Significance of Myocytes With Positive DNA In Situ Nick End-Labeling (TUNEL) in Hearts With Dilated Cardiomyopathy
Not Apoptosis but DNA Repair

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Background—The presence of apoptotic myocytes has been reported in human hearts with dilated cardiomyopathy (DCM) on the basis of a positive finding of DNA in situ nick end-labeling (TUNEL). However, ultrastructural evidence of myocyte apoptosis has not been obtained.

Methods and Results—A total of 80 endomyocardial biopsies were obtained from right and left ventricles of 20 patients with DCM and 20 normal control subjects. TUNEL-positive myocytes were found by light microscope in 15% of DCM specimens (controls, 0%, \( P < 0.05 \)), and the percentage of TUNEL-positive myocytes per section in DCM was 1.0 ± 2.7% (mean ± SD). According to TUNEL at the electron microscopic level (EM-TUNEL), immunogold particles, which label DNA breaks with 3'-OH terminals, were markedly accumulated in the bizarre-shaped nuclei, with widespread clumping of chromatin (so-called “hypertrophied nuclei”) of the myocytes obtained from DCM. Their ultrastructure was neither apoptotic nor necrotic but rather that of living cells. Taq polymerase–based DNA in situ ligation assay, which detects double-stranded DNA fragments more specifically than TUNEL, did not detect a positive reaction in any case. In mirror sections, all of the TUNEL-positive myocytes in DCM simultaneously expressed proliferating cell nuclear antigen, which is required for both DNA replication and repair, but Ki-67, a replication-associated antigen, was completely negative in all cases, which appeared to rule out cell proliferation activity.

Conclusions—Most of the TUNEL-positive myocytes in hearts with DCM are not apoptotic but rather living cells with increasing activity of DNA repair. (Circulation. 1999;99:2757-2764.)

Key Words: apoptosis ■ cardiomyopathy ■ cells ■ immunohistochemistry

Recently, it has been reported that apoptotic myocytes are observed in failing human hearts with dilated cardiomyopathy (DCM).\(^1\)\(^2\) These findings were based on positive DNA in situ nick end-labeling (TUNEL) at the light-microscopic level and the expression of proteins associated with apoptosis.\(^2\)\(^3\) However, ultrastructural evidence of myocyte apoptosis is still lacking in DCM. The positive TUNEL indicates not only the presence of double-stranded DNA breaks (DNA fragmentation, one of the most influential biochemical hallmarks of apoptosis\(^4\) but also that of single-stranded DNA breaks with free 3'-OH terminals. Thus, the positive TUNEL has been observed in necrotic cells as well as apoptotic cells.\(^5\) We recently reported that so-called apoptotic myocytes in the infarct area presenting positive TUNEL and DNA ladders are ultrastructurally oncocytic (necrotic) myocytes with DNA fragmentation.\(^6\) The incidences of apoptotic myocytes detected by TUNEL are too high for these myocytes to be considered dead via apoptosis in diseased hearts;\(^7\) considering that myocyte replication in adults is rare and that the apoptotic process progresses rapidly, such a continuous and massive myocyte loss without corresponding replication of myocytes would instantly cause clinical crisis in patients. However, no such clinical crisis is observed in most patients. In addition, a positive TUNEL reaction can be seen in living cells at the stage of active gene transcription.\(^8\) Therefore, we hypothesized that a positive TUNEL reaction in myocytes of DCM patients does not necessarily mean apoptosis or necrosis. To test this, we examined TUNEL at the electron microscopic level (EM-TUNEL) and the Taq polymerase–based DNA in situ ligation assay, which is considered to specifically detect double-stranded DNA breaks with single-base 3’ overhangs in cells.
undergoing apoptosis.\textsuperscript{9,10} in cardiac myocytes of DCM. We also investigated the expression of proliferating cell nuclear antigen (PCNA), an indicator of DNA replication and repair,\textsuperscript{11} and that of Ki-67, a replication-associated antigen,\textsuperscript{12} in the same myocytes using serial mirror sections at the light-microscopic level and sought a relationship between positive TUNEL reaction and DNA replication and/or repair.

Methods

Patients and Endomyocardial Biopsies

Forty endomyocardial biopsies were obtained from right and left ventricles of 20 patients with DCM (17 men and 3 women; age, 51±16 years [mean±SD]). Each endomyocardial biopsy was originally performed for differentiation between DCM and secondary myocardial diseases. The diagnosis of DCM was made principally according to the definition and classification proposed by the World Health Organization/International Society and Federation of Cardiology task force.\textsuperscript{13} Controls were 40 biopsies from 20 patients (13 men and 7 women; age, 53±18 years) who had been thought to have some diseases because of minimal ECG change, arrhythmia, or atypical chest pain but in whom no organic cardiac disease was found by either noninvasive or invasive examinations. Patients with DCM had a significantly more dilated left ventricular cavity (end-diastolic left ventricular volume index, 137±29 mL/m\textsuperscript{2}) and reduced contractility (ejection fraction, 35±11%) than control subjects (72±11 mL/m\textsuperscript{2} and 65±8%, respectively).

The largest specimens were fixed with 10% buffered formalin for 24 hours and embedded in paraffin for light microscopy. In 4-μm-thick paraffin sections stained with hematoxylin-eosin or Masson’s trichrome, myocyte size (mean diameter, 30 to 50 myocytes per specimen) and degree of fibrosis (from 0 to 3) were evaluated. Smaller specimens were fixed with 2.5% phosphate-buffered glutaraldehyde for 4 hours and 1% osmium tetroxide for 1 hour and embedded in epoxy resin (for EM).

EM-TUNEL was performed as described,\textsuperscript{6,14} and the specimens were observed under a Hitachi 700 electron microscope. Prostate tissue from a rabbit castrated 2 days before study was the positive control for TUNEL. Light-microscopic TUNEL was achieved for all sections between the 2 observers, with 5% difference between them.

Analysis of the Nuclear Ultrastructure of Myocytes

The nuclear ultrastructure of myocytes (~50 nuclei per specimen) was assessed by 2 hallmarks indicating “nuclear hypertrophy”: severe crenation of nuclear membrane (bizarre shape) and widespread clumping of chromatin.\textsuperscript{15} On the basis of these hallmarks, the nuclei were semiquantitatively scored from 0 to 3 (0, no hypertrophy; 1, 2, and 3, mild, moderate, and severe hypertrophy) according to the method proposed by Baandrup et al.\textsuperscript{15} The scoring was done independently by 2 observers, with <5% difference between them for each parameter.

\textit{Tag} Polymerase–Based DNA In Situ Ligation Assay

A 245-bp double-stranded DNA fragment was prepared by polymerase chain reaction with \textit{Tag} polymerase using primers 5'-CTCATAGCTACGCTTAGG-3' and 5'-AGTGTAGCGGATGTTAGCC-3', complementary to pBluescript SK(−) plasmid.\textsuperscript{9} The deparaffinized 4-μm-thick sections were subjected to \textit{Tag} polymerase–based DNA in situ ligation assay using the DNA fragment labeled with digoxigenin according to the method described by Didenko and Hornsby.\textsuperscript{9} Prostate tissue from a rabbit castrated 2 days before study was the positive control for in situ ligation.

Immunohistochemistry for PCNA and Ki-67

On the 4-μm-thick mirror section corresponding to TUNEL-stained sections, PCNA or Ki-67 was immunohistochemically stained by use of ABC Elite kits (Vector). The monoclonal anti–human PCNA antibody (DAKO, at a dilution of 1:100) and the rabbit polyclonal anti–human Ki-67 antibody (DAKO, at a dilution of 1:100) were used as the primary antibodies. Pretreatment by microwave irradiation at 400 W for 5 minutes was done twice to retrieve the antigens. Human tonsils were used as the positive control tissue sections. Unanimity on the positive immunohistochemical stainings as well as light-microscopic TUNEL was achieved for all sections between the 2 observers, who were unaware of which group the sections belonged to. We checked reproducibility of staining (TUNEL, PCNA, Ki-67) using mirror sections. Immunoreactivity (positive/negative) for each staining coincided between pairs of identical nuclei of myocytes on mirror sections.

Statistical Analysis

Data were expressed as mean±SD. Statistical comparisons were performed by Student’s \textit{t} test or ANOVA followed by a Newman-Keuls multiple comparison test. A value of \textit{P}<0.05 was considered significant.

Results

Conventional Light-Microscopic Findings

Biopsy specimens of DCM showed more significant hypertrophy of myocytes (mean diameters of 20±3.4 μm in the right and 23±3.0 μm in the left ventricle) than did controls (14±2.1 and 15±1.4 μm, \textit{P}<0.05). The degree of fibrosis was significantly higher in the DCM specimens (1.4±0.7 in the right and 1.4±0.7 in the left ventricle) than in the controls (0.3±0.5 and 0.2±0.4, \textit{P}<0.05).

TUNEL-Positive Myocytes at the Light-Microscopic Level

TUNEL-positive myocytes were found in 6 of 40 specimens (15%) from DCM patients and none of those from control subjects (\textit{P}<0.05): the mean percentage of TUNEL-positive myocytes per biopsy specimen was 1.0±2.7% among the total of 40 specimens of DCM and 7.9±1.5% among the 6 TUNEL-positive specimens of DCM. There was no difference in myocyte size and fibrosis between the DCM specimens with and without positive TUNEL reaction. When analyzed in the 6 TUNEL-positive specimens of DCM, the size of TUNEL-positive myocytes (26±7.8 μm) was greater than that of TUNEL-negative myocytes (21±4.9 μm, \textit{P}<0.05).

EM-TUNEL and Ultrastructural Features of Myocytes

Slight accumulation of immunogold particles (<10/μm\textsuperscript{2}) was seen in nuclei without hypertrophy of apparently normal myocytes from both control subjects and DCM patients (Figure 1A). Conversely, some nuclei of myocytes obtained from DCM patients showed marked accumulation of immunogold particles (>50/μm\textsuperscript{2}) (Figure 1C). The mean incidence
Figure 1. Electron photomicrographs of endomyocardial biopsy specimens from a control patient (A) and patients with DCM (B and C) treated with EM-TUNEL. Right panels show highly magnified photographs of portion indicated by arrows in corresponding left panels, printed lightly so as to contrast with immunogold particles. A, Nuclear structure appeared normal (score 0), and as shown in right panels, accumulation of immunogold was slight. However, in mildly (score 1, B) and severely (score 3, C) hypertrophied nuclei, more marked accumulation of immunogold particles was noted. N indicates nucleus. Bars=1 μm.
of the myocytes with marked immunogold accumulation was 0.0±0.0%, 0.3±0.5%, and 5.8±5.3% in control specimens, light-microscopic TUNEL-negative DCM specimens, and light-microscopic TUNEL-positive DCM specimens, respectively (Table 1).

Nuclear hypertrophy was classified as normal, mild, moderate, and severe. It was more severe in the light-microscopic TUNEL-positive DCM specimens than the light-microscopic TUNEL-negative DCM specimens and control specimens (Table 2). The incidence of the myocytes with severe nuclear hypertrophy was 0.0±0.0%, 0.7±0.8%, and 6.7±6.2%, respectively, in control specimens, light-microscopic TUNEL-negative DCM specimens, and light-microscopic TUNEL-positive DCM specimens. The accumulation of immunogold particles increased with a higher score of nuclear hypertrophy (Figures 1 and 2A). The incidences of myocytes with marked immunogold accumulation were 0.0±0.0%, 0.0±0.0%, 0.18±0.53%, and 98±1.7% in normal, mild, moderate, and severe nuclear hypertrophy, respectively (Figure 2A). Conversely, the incidences of myocytes with severe nuclear hypertrophy were 0.0±0.0%, 0.22±0.55%, and 100±0.0% for slight, moderate, and marked immunogold accumulation, respectively (Figure 2B). There was a significant positive correlation between nuclear hypertrophy score and grade of immunogold accumulation (r=0.977, P<0.0001).

No typical apoptotic or necrotic ultrastructure was observed in any myocytes, even in the myocytes with marked EM-TUNEL reaction. These myocytes were rich in mitochondria, lipofuscin granules, and glycogen deposits in the cytoplasm and showed myofibrillar derangement and slight edematous change of the cytoplasm. Their cytoplasmic organelles and plasma membranes were not disrupted (Figure 3).

**Taq Polymerase–Based DNA In Situ Ligation**

Taq polymerase–based DNA in situ ligation assay showed completely negative reactions for all myocytes in all cases, whereas positive reaction was noted in epithelial lining cells of the prostate tissue from a rabbit castrated 2 days before study as a positive control (Figure 4A and 4B).

**PCNA-Positive Myocytes and the Relationship With TUNEL at the Light-Microscopic Level**

A PCNA-positive reaction was noted in 25 of 40 control specimens (63%) and 27 of 40 DCM specimens (68%). The incidence of myocytes with a PCNA-positive nucleus per specimen was higher in DCM (8.1±9.7%) than in controls (2.0±2.2%, P<0.0001). Among the DCM specimens, the incidence of myocytes with a PCNA-positive nucleus was higher in the 6 TUNEL-positive specimens (29±4.9%) than the TUNEL-negative ones (4.7±4.8%, P<0.0001).

In the mirror sections from 6 TUNEL-positive specimens of DCM, a total of 401 pairs of myocytes cut at the level of the nucleus were recognized as identical, and their immunoreactions were evaluated (Figure 4C and 4D). The percentages of myocytes with TUNEL-positive/negative and PCNA-positive/negative nuclei are shown in Table 3. Notably, TUNEL-positive/PCNA-negative nuclei were never observed, that is, all of the TUNEL-positive nuclei simultaneously expressed PCNA.

**Ki-67 Immunohistochemistry**

Immunohistochemical Ki-67 expression was completely negative in myocytes in all cases, whereas it was evident in the follicular region of tonsil used as the positive control (Figure 4E and 4F).

**Discussion**

Limitation of TUNEL Method as a Marker of Apoptosis

TUNEL detects not only DNA fragmentation but also single-stranded DNA breaks with free 3'-OH terminals. Therefore,
TUNEL labels necrotic as well as apoptotic cells. A more recent study using vascular smooth muscle cells has shown a false-positive TUNEL reaction as apoptosis even in living cells when accompanied by active gene transcription. In the endomyocardial biopsy specimens of the present study, TUNEL-positive myocytes were seen in 15% of DCM specimens, but there was no evidence of positive myocytes on Taq polymerase–based DNA in situ ligation assay (0%, P < 0.05). The latter method is considered more specific for apoptotic DNA breaks because it detects double-stranded DNA breaks with single-base 3′-OH terminals. Therefore, the discrepancy indicates that TUNEL–positive myocytes do not have a sufficient number of double-stranded DNA breaks with single-base 3′-OH terminals (specific for apoptosis, as well as those with 2- to 4-base 3′-OH terminals) but do have double-stranded DNA breaks with multiple-base 3′-OH terminals or single-stranded DNA breaks with free 3′-OH terminals (not specific for apoptosis) whose numbers are sufficient to be detected by TUNEL. The previous reports and the present findings indicate that TUNEL is very unreliable as a method to detect apoptosis.

Ultrastructure of TUNEL-Positive Myocytes
EM-TUNEL revealed that immunogold particles accumulated only slightly in nuclei of the myocytes from control subjects or DCM patients whose structures were normal, whereas they were markedly accumulated in the bizarre-shaped nuclei, with widespread clumping of chromatin of the myocytes from DCM. In ultrastructural morphology, the latter nuclei were obviously different from those seen in apoptosis. They presented the morphology of hypertrophied nuclei, which are usually observed in hypertrophied or failing hearts. In fact, the hearts of our patients had failed and were hypertrophied. The cytoplasmic ultrastructure was compatible with that of hypertrophied cardiac muscle cells, but not apoptotic cells, because of the absence of cytoplasmic shrinkage, or necrotic cells, because of a lack of ruptured plasma membrane or disintegrated subcellular organelles. That is, the marked EM-TUNEL–positive myocytes in DCM as well as slightly and moderately EM-TUNEL–positive myocytes could be judged to be not dead, neither apoptotic nor necrotic, but alive in ultrastructure.

TUNEL-Positive Myocytes and DNA Repair or Synthesis
Next, we examined what was happening in the nuclei of the myocytes positive for TUNEL. Expression of PCNA in the myocytes of DCM is augmented. The present data confirmed this fact. In the analysis of mirror sections, we found that all of the TUNEL–positive myocytes simultaneously expressed PCNA. PCNA is a cofactor of DNA polymerase and is required for both S-phase DNA synthesis and DNA repair. However, we did not observe myocytes positive for Ki-67, a marker of DNA synthesis, either in control hearts or hearts with DCM. It has also been documented that PCNA expression can increase without a corresponding increase in S-phase DNA synthesis. These results suggest that PCNA positivity in the TUNEL–positive myocytes means that the myocytes are under DNA repair rather than S-phase DNA synthesis. It has been reported that DNA nicks are abundant in cells with DNA synthesis/repair. Thus, it could be assumed that positive TUNEL in myocytes of DCM may be detecting primarily the free 3′-OH terminals of single-stranded DNA breaks that could temporarily appear during repair of DNA, rather than DNA fragmentation due to apoptosis.

DNA Repair and Positive PCNA With Positive/Negative TUNEL
Light-microscopic positive PCNA in myocyte nuclei indicates DNA repair rather than DNA replication, as detailed in the previous section of the present article. Generally, PCNA and TUNEL at light-microscopic levels are evaluated not quantitatively but rather qualitatively. The present light-microscopic analysis using mirror sections showed that PCNA-positive nuclei of myocytes included all TUNEL-positive nuclei, but the reverse was not true. Therefore, tissue specimens were classified into 3 groups at the light-microscopic level: a group with PCNA-positive and TUNEL-positive nuclei, a group with PCNA-positive but TUNEL-negative nuclei, and a group with PCNA-negative and TUNEL-negative nuclei. The former group consisted of only DCM and the latter 2 of both DCM and control myocytes.
In EM-TUNEL, immunogold density reflects the number of DNA breaks with free 3'-OH terminals. Marked immunogold density was observed in 5.8% of myocyte nuclei in the group with PCNA-positive and TUNEL-positive nuclei at the light-microscopic level, and the incidence was similar to that of light-microscopic TUNEL-positive nuclei (7.9%). However, the immunogold density was less marked in the other 2 groups with negative TUNEL at the light-microscopic level. This suggests that myocyte nuclei with marked immunogold density at the EM level correspond to myocyte nuclei with PCNA-positive and TUNEL-positive nuclei at the light-microscopic level. The specific ultrastructure of myocyte nuclei with marked immunogold density almost coincided with the nuclei with bizarre shapes and marked nuclear hypertrophy scores (sensitivity of 98% and specificity of 100%), suggesting a greater increase in DNA repair activity in nuclei with marked immunogold density than in nuclei without such density. Thus, the positive PCNA and positive TUNEL reactions at the light-microscopic level may imply more increased DNA repair activity than the positive PCNA but negative TUNEL reactions.

**Limitations of the Present Study**

In the hearts with DCM, considerable myocyte loss (myocyte death) is evident, accompanied by diffuse interstitial fibrosis and rare infiltrated inflammatory cells, in addition to myocyte hypertrophy. This suggests that the mechanism of myocyte death involves apoptosis rather than necrosis. Electron microscopic and/or endomyocardial biopsy analyses are limited to small samples, and the apoptotic process generally progresses very rapidly. Therefore, the lack of evidence of cell death in the present study may result from the small samples associated with these analyses.

We did not perform DNA gel electrophoresis in the present study because of the small amount of each endomyocardial biopsy specimen. DNA ladders by gel electrophoresis are in general detectable when a high proportion of cells die simultaneously, and the sensitivity of ethidium bromide staining is known to be particularly low. Nevertheless, Olivetti et al reported that DNA ladders were detected in tissues of end-stage failing hearts containing only 0.24% (0.067% to 0.65%) TUNEL-positive myocytes, including hearts with DCM that were obtained at transplantation. Conversely, Narula et al failed to detect DNA ladders in some failing and transplanted hearts with high levels of TUNEL-positive myocytes (as high as 35.5%). In addition, it is impossible to differentiate by this method whether DNA fragmentation originated from myocytes or from other myocardial interstitial cells, such as infiltrating inflammatory cells, in which we recently reported the presence of typical...
Figure 4. Light photomicrographs of immunohistochemical preparations. A and B, Taq polymerase–based in situ DNA ligation assay. Positive reactions (arrows) were seen in prostate tissue from a rabbit castrated 2 days before study, which was used as a positive control (A). One positive cell (*) is shown in inset of A at a higher magnification. There was no positive reaction in endomyocardial biopsy specimen obtained from a patient with DCM (B). C and D, Serial mirror sections stained immunohistochemically with TUNEL (C) and PCNA antibody (D). T1 and P1 present same cell, as do T2 and P2, or T3 and P3. T1 was TUNEL-positive, and T2 and T3 were TUNEL-negative. P1 and P2 were PCNA-positive, and P3 was PCNA-negative. TUNEL-positive myocytes were always PCNA-positive. E and F, Positive Ki-67 immunostain was seen in human tonsillar tissue used as positive control (E), but Ki-67 immunostain was completely negative in endomyocardial specimen obtained from a patient with DCM (F). Magnification ×200.
apoptosis. Thus, the occurrence of DNA ladders in myocytes of the hearts with DCM is still unclear.

Conclusions
Most of the TUNEL-positive myocytes in hearts with DCM are not apoptotic but rather living cells with increasing activity of DNA repair.

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