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Characterization of myocardial hypertrophy by DNA content, PCNA expression and apoptotic index

Luigi Matturri[a], José Milei[b,*], Daniel Rodolfo Grana[c], Anna Maria Lavezzi[a]

[a]*Institute of Pathology, University of Milan, Milan, Italy*
[b]*School of Medicine, University of Buenos Aires, Tucumán 2163 4B (C1050AAQ), Buenos Aires, Argentina*
[c]*School of Medicine, University del Salvador, Buenos Aires, Argentina*

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Abstract

Background: At present little is known about the biological basis of cellular alterations in myocardial hypertrophy. The present study aims to analyze proliferating cell nuclear antigen (PCNA) expression, DNA content and apoptosis, in several types of myocardial hypertrophy in order to define the biological characteristics of this process. *Methods:* The biological parameters were investigated in normal hearts ($n=4$) and in 21 cases of left ventricular myocardial hypertrophy related to pressure overload ($n=7$), post-infarction remodeling ($n=8$) and hypertrophic cardiomyopathy (HCM) ($n=8$). *Results:* The analyzed biomarkers were similar in hypertension and in remodeling, with a very high apoptotic index (mean values: 8.1 and 8.5%, respectively), a low PCNA positivity (mean values: 1.8 and 1.6%) and a prevalent diploid DNA content (DNA index: 1.2). Conversely, HCM showed a high mean PCNA index (21.2%) associated with a prevalence of hyperdiploid myocytes (DNA index: 1.8) and a low number of apoptotic cells (mean value: 1.7%). *Conclusions:* There are significant biological differences between hypertrophy in HCM and that related to arterial hypertension and post-infarction remodeling. Therefore, the combined evaluation of DNA content, PCNA and apoptotic indices could provide a powerful diagnostic tool in doubtful cases of myocardial primary or secondary hypertrophy and open new avenues in the clinical treatment of these entities.

Keywords: Myocardial hypertrophy; DNA; Proliferating cell nuclear antigen; Apoptosis

1. Introduction

Myocardial hypertrophy is characterized by an increase in myocyte size in the absence of cell division. It may be considered a compensatory or an adaptative process, that is, a response to pressure or volume overload (for various physiological and/or pathological reasons such as systemic hypertension, valvular malformations, etc.), loss of contractile mass (in myocardial infarction), or it may itself be a primary alteration, generally of unknown origin, as in hypertrophic cardiomyopathy [1–5].

At present little is known about the biological basis of cellular alterations in myocardial hypertrophy. Some studies, mainly experimental, have demonstrated that the increase in nuclear size observed in hypertrophic myocytes is related to an increase in DNA content [6–9]. The hyperdiploidy was observed also in our previous paper on hypertrophic cardiomyopathy (HCM) [10]. However, these ploidy increases are difficult to explain since, after birth and specially at termination of the physiological growth of the organism, myocardial tissue loses its ability to synthesize DNA [11–14].

*Corresponding author. Tel./fax: +54-11-49-510-366.
E-mail address: drjosemilei@cardiopsis.com.ar (J. Milei).

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Proliferating cell nuclear antigen (PCNA) is a 36 kDa acidic, non-histone, nuclear protein required for DNA synthesis, and acts as the auxiliary protein of DNA-polymerase delta. Several studies carried out using PCNA antibodies have shown that PCNA accumulates in the cell nuclei during the S-phase and can be considered as a proliferation marker. Using a specific monoclonal method (PC10) has particular advantages over other techniques because of its relative simplicity and the rapidity of results [15].

The present study aims to analyze some biological parameters such as PCNA, DNA content and apoptosis, in various forms of myocardial hypertrophy (resulted from pressure overload, post-infarction remodeling and hypertrophic cardiomyopathy) in order to define the biological characteristics of this process and to determine whether differences exist in relation to the different pathologic conditions associated with it.

2. Materials and methods

The study was carried out in 21 cases of myocardial hypertrophy, obtained at necropsy from 13 males and eight females, aged from 38 to 88 years.

In eight cases hypertrophy was associated with left ventricular remodeling in post myocardial infarction, affecting the antero-lateral wall and/or the interventricular septum, in seven cases it was a consequence of arterial systemic hypertension and in eight it was related to HCM. Four of these cases were obstructive and four non-obstructive forms. An additional four hearts from three males and one female of 33, 51, 55 and 77 years respectively, who had not died from cardiovascular diseases, were used as controls.

For each case, five consecutive 5 μm sections were obtained from the left ventricle wall; fixed in buffered formaldehyde 10% and embedded in paraffin. Two of these were stained with hematoxylin/eosin and Azan for histologic examination, one was stained according to Feulgen for cytometric DNA analysis, and two were examined immunohistochemically for PCNA and apoptosis detection, respectively.

2.1. Cytometry

For evaluation of DNA content sections stained with Feulgen were examined. This method is based on the interaction between Schiff's reagent and the aldehyde groups of the deoxyribose molecules, previously unmasked by acid hydrolysis (5 N HCl at 22°C for 60 min) which removes the purinic bases.

Integrated optical density (IOD) was evaluated with a Zeiss CIRES image analyzer in 200 nuclei. The control reference value (diploid DNA content) was evaluated on the basis of 100 tissue lymphocytes. Nuclei that appeared to be overlapping or not clearly defined, were excluded from the assessment.

Ploidy is expressed as DNA Index=mean value ratio of DNA content of myocardial nuclei analyzed and that of reference lymphocytes.

DNA Index equal to 1 corresponds to diploidy, while DNA Index different from 1 corresponds to aneuploidy (value lower than 1 is hypodiploidy and higher than 1 hyperdiploidy).

2.2. PCNA evaluation

Sections were deparaffinized and brought to Tris–HCl-buffered saline solution (TBS; pH=7.6). After blocking endogenous peroxidase with 3% H_2O_2, the slides were incubated overnight with the primary antisera. Immunohistochemical staining was performed with the peroxidase–antiperoxidase method and avidin–biotin complex technique (ABC complex). Diaminobenzidine was used as chromogen substrate.

The sections were immunostained with PCNA monoclonal antibodies (DAKO) at a dilution of 1:200 and counterstained with light hematoxylin.

The PCNA index in every case was defined as the number of myocytes with strong unequivocal nuclear staining, corresponding to cells in S phase, divided by total number of cells counted, expressed as the percentage.

2.3. Apoptosis assay

The sections were deparaffinized and incubated with 20 μg/ml proteinase K. After the endogenous peroxidase treatment the deoxynucleotidyl transferase (TdT 0.3 U/ml), was used to incorporate digoxygenin-conjugated deoxyuridine (dUTP 0.01 mM/ml) to the ends of DNA fragments. The signal of TdT-mediated dUTP nick end labeling (TUNEL) was then detected by an anti-digoxigenin antibody conju-

gated with peroxidase. Apoptotic nuclei were identified by the presence of dark brown staining.

The apoptotic index was defined as the percentage of apoptotic nuclei on total number of myocardial cells evaluated in a minimum of 10 fields (×500). Of note, all the apoptotic cells counted were cardiomyocytes.

2.4. Statistical analysis

The differences between the mean values of ploidy, PCNA and apoptotic index in the different diseases studied and in normal hearts were calculated by variance analysis (*F*-test).

3. Results

Table 1 shows the densitometric and immunohistochemical results obtained for all cases studied.

The biological characteristics of myocardial hypertrophy secondary to hypertension and that associated with post-infarction remodeling were nearly the same. In both illnesses, the apoptotic index was very high (mean values 8.1 and 8.5%, respectively) with wide ranges of values, specially in the forms due to systemic hypertension (ranges: 0.7–16.6% and 1.5–11.9%) (Fig. 1). A different distribution of apoptotic nuclei was observed in both types. Apoptotic nuclei were widely distributed in cases of hypertrophy from volume overload but in cases of post-infarction remodeling they tended to be concentrated in fibrous areas rather than in the subendocardium.

On the other hand PCNA indices were low. For hypertensive hypertrophy the values ranged from 0 to 5.4% with a mean value of 1.8%. Similar PCNA results were obtained for post-infarction remodeling (range: 0–3.2%; mean value: 1.6%).

Regarding DNA content, analysis showed peaks mainly around diploidy with identical DNA index (1.2) and similar ploidy ranges (0.8–2.4 in hypertension and 0.9–1.8 in remodeling).

For HCM a contrasting opposite picture resulted. The apoptotic index was significantly lower than that observed in the other pathologic conditions (range: 0–4.5%; mean: 1.7%; $P<0.05$); conversely, the PCNA values were higher (range: 11.0–38.5%; mean: 21.2%; $P<0.05$) (Fig. 2) and DNA content almost tetraploid (DNA index=1.8).

In the control hearts the results were consistently lower than those observed in hypertrophy (mean values: PCNA=1.2%; apoptosis=0.9%; DNA=1.1). Of note, even if the expected number of mitotic cells is zero, a PCNA index positive value can exist in controls (see Discussion).

4. Discussion

The myocytes of the fetal heart and to some extent those of the neonatal heart have the ability to divide by mitosis [16–18]. Shortly after birth the myocardial tissue loses the ability to divide though it is still able to synthesize DNA.

In fact, the increase in heart size which accompanies the physiological growth of the organism occurs by hypertrophy of some of the pre-existing myocytes, which usually become hyperdiploid as it was stressed by other authors [1,11,12].

Hypertrophy may be more marked, affecting a large number of myocytes, as an adaptive or compensatory response in certain pathological processes resulting in an increase in cardiac mass [1–5]. However further clarification is required concerning the biological changes involved in hypertrophy, in particular the variations in the DNA content of

Table 1
PCNA, apoptosis and ploidy in hypertrophic and normal hearts

	PCNA index (%)		Apoptotic index (%)		DNA index	
	Range	Mean	Range	Mean	Range	Mean
Diseases						
HCM	11.0–38.5	21.2	0–4.5	1.7	1.0–2.6	1.8
Hypertension	0–5.4	1.8	0.7–16.6	8.1	0.8–2.4	1.2
Post-MI remodeling	0–3.2	1.6	1.5–11.9	8.5	0.9–1.8	1.2
Controls	0–3.0	1.2	0–2.5	0.9	0.9–1.8	1.1

HCM, hypertrophic cardiomyopathy; MI, myocardial infarction.

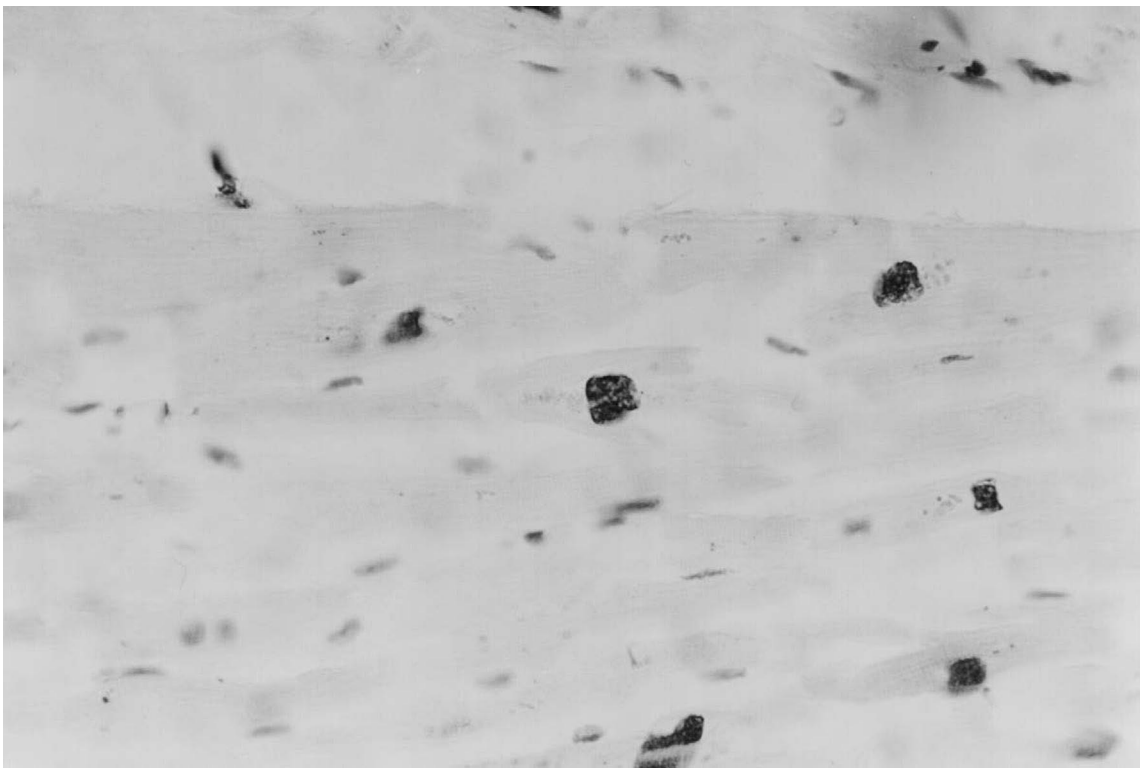

Fig. 1. High apoptotic index in secondary myocardial hypertrophy (×500).

myocytes, occurring outside the cell cycle, as demonstrated by the total absence of mitosis in the myocardium. Also hyperplasia could be the result of amitotic cell divisions of the nuclei, through longitudinal splitting of the hyperdiploid fibers, as shown by some authors [19–22] to be associated with a heart weight of over 500 or 250 g for the left ventricle.

Hypertrophy may be accompanied by qualitative and quantitative modifications of the expression of genes that codify the myocardial proteins [23–25]. Schaub et al. [26] have demonstrated, on cultured cardiomyocyte studies, that these modifications could be induced by factors such as anoxia, hormonal stimulation (thyroid hormones, catecholamines, angiotensin) and growth factors (Insulin-like Growth Factor, basic Fibroblast Growth Factor).

In the present study, by evaluating DNA content, PCNA and apoptosis, we aimed to characterize myocardial hypertrophy in different pathological conditions, namely arterial hypertension, post-infarction remodeling and hypertrophic cardiomyopathy.

Remodeling is the result of dynamic changes which affect the architecture of the left ventricle following myocardial infarction [27–30]. In this case, hypertrophy of viable myocytes quickly sets in, even within hours to few days of the acute event, and in segmental manner, mainly around the infarcted region [4,31,32]. On the other hand, hypertrophy that is secondary to arterial hypertension increases gradually, showing a homogeneous, concentric distribution [33].

However, in spite of the differences in the development process and also in the hypertrophic cell distribution pattern for post-infarction hypertrophy and that resulting from arterial hypertension, the biological picture which resulted in our study was similar in the two cases.

The most significant finding was the presence of a high apoptotic index in both forms of secondary hypertrophy, while the results related to PCNA index and DNA content were similar to those for normal heart myocytes.

Conversely, in the case of hypertrophic cardiomyopathy, the PCNA positivity was very high, and associated with a prevalence of hyperdiploidy and a low number of apoptotic cells.

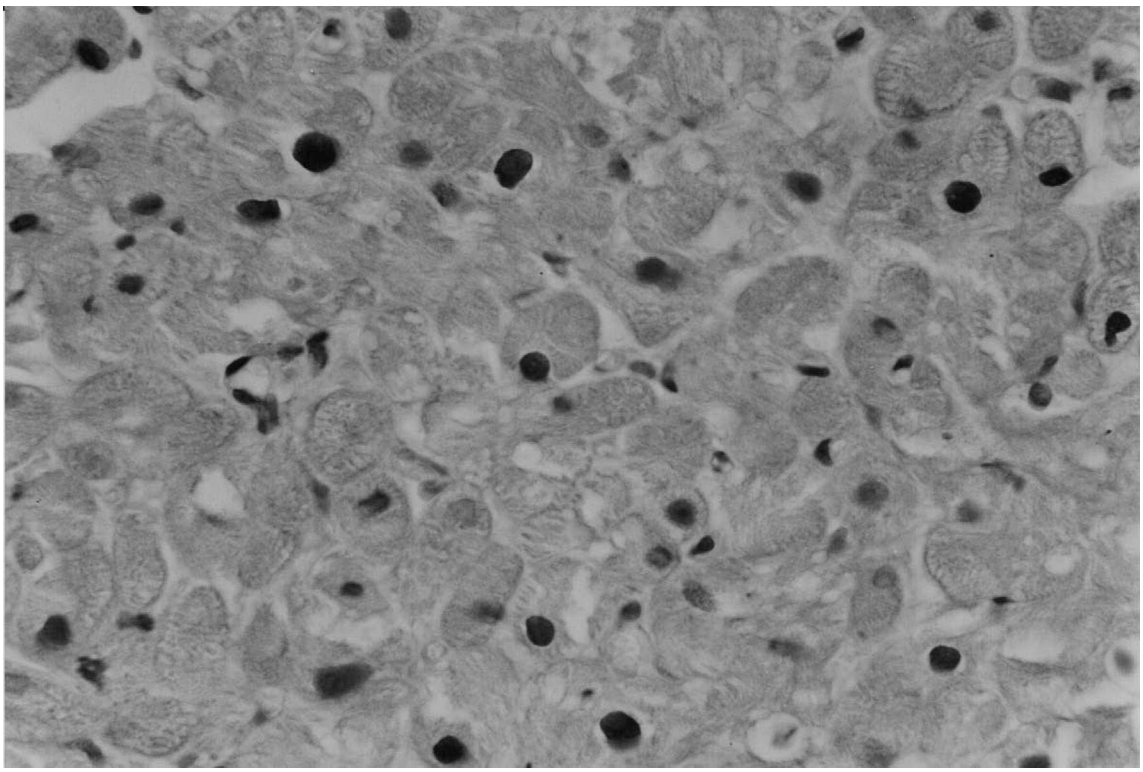

Fig. 2. High PCNA index in primary hypertrophic cardiomyopathy (×500).

The increased expression of PCNA in hypertrophic cardiomyopathy has been interpreted in one of our earlier studies [34] as a clear process of amitotic renewal of DNA, to counterbalance its marked instability. It may be stressed, that even if the expected number of mitotic cells is zero, a PCNA index positive value can exist in controls. In fact, the PCNA is a marker of the DNA content in interphase nuclei (particularly in the S phase) and it can be increased without the conclusion of the cell cycle in the mitosis. PCNA index is not equivalent of mitotic index.

A first consideration emerging from our present study concerns apoptosis. Although necrosis has been thought to be the principal form of cell death in the myocardium [35], we have observed that apoptosis is always present both in normal hearts and in pathological myocardial conditions, though the percentage of apoptotic myocytes varies, being lower in normal hearts and higher in the forms of hypertrophy resulting from pressure overload and infarction.

As apoptosis may play a compensatory role in tumor development [36–39], we believe that apoptosis could be an important regulator mechanism activated at the end of the hypertrophic process in order to eliminate the hyperdiploid myocytes. Conversely, Teiger et al. [40] have demonstrated in experimental studies that apoptosis appears in the early stages of myocardial hypertrophy. That is to say, its maximum value precedes the hypertrophic peak, which seems to be directly related to apoptotic cell fraction.

However, irrespective of the extent of apoptotic process and of the moment of onset, it can be considered that hypertrophy and apoptosis are two closely related processes. Moreover, some authors [41,42] have shown that when myocyte volume starts to increase the c-myc gene, an oncogene involved both in DNA synthesis and apoptosis, is expressed.

The most interesting feature of our study is the non-homogeneity of the results. It can be affirmed that, although the histologic picture for cardiac hypertrophy is very similar for the various diseases, with the exception of myocyte disarray in HCM, conflicting biological profiles nevertheless exist. In particular, there are substantial and significant differ-

ences between hypertrophy associated with hypertrophic cardiomyopathy and that related to arterial hypertension and to post-infarction remodeling.

If our data are confirmed by further studies involving a larger number of cases, the combined evaluation of DNA content and percentage of both PCNA and apoptotic positive cells could provide a valuable diagnostic tool, which would make it possible to distinguish between myocardial primary and secondary hypertrophy in doubtful cases. Of note, a thorough knowledge of the pathogenic mechanisms involved in these diseases may open new avenues in clinical treatments.

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