Original Article

Apoptosis in Endomyocardial Biopsies from Patients with Dilated Cardiomyopathy

(apoptosis / dilated cardiomyopathy / TUNEL method)

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Abstract. Apoptosis is an active energy-consuming mechanism of cell death, which may contribute to heart failure in patients with dilated cardiomyopathy. Dilated cardiomyopathy is a common clinical outcome of many prolonged cardiac insults, and therefore is considered as the most prevalent form of cardiomyopathy. Loss of heart mass is highly correlated with the heart failure and mortality, thus the purpose of this study was to define the apoptotic index in patients with dilated cardiomyopathy. Apoptosis was detected by the TUNEL method in 30 patients. Biopsies were obtained from the left ventricle, and at least three specimens were taken. TUNEL-positive cardiomyocytes were found in 26 of 30 cases (86.7 %) and the mean apoptotic index for the entire specimen series was 5.41 ± 1.70 %. The analysis showed that patients with dilated cardiomyopathy had significantly higher apoptotic index ($P < 0.001$) than healthy subjects. One subject (man, 41 years old) had a markedly elevated apoptotic index of 52.2 %. In the remaining subjects, the percentage of cardiomyocyte death ranged from 0 % to 15.5 %. The high percentage of apoptosis found in our study may be in accordance with the clinically manifested cardiac failure in patients with dilated cardiomyopathy since in most patients we recorded the left ventricular ejection fraction values below 30 %.

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Abbreviations: AI – apoptotic index, DCM – dilated cardiomyopathy, EMB – endomyocardial biopsies, ESD – extreme studentized deviate, IQR – inter-quartile range, LVEF – left ventricular ejection fraction values, HP – histopathological diagnosis, TUNEL – terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labelling technique.

Introduction

The heart exhibits a wide range of adaptive responses to different genetic and extrinsic factors to maintain the contractile function. These include activation of genes normally expressed during development, cardiac hypertrophy, and fibrous replacement of necrotic and apoptotic cardiomyocytes (Wang et al., 2010). When compensatory responses are not sustainable, cardiac dysfunction occurs, leading to cardiomyopathy. Dilated cardiomyopathy (DCM) is a common clinical outcome of many prolonged cardiac insults, and therefore is considered as the most prevalent form of cardiomyopathy. Enlargement of the ventricular chamber and thinning of the ventricular walls are the predominant morphological features in this disease. Loss of heart mass is highly correlated with heart failure and mortality. It was shown that 50 % of patients diagnosed with DCM die within five years of becoming symptomatic (Wencker et al., 2003; Harvey and Leinwand, 2011).

Although many pathways are associated with DCM, up-regulation of transcription and induction of apoptosis are major mediators of pathogenic responses in the heart (Olivetti et al., 1997; Krijnen et al., 2002; Hughes, 2003). Apoptosis is a regulated energy-consuming process and is physiologically found in embryogenesis and maturation of multiple cell systems, and pathologically in various diseases (Alter et al., 2001; Krijnen et al., 2002; Hughes, 2003). Apoptosis or programmed cell death is an actively regulated process, which is characterized by DNA fragmentation, membrane blebbing, cell shrinking, and condensation of chromatin (Alter et al., 2001; Krijnen et al., 2002).

The most widely used method to identify apoptosis in human histological material is the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique (Hughes, 2003). However, there are also many difficulties in the

interpretation of apoptotic index (AI) defined by the TUNEL technique, mostly due to the type of cardiac tissue sample (endomyocardial biopsy *vs*. autopsy tissue) and the quality of tissue fixation (Krijnen et al., 2002; Hughes, 2003; Koda et al., 2003). Apoptosis of cardiomyocytes in DCM and heart failure has been reported in several studies (Narula et al., 1996; Olivetti et al., 1997; Schaper et al., 1999; Krijnen et al., 2002; Zorc et al. 2003).

The aim of the present study was to determine the AI in endomyocardial biopsies (EMB) from patients with DCM and its influence in development of heart failure, which was expressed by left ventricular ejection fraction (LVEF) values.

Materials and Methods

Patients

Biopsies from 30 patients with DCM were obtained from the left ventricle, and at least three specimens were taken. EMBs were performed at the Institute of Cardiovascular Diseases Dedinje, Belgrade. The specimens were taken after obtaining the informed consent during a diagnostic or therapeutic procedure. The protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects).

The mean age of patients with DCM was 43.83 ± 6.42 years (median 43.0, IQR: 39.50–48.50 years) and the majority of them (28 subjects) were men. All patients with DCM had idiopathic dilatation of the left ventricular cavity and had been treated for congestive heart failure with various combinations of digitalis, diuretics, vasodilators and catecholamines. The LVEF was measured in all patients using echocardiography.

The histopathological diagnosis (HP) of DCM was made according to the definition and classification by the World Health Organization/International Society and Federation of Cardiology task force (Richardson et al., 1996). According to the degree of hypertrophy, attenuation, degenerative changes of cardiomyocytes and interstitial fibrosis, we divided all patients into three HP groups (initial, mild and advanced) of DCM.

Tissue specimens

Specimens were fixed in 10% buffered formalin for 24 h, embedded in paraffin, and cut into serial sections of 4 μm thickness. The sections were stained with haematoxylin-eosin and Masson trichrome. The detection of apoptosis was performed with the TUNEL method using an ApopTag kit (Integrene, Purchase, NY) according to the supplier's instructions. Sections were counterstained with haematoxylin.

For each myocardial specimen, all cardiomyocyte nuclei were counted. Cells with brown nuclear labelling were defined as TUNEL-positive (Fig. 1). We classified cardiomyocytes as TUNEL-positive when the stained nuclei were inside the cardiomyocyte contour. The nuclei counting technique was carried out using a simple grid graticule in the eyepiece. Total and labelled nuclei within the grid were counted at 400× magnification, and then the field of the view was moved for the width of the grid at a time to cover all the surface of the tissue. All the nuclei counts were performed by two histopathologists. The apoptotic index was defined as follows: AI (%) = 100 × apoptotic cells/total cells per biopsy case. TUNEL-positive cells in the border zone of the biopsy were excluded from counting to avoid assessment of artefacts.

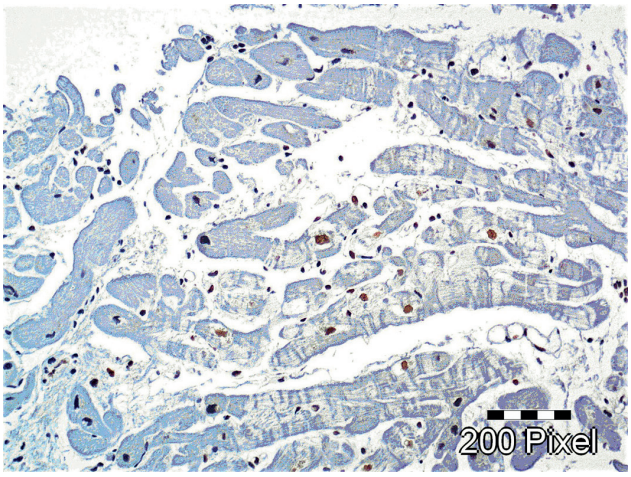

Fig. 1. TUNEL-positive cardiomyocytes in dilated cardiomyopathy (original magnification × 200)

Statistical analysis

Values are expressed as median and inter-quartile range (IQR). Departures from normal distribution were determined by Shapiro-Wilk normality test. For normally distributed values of LVEF and age variables, the parametric tests (*t*-test, one-way ANOVA, Pearson correlation) were used. The extreme AI value (52.2 %) was tested as an outlier by the ESD (extreme studentized deviate) method (Barnett et al., 1994).

After checking and confirming that the experiment was performed correctly and biological variability is a possibility (two necessary conditions), the value was not excluded from statistical analysis. Since AI values had non-Gaussian distribution, the nonparametric tests robust to the presence of outliers were used to test the AI variable (Wilcoxon signed-rank test, Kruskal-Wallis test, Spearman correlation). The P value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the GraphPad Prism statistical program.

Results

The values of LVEF and AI are shown in Fig. 2A. Comparison with the LVEF value of ≤ 40%, which is the validated border value as a measurement of heart failure, showed that DCM patients had a significantly lower LVEF level ($t_{(29)} = 13.97$, $P < 0.001$, *t*-test).

TUNEL-positive cardiomyocytes were found in 26 of 30 cases (86.7 %) and the mean AI for the entire

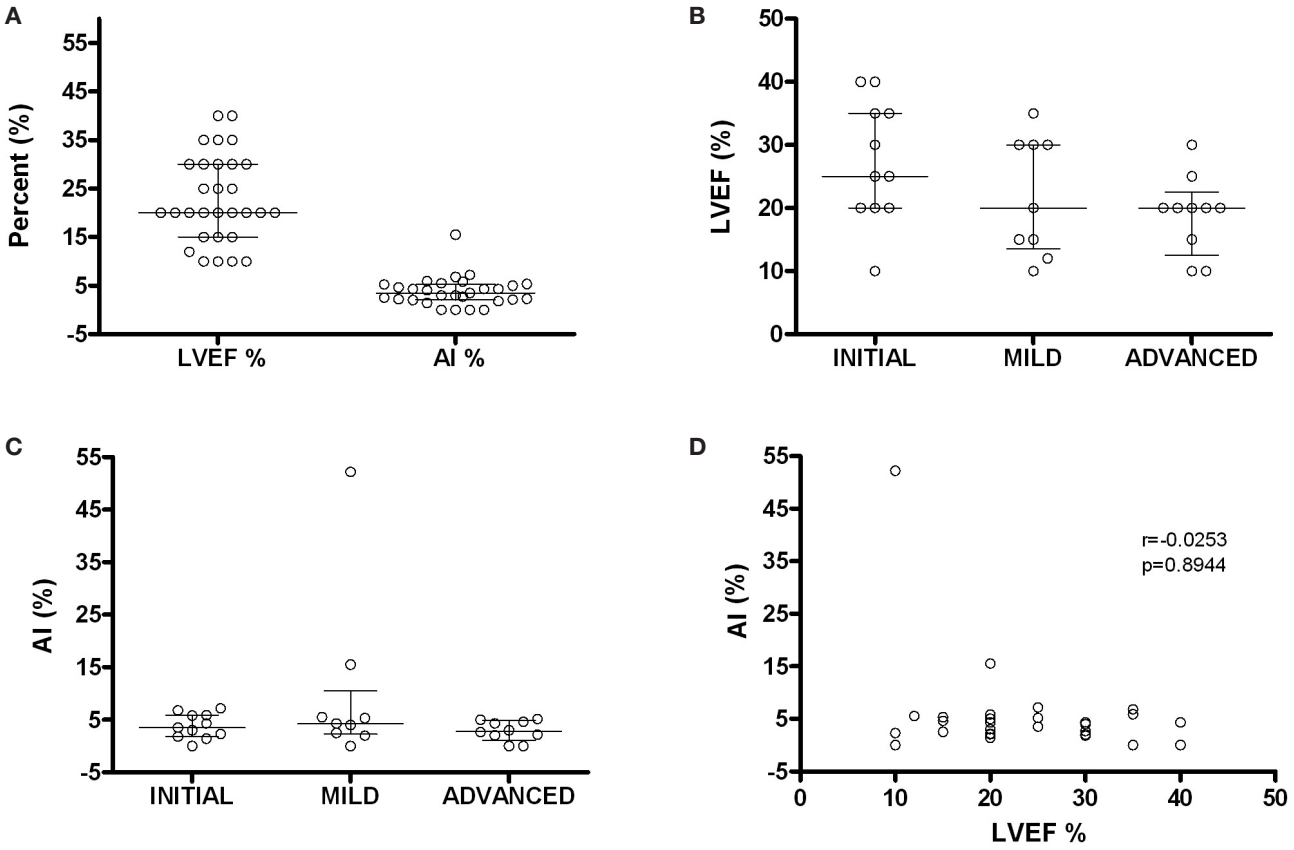

Fig. 2. **(A)** LVEF (%) and AI (%) in patients with DCM. Values are expressed as median and inter-quartile range (IQR). **(B)** LVEF (%) in patients with different HP diagnoses of DCM. Values are expressed as median and inter-quartile range (IQR). **(C)** AI (%) in patients with different HP diagnoses of DCM. Values are expressed as median and inter-quartile range (IQR). (**D**) Scatter plot of AI (%) against LVEF (%).

DCM series was 5.41 ± 1.70 %. Only one subject (man, 41 years old) had a markedly elevated apoptotic index of 52.2 %. In the remaining subjects, the percentage of cardiomyocyte death ranged from 0 % to 15.50 % (median, IQR: 3.50 %, 2.05–5.25 %, respectively). Our samples originated from EMB and control tissue could not be taken from healthy patients. Therefore, the obtained AI average of DCM patients was tested by Wilcoxon signed-rank test against the reference values of AI for normal heart. The maximum AI of normal heart, taken from Olivetti et al. (1997) and Mallat et al. (2001), were 0.0028 % and 0.043 %, respectively. The analysis showed that patients with DCM had a significantly higher AI ($P < 0.001$) than healthy subjects. We found no significant correlation between LVEF and the apoptotic index ($r = –0.03$, $P = 0.89$, Spearman).

Based on the HP diagnosis, all patients were classified into three groups and then tested for differences in LVEF (Fig. 2B) and AI (Fig. 2C) among them. The analysis showed that there was no significant difference in both examined variables (LVEF: $F = 2.55$, $P = 0.09$, one-way ANOVA; AI: $H = 1.58$, $P = 0.45$, Kruskall-Wallis). The percentage of LVEF or AI was not correlated with age ($r = 0.16$, $P = 0.38$; $r = 0.08$, $P = 0.66$, respectively).

Discussion

Studies attempting to identify and quantitate apoptotic cells in cardiac tissue have been carried out in ischaemia-reperfusion injury, mostly in experimental animals. In recent years, much evidence has implicated apoptosis in myocardial cell loss in DCM (Schaper et al., 1999; Alter et al., 2001; Zorc et al., 2003) and heart failure (Narula et al., 1996; Olivetti et al., 1997; Hughes 2003; Koda et al., 2003). There are conflicting results concerning the role of apoptosis of the myocytes in the progression of cardiomyopathy to heart failure (Narula et al., 1996; Olivetti et al., 1997; Schaper et al., 1999; Wencker et al., 2003; Koda et al., 2003). Apoptosis in the myocardium is complex and difficult to recognize (Olivetti et al., 1997). Cardiomyocytes are terminally differentiated cells and under physiological conditions, they should not undergo apoptosis.

We found relatively high AI that occurred in 86.7 % of DCM cases, and the mean AI value was also relatively high (5.41 %) compared to the data from previous studies (Olivetti et al., 1997; Okada et al., 2005). The high percentage of apoptosis found in our study may be in accordance with the clinically manifested cardiac failure in patients with DCM, since most of them had LVEF values under 30 %. The reason why these patients have not yet developed cardiac decompensation may lie

in the fact that cell proliferation expands the functioning myocardium in an attempt to delay the onset of terminal failure. Cell regeneration partially counteracts myocyte loss, thus interfering with the cell death mechanisms in the remodelling process of the pathologic heart (Kajstura et al., 1998).

Different levels of apoptosis have been reported so far. Extreme values of AI were recorded by Narula et al. (1996) and Valente et al. (1998): 5–35 % and 24.4 %, respectively. We found similarly high AI values only in two DCM patients (52.2 % and 15.5 %). These results are distinctly higher than the AI in other studies. One of the possible reasons for such high AI may be explained by the fact that the TUNEL method other than apoptosis also detects DNA repair, which can give false-positive results.

Contrary to these findings, Olivetti et al. (1997) reported 0.23 % of apoptotic cells in failing hearts, while in DCM, Schaper et al. (1999), Koda et al. (2003), and Alter et al. (2001) registered 0.0045 %, 1.82 %, and 0.03 % of apoptotic cells, respectively. In groups of DCM patients with different survival range, Zorc et al. (2003) observed 0.09 % and 0.05 % of apoptotic cells. The aforementioned large variations in apoptotic index in DCM may be due to different models of cell counting, relatively small series, as well as to various and nonstandard histological methods for the identification of apoptosis. Besides, the specificity of the TUNEL method may be challenged because of the following findings:

(i) DNA fragmentation can occur not only during apoptotic, but also during necrotic processes (Majno and Joris, 1995; Dong et al., 1997);
(ii) apoptosis includes various stages of DNA degradation, *i.e.* apoptotic nuclei are rich not only in double-stranded DNA fragments with single-base 3' overhangs, but also in single-stranded DNA fragments (Peitsch et al., 1993);
(iii) TUNEL labels the calcium-bound phosphate groups of the nucleotide non-specifically (Kockx et al., 1996);
(iv) TUNEL can be positive even in living cells, such as cardiac myocytes with increased DNA repair;
(v) due to varying lengths of apoptosis (Kanoh et al., 1999). Finally, the tissue fixation delay may affect sensitivity to TUNEL by activation of endogenous nucleases (Stahelin et al., 1998; Tateyama et al., 1998).

When we analysed the AI and LVEF values with respect to HP groups, we found no significant differences. Our results are in accordance with the findings of Narula et al. (1996) and Kanoh et al. (1999), who also reported the absence of correlation between the AI value and morphological stages of DCM. In contrast, some findings indicate that AI is higher in advanced stages of DCM when compared to the initial stage of the disease (Zorc et al., 2003). However, in the latter study other methods for detection of apoptosis besides TUNEL were used. One of the reasons for the lack of statistically significant difference between the LVEF value and AI in DCM is that the analysed material was obtained by EMB, which implicates analysis of small tissue samples and the fact that such material does not entirely reflect the condition of the whole heart. Also, sampling errors and other technical problems of the applied methods may affect this finding (Vasiljević and Mirić, 1995; Okada et al. 2005).

Although our study showed no significant difference when we analysed the correlation between the increase of apoptotic index and decrease of cardiac function, a relatively high AI that occurred in the majority of patients with DCM may imply that apoptosis is most likely one of the mechanisms responsible for the occurrence of heart failure.

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