribution of TUNEL-positive myocytes varies considerably in tissues from the same heart (as observed by the studies of autopsied and explanted hearts), our data are surprisingly similar to those of Saraste et al., rather than different. Although we agree that their percentages, which result from data obtained using large tissue sections, may be more precise than ours, which were determined from data obtained from very small tissue sections, the main purpose of our study was to examine whether the TUNEL-positive myocytes in DCM were apoptotic or not; we did not attempt to quantify TUNEL-positive myocytes in entire hearts with DCM. Therefore, we used all 6 specimens with light microscopic TUNEL-positive myocytes and 6 of 34 specimens with light microscopic TUNEL-negative myocytes for EM-TUNEL analysis. We found that the percentage of EM-TUNEL-positive myocytes was similar to that of the light-microscopic TUNEL-positive ones. Note that in each patient, the biopsied samples for light and electron microscopic examination were taken from similar portions. Thus, we could have observed many EM-TUNEL-positive myocytes.

Finally, as a positive control, we used prostate tissue from a rabbit castrated 2 days before the study in which many typical apoptotic cells were observed, as was discussed in the Methods section.1 We found good agreement in incidence between TUNEL-positive cells and ultrastructurally apoptotic cells. The incubation time of DCM specimens was adjusted with the degree of staining in the positive control section. Saraste et al. propose using adjacent cardiac tissue sections treated with DNase I as a positive control, without showing why their positive control (artificially fragmented DNA due to treatment with DNase I, which has not been proven to be the DNase responsible for the actual apoptotic process) is better than our control (true apoptotic DNA fragmentation). Further investigation is warranted regarding this issue.

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