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Expression and significance of cell immunohistochemical markers (HHF-35, CD-31, Bcl-2, P-53 and apopDETEC®) in hypertrophic cardiomyopathy

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Summary. There are several hypotheses concerning the pathogenesis of hypertrophic cardiomyopathy (genetic, ischaemic, immune, inflammatory and apoptosis induction). We have studied three types of cardiomyopathy in order to observe the expression and assess the significance of different immunohistochemical markers (muscular actin, CD-31, proliferation cell nuclear antigen -PCNA-, Ki-67, and markers related with programmed cell death, bcl-2, p-53 and apopDETEC®).

We studied different microscopic (haematoxylin-eosin and Masson's thrichrome) and immunohistochemical parameters (streptavidin-biotin-peroxidase and "in situ" hybridisation) of forty cases: ten each of hypertensive hypertrophic cardiomyopathy, essential hypertrophic cardiomyopathy, hypertrophic cardiomyopathy in patients treated with chemotherapy and morphologically "normal" hearts.

Our findings point to an absence of structural marker expression (actin and CD-31) in cases of hypoxic damage. The distribution and intensity of apoptosis markers, a seen by "*in situ*" hybridisation were irregular, and the rest of the markers studied showed negative results, with the exception of acridin orange (a marker of hypoxic damage).

In our opinion, the above immunohistochemical markers, especially actin and CD-31, could be used for differentiating hypoxic lesions in these three types of cardiomyopathy. Moreover, it is difficult to know the significance of the apoptosis markers, because the autolysis process produces cross reactions with false positive results. We think that there is a need for new studies on DNA breakdown processes during the post-mortem interval. To avoid autolysis problems the post-mortem material needs to be as fresh as possible.

Key words: Immunohistochemistry, Hypertrophic cardiomyopathy, Hypertensive cardiomyopathy, Toxic cardiomyopathy, Essential cardiomyopathy apoptosis

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Introduction

Hypertrophic cardiomyopathy is frequently associated with sudden death (Alfonso, 1996; Steimberg et al., 1996; Davies, 1999; Virmani 2001). Two types have been described classically: primary and secondary. Primary or essential hypertrophic cardiomyopathy may be due to genetic defects and mutations which codify, in the vast majority of cases, the structural proteins of the cardiomyocyte (Davies and Mackenna, 1995; Moolman et al., 1997; Tanaka et al., 1997). Secondary cardiomyopathy may have many causes, which are frequently of a hypertensive or toxic origin (Schoen, 1999; Gallo and D'Amati 2001). Toxic cardiomyopathy is increasingly common because of alcohol abuse (Walsh and Vacek, 1986) and the use of cardiotoxic cytostatic medication (Hochster et al., 1995; Lasbellaoui et al., 1997; Pai and Nahata, 2000).

In general, cardiomyopathies show-well defined microscopic characteristics, including interstitial fibrosis and the irregular disposition and hypertrophy of cardiomyocytes. All this, has classically been associated with myofibril alterations (Teare, 1958), usually due to genetic alterations which structurally damage the cardiomyocyte and which result in myocardial disarray.

This pathogenic mechanism, which is obvious in the case of essential cardiomyopathy, may be applicable to other types. Several histochemical studies have suggested that hypoxic mechanism operate in the pathogeny of cardiomyopathies, especially in toxic cardiomyopathies in the absence of coronary disease (Hochster et al., 1995). Immunohistochemical studies have pointed to immune mechanisms (Kuhl et al., 1992, 1995) or mechanisms which induce cell necrosis (Liu et al., 1995).

Despite all these studies, it remains very difficult to establish the true aetiology of cardiomyopathies and the mechanism involved in their pathogeny in patients who suffer sudden death as a result of cardiomyopathy. This study hopes to provide useful information in this respect by studying the expression and significance of different immunohistochemical markers, among them structural markers (muscular actin and CD-31), cell proliferation

(proliferation cell nuclear antigen –PCNA- and Ki-67) and markers related with programmed cell death (bcl-2, p-53 and apopDETEC®). We also and look for evidence of hypoxic-type lesions using histochemical markers such as acridin orange (Lachica et al., 1988). All the above were studied in different cardiomyopathy types to ascertain their usefulness as myocardial lesion markers and to throw some additional light on the pathogeny of these diseases.

Materials and methods

We studied forty hearts obtained during autopsies carried out by the Pathological Anatomy Service of the University General Hospital, Murcia (Spain) and in the Institute of Legal Medicine, Murcia. Of these forty cases, ten were cases of hypertensive hypertrophic cardiomyopathy, ten of essential hypertrophic cardiomyopathy (Fig. 1), ten of hypertrophic cardiomyopathy in patients treated by chemotherapy and ten were morphologically "normal" (250-349g in weight, free wall thickness of less than 13 mm in the left ventricle and equal to or less than 4 mm in the right ventricle) from patients who had died of non-cardiac causes.

The age, sex, personal and family records, underlying illness and immediate cause of death were recorded in all cases, as were several macroscopic morphological characteristics such as weight, ventricle thickness and valve sizes. Microscopic examination examined the disposition, cytoplasm and nuclear characteristics of the myocardic cells and recorded the presence of fibrosis, edema or congestion.

For this microscopic examination, samples were taken from the middle third of the free wall of both ventricles and interventricular partition. They were processed normally and included in paraffin. Sections (3-4 μm) were stained with haematoxylin-eosin, Masson's trichrome and immunohistochemical techniques using streptavidin-biotin-peroxidase, a modification of Hsu's method (Hsu et al., 1981). The following markers were used as primary antibodies:

1) Muscle markers: muscular actin (HHF-35, Dako diagnosis, Spain) to evaluate whether sarcomeres had suffered some type of damage that altered their expression.

2) Vascular markers: CD-31 (Dako diagnosis Spain), which specifically stains vascular endothelial cells and reveals any increase in vascular activity.

3) Cell proliferation markers: cell proliferation nuclear antigen (PCNA, Dako diagnosis Spain) to stain the G1, S and G2 stages of the cell cycle and ki-67 to stain the S stage.

4) Markers associated with apoptosis-regulating genes (bcl-2 and p-53, Dako diagnosis Spain) which can be considered indirect markers of the same.

The results were expressed on a scale of 0 to 3 to reflect the degree of positivity, and all the mycroscopical studies were performed by two different observers.

"In situ" hybridisation techniques were used to detect DNA fragments overproduced by apoptosis, using the apopDETEC® Kit Cell Death Assay Systems (ENZO diagnosis USA). Positive cells were graduated semiquantitatively by counting four fields x200, the results being expressed on a scale of 0 to 4 where 0 =negative cases; 1+ for <10% cells; 2+ for 10-25%; 3+ for 26-50%, and 4+ >50%.

One of the sections was stained with acridin orange to evaluate the presence of any unspecific cell damage. The observations were graded as very positive (+++), doubtful (+/-) or negative (-).

The statistical study consisted of calculating and observing the distribution of relative and absolute frequencies for each of the variables, any statistical significant relationship being established by Pearson's Chi-square test and Fisher's exact test in 2x2 tables.

Fig. 1. Hypertrophic cardiomyopathy. Cardiac section where a diffuse thickness of the free and interventricular wall of the heart can be seen.

Results

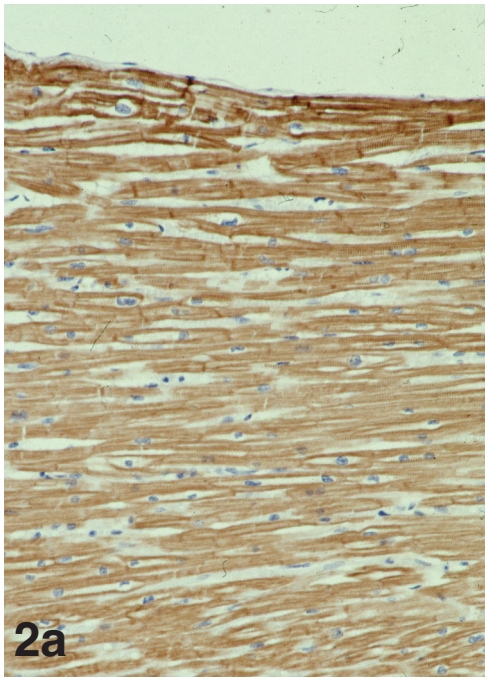
The age, heart weight and ventricle thicknesses are shown in Table 1. There data were significantly associated ($p<0.001$) to the different types of cardiomyopathy studies. The highest mean values corresponded to the hypertensive cardiomyopathy (age, 69.6 years; weight, 519.5 g and ventricle thicknesses 5.7 mm (right) and 19.7 mm (left). Essential cardiomyopathies occurred earlier (28.1 years) and showed similar but slightly lower weights and mean ventricle thicknesses. The values corresponded to the control heart were in a normal range (Table 1).

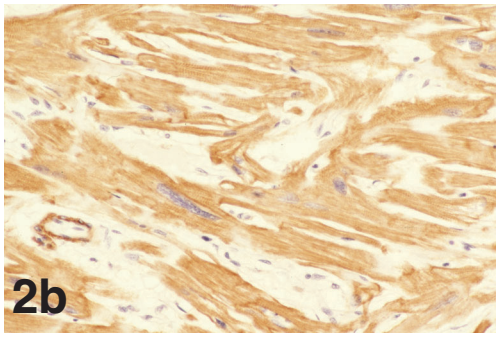
The histological study using conventional methods pointed to the presence of cardiomyocytes with spicular nuclei and a certain degree of nuclear pycnosis, which was sometimes absent. Such alterations, which were interpreted as a sign of autolysis, affected most of the histological sections. All the cases showed histological signs of hypertrophic cardiomyopathy with a huge nucleus and myocardial disarray.

Structural markers showed intense cytoplasmic

Table 1. Mean age, heart weight and ventricle thickness. Distribution accordingto type of cardiomyopathy and control group.

	Age (years) ø/Range		Weight (g) ø/Range		Thickness LV (mm) ø/Range		Thickness RV (mm) ø/Range	
Control group	33	23/60	288.5	180/350	10.8	10/12	3.3	2/4
Hypertensive HCM	69.6	33/92	519.5	351/685	19.7	14/25	5.7	3/9
Essential HCM	28.1	10/46	468.9	210/595	17.8	14/25	5.3	3/8
Toxic HCM	48	19/71	390	255/520	14	10/20	3.8	3/5





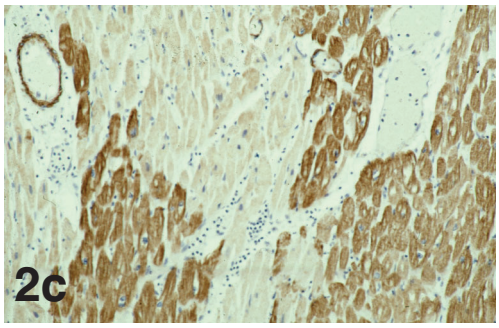
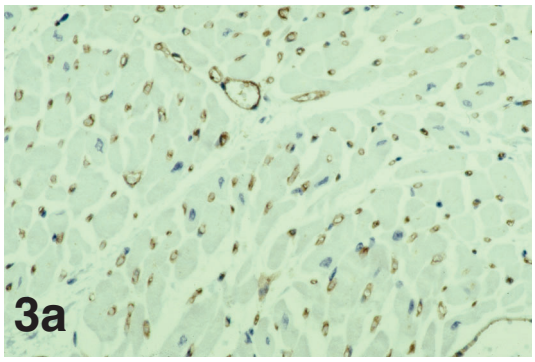
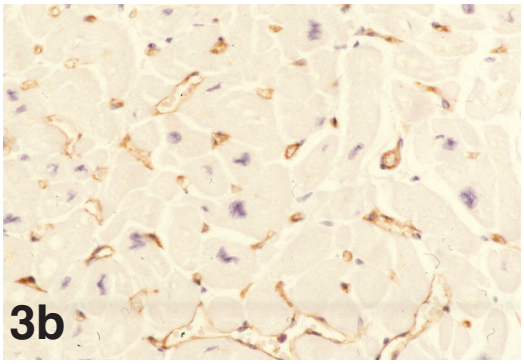
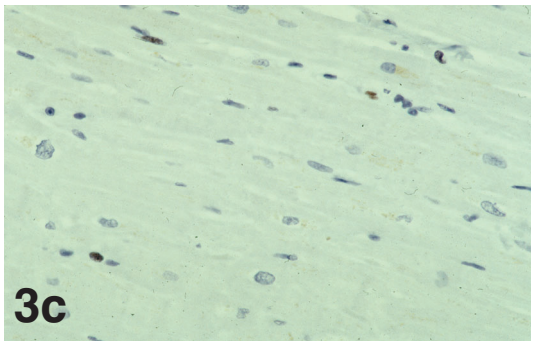


Fig. 2. Cardiomyocytes stained with muscle actin (SBP). **a.** A panoramic view of myocardial cells with diffuse staining in a control heart. x 150. **b.** A detail of myocardial disarray in a case of hypertrophic cardiomyopathy. x 325. **c.** Cardiomyocyte groups with a lack of staining which reveals a hypoxic-type lesion. x 125







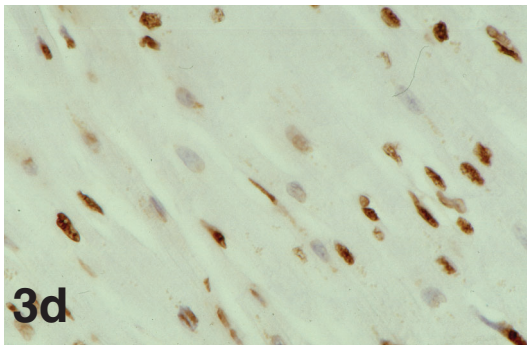


Fig. 3. a. Capillary vessels stained with CD-31 accompanying muscle cells (SBP). x 125. **b.** Greater staining intensity with CD-31 in a case of hypertrophic cardiomyopathy (SBP). x 300. **c.** Nucleus of stromal cells strongly stained with Ki-67 (SBP). x 250. **d.** Cardiomyocyte nucleus and stromal cells irregularly stained with apopDETEC® by "in situ" hybridisation. x 325

positivity for muscular actin, both in normal hearts (Fig. 2a) and all cardiomyopathies (Fig. 2b), except in two cases where the intensity was lower, reflecting hypoxic-type lesions (Fig 2c). There was no statistical association with the different types of cardiomyopathy. CD-31 specifically marked the capillary vessels which accompanied the myocardial cells (Fig. 3a) and there was a significant association ($p<0.001$) between the different types of cardiomyopathy studied and staining intensity (Fig. 3b) (Table 2).

As regards the cell proliferation markers, both PCNA and Ki-67 were negative (in the cardiomyocytes), except for an isolated positivity in the stromal and/or inflammatory cells (Fig. 3c). Only the protein Bcl-2 showed a weak positivity (in three cases) while p-53 was not expressed.

However, when *"in situ"* hybridisation was carried out to mark the DNA fragments released during apoptosis, the myocardial cells showed irregular nuclear staining (as did adjacent cells to a lesser degree) (Fig. 3d). The high percentage of positive cells led us to re-evaluate the results and, after another count of positive cells in four fields x200, we considered a scale of 0 to 4 (0 = negative; 1+ = <80% positive cells; 2+ = 80-89% positive; 3+ = 90-95% positive cells and 4+ = >95%). According to this graduation, most cases showed a high percentage of positive cells (80-100%), while there was no relation between the percentages and the cardiomyopathy types studied (Table 3).

For this reason, we evaluated the staining intensity, which, along with the percentage of cells, was seen to be very high, except in a few cases (although always present) (Table 4).

Acridin orange showed a yellowish fluorescence of varying insensitivity in 17 out of the 40 cases, which did not affect the nucleus and cytoplasm to the same extent, for which reason they were evaluated separately. The results pointed to statistically significant differences ($p>0.001$) in cytoplasmic staining intensity, between different cardiomyopathy types and normal hearts. Staining was not so widespread in the hypertensive and essential cardiomyopathies as in the toxic group. No association was observed between positive fluorescence in the nucleus and the different types of cardiomyopathy (Table 5).

Table 2. Distribution of CD-31 expression in different types of cardiomyopathy.

	CD-31			TOTAL
	+1	+2	+3	
Normal heart	6	2	2	10
Hypertensive HCM	-	3	7	10
Toxic HCM	-	2	8	10
Essential HCM	2	6	2	10
Total	8	13	19	40

χ^2 Test	Value	G l	Bilateral Asint. Sig.
χ^2 (Pearson's coefficient)	21,781	6	0.001
Cramer's coefficient	23,026	6	0.001
Line by line association	1,375	1	0.241
Valid cases	40	-	-

Table 3. Percentage of cells staining positively with apoptosis marker.

	% APOPTOSIS				TOTAL
	<80	80-89	90-95	>95	
Normal heart	-	3	-	7	10
Hypertensive HCM	-	7	1	2	10
Toxic HCM	1	-	2	7	10
Essential HCM	-	3	4	3	10
Total	1	13	7	19	40

χ^2 Test	Value	G l	Bilateral Asint. Sig.
χ^2 (Pearson's coefficient)	19,984	9	0.018
Cramer's coefficient	23,222	9	0.006
Line by line association	0.022	1	0.882
Valid cases	40	-	-

Table 4. Distribution of positivity recorded with apoptosis marker according to staining intensity.

	APOPTOSIS STAINING INTENSITY			TOTAL
	+1	+2	+3	
Normal heart	4	4	2	10
Hypertensive HCM	-	6	4	10
Toxic HCM	-	4	6	10
Essential HCM	1	5	4	
Total	5	19	16	

χ^2 Test	Value	G l	Bilateral Asint. Sig.
χ^2 (Pearson's coefficient)	11,179	6	0.083
Cramer's coefficient	11,159	6	0.074
Line by line association	3.135	1	0.077
Valid cases	40	-	-

Table 5. Distribution and association between expression of acridin orange in the cytoplasm and types of cardiomyopathy.

	ACRIDINE ORANGE CYTOPLASMIC STAINING			TOTAL
	+1	+2	+3	
Normal heart	4	7	4	10
Hypertensive HCM	1	7	2	10
Toxic HCM	-	4	6	10
Essential HCM	-	10	-	10
Total	5	23	12	40

χ^2 Test	Value	G l	Bilateral Asint. Sig.
χ^2 (Pearson's coefficient)	21,658	6	0.001
Cramer's coefficient	24,550	6	0.000
Line by line association	0.309	1	0.578
Valid cases	40	-	-

Discussion

The age and weight/thickness measurements of both ventricles were significantly ($p<0.001$) associated with the different types of cardiomyopathy, as has been mentioned elsewere (Litvosky and Rose, 1988), although the weight of the essential hypertrophic cardiomyopathy hearts was much less than that classically described and similar to the weight of hypertensive hypertrophic cardiomyopathy hearts (Schoen, 1999). However, the mean thickness of both ventricles was similar to that described by other authors (Arola et al., 1997; Vikstrom et al., 1998).

Histologically, the presence of nuclear retraction with pycnosis and cariolysis can only be interpreted as a sign of autolysis, even if the sample is taken within a few hours of death, with no statistical differences existing between the normal and pathological hearts.

The immunochemical expression of the muscle marker HHF-35 was strongly positive in most cases except in one case of death by disseminate intravascular coagulation, when it was much weaker in areas of localised ischaemia (Fig. 2b). It was also weaker where microscopic signs of autolysis existed. Both hypoxia and autolysis are two factors that may affect actin expression although it may also be affected by alterations of the sarcomere in cardiomyopathies of a genetic origin (Davies and McKenna, 1995; Vikstrom et al., 1998).

CD-31 expression was weaker in cases where autolytic processes were evident, as in areas of fibrosis. The fact that other authors (Tomita et al., 1997) detected an increase in the number of capillaries in essential hypertrophic cardiomyopathies associated to areas of fibrosis is probably related to the evolution of fibrosis.

The negative expression of the cell proliferation markers (Ki-67 and PCNA) in myocardic cells contrasts with the finding of other authors (Matturri et al., 1997) using PCNA, although we did find a degree of positivity in stromal cells, which are capable of proliferating.

The presence of apoptosis in the myocardial cells is more controversial since no similar results have been found in the literature, although some authors have suggested that apoptosis may play an important role in the production of toxic cardiomyopathies (Wang et al, 1998; Kavantzas et al., 2000), cardiomyopathy resulting from viral infection (Huber, 1997), some primary cardiomyopathies (Valente et al., 1998) or even in experimentally-induced cardiomyopathy in animals (Zhang et al., 1996).

Other authors, on the other hand, have seen no sign of apoptosis in cardiomyocytes despite attempts to induce it by the administration of strongly cardiomyotoxic drugs in animals (Narula et al., 1996).

In our study, the indirect markers of apoptosis (Bcl-2 and P-53) were negative, while with "*in situ*" hybridisation most cells (cardiomyocytes, stromal and endothelial cells) showed strong but patchy nuclear staining, although some nuclei remained unstained (Fig. 2d). This observation has not been described previously, although some studies report between 18.5% and 35.5% of cells being stained by apoptosis markers (Colucci, 1996; Kavantzas et al., 2000).

These findings, together with the systematic appearance of signs of autolysis, both in the cardiomyopathies and normal hearts, led us to think that there might be some sort of cross reaction between the broken DNA terminal fractions and the autolysis processes. DNA breakdown also occurs in autolysis in a somewhat anarchic and random way. This conclusion is also supported by other authors (Granville et al., 1988).

The strong fluorescence observed with acridin orange, which has been described as a histochemical marker of hypoxic lesions (Lachica et al., 1988), might suggest that this mechanism is involved in most of the cases studied. However, we are not of this opinion and consider this technique of little use when there are signs of autolysis.

In conclusion, we think that immunohistochemical determination of muscle actin and CD-31 is a good method for discriminating between cardiomyocyte lesions, while "in situ" hybridisation can provide important information on the pathogeny and identification of the different types of cardiomyopathy, (bearing in mind, however, that autolysis processes may seriously affect any results and invalidate the usefulness of some techniques). In addition, we must look more carefully at the processes of DNA breakdown during autolysis and apoptosis to determine the validity of results obtained using necropsy material, and, above all, the time elapsing between death and the fixing and processing of samples must be as short as possible.

Finally, all the above may be used to define the three types of cardiomyopathy and to differentiate them from normal hearts. Furthermore, the findings can be used "in vivo" thanks to modern imaging and endomyocardial biopsy techniques.

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