The Effects of Enalapril on p53 Expression in Left Ventricular Cardiomyocytes after Aortic Stenosis

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Abstract. ACE-inhibitors prevent the development of left ventricular hypertrophy (LVH). The tumor suppressor gene p53 up-regulates the cellular renin-angiotensin system, resulting in ANG II synthesis, which activates p53 creating a positive feedback loop. One hundred and fourteen rabbits were separated into groups A (control), B (sham-operated), C and D. In groups C and D, an aortic stenosis was performed, and in group D the animals were treated with enalapril. For p53 determination, LV specimens were examined by Western blot analysis and an immunohistochemical study was performed, except for samples from group D. In conclusion, LVH was significantly induced at 7 and 28 days after aortic stenosis with no difference between the two periods, while enalapril prevented hypertrophy in these two groups. p53 was transcriptionally activated and immunoreactively present after acute pressure overload as well as in the sham-operated group. Enalapril decreased the p53 expression at 180 min, 7 and 28 days following aortic stenosis.

The myocardium is composed of various cell types of which the cardiomyocytes comprise only one-third (1). Ventricular remodeling in response to a pressure overload classically implies the hypertrophy of muscle cells and the proliferation of non-muscle cells (2, 3). During the early phase of pressure overload, several genes, including hsp70 and the oncogenes c-myc, c-fos and c-jun, have been shown to be transiently expressed and are thought to participate in the cardiac adaptive response (4). Some of the changes in contractility can be attributed to alterations in the expression of such contractile proteins as actin and myosin (5, 6). Furthermore, there is emerging evidence from a number of studies for the existence of an independent local (tissue) renin-angiotensin system in the heart (7, 8). Increased-angiotensin converting enzyme activity and mRNA expression were observed in rat hearts with pressure overload (9) or volume-overload-induced hypertrophy (10) and this tissue-specific induction of the angiotensin-converting enzyme gene may contribute to the harmful effects of the renin-angiotensin system in cardiac diseases.

Strong evidence that the renin-angiotensin system is involved in the regulation of cardiac growth comes from several studies using inhibitors of the system, which not only induce the regression but also prevent the development of cardiac hypertrophy (11-16). p53 induces myocyte apoptosis via the activation of the renin-angiotensin system, while p53 inhibition prevents renin-angiotensin system activation and stretch-mediated myocytes apoptosis (17, 18). Ikeda et al. (19) demonstrated that the occurrence of apoptotic cardiomyocytes in right-sided acute pressure overload, accompanied by enhanced expression of apoptosis, induces p53 and Bax. Enhanced expression of Bax with right ventricular pressure overload suggests that the myocardium exposed to such acute pressure overload becomes pro-apoptotic.

The purpose of the present study was to examine and estimate the degree of left ventricular hypertrophy (LVH) and the time course of changes in the expression of the p53 gene following experimentally-induced aortic stenosis in rabbits, with and without the administration of an angiotensin-converting enzyme inhibitor (enalapril).

Materials and Methods

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divided into six subgroups according to the time of follow-up (15, 45, 90, 180 min, 7 and 28 days) and underwent a sham operation. The animals in groups C (n=36) and D (n=36) were similarly subdivided into six subgroups employing the same criteria. All animals in groups C and D had experimentally-induced aortic stenosis, while the animals of group D were treated with a converting enzyme inhibitor (enalapril).

The animals were housed in single metal cages and had access to tap water and standard balanced rabbit chow ad libitum. The room temperature ranged between 18°C and 22°C, relative humidity between 55% and 65% and the light/dark cycle was 6 a.m./6 p.m. The care and treatment of the experimental animals complied with the guidelines of the Presidential Decree 160/1991 issued after the 609/86 directive of the European Union and conformed with the Guide for the Care and Use of Laboratory Animals of the United States National Institute of Health.

Enalapril (trade mark Angioten, product and donation of Gerolimatos Company, Greece) was dissolved in 3 ml water and given to all animals in group D orally by catheter in a single dose. The administration of enalapril started 4 days before surgery at a single dose of 10 mg/kg body weight (BW)/daily and in the 7- and 28-day subgroups was continued as a half dose (5 mg/kg BW/daily) during the whole experimental period.

The animals were premedicated with an intramuscular injection of 5 mg/kg BW xylazine and 40 mg/kg BW ketamine. Anesthesia was initiated with sodium thiopentone (28 mg/kg BW) given i.v. and maintained with occasional small doses to keep a stable anesthetic stage. The animals were intubated and connected to a volume-controlled ventilator (Rodent Ventilator, model 683, Harvard Apparatus Ltd.). Mechanical ventilation was adjusted to 16 breaths/min. Fluid loss was replaced with an infusion of 0.5 ml/min during the whole experimental period.

The animals were performed through the third intercostal space and the ascending aorta was exposed and dissected free from the pulmonary artery and surrounding tissues. Aortic stenosis was produced by placing a surgical thread (1-0 silk) around the ascending aorta between the base of the heart and the right carotid artery (3, 20). Using a caliper, the diameter of the aorta was determined. A small piece of a non-elastic tube, with an external diameter 40% of the aortic diameter, was tied on top of the aorta by the thread. The plastic tube was removed rapidly and the aortic diameter was reduced by 60% approximately (20). In the 7- and 28- day subgroups, the thoracic cavity was closed in layers. Animals in group C underwent a sham operation, without constricting the aortic root. In the 7- and 28-day subgroups of groups B, C and D, a single dose of cefamandole (30 mg/kg BW) was also administered i.v. before surgery and repeated i.m. on the first post-operative day.

All the animals were sacrificed with a bolus dose of 300 mg of sodium thiopentone at either 0, 15, 45, 90, 180 min, 7 or 28 days after the surgical procedure. In the 7- and 28-day subgroups, a left thoracotomy was performed, the heart excised and the LV wall separated free from the right ventricle and the interventricular septum, weighed to estimate hypertrophy and a specimen was separated free from the right ventricle and the interventricular septum. Aortic stenosis was maintained in liquid nitrogen and stored at -80°C for Western blot analysis. Another sample was fixed in 10% buffered formalin for immunohistochemical examination.

LVH was estimated from the weight of the free wall of the LV of the animals.

Western blotting. Total proteins were extracted from ventricular myocytes. The myocytes were suspended in 150 μl to 200 μl of lysis buffer (0.1 M Tris-HCl, pH 7.4, 15% glycerol, 2 mM EDTA, 2% sodium dodecyl sulfate (SDS), containing the protein inhibitors, Phosphatase Inhibitor Cocktail I and Protease Inhibitor Cocktail I (Sigma, USA), incubated on ice for 30 min and spun down at 14000 rpm for 10 min.

The protein concentration was measured by the Bio-Rad protein assay and samples containing 80 μg of total proteins were mixed with loading buffer (20% glycerol, 3% SDS, 3% DTT, 10 mmol/l EDTA and 0.05% bromophenol blue), boiled for 4 min and loaded onto 4-10% gradient SDS-polyacrylamide gel and separated by 4-10% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (21). The proteins were transferred to nitrocellulose membranes in the presence of the glycine/methanol transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% (v/v) Methanol, pH 8.3). The nitrocellulose membranes were blocked with 1% BSA in TBST buffer (0.1 M Tris-HCl, 1.5 M NaCl, 0.05% Tween-20) for 1 h at room temperature and were incubated overnight at 4°C with the primary mouse monoclonal antibody p53 (Clone PAB421, monoclonal mouse IgG, Oncogene Research Products, USA) diluted 1:300 with TBST. The blots were subsequently washed in TBST. The bound antibodies were identified by horseradish peroxidase-conjugated anti-mouse IgG (Sigma, USA) at 1:4500 dilution in TBST, incubation for 1 h at room temperature and recognized by a peroxidase chemiluminescent detection reagent (ECL detection reagents, Pierce, USA). p53 was detected as a 53- kDa band. The same procedure was followed using an actin-targeted antibody (monoclonal anti-actin/amoeba, Clone KJ43A, Sigma) in order to confirm equal loading.

Immunohistochemical examination. Transmural LV tissue sections from all animals, except for those of group D, were cut in a transverse plane. The slices were then processed conventionally for histochemical examination (dehydrated in graded alcohols and paraformaldehyde).

a) Antibodies: For immunohistochemical analysis, the anti-p53 (L21) mouse monoclonal antibody with reactivity in rabbit was used (Oncogene Research Products, USA). The L2 anti-p53 clone is raised against the amino acid 371-380 of the human p53 protein. b) Method: Representative 5-μm sections were mounted on poly-L-lysine–coated slides. The sections were dewaxed, rehydrated in a gradient of alcohol solutions and incubated for 15 min with 0.3% hydrogen peroxide to quench the endogenous peroxidase activity. Antigen unmasking was carried out using the heat-mediated antigen retrieval (HMAR) method (21), consisting of incubation in 10 mM sodium citrate buffer, pH 6.0, in a microwave oven at 700 W for five cycles of 5 min each. The level of the fluid was constantly maintained during the procedure by adding retrieval solution after each cycle. After completion of the fifth cycle, the sections were allowed to cool at room temperature for approximately 20 min and were then rinsed in Tris-buffered saline (TBS), at a pH of 7.6. The sections were then incubated with 10% (v/v) normal rabbit serum (DAKO) for 30 min at room temperature, followed by incubation with a 1:10 dilution of the primary antibody at 4°C overnight.

The biotin-conjugated secondary antibody (biotinylated rabbit anti-mouse immuno-globulins; DAKO) was added at a 1:200 dilution for 30 min at room temperature. The next stage included a 30-min incubation in StreptABComplex (1:100 stock biotin
less than 0.05 was considered to be statistically significant.

Aortic stenosis effect on left ventricular weight. The detailed results of the body weight, the LV wall weight and their ratio from all the included groups are presented in Table I.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>Days</th>
<th>BW, kg</th>
<th>LV, g</th>
<th>LV/BW, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>–</td>
<td>3.57±0.07</td>
<td>3.12±0.11</td>
<td>0.87±0.04</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>7</td>
<td>3.50±0.11</td>
<td>3.01±0.13</td>
<td>0.86±0.04*</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>7</td>
<td>3.32±0.08</td>
<td>3.53±0.25</td>
<td>1.05±0.06</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>7</td>
<td>3.31±0.30</td>
<td>2.87±0.16*</td>
<td>0.86±0.04*</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>28</td>
<td>3.45±0.11</td>
<td>2.72±0.11*</td>
<td>0.79±0.04*</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>28</td>
<td>3.60±0.15</td>
<td>3.92±0.24</td>
<td>1.08±0.04</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>28</td>
<td>3.41±0.05</td>
<td>2.97±0.21*</td>
<td>0.87±0.06*</td>
</tr>
</tbody>
</table>

Values are expressed as means±SEM; n=number of animals; BW=body weight; LV=left ventricular weight; LV/BW=left ventricular weight to body weight ratio; Groups: A=normal, B=sham-operated, C=aortic stenosis and D=aortic stenosis with enalapril animals. *p<0.05 vs. group C.

solution, 1:100 stock streptavidin-hyperoxidase solution; DAKO). For color development, 3,3’-diaminobenzidine tetrahydrochloride (DAB) was used with hematoxylin was used as counterstain.

c) Controls: The MCF-7 cell line (derived from breast cancer), as well as LV aorta-banding specimens overexpressing the p53 protein, were used as positive controls.

d) Evaluation: For scoring the p53 staining patterns, cytoplasmic reactivity was dis-regarded and only nuclear staining above any cytoplasmic background was considered to be evidence of p53 protein expression. The tissue sections from each group were examined microscopically at ×400 magnification and at least ten fields were observed. The p53 status was assessed as the percentage of stained nuclei. Two independent observers (VG and HHZ) carried out the slide examination. Inter-observer variability was minimal (p<0.01). The staining was classified according to the following semi-quantitative method: –, 0%; +, ≤25%; ++, ≤ 50%; ++++, ≤75%; +++++, ≤100%.

Statistical analysis. The results are expressed as mean±SE. For statistical comparisons between normal, sham-operated and aorta banding groups, analysis by the ANOVA test was done. A p value less than 0.05 was considered to be statistically significant.

Results

A total mortality of 14% at 7 days was observed in all groups. At 28 days, the total mortality was 5% in the sham-operated animals, 33% in the aortic stenosis group and 25% in the aortic stenosis with enalapril group. All the dead animals were replaced.

Aortic stenosis effect on left ventricular weight. The detailed results of the body weight, the LV wall weight and their ratio between the different groups. The mean LV weight of group A was 3.12±0.11 g. After 7 days of pressure overload, the mean LV weight of group C increased to 3.53±0.25 g, a statistically significant change when compared with group D. No significant change was noted in the mean LV wall weight between groups B and C. The mean LV weight of animals in group B at 28 days was 2.72±0.11 g, in group C 3.92±0.24 g and in group D 2.97±0.21 g. A significant difference was observed between groups B, D and C.

The LV weight normalized to BW was significantly higher at 7 and 28 days in the aortic stenosis group in comparison to the sham-operated and aortic stenosis with enalapril animals (Table I).

p53 expression after Western blot analysis. As shown in Figure 1, the p53 expression levels were elevated (p<0.05) at all time-points (15, 45, 90, 180 min and 7 and 28 days) and in all groups (B, C, D) when compared to the control group (A). In animals with aortic stenosis treated with enalapril (group D), the levels of p53 expression, although higher than those of the control group (A), were lower (p<0.05) at 180 min, 7 and 28 days when compared with the values obtained for groups B and C (Figures 1 and 2).

p53 immunohistochemical findings. The results of the immunohistochemical analysis demonstrated that p53 expression was specifically restricted to the myocytes and was not observed in any other stromal elements of the cardiac muscle (Figure 3). These analyses were performed in all groups except for group D, because this technique is semi-quantitative and, therefore, the results cannot be statistically analyzed to reveal any quantitative differences between the groups. p53 immunoreactivity in the LV free wall was present at all experimental time-points (15, 45, 90, 180 min, 7 and 28 days) in all animals of the sham-operated group (B) and the aortic stenosis group (C). However, in all animals of the control group (A), p53 immunoreactivity was absent. In group B, p53 immunoreactivity was less than or equal to 50% at all time-points, except at 28 days where it was 50-75%. In group C, p53 immunoreactivity was less than or equal to 50% at 15 min and 7 days and 50-75% at 45, 90 and 180 min and 28 days. The immunoreactivity was the same (≤50%) between groups B and C at 15 min and 7 days as well as at 28 days (50-75%), but was higher in group C (50-75%) compared to that of group B (≤50%) at 45, 90 and 180 min.

Discussion

Cardiac hypertrophy may be regarded as an adaptive physiological response to increased functional demands on the heart (22, 23). It is known that during the development of hypertrophy, cells within the ventricular wall undergo ischemic injury (3). The p53 protein is a transcriptional...
factor that enhances the rate of transcription of target genes that carry out the p53-dependent functions in a cell (24,25). Long et al. (26) reported that the intracellular signaling pathways activated by p53 play a critical role in the regulation of hypoxia-induced apoptosis of cardiomyocytes.

p53 effects cell cycle arrest or apoptosis in response to a variety of genotoxic and physical stresses (27, 28). The protein levels of p53 increase in response to DNA damage (28), oxidative stress (29) and, significantly, to hypoxia (24). Cardiac hypertrophy is associated with elevated intracardiac angiotensin-converting enzyme activation, which may contribute to diastolic dysfunction (30). The intracoronary infusion of the angiotensin-converting enzyme inhibitor (enalapril) induced improvement of the LV function, this observation supporting the hypothesis that, in patients with concentric pressure-overload hypertrophy (30), the cardiac renin-angiotensin system is activated (31, 32).

In previous studies (33-35) in adult ventricular myocytes, p53 was reported to up-regulate the cellular renin-angiotensin system, resulting in the synthesis of the

Figure 1. Quantitative analysis of p53 expression of the left ventricle in animals of the control (A), sham-operated (B), aortic stenosis (C) and aortic stenosis with enalapril (D) groups. Values are mean±SEM.

Figure 2. Representative autoradiograms of Western blots of p53 from left ventricle. Sham: sham-operated; AoS: aortic stenosis; AoS+En: aortic stenosis treated with enalapril.
octapeptide angiotensin II both in vitro and in vivo. Angiotensin II, in turn, activates p53, creating a positive feedback loop in which hormone production and p53 function are intimately related in myocytes. Although p53 mRNA was readily detectable in the embryonic heart, expression decreased at birth and transcripts were barely detectable in the hearts of adult animals (36). Kim et al. (36) observed that the tumor suppressor gene p53 was not transcriptionally-activated during acute myocardial overload or isoproterenol-induced myocardial hypertrophy in mice models. Neither acute nor chronic myocardial overload significantly induced tumor suppressor gene transcription. The lack of p53 induction following isoproterenol-induced hypertrophy is consistent with absence of DNA synthesis in similarly treated animals (37). p53 expression was also inversely-related to cardiomyocyte terminal differentiation. Transcripts were readily detected in proliferative embryonic cardiomyocytes and were markedly down-regulated in the adult myocardium (36).

In the present study, enalapril prevented LVH at 7 and 28 days after aortic stenosis (Table I), in accordance with other investigators’ findings (11-16). LVH was greater (p<0.05) in animals of group C at 7 days compared to that of group D and at 28 days compared to groups B and D, but there was
not a significant increase from the 7th to the 28th day in group C, in contrast to a previous report where hypertrophy increased linearly up to 30 days of aortic stenosis (3) (Table I). The p53 expression levels were elevated \( p<0.05 \) in all groups compared to those of the control group (A). Furthermore, p53 was consistently expressed in the sham-operated (B) and aortic stenosis (C) groups, a finding that contrast with other results (19, 36).

In relation to the 7- and 28-day time-points in groups B, C and D, the question arises as to whether the p53 expression levels remained elevated during all the intervals or not. In the last case, surgery stress may supersede the new p53 expression increase, taking into account that the readiness and/or the sensitivity processes of p53 expression were activated during the first operation (Figure 1). The p53 quantity increase in groups B, C and D could be a consequence of an external death stimulus due to DNA damage, hypoxia, etc. (24, 28, 29, 33).

The p53 expression levels in group D remained high at all time-points compared to those of the control group (A). The levels were the same between groups B and C at 15, 45 and 90 min, while they decreased \( p<0.05 \) in group D at 7 and 28 days compared to groups B and C, respectively, as well as in group D at 180 min compared to those of group C (Figure 1). In this case, either the 90-min time-course might have been inadequate for enalapril to act on the p53 expression, despite the fact that the drug administration was started 4 days before the operation, or the acute and intense stimulus of the aortic stenosis could have predominated in the initial stage of LV remodeling (Figure 1). Low p53 expression levels at 180 min, 7 and 28 days in the group D animals could be attributed to a number of factors. It is possible that the feedback mechanism was activated, despite the fact that enalapril impeded angiotensin II production by fully disrupting the renin-angiotensin axis, while leaving the chymase-dependent angiotensin II formation pathway intact (17, 35, 38-40).

Enalapril could also influence p53 expression through an unknown mechanism. Finally, another feed-back loop may be created, given that the p53 protein protects against DNA damage, either by inhibiting cell growth until the damage has been repaired or by leading the cell to commit suicide through apoptosis (41). In summary, we demonstrated that LVH was induced after aortic stenosis at 7 and 28 days, although the difference between the two time-points was not significant, and that enalapril prevented LVH. The tumor suppressor gene p53 was transcriptionally activated and immunoreactively present in acute and chronic myocardial overload, as well as in the sham-operated animal model. Enalapril significantly decreased the p53 expression in response to aortic stenosis at 180 min, 7 and 28 days by an unknown mechanism that remains to be elucidated.

References


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