PHARMACOLOGY AND CELL METABOLISM
Evaluation of Myocyte Proliferation in Alcoholic Cardiomyopathy: Telomerase Enzyme Activity (TERT) Compared with Ki-67 Expression

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Abstract — Aims: Although the human heart was classically considered a terminal organ, recent studies have reported a myocyte proliferation response versus some aggressions. Excessive ethanol consumption induces development of cardiomyopathy (CMP) through myocyte apoptosis. We evaluated myocyte proliferation response in the heart of chronic alcoholic donors with telomerase activity (telomerase reverse transcriptase (TERT)) compared with Ki-67 nuclear expression. Methods: Heart samples were prospectively obtained from organ donors on life support. We included donors with (1) high lifetime alcohol consumption (n=15), (2) longstanding hypertension (n=14), (3) other causes of CMP (valve, coronary or idiopathic) (n=8) and (4) previously healthy donors (n=6). Groups 2 and 3 were subdivided according to the presence of CMP. Evaluation comprised parameters of ethanol consumption, left ventricular function by chest X-ray and 2D echocardiography, and histology and immunohistochemical studies. Myocyte proliferation was evaluated using an assay for Ki-67 expression and measuring telomerase gene activity by real-time PCR. Results: Forty-three donors were included in the study, 35 having CMP. Nuclear Ki-67 activity was low in healthy controls and significantly increased in the other groups, mainly in those with CMP. Alcoholics with CMP had a non-significantly lower proliferation response than the other CMP groups. No proliferation activity was detected with TERT in any case. Conclusion: Heart Ki-67 proliferation activity increases in organ donors with CMP, independently of its origin. Alcoholics presented non-significant lower myocyte proliferation capacity compared with the other groups of CMP. TERT activity was not a useful marker of proliferation in this model. Ki-67 is a better procedure to evaluate proliferation than TERT expression in alcohol-induced heart damage.

INTRODUCTION

Recent scientific knowledge has shown that the human heart is not a terminal organ (Anversa and Kajstura, 1998; Nadal-Ginard et al., 2003a; Quaini et al., 2004; Buja and Vela, 2008). The heart exhibits plastic response versus diverse physiologic or pathologic stimuli (Gerder, 2002; Kajstura et al., 2004; Hill and Olson, 2008). Cardiac myocyte renewal has been observed in different animal species (Soonpaa and Field, 1998; Jopling et al., 2010), and also in human myocardial areas surrounding necrotic tissue (Beltrami et al., 2001; Urbanek et al., 2005). Therefore, a relationship between myocyte necrosis and subsequent proliferative response has been established (Anversa and Nadal-Ginard, 2002; Beltrami et al., 2003; Anversa et al., 2004).

In the last decades, a dose-dependent relationship between excessive alcohol consumption and diffuse myocardial damage has been corroborated (Urbano-Márquez et al., 1989; Molina et al., 2002; Nicolás et al., 2002; Piano, 2002; Urbano-Márquez and Fernández-Solà, 2004). Mechanisms underlying this effect are diverse, with direct induction of apoptosis and myocyte cell death by ethanol (Jánkalá et al., 2002; Molina et al., 2002; Fernández-Solà et al., 2006). However, not all excessive alcohol consumers develop significant myocardial damage, with some discordance between the high prevalence of alcohol consumption and the relative low incidence of alcoholic cardiomyopathy (CMP) (Urbano-Márquez et al., 1995; Fernández-Solà et al., 2002). It has been suggested that some mechanisms may compensate the degree of alcohol-induced myocyte damage (Urbano-Márquez and Fernandez-Solà, 2004).

We previously analyzed factors influencing death and proliferation of cardiac myocytes and observed a higher apoptosis index and myostatin activity in alcoholic compared with healthy donors (Fernández-Solà et al., 2006). Alcoholics with CMP showed higher apoptotic and proliferative Ki-67 activity compared with their partners without CMP. Chronic ethanol consumption increases myostatin activity, a factor that favors the loss of cardiac myocytes and impairs their proliferation (Fernández-Solà et al., 2008).

Several morphological, immunohistochemical and molecular markers have been used to evaluate myocyte proliferation (Leri et al., 2001). Ki-67 is a nuclear antigen expressed only when cells are in the replication cycle. It is commonly used as a marker of cell proliferation either in tumoral (Magdelénat, 1992) or non-tumoral tissues, including cardiac myocytes (Kajstura et al., 1998; Anversa and Nadal-Ginard, 2002; Nadal-Ginard et al., 2003b). Telomerase is a DNA polymerase constituted by two RNA subunits (telomerase reverse transcriptase (TERT)) that maintains the telomere length stable and protects chromosomes from degradation and molecular recombination. When telomerase activity decreases, telomere length shortens to a critical size, activates cell apoptosis and stimulates TERT expression as a compensatory mechanism. This process physiologically appears along senescence (Anversa et al., 2005), being implicated in ischemia (Serrano and Andres, 2004), heart failure (Leri et al., 2003), ventricular hypertrophy (Urbanek et al., 2003), infertility and sarcopenia (Blasco, 2005). In contrast, telomerase exhibits a high activity in progenitor stem cells (Dijoosubroto et al., 2003) and in tumoral cells (Blasco, 2005).
All these facts led us to consider the possible increase in telomerase activity and proliferation response in the setting of alcoholic CMP. This myocyte proliferation would partially compensate the ethanol-induced myocyte loss (Nadal-Ginard et al., 2003a; Hotchkiss et al., 2009). Differences in the myocyte proliferation response may explain discordances in the relation between ethanol intake and the degree of cardiac lesion.

In the present study, we evaluated proliferation mechanisms similar to those described in ischemic CMP in the heart of chronic alcoholics. In a prospective case–control study using heart samples from human donors, we assessed the effect of alcohol consumption on myocyte proliferation, evaluated by cardiac myocyte telomerase activity and the expression of Ki-67 nuclear antigen.

MATERIALS AND METHODS

Patient selection

Over a 2-year period (September 2006 to November 2008), we consecutively studied hearts from subjects who had brain death either of traumatic or cerebrovascular origin and had been considered suitable as organ donors by the transplant team of the Hospital Clinic of Barcelona.

Of 127 cadaveric donors younger than 70 years of age, 43 hearts were not suitable for transplantation. Of these latter organs, we selected 14 with chronic hypertension, 15 cases with a history of ethanol intake (≥ 60 g/day, >10 years) and 10 hearts from healthy people who were not eligible for implantation because of a lack of matched receptor or size inadequacy. Additionally, eight specimens from patients with heart disease (three coronary diseases, three with idiopathic dilated CMP and two with valve-heart disease) were selected. Alcoholic and hypertensive donors were subdivided in two groups depending on the existence or not of CMP.

Exclusion criteria

According to the general protocol of organ donation, subjects with drug misuse, transmissible infections (HIV, hepatitis B or C), sepsis, disseminated neoplasms or metabolic diseases (diabetes or other endocrine diseases), or other diffuse structural diseases were excluded as were those with coexistent hypertension and alcohol consumption.

All patients were white Caucasians of Spanish descent, who lived with their families in or around Barcelona and none was indigent. The study protocol was approved by the Ethics Committee of the Hospital Clinic (reference # JFS/17/04/2008) and included informed consent from the families of the donors concerning the use of myocardium tissue for this research protocol study. One-third of these subjects had been evaluated in previous studies on cardiac apoptosis (Fernández-Solà et al., 2006) and myostatin activity (Fernández-Solà et al., 2008).

All cases had been admitted to the Intensive Care Unit, and ventilatory and hemodynamic parameters were appropriately maintained at normal values throughout hospitalization: $P_{O_2} >60$ mmHg, systolic blood pressure $>100$ mmHg and arterial pH within the normal range. None of the patients required in-hospital cardiopulmonary resuscitation maneuvers.

Clinical and laboratory evaluation

Detailed history of ethanol intake was retrospectively obtained by consultation with family members using a structured questionnaire (‘time-line follow-back method’) (Sobel et al., 1979) as previously reported (Urbano-Márquez et al., 1989; Nicolas et al., 2002). Duration of ethanol intake was calculated in each group as the total cumulated period of alcohol consumption in years, either recent or previous. Body mass index (BMI, kg/m²) was determined as the actual body weight relative to the square of the body height. Patients were considered to have caloric malnutrition if the BMI was <17 kg/m².

Cardiac studies

Past and present signs and symptoms of heart failure were evaluated in consultation with medical records and family members of the donors, and the New York Heart Association (NYHA) functional class was determined according to the Goldman activity scale (Goldman et al., 1981). Chest X-rays with measurement of cardiothoracic index and conventional electrocardiography were performed in all cases. Moreover, a bi-dimensional echocardiography was performed (Hewlet Packard Sonos 2500, USA) in 14 patients compared with none of the controls with a cardiotoracic index ≥0.48. End-diastolic and end-systolic diameters, the shortening fraction, the left ventricular (LV) mass and ejection fraction were measured according to the standards of the American Society of Echocardiography (Gottdiener et al., 2004). CMP was defined in the presence of an LV ejection fraction <50% and LV dilatation in addition to the presence of structural histological heart damage. We observed a good correlation between the cardiotoracic index and the left-ventricle end-diastolic diameter ($r=0.68$; $P<0.01$). The personnel who performed and evaluated these tests had no knowledge of the alcoholic history of the patients.

Myocardium histological studies

A distal 3 cm sample of the left-ventricle apex was surgically excised (total weight of 4–5 g) at the time the donor was under cold perfusion. The specimen was cut into fragments, and one of these was processed for further histological analysis. The remaining fragments were immediately frozen (~80°C) under liquid nitrogen until telomerase and Ki-67 studies were performed. Specimens were stained with hematoxilin-eosin and toluidine-blue in semithin sections for histological studies. Two independent observers (J.F.-S. and A.U.-M.) evaluated the degree of myocardial cell and nuclear damage. We observed a good correlation between the cardiotoracic index and the left-ventricle end-diastolic diameter ($r=0.68$; $P<0.01$). The personnel who performed and evaluated these tests had no knowledge of the alcoholic history of the patients.

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Myocardium proliferation studies

**TERT protocol.**

(a) RNA extraction: The RNAqueous-4PCR kit (Ambion, Austin, TX, USA) was used to extract RNA from small tissue samples (0.5–75 mg). From each cryopreserved sample, a fragment of 5 mg was obtained and RNA was isolated following the manufacturer’s instructions. Finally, in order to eliminate trace amounts of DNA, a DNase inactivation treatment was performed. RNA samples obtained were preserved at −80°C. RNA quantity in each sample should be around 1–10 µg per mg of tissue. Since we considered a minimum of 50 ng/µl adequate, 10 of 53 samples were rejected, remaining 43 valid samples to study. Adequate RNA integrity and concentration were corroborated using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

(b) Retrotranscription from RNA to cDNA: The High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA) was used to obtain cDNA from each sample using 1 µg of RNA according to the manufacturers’ instructions. Just a minor modification was performed with the addition of RNAse Inhibitor (Applied Biosystems, Foster City, CA, USA) at a final concentration of 0.4 U/µl. Samples were incubated at 25°C for 10 min and 37°C for 120 min.

(c) TERT expression quantification by real-time PCR: The expression level of TERT was measured by real-time quantitative PCR using the TaqMan technology on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers and FAM dye-MGB probe were developed by the manufacturer as a TaqMan Gene Expression Assay bridging exons 11 and 12 of the TERT mRNA sequence (NM_198253.2). As positive technical control in RT-PCR, TATA-binding protein (TBP) expression was used (Foldager et al., 2009; Valente et al., 2009). TBP expression was monitored as the endogenous control gene in a duplicate of the same samples, being the assay available from the manufacturer as a VIC dye-MGB probe.

PCR reactions were prepared using 5 µl of cDNA in a final volume of 25 µl with final concentrations of 1× TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) with uracil DNA-glycosylase (AmpErase UNG), and the mentioned specific TERT assay. Amplification conditions comprised an initial UNG incubation at 50°C for 2 min, AmpliTaq Gold® DNA Polymerase activation at 95°C for 10 min, 50 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

Each measurement in a sample was performed in triplicate for both TERT and TBP, and the threshold cycle (Ct), the fractional number at which the amount of amplified target reached a fixed threshold, was determined. The standard deviation in sample triplicates was always <0.2. Relative amounts of both genes were also normalized to a pool of control colonic tissue RNAs acting as calibrator to allow comparison across all tested samples. The comparative Ct method (Livak and Schmittgen, 2001), also known as the $2^{-\Delta\Delta C_t}$ method, was calculated from

$$\Delta C_t = C_{t,\text{sample}} - C_{t,\text{calibrator}}$$

where $\Delta C_{t,\text{sample}}$ was the TERT Ct value for any sample normalized to TBP and $\Delta C_{t,\text{calibrator}}$ was the TERT Ct value for the calibrator also normalized to TBP. For the $\Delta\Delta C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal, being previously confirmed by checking how $C_t$ varied with template dilution for each tested genes. The investigator performing the real-time quantitative PCR experiments was blinded with respect to the clinical characteristics of the patients.

**Immunohistochemical Ki-67 studies**

Ki-67 myocardium activity was evaluated by immunohistochemical assay on frozen myocardium tissue. Myocardium samples were fixed by ketone at −4°C for 10 min, followed by PBS washing and serum blockade for 30 min. We used the commercial Ki 67-hq-ap Monoclonal Mouse Anti-Human kit (TechMate/chemMate, Dako Cytomation, Carpenteria, CA, USA). Immunohistochemical lecture was performed by means of a phosphatase staining. Primary antibody incubation was performed with the nuclear Ki-67 marker in a wet chamber, followed by washing and incubation with the secondary antibody linked to alkaline phosphatase for 30 min in a wet chamber. Finally, the sample was submitted to gentle washing, revealed with substrate and counterstaining with hematoxilin. Evaluation of Ki-67 activity was performed by means of a semiquantitative study, evaluating the percentage of positive cells with respect to total evaluated myocardial cells. In each case, a minimum of 3000 myocytes were evaluated. We compared results from cases (alcoholics) with healthy donors and also pathological controls either with hypertension or other causes of CMP. All these procedures were supervised by an experienced pathologist.

**Statistical analysis**

Standard statistical methods with the SPSS Statistical Analysis System V-16.0 were used. Differences between groups were analyzed using the ANOVA, Fisher’s exact test and the two-tailed Student’s t-test. Correlation studies were obtained by Pearson’s correlation coefficient. Since the variables followed a normal distribution, data are expressed as mean ± SD, and a significance level of $P<0.05$ was used. Statistical software was Stata Corp. 2003. (Statistical Software: Release 8.1. College Station, TX: Stata Corporation, USA).

**RESULTS**

**Clinical data**

After the selection period, we finally included 43 heart donors in the study. Fourteen heart donors had chronic hypertension, and 10 had CMP. Fifteen donors had a history of excessive alcohol consumption (>60 g/day, along >10 years), with seven having CMP. Eight donors presented other causes of CMP (three valve disease, three coronary and two of idiopathic origin). Finally, the other six donors did not report previous ethanol consumption, and did not have arterial hypertension or other causes of cardiovascular disease (healthy controls).
The main clinical and epidemiological characteristics of cases and controls are reported in Table 1. The mean age of the donors was 55.8 ± 16.2 years, 32 cases (74.4%) being male and 11 (25.6%) female. There was a similar age and male/female ratio in the different groups of donors, with male predominance in all the groups. The BMI was similar in all groups, and no subjects fulfilled the criteria of caloric malnutrition. The cardiothoracic index evaluated on chest X-ray was normal in the control group (0.48 ± 0.01) and enlarged in the other groups of donors, with donors with other causes of CMP being those with higher values (0.50 ± 0.06). Alcoholics and donors with hypertension showed comparable CTI (0.55 ± 0.06 and 0.56 ± 0.04, respectively).

Cardiac echo sonography data in the control group showed normal left ventricle ejection fraction (LVEF) (60.2 ± 5.1). Hypertensive donors showed a slight decrease in the LVEF (55.0 ± 14.4), that was clearly significantly decreased in the groups of alcoholics (33.1 ± 20.7) and donors with other causes of CMP (34.6 ± 19.0), \( P < 0.01 \) both. Evaluating NYHA functional class, all controls showed NYHA class I. NYHA II functional class predominated in the group of hypertensive donors, whereas NYHA I functional class predominated in alcoholics and other causes of CMP.

In the alcoholic donor group, the mean daily alcohol consumption was 104.7 ± 48.4 g/day, being up to 60 g/day in all cases. The mean total lifetime dose of ethanol was 12.2 ± 5.4 Kg ethanol/kg body weight. In all cases, the period of alcohol consumption lasted >10 years, with a mean of 24.5 ± 6.4 years. Ethanol consumption in the other groups was significantly lower (\( P < 0.01 \)).

Ischemic or hemorrhagic stroke was the mean cause of death in the groups of hypertensive and alcoholic donors. In the other groups of donors, death was of diverse origin.

No signs of myocardial infarction or other focal lesions were detected in the macroscopic examination of heart donors. In the histological study of heart samples, we did not observe fibrosis or other structural lesions in control donors, and the extent of myocardial fibrosis, hypertrophy and necrosis was slight to moderate and of similar degree in the different groups of donors affected of CMP.

### Table 1. Epidemiologic, clinical and heart function data of the different groups of donors

<table>
<thead>
<tr>
<th></th>
<th>Healthy control donors (n = 6)</th>
<th>Donors with other causes of cardiomyopathy (n = 8)</th>
<th>Hypertensive donors (n = 14)</th>
<th>Alcoholic donors (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female ratio)</td>
<td>4:2</td>
<td>6:2</td>
<td>10:4</td>
<td>12:3</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.8 ± 16.2</td>
<td>57.9 ± 13.3</td>
<td>61.3 ± 11.2</td>
<td>55.5 ± 11.2</td>
</tr>
<tr>
<td>Body mass index (kg/cm²)</td>
<td>25.9 ± 3.60</td>
<td>26.9 ± 3.05</td>
<td>27.4 ± 3.33</td>
<td>27.4 ± 4.99</td>
</tr>
<tr>
<td>Cardiothoracic index</td>
<td>0.48 ± 0.01</td>
<td>0.58 ± 0.06</td>
<td>0.56 ± 0.04</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>60.2 ± 5.1</td>
<td>34.6 ± 19.0**</td>
<td>55.0 ± 14.4</td>
<td>33.1 ± 20.7**</td>
</tr>
<tr>
<td>NYHA function class (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>III and IV</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Daily ethanol consumption (g/day)</td>
<td>0</td>
<td>7.5 ± 21.2</td>
<td>5.3 ± 12.9</td>
<td>104.7 ± 4.4*</td>
</tr>
<tr>
<td>Total lifetime dose of alcohol (kg EtOH/kg body weight)</td>
<td>0</td>
<td>1.22 ± 3.46</td>
<td>0.82 ± 1.30</td>
<td>12.2 ± 5.40*</td>
</tr>
<tr>
<td>Period of ethanol consumption (years)</td>
<td>0</td>
<td>2.50 ± 7.07</td>
<td>3.22 ± 1.23</td>
<td>24.5 ± 6.41*</td>
</tr>
<tr>
<td>Cause of death (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrovascular</td>
<td>3</td>
<td>3</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Cranial trauma</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD.

\* \( P<0.01 \) compared with the other groups.

\** \( P<0.01 \) compared with controls.

### Table 2. Cardiac myocyte Ki-67 expression and TERT activity in different subgroups of donors

<table>
<thead>
<tr>
<th></th>
<th>Healthy control donors</th>
<th>Hypertensive donors without CMP</th>
<th>Hypertensive donors with CMP</th>
<th>Alcoholic donors without CMP</th>
<th>Alcoholic donors with CMP</th>
<th>Donors with other causes of CMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 (% of positive cells)</td>
<td>3.92 ± 0.99*</td>
<td>9.90 ± 5.80</td>
<td>13.5 ± 3.56**</td>
<td>9.92 ± 5.36</td>
<td>12.8 ± 4.99***</td>
<td>15.9 ± 2.21</td>
</tr>
<tr>
<td>Increasing of ki-67 expression with respect to healthy controls</td>
<td>x2.5</td>
<td>x3.4</td>
<td>x3.4</td>
<td>x2.5</td>
<td>x3.2</td>
<td>x4.0</td>
</tr>
<tr>
<td>TERT activity (% of positive cells)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD.

CMP, cardiomyopathy.

\* \( P<0.01 \) compared with the other groups with CMP.

\** \( P<0.01 \) compared with the other groups with CMP.

\*** \( P<0.01 \) compared with the other groups with CMP.

Myocardial proliferation studies

TERT expression

All 43 heart donor samples obtained were submitted to RNA extraction and subsequent monitorization of the TERT gene by real-time PCR, using the TBP gene (TATA-binding protein) as endogenous control.
protein), as endogenous control. All these procedures were carefully controlled by an experienced technician. Purity and quality of samples were corroborated with the previously described procedures. The quantity of obtained RNA in each case was over 50 ng/μl, which is the minimum quantity considered to continue the study. Similarly, purity of samples was adequate in all cases (RNA integrity number (RIN) >8).

Real-time PCR amplifications in triplicate showed clear TBP gene expression in all cases. Notably, none of the samples showed significant TERT expression. The fact that the endogenous TBP gene was expressed in all samples can be considered a guarantee of good technical procedure, thus confirming that the lack of detection of TERT expression is real, not a technical error. In addition to the heart samples, as positive technical control, we also used human colonic cancer tissue, being TERT expression clearly increased.

Table 2 shows the results of Ki-67 nuclear immunohistochemical expression. Healthy controls were the group of donors with lower nuclear Ki-67 expression (3.92 ± 0.99% of positive myocytes). The group of alcoholics and hypertensive donors showed a significant increase in Ki-67 expression, mainly in the subgroup of donors with CMP (Fig. 1A). Similarly, the group of other causes of CMP showed a significant increase in Ki-67 expression compared with controls, in a similar range as the subgroups of alcohol and hypertensive donors with CMP. Table 2 also reflects the degree of increase in Ki-67 expression with respect to controls in each group of donors. Thus, we observe a 2.5-fold increase in the group of hypertensive and alcoholic donors without CMP. The presence of CMP, independently of the cause, was a clear factor of increase in Ki-67 nuclear expression. Thus, alcoholics with CMP showed a 3.2-fold increase, hypertensive donors with CMP a 3.4-fold increase and other causes of CMP a 4-fold increase with respect to healthy controls (Table 2).

Healthy control donors showed a significantly lower Ki-67 expression compared with all the other groups of donors with CMP, either of alcoholic, hypertensive or of other causes (P < 0.01 in all cases; Fig. 1B). On comparing the different groups of donors with CMP, alcoholics were those with the lowest Ki-67 nuclear expression, followed by hypertensive CMP and other causes of CMP. However, differences between groups of donors with CMP did not achieve significance (P > 0.800 in all cases).

Fig. 1. Cardiac Ki-67 expression in the different groups of heart donors. (A) Expression of Ki-67 antigen in healthy control donors compared with donors with cardiomyopathy of alcoholic, hypertensive or other causes. (B) Ki-67 expression in healthy controls compared with all donors with cardiomyopathy.

Fig. 2. Ki-67 heart immunohistochemical assay. (A) Control donor with low Ki-67 nuclear activity (magnification ×250). (B) Alcoholic donor with cardiomyopathy. Increased Ki-67 activity is evident in some nuclei (arrows) (magnification ×250).
In the groups of hypertensive donors, those with CMP showed a higher but non-significant increment in Ki-67 nuclear expression compared with those without CMP (13.51 ± 3.56 versus 9.90 ± 5.80%, respectively, \( P = 0.805 \)). Figure 2 compares the low Ki-67 nuclear activity in a control donor (Fig. 2A) with the increased Ki-67 activity in an alcoholic donor with CMP (Fig. 2B). Similarly, in the groups of alcoholics, those with CMP showed a non-significant increase in Ki-67 nuclear expression (12.81 ± 4.99 versus 9.92 ± 5.36%, respectively, \( P = 0.859 \)).

According to the present results, the absence of significant telomerase gene expression in these samples does not allow a possible correlation with the Ki-67 nuclear antigen expression, the other marker of cardiac myocyte proliferation, to be established.

**DISCUSSION**

The present study demonstrates the activation of the Ki-67 cell proliferation marker in cardiac myocytes from donors with alcoholic and other causes of CMP such as those of hypertensive, valve, coronary or idiopathic origin. However, TERT expression was not useful to detect this proliferation activity in this specific biological model.

Previous studies have clearly corroborated the role of alcohol in inducing apoptosis and cell-death mechanisms in cardiac myocytes (Molina et al., 2002; Fernández-Solà et al., 2006). Although a clear dose-dependent effect of ethanol-induced left ventricular dysfunction has been described, not all alcohol misusers develop dilated CMP. Thus, subjects with similar quantity of cumulated lifetime alcohol consumption may develop diverse degrees of ventricular dysfunction. Therefore, in addition to the toxic effect of ethanol causing apoptosis, necrosis and cell loss, other mechanisms may influence the development of cardiac functional and structural damage (Urbano-Márquez et al., 1995; Fernández-Solà et al., 2002, 2008).

Recent evidence of cardiac myocyte proliferation led us to consider the possibility that some repair mechanism may modulate the degree of ventricular damage induced by ethanol. The concept of cardiac homeostasis and plasticity would consider an equilibrium between both damaging and repair mechanisms, sometimes acting synchronously (Nadal-Ginard et al., 2003a; Buja and Vela, 2008). In fact, cell-death mechanisms probably activate the proliferation response themselves (Hotchkiss et al., 2009).

Previous studies have shown the influence of ethanol decreasing myocyte proliferation after heart damage, as evaluated by myocyte Ki-67 immunohistochemical activity. This effect may act through diverse mechanism, implicating up-regulation of myocyte myostatin activity (Fernández-Solà et al., 2008). Other studies in animal and human models have evidenced the relevance of TERT expression and telomere function in maintaining the cell replication potential (Djiojsobrotu et al., 2003; Anversa et al., 2005). Human telomere length shortens in diverse situations such as endothelial cells from atherosclerotic plates, in hypertrophic myocardium tissue, in end-stage heart failure and in leukocytes from subjects with cerebrovascular disease, hypertension, diabetes or acute myocardium infarction (Fuster and Andrés, 2006). Other *in vitro* studies with dog cardiomyocytes showed a relationship between TERT activity, Ki-67 expression and cardiac dysfunction (Leri et al., 2001).

In the present study, we hypothesized that TERT expression could be a useful approach to evaluate the degree of heart myocyte proliferation. Although TERT has been used to evaluate cell proliferation in diverse experimental and clinical situations (Leri et al., 2000, 2003; Oh et al., 2001; Djiojsobrotu et al., 2003; Urbanek et al., 2005), no specific studies on TERT expression in alcoholic CMP have previously been performed.

Studies of TERT and other proliferation markers such as Ki-67 expression performed in ischemic CMP, hypertension and atherosclerosis showed paradoxical results. In fact, it is debated whether TERT activity and telomere shortening are independent factors or only a consequence of cardiovascular tissue damage (Fuster and Andres, 2006). Some of these studies evaluate TERT expression in focal areas of a heart lesion, for instance in the surrounding areas of myocardial infarction (Beltrami et al., 2001, 2003; Serrano and Andrés, 2004), but not in situations of diffuse heart damage as alcoholic CMP. This discrepancy in results led Anversa et al. (2005) to suggest the use of more than one proliferation marker in myocyte studies.

In cultured human embryo cardiomyocytes, TERT-independent mechanisms such as the expression of p16 protein and beta-galactosidase activity were able to accelerate or decrease the process of cell senescence and death (Ball and Levine, 2005). In healthy adult heart tissue, TERT expression is not present or is slightly expressed because of its low proliferation capacity. The cell renewal myocyte index in the human heart has not been clearly established, and some factors such as individual age and the presence of heart damage may influence its presentation (Hosoda et al., 2010). In an interesting study, using DNA-integrated C14, Bergmann et al. (2009) calculated a renewal percentage of 0.2–2% per year in healthy individuals. This percentage clearly diminishes along the senescence process. Thus, a 25-year-old subject has 1%/year renewal percentage and a 75-year-old subject 0.45%. Buja and Vela (2008) approximated a regenerative percentage of healthy cardiac myocytes of 0.0014% (14 myocytes per 1 million). In end-stage heart failure, this index is 0.013–0.015%, and is 0.03% in neighboring necrotic areas after myocardial infarction.

Thus, several studies have clearly corroborated the existence of some capacity of the adult human myocardium to establish proliferation response. However, the degree of this myocyte proliferation response may be modified by different factors such as age, toxic habits, the presence of cardiac disease such as hypertension or coronary disease (Beltrami et al., 2003; Anversa et al., 2004, 2005).

In the present study, we expected to find increased myocyte proliferation either shown by Ki-67 expression or TERT activity in donors, mainly in those affected by CMP. We found significant increase in Ki-67 activity in donors with CMP of diverse origin, with a relatively lower increase in activity in alcoholics compared with other causes of CMP. This result is in concordance to that described in a previous study (Fernández-Solà et al., 2008). However, we were not able to detect TERT expression in any of the 43 heart samples studied. We detected a clear activity of the control TBP gene and also in colon cancer tissue, a fact that validates the technical procedure. Notably, real-time PCR
constitutes a more sensitive technology than the telomeric repeat amplification protocol, used in previous similar studies (Oh et al., 2001). The quantity, purity and adequacy of RNA samples were clearly corroborated and validated. In relation to this subject, a previous study of de Kok et al. (2000) evaluating TERT expression by RT-PCR in human tissues detected increased TERT expression in diverse tumoral tissues, but low activity in healthy tissues (lung, esophagus and colon), and null expression in pancreas and bladder tissues. Since myocardium has lower proliferation activity than the healthy tissues examined, null TERT expression in heart tissue was also expected.

According to the results obtained, we can conclude that TERT expression evaluated by real-time PCR in human heart samples is not a good marker of myocyte proliferation in this biological model, since no activity was detected in any of the samples that were otherwise positive for Ki-67 proliferation activity. Diverse reasons may explain these results. One is the possibility that proliferation is a limited tissue response that may be exhausted after a period of persistence of lesion. In the case of ethanol cardiac damaging effect, diverse mechanisms regulate the interaction between induction of apoptosis and cell death and the reparative proliferation response (Molina et al., 2002). Some of these mechanisms may even be counterpoised. Finally, we have shown evidence that ethanol decreases the compensatory proliferative response that may be inhibited in these cases. Proliferative response of myocardium also depends on the type of lesion. Thus, in human cardiac myocytes, Kubo et al. (2008) found an increase in cardiac stem cells in the more affected hearts. However, in the situation of heart ischemia, inflammation or oxidative stress, this endogenous myocardial renewal was clearly limited.

Since one of the limitations in the present study is the relative small number of samples because of the difficulty in obtaining human heart tissue, it is possible that an increase in the sample number might show more evident and significant results. However, the homogeneity of the samples and the clear significance of results allow us to validate the obtained data. Although the macroscopic examination of the hearts from donors do not exhibit focal lesions, this study is based on samples obtained from the left ventricle apex and, therefore, we cannot exclude structural involvement in other sites of the heart.

It is of note that the model of alcoholic CMP in donors is different from that performed in clinical series of subjects, in which the degree of alcohol consumption and the clinical relevance of the CMP was clearly higher (Urbano-Márquez et al., 1989; 1995; Fernández-Solá et al., 1997, 2002; Nicolás et al., 2002). Another difference may be that our study analyzed causes of diffuse heart damage either of alcoholic, hypertensive or other origin. Most previous studies have been performed in the model of ischemic heart damage where localization of damage is more focal and intense.

Nuclear Ki-67 antigen only expressed when cells enter in replication cycle. However, Ki-67 activity detection may show some pitfalls, since not all cells expressing Ki-67 activity are dividing in proliferation or regenerative processes (with karyorrhexis and cytokinesis). Some Ki-67 positive cells may just be bi-nucleated cells with karyorrhexis without cytokinesis. In fact, all mammalian cells may be mono or bi-nucleated in different proportion (Nadal-Ginard et al., 2003b). For instance, 25–57% of human myocytes 3-week from birth are bi-nucleated. In the case of rats, this proportion is 85–90% (Ahuja et al., 2007). In addition, DNA replication may also be detected in heart hypertrophy or after heart injury or blunted cell division leading to apoptosis (Bicknell et al., 2007).

In the present study, we also consider the necessity to evaluate cardiac regeneration with more than one marker. Since confocal microscopy was not available, we used TERT activity in addition to Ki-67 as a second marker of regeneration. This combined method was previously used by other authors with positive results. However, in the present study, all heart samples show the absence of significant TERT activity.

Because of these negative TERT results, Ki-67 activity was the only marker of heart regenerative activity in the present study. This is a clear limitation, since we cannot clarify whether all Ki-67 positivity is due to cardiac regeneration or is secondary to cardiac hypertrophy, bi-nucleation of cells or blunted cell division. However, these limitations are common and homogeneously affected to all studied groups. Finally, Ki-67 and TERT activities may be measured by different technical procedures that may provide somewhat different results.

Considering the results from the present as well as other previous studies in alcoholic CMP, we suggest the necessity to design and develop further approaches to explain the complex mechanisms that regulate cell death and proliferation response in alcoholics with dilated CMP. Other regulatory mechanisms such as myostatin (McNally, 2004; Yang et al., 2005; Wagner et al., 2005) or IGF-1 activities (Ahuja et al., 2007; Ge et al., 2010) probably contribute to maintain cardiac homeostasis and plasticity in the heart of alcohol misusers. Better knowledge of myocyte cell cycle control may allow the use of stem cells therapy that could allow myocardial renewal (Regula et al., 2004; Taylor, 2004; Von Harsdorf et al., 2004).

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Myocyte proliferation in alcoholic cardiomyopathy


