

Apoptosis in Pressure Overload-induced Heart Hypertrophy in the Rat

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Abstract

Pressure overload induces cardiac growth in the rat, which implies the hypertrophy of cardiac muscle cells and proliferation of nonmuscle cells. The cardiac cell loss observed in parallel has generally been attributed to necrosis. Using an *in situ* assay, we demonstrated a phase of apoptosis or programmed cell death during the first 7 d after pressure overload with a peak at day 4 while cardiac growth continued for over 30 d. The increase in apoptosis was confirmed by quantification of 180–1500-bp DNA oligonucleosomes with agarose gel electrophoresis and *in situ* labeling via 3'-terminal deoxynucleotidyl transferase assay. While some apoptosis was observed in the basal state in nonmuscle cells, pressure overload induced apoptosis mainly in cardiomyocytes. These data suggest that cardiac hypertrophy is initiated by a wave of apoptosis of cardiomyocytes. Thus, apoptosis may be involved in the pathogenesis of heart remodeling. (*J. Clin. Invest.* 1996. 97:2891–2897.) **Key words:** apoptosis • heart • hypertrophy • aortic stenosis • pressure overload

Introduction

Apoptosis is an ubiquitous and physiological mode of cell death which plays a complementary but opposite role to mitosis in the regulation of tissue homeostasis. Its occurrence is a major factor in the control of shape and size during normal and abnormal growth processes (1–3). Apoptosis is distinct from necrosis, the other type of cell death. Unlike necrosis, apoptosis or programmed cell death (PCD) proceeds in an orderly and controlled manner without loss of membrane integrity and release of cell content. The morphological changes observed are remarkably similar between tissues and species. They include shrinkage of the cell, condensation of the cytoplasm and chromatin, “blebbing” of the cell membrane, and fragmentation of the nucleus. The major biochemical event

considered a hallmark of apoptosis is the internucleosomal cleavage of genomic DNA. This DNA degradation is due to cleavage by $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent and -independent endonucleases at linker DNA sites between nucleosomes (4). The fragments generated are multimers of 180–200-bp oligonucleosomal units which produce a typical “DNA ladder” after agarose gel electrophoresis.

The importance of apoptosis is seen in a wide variety of physiological and pathological processes such as morphogenesis (5), neoplasia (6), hormonal regulation of cell function (7, 8), mediation of immunological responses and neurodegenerative disorders like Alzheimer's and Parkinson's disease (9). Apoptosis in the heart and cardiomyocytes is just now being recognized (10–13).

Ventricular remodeling in response to pressure overload classically implies the hypertrophy of muscle cells and proliferation of nonmuscle cells (14). Cardiac cell loss has been observed, but was attributed to necrosis (15, 16). The hypertrophic/hyperplastic part of this complex physiological phenomenon has been the subject of intense research and is of recognized clinical relevance (14, 17) but the possible involvement of its counterpart, apoptosis, has remained unexplored until now. We hypothesize that remodeling of cardiovascular organs requires a PCD (apoptosis) phase followed or paralleled by cell growth (10). Remodeling of the heart is frequently considered a somewhat passive tissue rearrangement or a simple growth event. It is now increasingly evident that cell growth and PCD are in fact two linked processes. The aim of this study was to test for the presence of the apoptotic process in the development of ventricular hypertrophy after experimental pressure overload.

Methods

Reagents. Proteinase K, RNase A, dithiothreitol (DTT), bovine serum albumin (BSA), dATP and dCTP were obtained from Boehringer Mannheim Canada (Laval, Québec, Canada). 3'-terminal deoxynucleotidyl transferase (tdt) was purchased from Pharmacia Biotech Inc. (Baie d'Urfé, Québec, Canada). Nylon membrane Hybond N⁺ and ³²P-dCTP (3,000 Ci/mmol) were from Amersham Canada Ltd. (Oakville, Ontario, Canada). Polysine slides were from Baxter Diagnostics Inc. (Deerfield, IL). Anti-skeletal myosin and TRITC-conjugated goat anti-rabbit IgG were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The Apoptag kit was furnished by Oncor (Gaithersburg, MD). All other agents were purchased from usual local suppliers.

Animals and surgical procedures. Female Wistar rats (23–24 d old) were obtained from Iffa-Credo (Lyon, France). They weighed ~50 grams at the time of surgery which was performed under Brietal anesthesia (3 mg/kg, ip). Respiratory assistance was maintained throughout the operation.

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Aortic stenosis was produced by placing a partially calibrated, occluded Weck hemoclip around the ascending thoracic aorta between the base of the heart and the right carotid artery (18). Sham-operated controls underwent an identical procedure except for placement of the hemoclip. Both groups were killed 1, 2, 4, 7, 15, and 30 d after surgery, including three rats for each time period and group. Their bodies were weighed and their hearts were rapidly excised. Right and left atria, extraneous tissue, and large vessels were then dissected from the base of the heart, and the right ventricular free wall was separated from the remaining portion of the heart. Left ventricle (including interventricular septum) samples were weighed to estimate hypertrophy. For the study of apoptosis, whole hearts were collected and immediately frozen in isopentane (-18°C) for 30 s, then kept at -80°C until DNA extraction or tissue section preparation.

In vitro DNA extraction and labeling. A portion containing both ventricles was removed from the heart. This tissue was homogenized in buffer containing 20 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris-HCl, pH 8.0, 0.5% sodium dodecyl sulfate (SDS), and 100 $\mu\text{g}/\text{ml}$ proteinase K, incubated for 3 h at 50°C and treated with RNase A (final concentration 150 $\mu\text{g}/\text{ml}$) for 1 h at 37°C . After extraction with phenol and chloroform, DNA was precipitated with ethanol, washed with 70% ethanol and resuspended in deionized H_2O . DNA concentration was quantitated by spectrophotometry.

1 μg of extracted DNA was labeled by enzymatic assay, using tdt as described previously (13). It was labeled in buffer containing 2 mM CoCl_2 , 0.5 mM DTT, 100 mM potassium cacodylate, 166 nM ^{32}P -dCTP (3,000 Ci/mmol), 664 nM dCTP and 20 U of tdt. The samples were incubated for 60 min at 37°C . One-tenth of the total reaction volume was loaded on 1.5% agarose gel, run at 90 V for 3.5 h, transferred onto a Hybond N^+ nylon membrane, exposed to a phosphor-sensitive screen and analyzed with the PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

DNA fragmentation after labeling by tdt assay was quantified by the summation of radioactivity incorporated into 180–1500-bp DNA fragments.

In situ DNA fragmentation labeling. To detect DNA fragmentation in situ, 3'-end tail labeling was performed by the method of Gavioli et al. (19) which we have adapted for tissue sections (20). Heart sections (5 μm) were cut with a cryostat and mounted on polysine slides. The slides were fixed in 4% paraformaldehyde in phosphate buffer (24 mM, pH 7.4) for 30 min at room temperature, washed three times for 15 min in phosphate-buffered saline (PBS, 12 mM) and steam-heated at 90°C for 30 min. For a typical enzymatic reaction, 20 μl of a solution containing 2 mM CoCl_2 , 0.5 mM DTT, 100 mM potassium cacodylate, 30 mM Tris-HCl, pH 7.4, 0.05 mg/ml BSA, 0.35 mM dATP, 50 nM ^{32}P -dCTP (3,000 Ci/mmol) and 4.5 U of tdt were applied on slides and covered with a plastic cover-slip. Controls were prepared with the same solution but without tdt. The slides were then incubated for 30 min at 37°C in a humidified atmosphere. The cover-slips were removed and the slides were washed overnight in PBS containing 1% SDS at room temperature, rinsed twice for 30 min in PBS and air dried. They were finally exposed to a phosphor-sensitive screen, analyzed and quantified with the PhosphorImager system (Molecular Dynamics). The instrument is a computing densitometer which is more sensitive and linear than the conventional autoradiographic technique using x-ray film. With the PhosphorImager, the film is replaced by a photo-sensitive plate of BaFBr:Eu^{2+} capable of detecting isotopes emitting β , γ or x-rays. These radiations excite the Eu^{2+} ion of the BaFBr:Eu^{2+} complex into Eu^{3+} which, in turn, can

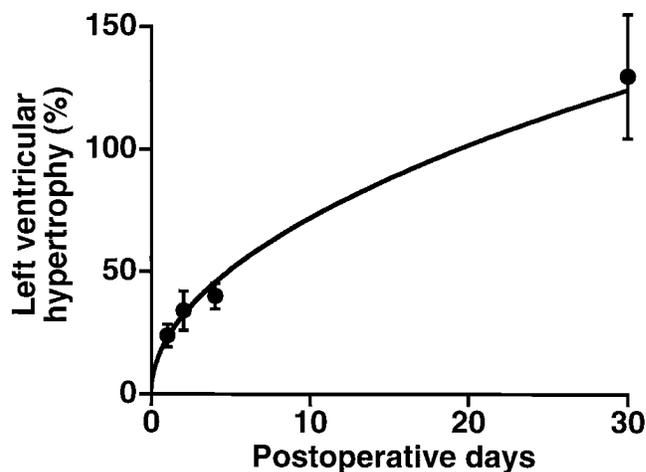


Figure 1. Ventricular hypertrophy and apoptosis in rats with thoracic aortic stenosis. Aortic stenosis was produced as described in the Animals and Surgical Procedures section. Rats were killed 1, 2, 4, and 30 d after surgery. Left ventricles were removed and weighed for the estimation of hypertrophy expressed as a percent of sham-operated control values.

be detected by the PhosphorImager equipped with a helium-neon laser beam. The sensitivity of these plates is more than 100 times that of x-ray films, thus reducing exposure time. Uptake is not saturable, leading to more precise quantification of the radioactive gels or tissue section slides. The results are accumulated and can be quantitated in the form of units per pixels. For the quantitation of oligonucleosomal laddering, pixels corresponding to 180 bp and their multiples of small molecular weight (up to 1500 bp) were accumulated and computed.

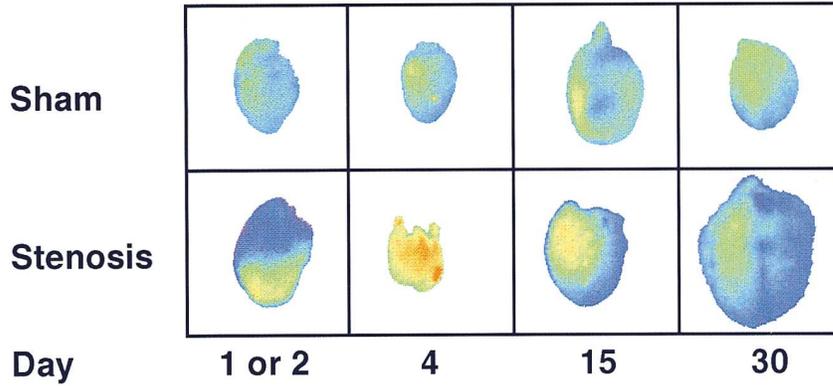
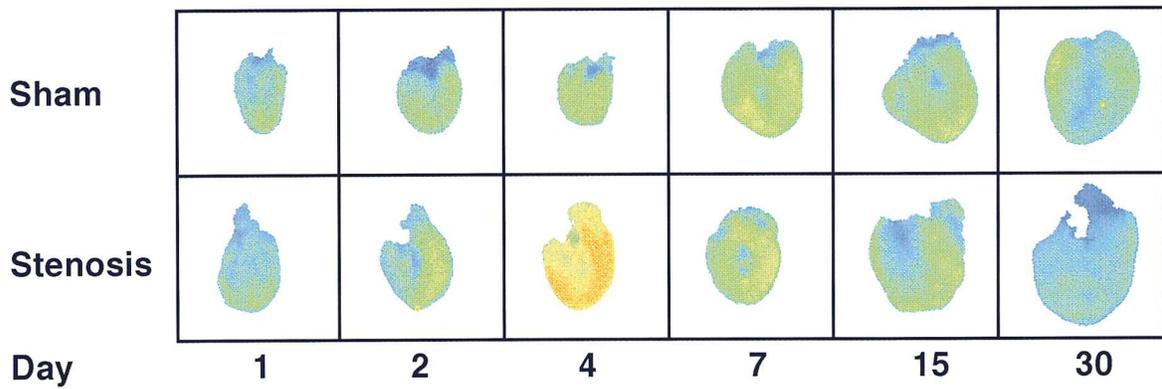
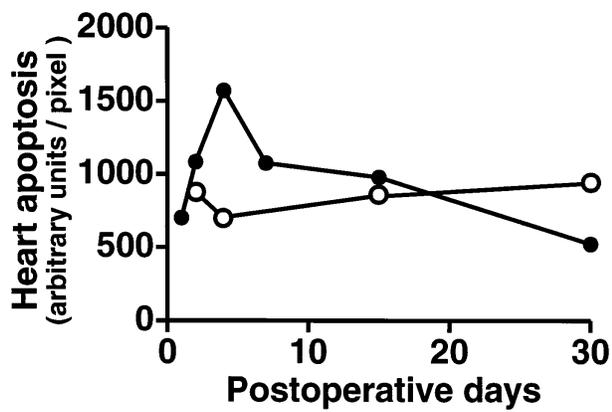
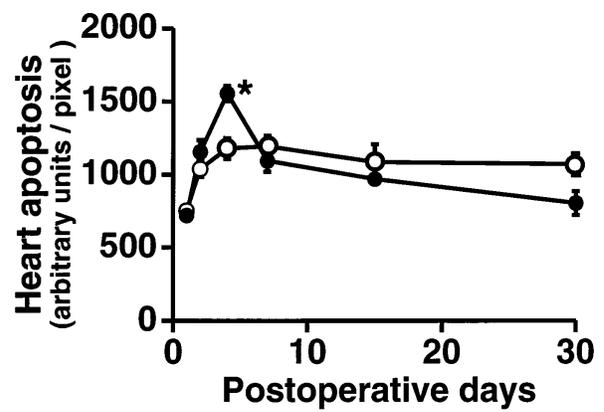
Confocal microscopy of apoptotic cardiomyocytes. We also used the Apoptag kit for in situ apoptosis detection. The slides were fixed in 10% buffered formaldehyde for 10 min and postfixed in ethanol:acetic acid (2:1) for 5 min at -20°C . They were incubated with tdt enzyme and digoxigenin -11-dUTP and dATP in buffer provided with the kit for 1 h at 37°C . The reaction was terminated with the addition of stop buffer. The slides were washed in PBS before the addition of anti-digoxigenin-fluorescein for 30 min at room temperature. Negative controls were routinely obtained by omission of the enzyme.

To further demonstrate that apoptosis labeling occurred in nuclei of cardiomyocytes, sections from a number of samples were first labeled with the Apoptag kit for in situ identification of apoptosis. Subsequently, they were incubated for 30 min with rabbit anti-skeletal myosin diluted 1:20 at 37°C , followed by anti-rabbit IgG-rhodamine (dil 1/64) at 37°C for 30 min. By this approach, tdt-apoptosis-labeled nuclei appeared greenish and skeletal myosin staining of the cytoplasm appeared reddish under fluorescence confocal microscopy (Bio-Rad, Mississauga, Ontario, Canada).

Results

As expected, thoracic aortic stenosis induced left ventricular hypertrophy (18). After 1 d of pressure overload, the left ven-

Figure 2. Apoptosis in rats with aortic stenosis. (A) Representative tissue slices used for the in situ detection of apoptosis. Slides were prepared as described in the In situ DNA Fragmentation Labeling section of Methods. In situ tdt assay was performed using ^{32}P -dCTP. Results from two different experiments are shown: Heart apoptosis in stenotic and sham-operated rats killed at 1, 2, 4, 15, and 30 d (experiment I) or 1, 2, 4, 7, 15, and 30 d (experiment II) after the surgical intervention. The color representation of density magnitude is blue < green < yellow < red. (B) Apoptosis was quantified from tissue slices with the PhosphorImager system. 1 pixel = $77.44 \times 10^{-4} \text{ mm}^2$. Experiment II represents the means \pm SEM of three heart sections from three different rats. * $P < 0.005$ by unpaired Student's t test.

A**Experiment I****Experiment II****B****Experiment I****Experiment II**

○ Sham
● Stenosis

tricles of aortic stenotic rats were 20% heavier than those of sham-operated controls. Hypertrophy increased linearly up to day 30, reaching a maximum of 130% (Fig. 1).

In situ labeling revealed a rapid increment of apoptosis which peaked around day 4 (red color indicating maximum apoptosis) as compared with sham-operated controls (Fig. 2 A). Two separate experiments showed a narrow apoptotic window around day 4 in the aortic stenotic rats. At day 30, the apoptotic process was back to control levels while hypertrophy was clearly evident (Fig. 2 A). Quantification of in situ 3'-end DNA labeling (by PhosphorImager) is depicted in Fig. 2 B. It clearly illustrates in both experiments that peak apoptosis is an early event in the hypertrophic process of hearts subjected to pressure overload.

After ventricular DNA extraction and labeling with tdt, DNA laddering representing inter-oligonucleosomal DNA fragments typical of apoptosis could be detected, even in the basal state and in sham-operated animals. Since we used the 3'-end DNA labeling method, which is far more sensitive than ethidium bromide DNA staining, we could easily detect basal DNA laddering and apoptosis in normal hearts. Basal, low level apoptosis was also demonstrated by the [³H]thymidine release technique in vascular smooth muscle cells from normal rats (13). For equal amounts of DNA loaded in each lane, samples from aortic stenotic rats showed a massive increase of 3'-end labeling with maximum values between 4 to 7 days ($P < 0.03$ by student's t test) (Fig. 3). Thereafter, labeling declined to control levels. Quantification of the radioactivity incorporated into 180–1500-bp DNA fragments (seen at the bottom) illustrates the temporal evolution of this phenomenon.

In situ 3'-end labeling with the Apoptag kit in heart sections (Fig. 2) demonstrated essentially the same pattern as the DNA fragmentation observed on agarose gel (Fig. 3). To confirm the specificity of DNA fragment labeling, we extracted and tdt-labeled heart DNA 4 d after sham or aortic stenosis surgery. The data presented in Fig. 4 clearly show higher oligonucleosomal laddering in hearts of aortic stenotic rats as compared with sham-operated animals with each amount of DNA loaded on agarose gel (Fig. 4 A). The slope of labeled DNA as a function of total DNA loaded was higher in stenotic rats, demonstrating augmented DNA fragmentation (180–1500 bp) for the same amount of DNA loaded (Fig. 4 B).

To further ascertain whether cells undergoing apoptosis are indeed cardiomyocytes, fluorescent confocal microscopy was used. Heart sections were first labeled with the fluorescein Apoptag kit followed by anti-digoxigenin fluorescein for detection of apoptotic nuclei and then with rabbit anti-skeletal myosin followed by anti-rabbit IgG-rhodamine for detection of cardiomyocytes. As shown in Fig. 5, apoptotic nuclei appeared greenish and the cytoplasm of cardiomyocytes appeared reddish under fluorescence confocal microscopy. The majority of labeled cells seemed to be cardiomyocytes. It has to be noted that heart sections from stenotic rats at day 4 (Fig. 5 C) showed always higher apoptotic signals than those from sham-operated controls (Fig. 5 B). However, this method does not allow quantification since apoptosis appeared patchy, i.e., areas of intense apoptosis neighbored areas without apoptosis.

Discussion

It is now apparent that, similarly to nondividing cells of the nervous system (9), long-lived, terminally differentiated cells

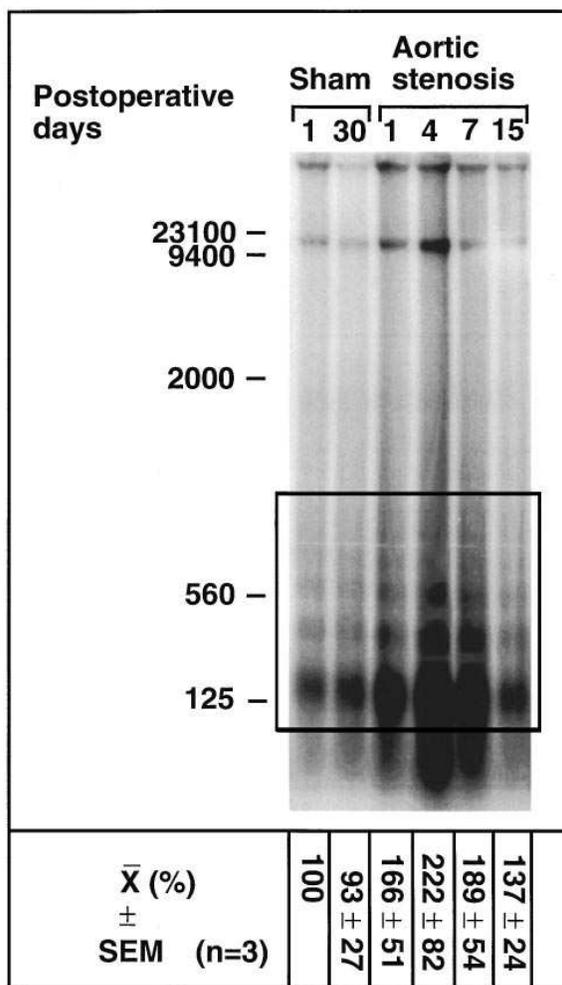


Figure 3. DNA laddering in stenosis-induced hypertrophic rat hearts. Extracted DNA was labeled with tdt and ³²P-dCTP and loaded on 1.5% agarose gel as described in the text. Equal amounts of DNA were loaded in each lane. The first two lanes represent tdt-labeled DNA extracts from sham-operated rats at days 1 and 30, respectively, and the following lanes represent tdt-labeled DNA extracts at days 1, 4, 7, and 15 after aortic stenosis. The laddering typical of apoptosis represents multiples of 180-bp internucleosomal DNA fragments. The lower panel shows the quantification of 180–1500-bp DNA fragments expressed as means ± SEM of three different extractions from three different animals for each time point ($n = 3$).

of the heart retain the ability to die via the apoptotic mechanism. This intrinsic property of adult cardiomyocytes is suggested by the observation of severe myocardial damage in individuals undergoing chemotherapy, as with adriamycin, which is now known to be a potent inducer of apoptosis (21), although it has not directly been shown to be elicited by this agent in cardiomyocytes. The present study provides proof of an apoptosis phase as an initial part of heart remodeling.

The thoracic aortic banding procedure used in this investigation resulted in strong, acute pressure overload that rapidly led to a dramatic increase of left ventricular mass. DNA laddering experiments demonstrated a basal level of apoptosis in sham-operated animals with a strong increase after aortic stenosis. The tdt assay is very sensitive and can detect even very low apoptosis. The in situ assay showed a few apoptotic

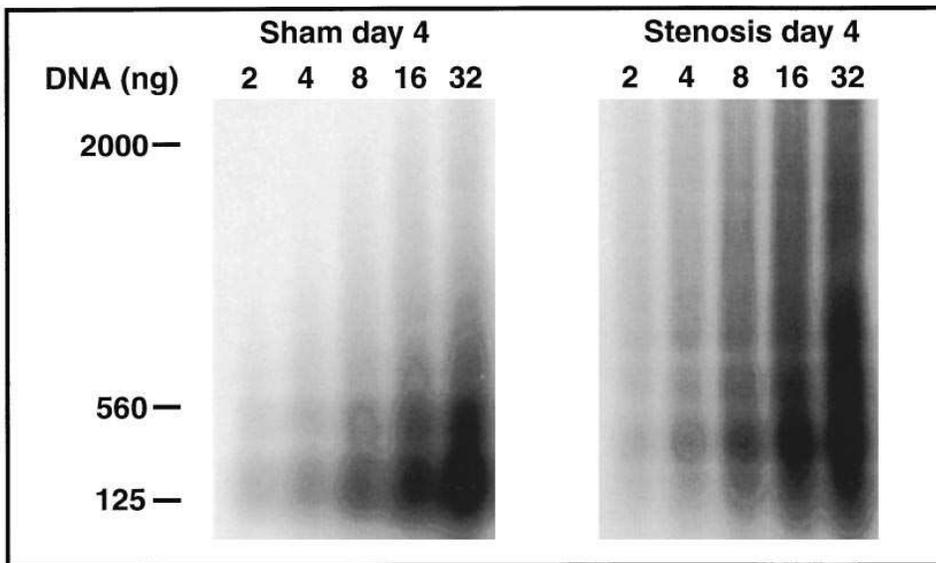
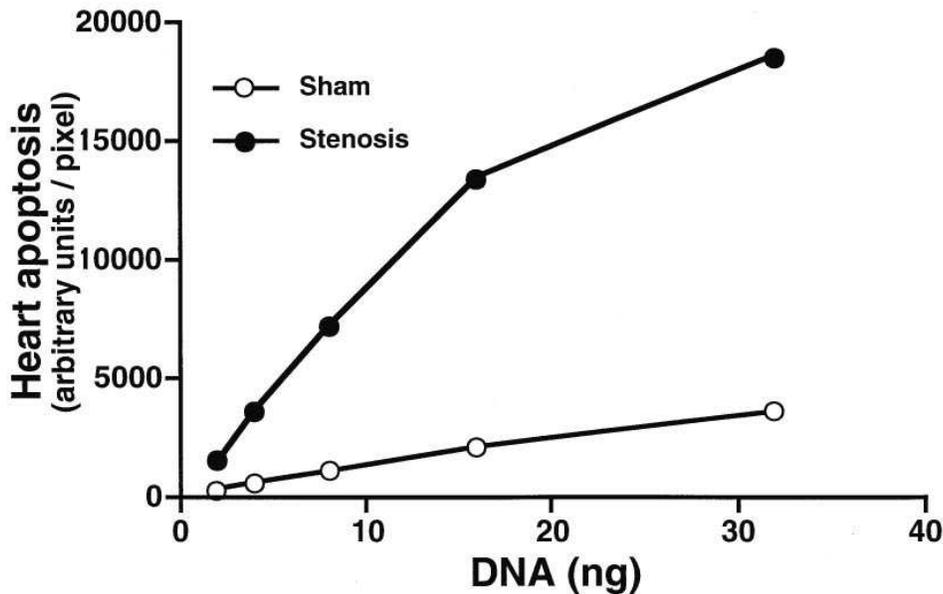
A**B**

Figure 4. Oligonucleosomal labeling as a function of increasing amounts of total DNA. DNA was extracted from stenosis-induced hypertrophic rat hearts and corresponding controls at day 4 after surgery. Increasing amounts (2, 4, 8, 16, 32 ng) of tdt-labeled DNA were electrophoresed as described in Methods. (A) Electrophoretic typical pattern of DNA laddering of sham-operated and stenotic rats at day 4. (B) Quantification of 180–1500-bp DNA labeled fragments as a function of the amount of DNA loaded.

cells in the basal state. However, Fig. 5 clearly indicates that the apoptotic cells present after pressure-induced overload were cardiomyocytes which were much more numerous than in sham-operated controls. Apoptosis was transient, and its maximum (day 4) by far preceded the peak of hypertrophy (Figs. 1 and 2 A), illustrating by itself that the method of apoptosis detection specifically distinguished this process from cell growth, which continued for the next 30 d. 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 car-

diac adaptive response (22). Among these factors, *c-myc* is involved both in proliferation and apoptosis, the two processes being closely related (23). We have previously reported heightened hsp70 expression in vascular smooth muscle cells of spontaneously hypertensive rats (SHR) which paralleled their increased growth potential (24). More recently, we have documented an increment of apoptosis in cultured vascular smooth muscle cells and hearts from SHR, a well-described model of cardiac hypertrophy (10, 13). Furthermore, during prostatic involution, a good model of apoptosis, an increase of *c-myc*, *c-fos* and hsp70 has also been reported (25).

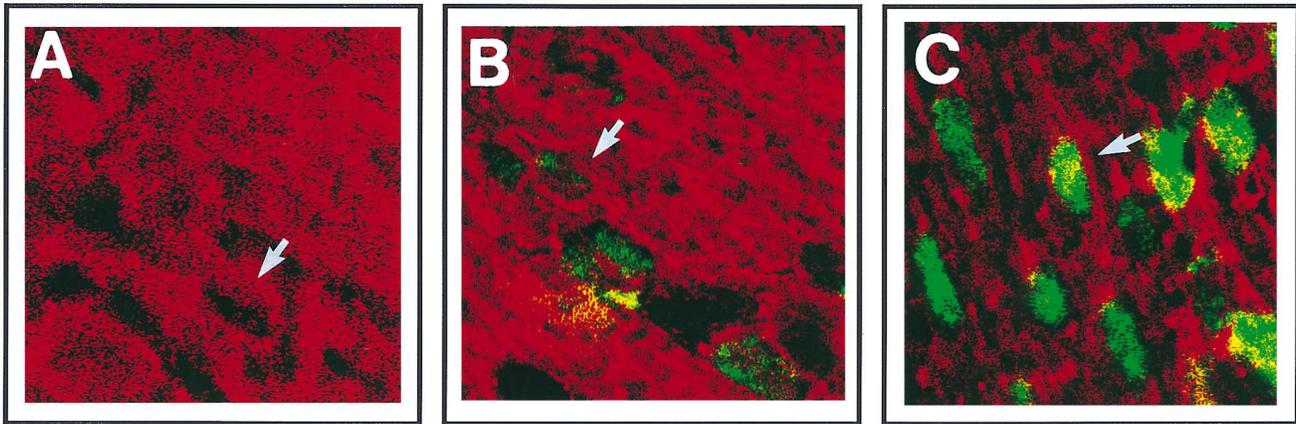


Figure 5. Identification of aortic stenosis-induced apoptotic cells in the heart. Confocal microscopy of representative tissue slices from sham-operated and pressure overload-induced heart hypertrophied rats 4 d after surgery. Heart slices were prepared as described in Methods. Apoptotic nuclei were labeled with a fluorescein Apoptag kit and myosin detected by immunofluorescent staining with anti-skeletal myosin antibody. (A–C) Confocal microscopy of heart tissue sections staining for myosin (red) and apoptotic nuclei (green). Original magnification of all photomicrographs is 1200 \times . Arrows indicate representative cardiomyocytes. (A) Control; pressure overload-induced hypertrophied heart, without tdt enzyme. (B) Heart section from a sham-operated rat 4 d after surgery. (C) Heart section from a rat 4 d after aortic stenosis.

The mechanism triggering apoptosis in aortic stenosis should be further investigated, but in vitro experiments have shown that hypoxia can specifically induce apoptosis in cardiomyocytes and the expression of Fas antigen mRNA (11). However, Gottlieb et al. (12) have demonstrated the appearance of apoptosis after reperfusion while DNA laddering remained totally absent when ischemia only was applied. The authors attributed the mechanism of this cell death to apoptosis. Yet, since the latter occurs only after reperfusion, it may also be part of a compensatory mechanism. The link between apoptosis and growth is underlined in the present study. Reperfusion has been viewed as a double-edged sword with its positive and negative impact on tissue salvage (26). Whether or not the calcium paradox and/or free radical generation are involved in apoptosis after reperfusion and stenosis-induced hypertrophy needs further investigation. It is known that during the development of hypertrophy, cells within the ventricular wall undergo ischemic injury. We propose that part of the cell death occurring during hypertrophy, which was previously attributed to necrosis, is in fact due to the apoptotic process. Recent understanding of neoplastic growth suggests that apoptosis may play a growth compensatory role (27–29). Apoptosis is also an essential requirement for progression of growth in utero: its arrest results in phocomelia (30). The present study establishes that in cardiovascular hypertrophy, initial apoptosis is also linked to growth. Further studies will have to examine whether apoptosis is a prerequisite for remodeling and whether hypertrophy represents a failure of compensatory apoptosis.

Our results indicate that apoptosis may be an important regulatory mechanism involved in the cardiac adaptive response to pressure overload. This notion modifies our understanding of cardiac remodeling and may offer a new target for therapeutic intervention.

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