Cardiac hypertrophy and ensuing heart failure are among the most common causes of mortality worldwide, yet the triggering mechanisms for progression of hypertrophy to failure are not fully understood. Tissue homeostasis depends on proper relationships between cell proliferation, differentiation, and death and any imbalance between these results in compromised cardiac function. Recently, we developed a transgenic (Tg) mouse model that overexpress myotrophin (a 12-kDa protein that stimulates myocyte growth) in heart resulting in hyper trophy that progresses to heart failure. This provided us an appropriate model to study the disease process at any point from initiation of hypertrophy end-stage heart failure. We studied detailed apoptotic signaling and regenerative pathways and found that the Tg mouse heart undergoes myocyte loss and regeneration, but only at a late stage (during transition to heart failure). Several apoptotic genes were up-regulated in 9-month-old Tg hearts compared with age-matched wild type or 4-week-old Tg hearts. Cardiac cell death during heart failure involved activation of Fas, tumor necrosis factor-\( \alpha \), and caspases 9, 8, and 3 and poly(ADP-ribose) polymerase cleavage. Tg mice with hypertrophy associated with compromised function showed significant up-regulation of cyclins, cyclin-dependent kinases (Cdks), and cell regeneration markers in myocytes. Furthermore, in human failing and nonfailing hearts, similar observations were documented including induction of active caspase 3 and Ki-67 proteins in dilated cardiomyopathic myocytes. Taken together, our data suggest that the stress of extensive myocardial damage from longstanding hypertrophy may cause myocytes to reenter the cell cycle. We demonstrate, for the first time in an animal model, that cell death and regeneration occur simultaneously in myocytes during end-stage heart failure, a phenomenon not observed at the onset of the disease process.

Cardiac hypertrophy and resulting heart failure are the most common cause of mortality in the world. The triggering mechanisms for progression of cardiac hypertrophy to heart failure are still not fully understood, but many observers have suggested that programmed cell death (PCD), that is, apoptosis, is a major contributor to heart failure. Although apoptosis in the myocardium is a complex process and difficult to recognize, there is evidence that potential mechanisms of induction of apoptosis at the cellular level may involve interplay between mechanical factors and elevated levels of neurohumoral factors. Volume overload and elevated left-diastolic ventricular pressure may initiate the events of myocyte apoptosis (1). Recently, myocyte apoptosis has been demonstrated after injury because of ischemia, reperfusion, myocardial infarction, ventricular pacing, cardiac aging, and coronary embolization (2–4). Furthermore, Olivetti et al. (5) demonstrated that cell death accompanies congestive heart failure in humans.

Tissue homeostasis depends on proper relationships between cell proliferation, differentiation, and death. The balance between proliferation and apoptosis must be maintained to sustain tissue homeostasis. As a cell progresses through the cell cycle, it must determine whether to complete cell division, arrest growth to repair cell damage, or undergo apoptosis if the damage is too severe to be repaired. Whether the heart can grow by multiplication of myocytes has been controversial. Recently, Beltrami et al. (6) provided convincing proof of myocyte replication in the failing human heart and showed that this form of cell growth could compensate for exhaustion of myocyte hypertrophy. A myocyte mitotic index of 0.015% was measured in explanted hearts from patients in terminal stages of cardiac decompensation.

One major limitation to examining molecular changes during the progression of hypertrophy to heart failure has been the availability of a suitable animal model. We have identified a factor, myotrophin, from spontaneously hypertensive rat hearts and cardiomyopathic human hearts, which stimulates myocyte growth (7). Myotrophin is a novel gene, localized in human chromosome 7q33 (8). Recently, we have developed a transgenic mouse model overexpressing myotrophin in the heart under the transcriptional regulation of the \( \alpha \)-myosin heavy chain promoter. This model is associated with increased expression of proto-oncogenes, hypertrophy marker genes (\( \beta \)-myosin heavy chain and atrial natriuretic factor), and rapid organization of myofibrils (9). This transgenic mouse model showed hypertrophy as early as 4 weeks of age that progressively led to heart failure with severe compromised function (Fig. 1A). All the symptoms in this model mimic human heart failure.

Using DNA microarray analysis, we compared a differential expression profile of several gene clusters in wild-type and myotrophin overexpressing transgenic; WT, wild-type; FADD, Fas-associated death domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase; pCNA, proliferation cell nuclear antigen; DCM, dilated cardiomyopathic; NF, nonfailing; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \); RPA, RNase protection assays; DAPI, 4,6-diamidino-2-phenylindole; Cdks, cyclin-dependent kinases.

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‡ The abbreviations used are: PCD, programmed cell death; Tg, transgenic; WT, wild-type; FADD, Fas-associated death domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, PARP, poly(ADP-ribose) polymerase; pCNA, proliferation cell nuclear antigen; DCM, dilated cardiomyopathic; NF, nonfailing; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \); RPA, RNase protection assays; DAPI, 4,6-diamidino-2-phenylindole; Cdks, cyclin-dependent kinases.
transgenic animals during initiation (about 4 weeks of age) and transition of hypertrophy to heart failure (around 36 weeks of age). A cluster of apoptotic genes, as well as genes involved in cellular regeneration, was found to be significantly up-regulated in 36-week-old Tg mice heart samples but not those from 4-week-old mice (9). Therefore, we chose to study the molecular changes for both cardiac cell death and regeneration during initiation of cardiac hypertrophy and during the transition from hypertrophy to heart failure, the later still being an open question. To establish the relevance of our findings in murine model, we also studied some key genes in these processes (active caspase 3 for cell death and Ki-67 for cell regeneration) in human dilated cardiomyopathic (DCM) and nonfailing (NF) hearts. Our data showed that both cell death and regeneration occur simultaneously during heart failure that is not evident during onset of this disease.

**EXPERIMENTAL PROCEDURES**

**Animals Used**—All mice used in this study were obtained from Harlan Sprague-Dawley (Indianapolis, IN). This investigation conformed to the “Guide for the Care and Use of Laboratory Animals” (29). For each experiment discussed in this article, at least five different animals, both wild-type (WT) and transgenic (Tg), from each age group (4 weeks and 9 months), were used. The Tg animals used represent all four founder lines that overexpress myotrophin protein in the heart. Our data represent both male and female Tg and WT mice. No difference was observed between males and females on the parameters studied.

**Human Samples**—Human DCM and NF heart samples were obtained from the cardiac transplantation core at the Cleveland Clinic Foundation. NF human hearts were obtained from 5 organ donors not suitable for transplantation but with no history of cardiac diseases and were victims of either motor vehicle accidents or gunshot wounds. Failing hearts were obtained from 6 transplant patients diagnosed with DCM. All heart samples were transported to the laboratory in cold cardioplegia and were snap frozen instantly for future use. Protocols for tissue procurement were approved by the Cleveland Clinic Foundation Institutional Review Board. The clinical characteristics of these heart samples are tabulated in Table I.

**TUNEL Assay**—DNA fragmentation was detected in left ventricular sections of 9-month-old WT and Tg mice by TUNEL staining using the APO-BRDT kit (BD Pharmingen, San Diego, CA). Briefly, the sections were passed through graded alcohol and labeled with bromodeoxyuridine triphosphate, washed twice with phosphate-buffered saline, and labeled with bromodeoxyuridine triphosphate by the terminal deoxynucleotidyl transferase enzyme for 2 h at 37 °C. After labeling, sections were washed and stained with fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine monoclonal antibody for 30 min in a low-light environment. RNase was added and samples were incubated for an additional 30 min at room temperature. Slides were rinsed 3–4 times with 1× phosphate-buffered saline before being mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA). The percentage of fluorescein isothiocyanate-positive cells was analyzed by fluorescence microscopy using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green). Negative controls included sections incubated in the absence of substrate.

**RNase Protection Assay**—Total RNA was isolated from WT and Tg mice hearts and human heart samples using TRIzol reagent (Invitrogen). RNase protection assays (RPAs) were done using the RiboQuant system with a multiprobe template set from BD Pharmingen. For mice, the mAPO-1, mAPO-2, mAPO-3, and mCYC-1 template sets were used for T7 polymerase directed synthesis of high specific activity [32P]UTP-labeled antisense RNA probes. The probe sets contained 13 probes including two housekeeping genes, GAPDH and L32. Probes (4 × 105 cpm) were hybridized with each RNA (10 μg) sample overnight at 56 °C. RNA samples were digested with RNase A and T1, purified, and resolved on 6% denaturing polyacrylamide gels. Internal housekeeping genes were analyzed to confirm equal RNA loading. For failing and nonfailing human heart samples (n = 5), multiprobe template sets hAPO-1c, hAPO-2c, hAPO-3, and hCYC-1 were used for RPA, following the manufacturer’s protocol.

**Immunohistochemistry**—Myocardial sections were stained with antibodies against Fas, Fas-associated death domain (FADD), the cleaved active form of caspase-8, -7, and -3, or the macrophage markers CD13 and CD14 (BD Pharmingen). The sections were then stained with propidium iodide and analyzed by fluorescent microscopy (26).

**Caspase Activity Assay**—The activity of caspase-3, -8, and -9 was measured using a commercially available caspase assay kit (Clontech). Briefly, tissues were washed twice with cold phosphate-buffered saline and lysed on ice in 1× lysis buffer (50 mM Tris-Cl, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 μg/ml pepstatin A), and aliquoting containing 40 μg of protein were fractionated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were subsequently incubated with monoclonal antibody to Bax, Bcl2, Fas, tumor necrosis factor (TNF)-α RI and RII (Santa Cruz Biotechnology Inc.), polyclonal antibody to active caspase-3 (BD Pharmingen), caspase-8 (Stressgen Biotechnologies Corp., Victoria, BC, Canada), Bcl-X (Transduction Laboratories, San Diego, CA), and polyclonal antibody to cyclin A, B1, or B2 (Santa Cruz Biotechnology) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce). Immunoreactive bands were visualized using enhanced chemiluminescence (PerkinElmer Life Sciences). Equal protein loading was confirmed by staining the gel with Coomassie Blue and probing the nitrocellulose GAPDH (Amersham Life Sciences, Arlington Heights, CO).

**Cyclin-dependent Kinase Activity Assay**—Six hundred micrograms of tissue lysate from Tg and WT hearts lysed in buffer (50 mM Heps, pH 7.0, 150 mM sodium chloride, 10% glycerol, 0.1% Tween 20, protease inhibitor mixture (Calbiochem, San Diego, CA), 0.5 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, and 5 mM sodium fluoride) were immunoprecipitated with polyclonal antibody to Cdk2, Cdk2, or Cdk4 (Santa Cruz Biotechnologies) for 2 h at 4 °C. The protein A-Sepharose beads containing the immunocomplexes were incubated with 25 μl of kinase buffer, 2 μg of histone H1 (as substrate for Cdk2) or 0.5 μg of retinoblastoma p105 (as substrate for Cdk4, Cdk2, or Cdk4) (Santa Cruz Biotechnologies) for 1 h at 30 °C. The kinase reactions were stopped by the addition of 50 mM EDTA and probed by SDS-PAGE and immunoblotting with anti-Cdk2 or anti-Cdk4 antibody. The results were revealed by enhanced chemiluminescence (PerkinElmer Life Sciences).
involved in the death receptor pathway were analyzed using a matched WT or 4-week-old Tg hearts. Transcript levels of genes in the 9-month-old Tg hearts compared with either the age-matched WT and Tg mouse hearts (n = 6). Hearts were perfused with perfusion buffer (glucose, 1 g; NaHCO3, 0.58 g; and pyruvic acid, 0.27 g, pH 7.3) with 95% O2 and 5% CO2 on a Langendorff apparatus. After perfusing the heart for 10 min in EGTA-supplemented perfusion buffer, hearts were digested using collagenase (2 mg/ml) for 28 min, with gradual enhancement of CaCl2. After 28 min of digestion with collagenase, the heart was taken out and incubated in a digested collagenase solution for 10 min in a shaking water bath at 37°C. The ventricles were separated from the atria, triturated for 30 s, and subsequently filtered through cheesecloth. The filtrate was centrifuged at 400 rpm for 2 min, the supernatant was removed, and the pellet was resuspended in 4% bovine serum albumin solution and observed under a phase-contrast microscope. Preparations with 80–85% beating rod-shaped cells were used for experimental purposes.

Isolation of Nuclear Protein from the Isolated Myocytes—Nuclear protein was prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce), using the manufacturer’s protocol. Both cytoplasmic and nuclear fractions were collected, the amount of protein was measured using standard techniques, and Western blots were performed as described earlier with primary antibodies. Twenty micrograms of nuclear protein was used to detect proteins using monoclonal antibodies to poly(ADP-ribose) polymerase (PARP) (Biomol, Plymouth Meeting, PA), pCNA (Santa Cruz Biotechnologies), and phosphohistone H3 (Cell Signalling Technology, Beverly, MA). Twenty micrograms of cytoplasmic proteins was used to detect c-kit and Sca-1 (R&D Systems, Minneapolis, MN) using the respective antibodies. These blots were probed with GAPDH antibody as a loading control.

Statistical Analysis—Each experiment was repeated at least five times. Results were expressed as mean ± S.E. Data were analyzed by two-way analysis of variance, and differences between groups were determined by the least-square means test (SUPERNOVA). Significance was evaluated using the analysis of variance test. A value of p < 0.05 was considered significant.

RESULTS

Detection of Cell Death in Heart Samples of Tg and WT Mice: TUNEL Analysis

Cell death (apoptosis) was compared in heart tissue (n = 6) from 4-week-old and 9-month-old Tg mice with significant hypertrophy (heart weight/body weight ratio of 10.4 ± 0.4 compared with 4.7 ± 0.1 in WT) and age-matched WT mice by TUNEL staining. In 9-month-old WT hearts, only 5–8 nuclei per 105 cells were TUNEL positive (Fig. 1A). Apoptotic nuclei were absent in young (4-week-old) transgenic heart sections. This value was markedly increased in ventricular sections of failing hearts from 9-month-old Tg mice in which TUNEL-positive cells appeared to be distributed toward the distal end of the myocardium. The number of TUNEL-positive nuclei varied between 85 and 185 per 105 cells among different heart sections from 9-month-old Tg mice (n = 12). There were an average of 130 TUNEL-positive nuclei per 105 cells (Fig. 1B), resulting in an almost 12-fold increase in the number of apoptotic cells in failing hearts compared with nonfailing hearts of the same age group.

Comparison of Apoptotic Gene Expression between Initiation and Progression of Disease Process

RNA Profiling: by RNase Protection Assay—RPA studies were performed using RNA from 4-week-old and 9-month-old WT and Tg mouse hearts (n = 5) with mouse multiprobes mAPO1, mAPO2, and mAPO3 (BD Biosciences Pharmingen; Fig. 2). Several apoptosis-regulating genes were up-regulated in the 9-month-old Tg hearts compared with either the age-matched WT or 4-week-old Tg hearts. Transcript levels of genes involved in the death receptor pathway were analyzed using a mAPO1 probe set (caspase-8, Fas, FADD, Fas-associated phosphatase, Fas-associated factor, TNF-α-related apoptosis-inducing ligand, TNF-α Rp55, TNF-α receptor-1-associated death domain protein, RIP, L32, and glyceraldehyde-3-phosphate dehydrogenase). Four genes were up-regulated in 9-month-old Tg mice compared with the age-matched wild-type controls or 4-week-old Tg mice: Fas >5-fold, FADD >4-fold, TNF-related...
apoptosis inducing ligand >2-fold, and TNF-α receptor (Rlp55) >4-fold (Fig. 2A, p < 0.01).

Changes in the expression of mitochondrial genes in the Bcl2 family (mAPO2) were compared in 4-week-old and 9-month-old Tg mice and WT controls. Bcl-1, Bcl-w, Bax, and Bcl-X transcripts were up-regulated significantly (>4-fold) in 9-month-old Tg mice compared with the WT or 4-week-old Tg mice (Fig. 2B, p < 0.01). Bcl2 transcripts were up-regulated by 2-fold in failing heart samples. No significant difference was observed in levels of Bax and Bad transcripts during initiation and transition phases of the disease.

Significant up-regulation of the initiator and effector caspases, namely, caspase-3, -7, -8, and -12, were observed in failing hearts compared with normal or 4-week-old hearts during initiation of hypertrophy (Fig. 2C). No significant differences were observed in caspase-6, -2, and -1 transcripts between Tg and WT hearts. On the other hand, a >2-fold increase in caspase-X and -11 transcripts was observed in failing heart samples. No significant difference was observed in expression levels of the caspases between 4-week-old WT and Tg. However, a significant increase in expression of active caspases was observed in 9-month-old Tg mice hearts compared with either age-matched WT or 4-week-old Tg mice. Induced expression of Bax protein was observed by Western blot analysis during the initiation phase of hypertrophy (4-week-old Tg compared with age-matched WT), although induced Bax protein was not observed in these hearts by immunohistological staining.

(iii) Fig. 4A shows immunohistochemistry using antibodies against active fragments of caspase-3, -7, and -8 (n = 5). Data showed no difference in expression levels of the caspases between 4-week-old WT and Tg. However, a significant increase in expression of active caspases was observed in 9-month-old Tg mice hearts compared with either age-matched WT or 4-week-old Tg mice. Cleavage of caspase-3 and -8 was further confirmed by immunoblot analyses. Active fragments (p17 and p32) were detected in the failing hearts (9-month-old Tg) only but not in nonfailing hearts from WT or 4-week-old Tg mice (Fig. 4B).

(iv) Induction of infiltrating macrophages, CD13 and CD14, was detected by immunohistological staining in failing heart sections only (9-month-old Tg; Fig. 4C, n = 5). Infiltration of macrophages was not detected in any of the nonfailing heart samples (9-month-old WT or 4-week-old Tg). High numbers of infiltrating macrophages in failing hearts may be involved in phagocytosis of dead cells in the tissue.

Biochemical Analysis of Activity of Caspases in Cellular Extracts of Tg and WT Hearts—Because immunoblot analyses confirmed the presence of active caspases in the Tg hearts, caspase activity was measured in hearts from both Tg and WT mice using specific fluorogenic substrates (ApoAlert; Clontech, Palo Alto, CA) for caspase-3, -8, and -9. No difference was observed in caspase-3 activity levels between heart samples of 4-week-old WT and Tg mice. A slight but nonsignificant in-
crease in caspase activity was also detected in 4-week-old Tg hearts compared with age-matched wild-type hearts, especially for caspase-8 and -9. Activity of the executor caspase, caspase-3 was increased 92.5% in hearts from 9-month-old Tg mice, whereas the activities of the initiator caspases, caspase-8 and -9, were increased by 59 and 79%, respectively, compared with...
Consequence of Long-standing Hypertrophy on the Cell Cycle Regulator Genes in Mice

Despite significant cell death in Tg mice hearts, the heart weight:body weight ratio was $>12$, implicating either increased cell division (mitosis), cell enlargement, or both. To analyze how the transition from hypertrophy to heart failure in 9-month-old Tg hearts affects the expression of cell cycle regulatory genes, both RPA and immunoblot analyses were performed.

RPA Analysis Using Cyclin Multiprobes—RPA studies using the mouse mCdk3b multiprobe of cell cycle regulatory genes showed significant up-regulation of different cyclin transcripts in the hearts of Tg mice. Maximum induction was observed for cyclin B1 and B2 transcripts ($>4$-fold) in failing heart. The cyclin D family (D1, D2, and D3) was up-regulated by 2-fold, cyclin A2 by 3-fold, and cyclin C by 1.2-fold in hearts of 9-month-old Tg mice, compared with 9-month-old WT ($n = 5$; Fig. 6A). Some of the cyclin genes (cyclin C, D2, and D3) were induced in the 4-week-old Tg heart samples (although to a much lesser degree than 9-month-old Tg) when compared with age-matched WT samples.

Immunoblot Analyses—Changes in the cell cycle regulator genes (cyclin A, B1, D1, and D3) between WT and Tg heart samples were further confirmed at the protein level. Western blot analysis showed maximum up-regulation of cyclin A as $>4$-fold, cyclin B1 as $>3$-fold, cyclin D1 as $>5$-fold, and cyclin D3 as $\geq 2$-fold ($n = 5$; Fig. 6B) in 9-month-old Tg animals compared with age-matched WT or 4-week-old Tg hearts. Some induction (although nonsignificant) in the protein level of these cyclins was also observed in Tg mice at as early as 4 weeks, during initiation of hypertrophy, when compared with their age-matched WT.

Changes in Cyclin-dependent Kinases—Cdks regulate the action of the cyclin genes. Cdk-1 (Cdc2), Cdk-2, and Cdk-4 bind to cyclin A, B, and D, respectively. Once it was determined that cyclin genes were up-regulated in hypertrophic heart, immunoblot analyses were done to analyze the protein expression of...
different Cdk. However, only Cdk-1 (not Cdk-2 and -4) showed a significant change in protein expression levels in 9-month-old Tg animals, compared with either age-matched WT or 4-week-old Tg (Fig. 6C).

However, because kinase activity is regulated during the cell cycle, in vitro kinase assays were performed to assess changes in kinase activity during the transition from hypertrophy to heart failure. The kinase activities for Cdk-1, -2, and -4 were significantly elevated in failing hearts compared with nonfailing animals. Maximum induction of kinase activity was observed for Cdk-2 (about 4-fold) and 2.5-fold increase in activity was documented for Cdk-4 and Cdk-1 in hearts from 9-month-old Tg mice (Fig. 6D, n = 5, p < 0.01). No change in kinase activity was documented in 4-week-old Tg and age-matched WT.

**Cell Death and Regeneration Occurs in Myocytes of Failing Murine Hearts**

Because we had already shown that apoptosis and cellular regeneration occur during the transition of hypertrophy to heart failure, our next goal was to confirm whether cell death and regeneration occurs in cardiac myocytes in the failing heart. As shown in Fig. 7A many myocytes in 9-month-old Tg heart sections detected by α-actinin (red) were also positive for active caspase-3 protein (green; panel b). WT sections (panel a) did not show the presence of active caspase-3 proteins in myocytes stained with α-actinin. The subcellular localization of active caspase-3 and α-actinin varied between each other, whereas both of them are present in the cytoplasm of the myocytes. Analysis of confocal microscopic studies involved myocardial sections from five different WT and Tg mice.

Nuclear protein isolated from myocytes of WT and Tg mouse hearts during initiation of hypertrophy as well as transition from hypertrophy to heart failure (4-week-old and 9-month-old mice) showed intact PARP protein (116 kDa) in protein samples of both 4-week-old WT and Tg as well as 9-month-old WT myocytes. Cleavage of the PARP protein (89 kDa) was observed in 9-month-old Tg myocytes (n = 5; Fig. 7B). The induced expression of active caspase-3 protein and the appearance of an 89-kDa cleaved product of PARP protein in cardiomyocytes of the 9-month-old Tg heart document the occurrence of PCD in myocytes during heart failure.

Some of the myocyte nuclei stained positive for the Ki-67 protein in the same myocardial sections of failing mouse heart (Fig. 8A, panel A–D). No Ki-67-positive myocytes were documented in myocardial sections of age-matched WT or in 4-week-old Tg mice. The nuclei in these sections were counterstained with DAPI (blue). Ki-67 immunostaining (green) was observed in nuclei of some myocytes, whereas the cytoplasm was labeled with α-actinin (red) antibody. The Ki-67-positive nuclei in myocytes were few in the failing heart sections (the number of Ki-67-positive myocytes in 9-month-old Tg hearts was 23 ± 0.89 in 2253 ± 53.3 myocytes examined, n = 5; p < 0.01).

Significant induction of several other cell cycle marker proteins by Western blot was observed in isolated cardiac myocytes from 9-month-old Tg mice compared with either age-matched WT or 4-week-old Tg or WT mice (Fig. 8B). Proliferation of cell nuclear antigen (pCNA, panel 1) and phosphohistone H1 (Ser-10) protein (panel 2) showed maximum induction in myocytes during heart failure (9-month-old Tg) compared with either age-matched WT mice or during the initiation phase of hypertrophy (≈5-fold, n = 5, p < 0.01). An almost 2-fold induction for c-kit (panel 3) and Sca-1 (panel 4) proteins was also observed in 9-month-old Tg myocytes compared with myocytes isolated from nonfailing heart samples (Fig. 8B). Furthermore, we colocalized overexpression of cyclin B1 protein and phosphohistone H3 in the myocardial sections from 9-month-old Tg mice. We also confirmed the presence of these cell cycle marker proteins in myocytes that stained positive with α-actinin, as shown in Fig. 8C. Induction of these proteins in 9-month-old Tg myocytes clearly indicates that the myocytes are beginning to undergo or actually undergoing cell division during the transition of hypertrophy to heart failure, a state that is not documented during onset of hypertrophy or during progression of this disease process.
Changes in Apoptotic and Cell Cycle Regulator Genes in DCM Human Hearts

RPA Analysis—Several apoptotic genes both upstream and downstream and cell cycle regulator genes (similar to the murine heart failure model) were found to be up-regulated in DCM hearts compared with NF samples. Among the apoptotic genes, FAS, TRAIL, several death receptors (DR3 and DR4), BCL-X, BAX, and BCL2 were significantly up-regulated in DCM hearts compared with nonfailing samples. Among several caspase transcripts, caspase 3 and caspase 9 were maximally up-regulated in failing hearts similar to what was observed in the myotrophin-overexpressed Tg mouse model. Cell cycle regulator genes like cyclin A, cyclin C, and cyclin D were also turned on in DCM hearts. Immunohistology and Confocal Microscopy—To confirm our observation that apoptosis and cell regeneration occur simultaneously in cardiomyocytes during heart failure in the Tg mouse model, confocal microscopy studies were performed with active caspase 3 (marker for apoptosis) and Ki-67 (marker for cell regeneration) on sections of DCM and nonfailing heart samples. Our results clearly show that active caspase 3 protein was found to be significantly up-regulated in failing cardiomyocytes (positive for α-actinin) of DCM heart sections compared with nonfailing sections. 1.2% of Ki-67 positive cardiomyocyte nuclei were also observed in DCM heart sections, whereas no Ki-67 positive myocytes was found in nonfailing sections (Fig. 9B). Induced active caspase 3 and presence of Ki-67 positive nuclei in myocytes of DCM hearts corroborates our claim that apoptosis and cell regeneration occur simultaneously during heart failure only, as seen in the myotrophin overexpressed Tg mouse model that closely mimics human heart failure.

DISCUSSION

In this study, we have demonstrated a prevalence of DNA damage in hearts from 9-month-old Tg mice during the transition from long standing hypertrophy to heart failure, compared with the initiation phase, despite the presence of significant hypertrophy. We have also shown the induction of the cell regenerative machinery, especially in cardiac myocytes of the failing hearts. Importantly, human DCM heart samples also show similar changes in several genes for apoptosis and cell

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regeneration, compared with NF heart. Our data in the Tg mouse model (which specifically overexpresses myotrophin in the heart, develops hypertrophy at as early as 4 weeks of age and gradually progresses to heart failure around 36 weeks of age) convincingly suggest that cell death and regeneration take place simultaneously during the transition of hypertrophy to heart failure. In this model of heart failure, TUNEL-positive nuclei were mostly localized in the subendocardial region of the left ventricle. In addition, the apoptotic nuclei were concentrated in areas where interstitial fibrosis was more prevalent. In left ventricular hypertrophy, apoptosis was confined within the free walls of the left ventricle, and only a few TUNEL-positive nuclei were observed in the septal region. Our data share similarities with data from the pathological analysis conducted on spontaneously hypertensive rats, in which left ventricular dysfunction was accompanied by increased cardiac apoptosis that was more frequent in free walls than in the septal region of the left ventricle (10). The number of apoptotic cells reported by these investigators and others (11) was similar to our findings.

Because it is difficult to discriminate between the individual contributions of apoptosis and necrosis during transition from hypertrophy to heart failure, quantification of the expression of different genes involved in the apoptotic pathway provides a good index of the probability that a cell will undergo apoptosis. We observed changes in some of the key apoptotic genes at the level of transcription and translation during the transition to heart failure. This result not only confirms our claim that PCD is active in this end stage of the disease process, but also implicates the probable death signal pathway in failing hearts.

Our data show that PCD or apoptosis proceeds through the classical pathway involving the upstream activators TNF-α or Fas. Significant up-regulation of adaptor molecules such as Fas or TNF-α was observed in the failing heart samples compared with the WT samples. The receptor molecules and the corresponding death domains were also induced, as well as the initiator (caspase-8) and effector caspases (caspase-3 and -7). Cleavage of caspase-3 was observed in 9-month-old Tg hearts, but this 17-kDa cleaved product was absent in the nonfailing heart samples (Fig. 4B). Caspase activity was significantly higher for caspase-9, -8, and -3 in failing hearts (Fig. 5). Although PCD was confirmed and possible apoptotic pathways were determined for the transition from hypertrophy to heart failure in this Tg mouse model, we could not determine exactly when this process is initiated in a hypertrophic heart. Our studies point toward the fact that PCD does not begin with the onset of hypertrophy but rather is more predominant as the hypertrophic heart progresses toward failure. It was also shown, by confocal microscopy, that active caspase-3 was present in the cytoplasm of myocytes of failing hearts (Fig. 7A). A cleaved product of the PARP protein (89 kDa) was also detected in myocytes of failing heart samples (Fig. 7B). The observed increase in caspase-3 activity, a key intermediate in the activation of apoptosis in many cell types (12) (including myocytes) (13), as well as its cleavage in the failing heart, is consistent with the conclusion that apoptosis is prevalent in the remote myocardium, especially in myocytes, during transition from hypertrophy to heart failure.

Apoptosis is initiated by the withdrawal of specific factors, and the addition of other relevant factors may prevent cell death. Certain oncogenes modulate apoptosis; Bcl2 expression has been reported to inhibit apoptosis (14), whereas p53 (15) and c-myc protein (16) may induce its development. We have also observed a significant increase in expression of c-myc and p53 protein in the failing heart samples. Some earlier reports have examined changes in the pro-apoptotic gene Bax and the anti-apoptotic gene Bcl2. In failing human hearts, Olivetti et al. (5) showed enhanced expression of BCL2 in decompensated hearts compared with normal hearts, but the expression of Bax

[Fig. 7A. Confocal microscopic analysis of 9-month-old WT and Tg mice heart shows the presence of active caspase-3 (green) and α-actinin (red) immunoreactivity in Tg heart sections (scale bar = 20 μm). Panel a, 9-month-old WT heart sections showed little or no caspase-3 immunoreactivity, but were stained positive for α-actinin antibody (red). Panel b, age-matched Tg sections show the presence of active caspase-3 protein in cells positive for α-actinin (red). The nuclei are stained with DAPI (blue). B. Western blot analysis using anti-PARP antibody to the nuclear protein isolated from myocytes from WT and Tg mice hearts. Myocytes were isolated from 4-week-old and 9-month-old WT and Tg mice hearts (n = 5). Nuclear protein, from these cells was isolated as described earlier. The blot shows a cleaved PARP fragment of 89 kDa, in the 9-month-old Tg heart samples only during the transition from hypertrophy to heart failure. The cleaved product was absent in all nonfailing myocytes. The blot represents results of five independent experiments.

FIG. 8. A, confocal microscopic analysis of transgenic mice heart sections shows triple staining for Ki-67, α-actinin, and DAPI. Ki-67 positive nuclei were observed in myocytes of 9-month-old Tg mice ventricular sections only. Ki-67 positive nuclei were absent in myocytes from 4-week-old Tg mice during the initiation phase of hypertrophy. Panel a, immunohistochemistry for Ki-67 (green); panel b, double staining for Ki-67 (green) and DAPI (blue); panel c, double staining for α-actinin (red) and Ki-67 (green); panel d, triple staining for α-actinin (red), Ki-67 (green), and DAPI (blue). Some of the nuclei, but not all, present in the scanned area were positive for Ki-67-immunoreactivity (0.08%). None of the myocytes in 9-month-old WT heart sections stained positive for the Ki-67 antibody. Magnification is the same among panels of Fig. 8A (scale bar = 30 μm). B, Western blot analysis of several cell-regeneration marker proteins in myocytes isolated from hearts from 4-week-old and 9-month-old WT and Tg mice (n = 5). Nuclear protein from WT and Tg myocytes from different age groups shows significant induction of pCNA (panel 1) phosphohistone H3 (Ser-10) proteins (panel 2) in failing hearts of 9-month-old Tg compared with either 4 Tg or 9 WT samples. Induction of phosphohistone H3 in isolated myocytes of failing hearts indirectly confirms that some myocytes are undergoing cell division in failing hearts. Significant induction of c-kit (panel 3) and Sca-1 (panel 4) protein levels detected in the cytoplasmic fraction of myocytes isolated from failing heart samples compared with 9 WT or 4 Tg. GAPDH antibody was used as a loading control. The picture represents results from five independent experiments. C, confocal microscopic analysis of 9-month-old transgenic mice heart sections showing triple staining for cyclin B1 (red), phosphohistone H3 (green), and DAPI (blue). Cells overexpressing cyclin B1 also found positive for phosphohistone H3. Phosphohistone H3-positive nuclei were observed in myocytes (actinin positive, shown in inset) of similar sections.
protein was not altered. The enhanced expression of the anti-apoptotic gene in failing hearts, as the authors suggested, was because of compensatory activation mechanisms in overloaded myocardium attempting to maintain cell survival. Ikeda et al. (17) reported no change in the expression of either Bax or Bcl2 proteins between the stages of hypertrophy and failure in spontaneously hypertensive rats. Induction of Bax, but not Bcl2, was observed during the transition to left ventricular dysfunction during chronic pressure overload in rats (11). Our study in 9-month-old Tg mice overexpressing myotrophin showed a significant increase in both the pro-apoptotic gene Bax, as well as the anti-apoptotic gene Bcl2, at both RNA and protein levels during transition from hypertrophy to heart failure. Levels of Bcl-xl and Bf-1 transcripts were also elevated in the failing heart (Fig. 2). Considerable increases in Cd13 and Cd14 macrophages were also observed in failing hearts, suggesting that the dying cells are ingested by infiltrating macrophages in the failing myocardium (Fig. 4C).

Like apoptosis, cell division is a fundamental and ubiquitous process in multicellular organisms. The molecules that regulate cell cycle progression, cyclins and Cdks (18, 19), are well characterized. The kinase activity of Cdks is dependent on the
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presence of activating subunits, the cyclins. Evidence exists to suggest that apoptosis and the cell cycle may be interconnected (20). For example, expression of the proto-oncogene c-myc stimulates cell proliferation and can also predispose cells to apoptosis when growth factors are limiting (16). Recent work now indicates that the apoptotic regulatory proteins themselves can directly impinge on the cell cycle machinery (21, 22). Thus as a cell progresses through the cell cycle, it must determine whether to complete cell division, arrest growth to repair cellular damage, or undergo apoptosis if the damage is too severe or if the cell is incapable of repairing the DNA. Our data show significant up-regulation of different cyclin genes (A2, B1, B2, D1, and D2) in failing hearts compared with the age-matched WT hearts, at the transcription and translation levels. Some increase in these proteins in 4-week-old Tg mice compared with age-matched WT mice seems logical, given the need for growth during this period. We also observed significant induction of Cdk activity in hearts from 9-month-old Tg mice. Cdk1 and Cdk2 were induced more than 4-fold in failing hearts compared with nonfailing hearts (Fig. 6D). So the question remains, why is remodeling needed, especially at the end stage? We postulate that, after longstanding hypertrophy, the myocardial response to compensate for cell loss causes more cardiac cells to reenter the cell cycle. Our results suggest that c-myc and cyclins are involved at an important nodal point shared by pathways regulating cellular proliferation and apoptosis. It is possible that protection against cell death by Bcl2 indirectly augments the induction of multiple cyclins and Cdns.

In this study, we have documented significant changes in several cell cycle regulatory proteins and regulatory kinases in failing hearts. The expression of pCNA, proliferation associated nuclear antigen Ki-67, has been demonstrated in heart sections undergoing severe stress (23). Phosphorylation at Ser-10 of histone H3 is tightly correlated with chromosome condensation during both mitosis and meiosis (24). We have documented the presence of the Ki-67 protein in the myocyte nuclei of failing Tg hearts (Fig. 8A). Bromodeoxyuridine-positive nuclei (34 ± 5.54 out of 3893 ± 423 cells examined) were identified in some myocytes of failing heart sections. Mitosis markers like phosphohistone H3 (Ser-10), pCNA, c-kit, and Sca-1 were detected in much higher amounts in isolated cardiac myocytes of 9-month-old Tg mice, compared with myocytes from nonfailing (WT and 4-week-Tg) heart samples (Fig. 8B). More importantly, colocalization of overexpressed cyclin and phosphohistone H3 was observed in myocytes from failing myocardial sections of Tg mice (Fig. 8C). Significant up-regulation of these cell cycle marker proteins in myocytes of 9-month-old Tg hearts convincingly points toward a regeneration process in failing hearts and specifically provides evidence of proliferation of cardiac myocytes in response to stress. Although one can argue that the up-regulation of cell regeneration markers may be because of nuclear division in myocytes, as myocytes are known to be multinucleated. However, we have always compared our data to age matched WT mice where no evidence of such cell regeneration was observed. Our data thus suggest, for the first time, that both cell death (Fig. 7) and cell regeneration (Fig. 8) occur in the myocytes (although the frequency is small, ~0.08%) during the transition from hypertrophy to heart failure, although the origin of cycling myocytes in heart failure is an interesting and yet unsolved and debatable issue. To validate our data that myocytes do undergo simultaneous apoptosis and cell regeneration during heart failure in Tg mice hearts, we have compared the gene profiles of several apoptotic and cell cycle regulator genes in DCM human hearts, compared with nonfailing ones. The transcript profiles of several apoptotic as well as cell cycle regulator genes have shown similar changes between DCM hearts and murine heart failure model overexpressing myotrophin. We have also shown up-regulation of active caspase 3 protein and Ki-67 positive nuclei in myocytes of DCM heart samples (Fig. 9B). Dividing myocytes may also originate from cardiac stem cells or from migratory stem cells. As reported by Beltrami et al. (25) stem cells may regenerate myocytes that have been lost by severe stress, then go into “overdrive” in response to significant myocyte loss. Myocyte proliferation may be a component of the growth reserve of the heart upon demand, and there is evidence that regeneration in myocytes may challenge the dogma that the heart is a post-mitotic organ (6, 23). Furthermore, the ability of the heart to replace damaged myocardium and induce cell division during failure suggests that there is a continuous turnover of cells during the lifespan of the organism. Because the heart ultimately goes to failure, it can be postulated that under severe stress, the cell death process ultimately overtakes the regeneration machinery in the defective myocardium during heart failure. Apoptosis and cell regeneration thus can be because of a combined effect of neurohumoral changes and mechanical factors in addition to increased cardiac mass, which triggers the heart to go to failure with severely compromised cardiac function. Studies are underway to explore the frequency of cell death in cell types other than cardiac myocytes. Because our data showed that cell death and regeneration do not occur during the early phase of hypertrophy (onset of the disease), it would also be important to define the precise time point at which apoptosis and particularly the cell regeneration process starts during the transition of hypertrophy to heart failure in both murine and human heart failure model. This information would be necessary to determine the optimal time to start treatment to prevent this deadly disease.

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REFERENCES
