Programmed cell death is controlled by 2 main signaling pathways that process apoptotic stimuli—the intrinsic mitochondrial and extrinsic death receptor–mediated pathways (1). Regardless of the specific induction pathway, a common proteolytic cascade of caspases is activated. These proteases regulate the execution of the mammalian cell death program.

Caspases represent a family of aspartate-specific proteases that exist as precursor molecules in the cytosol and mitochondrial intermembrane space. Caspase-3, -8, and -9 are thought to be important in cardiomyocyte apoptosis (2). Activation of caspase-8 occurs through death receptors, including Fas and tumor necrosis factor receptor, which contain an intracytoplasmic part known as the death domain (3). After binding of adapter proteins and interaction with death effector domains, they are able to induce cleavage of procaspase-8 into its active form. Activation of caspase-9, however, is ultimately controlled by the proapoptotic and antiapoptotic members of the bcl-2 family, which modulate the mitochondrial response to apoptotic signals (4). Subsequent opening of mitochondrial permeability transition pores and formation of the apoptosome results in cleavage of bound procaspase-9 into its active moiety (5). Both of these upstream initiator caspases, 8 and 9, induce activation of the downstream caspase-3, which affects the nuclear apoptotic degradation process by cleaving caspase-activated deoxyribonuclease from its inhibitor. This inactivates proteins involved in deoxyribonucleic acid (DNA) repair and replication such as poly-adenosine diphosphate-ribose polymerase and directly disassembles cell structures such as nuclear lamins (6).

Several studies have previously examined caspase activities in heart failure, with varying results. In the first, increased cleaved caspase-3 was observed in patients with dilated and ischemic cardiomyopathy in comparison to those with “normal” hearts (7). These investigators further...
reported that although caspase-3 undergoes activation, it gets inhibited by up-regulation of antiapoptotic factors, such as XIAP (8). Inhibition of caspase-3 is consistent with results of subsequent studies showing that up-regulation of caspase-8 and -9 was shown in the failing human heart, caspase-3 activity was not seen (9), or only minimal activation of caspase-3 activity was observed in failing human hearts (10–12).

In this study, left ventricular (LV) endomyocardial tissue was obtained serially from an ovine model of pressure overload during the transition to heart failure. These biopsies were used to correlate caspase activity and presence of apoptosis with LV echocardiographic changes.

**METHODS**

**Animal model.** A total of 10 adult male Naemy sheep weighing 34 to 42 kg were used in the study. After induction of general anesthesia, a variable aortic constriction device was passed around the ascending aorta just proximal to the origin of the innominate artery (13). The device consisted of a Gore-Tex cuff enclosing the balloon of a Foley catheter (Fig. 1). The proximal port of the catheter was brought out subcutaneously, caudal to the wound, for subsequent inflation and deflation of the balloon. The balloon of the variable constriction device was inflated during the operation to achieve an aortic transcoarctation gradient of approximately 20 mm Hg. Four weeks and 6 weeks postoperatively, the balloon was inflated further to produce an aortic transcoarctation gradient of approximately 40 mm Hg and 80 mm Hg, respectively. The LV tissue was obtained endomyocardially at the initial operation before insertion of the aortic band, at 4 and 6 weeks postoperatively before balloon inflation, and when clinical and echocardiographic signs of LV failure developed in the animals. The biopsies were carried out using a 7-F endoluminal biopmtome (Cordis, Miami, Florida) passed via the right external carotid artery under fluoroscopic guidance. These biopsy samples were stored in 10% neutral buffered formalin for histological studies or snap frozen in liquid nitrogen and then stored at −80°C for biochemical analyses. All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (14) published by the
National Institutes of Health and with full approval of the local Animal Ethics Committee.

**Echocardiographic monitoring.** The sheep underwent echocardiography daily for the first few postoperative days, and then twice weekly. A Vingmed 720 CFM echocardiography machine (Vingmed Holdings AS, Oslo, Norway) was used with a 3.25-MHz and a 5-MHz transducer and a continuous-wave Doppler probe. The sheep were placed in the left lateral decubitus position without sedation or anesthesia. Standard 2-dimensional long- and short-axis transthoracic views from the right sternal edge were obtained to measure LV cavity and wall dimensions in systole (s) and diastole (d). These included LV internal diameter (LVIDd and LVIDs), posterior wall thickness (PWTd and PWTs), and interventricular septal thickness (IVSd and IVSs). The measurements were taken in M-mode at the level of the mitral valve papillary muscles. This allowed the LV mass index (LVMI) (15) and fractional shortening (FS), as a measure of ventricular contractility, to be calculated.

\[
\text{LVMI} (g/m^2) = \frac{LVM (g)}{BSA (m^2)}
\]

where \(LVM\) = LV mass, \(BSA\) = body surface area.

The peak aortic transvalvular gradient was obtained from the suprasternal position by measuring Doppler velocity flow and using the modified Benoulli equation.

**Caspase activity assays.** Total protein was extracted by homogenizing 50 mg LV endomyocardial tissue in ice-cold lysis buffer A (25 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) [pH 7.5 at 25°C], 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM ethylenediamine tetra-acetic acid, 10 mM phenylmethylsulfonyl fluoride) containing 1% (v/v) Triton X-100. After centrifugation for 60 min at 4°C, the supernatant was boiled with 0.33 volume sodium dodecyl sulfate polyacrylamide gel electrophoresis sample remaining supernatant was boiled with 0.1 M methanesulfonic acid pH 6.5, 10 mM DTT for 10 min. The sample was then centrifuged (100,000 \(g\) for 60 min at 4°C) and total protein concentration was determined by the BioRad Bradford method (16). The remaining supernatant was boiled with 0.33 volume sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer and stored at \(-20^\circ\text{C}\). Protein extract (50 \(\mu\)g) and 50 \(\mu\)l specific reaction buffer (0.1 M HEPES pH 7.0, 10% polyethylene glycol (PEG), 0.1% chlormidopropyl dimethylammonium propanesulfonate (CHAPS), 10 mM DTT for caspases 3 and 8 and 0.1 M methanesulfonic acid pH 6.5, 10% PEG, 0.1% CHAPS, 10 mM DTT for caspase-9) were mixed with 2 \(\mu\)l 5 mM specific fluorogenic substrates (DEVD-AFC for caspase-3, LETD-AFC for caspase-8 and LEHD-AFC for caspase-9; Enzyme Systems, Livermore, California) and incubated at 37°C for 2 h. Specificity of these fluorogenic substrates has been described previously (17). Caspase activity was measured in a spectrofluorometer (Perkin Elmer, Boston, Massachusetts) at 400 nm excitation and 505 nm emission wavelengths. Relative caspase activity in the experimental stages was expressed as fold stimulation relative to controls.

**Immunohistochemistry.** Biopsy samples of 4 animals from each stage were analyzed histologically. Samples were immediately fixed in 10% neutral buffered formalin at surgery and then paraffin-embedded within 24 h. Myocardial sections (5 \(\mu\)m) were obtained, mounted on silan-coated slides, deparaffinized, and rehydrated. To optimize antigen retrieval, the sections were placed in 10 mM modified citrate buffer (pH 6.1) and exposed to microwave fixation at 750 W for 10 min. Endogenous peroxidase activity was inhibited by 4% hydrogen peroxide. Sections were then incubated with mouse monoclonal antibody specific for the active p11 fragment of caspase-3 (1:500 dilution, Oncogene Research Products, San Diego, California) for 30 min at room temperature. After rinsing with wash buffer (Dako, Carpinteria, California), the sections were incubated with peroxidase labeled anti-mouse immunoglobulin G secondary antibody (1:200 dilution, Dako) for 30 min. After further rinsing, the sections were stained with 3% diaminobenzidine tetrahydrochloride for 8 min. Counterstaining with 2% methoxylin for 15 s and fixing with 1% lithium carbonate for 15 s allowed the proteins to be identified using light microscopy under \(\times\)40 magnification. Caspase-3 activity was assessed by grading cytoplasmic staining intensity as negative, weak, moderate, or strong. Omission of the primary antibody served as a negative control for each sample. Sheep lymph nodes were used as positive controls.

**Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay.** The TUNEL assay was performed using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, California). Myocardial sections (5 \(\mu\)m) were pretreated with 20 \(\mu\)g/ml proteinase K in phosphate-buffered solution. Endogenous peroxidase activity was inhibited by 3% hydrogen peroxide. The sections were then incubated with 60 \(\mu\)l Terminal Deoxynucleotidyl Transferase (TdT) enzyme and reaction buffer containing digoxigenin-labeled dUTP at 37°C for 60 min. After incubation with 7 ml stop buffer, the sections were incubated with 65 \(\mu\)l anti-digoxigenin antibody conjugated with peroxidase for 1 min at room temperature. The sections were then stained with 100 \(\mu\)l of ready-to-use 3% diaminobenzidine tetrahydrochloride for 8 min. Counterstaining with 2% hematoxylin for 15 s and fixing with 1% lithium carbonate for 15 s allowed the nuclei to be identified using light microscopy under \(\times\)40 magnification. The nuclei were considered to be TUNEL-positive when the nuclei were identified staining dark brown. For negative controls, the TdT enzyme was omitted. Sheep lymph nodes were used as positive controls.
In situ oligoligation (ISOL) assay. This assay was performed using the ApopTag In Situ Oligoligation Kit (Chemicon International). Myocardial sections (5 μm) were initially incubated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity, followed by pretreatment with 20 μg/ml proteinase K in phosphate-buffered solution. After incubation with equilibration buffer, the sections were incubated with a 60-μl solution of T4 DNA ligase and biotinylated oligo A, overnight at 16°C to 22°C. The sections were then incubated with 60 μl streptavidin-peroxidase conjugate for 30 min followed by staining with 100 μl 3% diaminobenzidine for 10 min. Finally, tissue sections were counterstained in 2% hematoxylin for 15 s and fixed with 1% lithium carbonate for 15 s. From each section, 10 light microscopic fields (under ×40 magnification) were used to identify ISOL-positive cells. A total of ~2,500 nuclei per section were examined by a blinded observer. Myocytes were considered to be ISOL positive when the nuclei were identified staining dark brown. For negative controls, the T4 DNA ligase enzyme was omitted. Sheep lymph nodes were used as positive controls.

Statistical analysis. All values are expressed as mean ± SEM. The statistical difference between the 4 stages was determined using repeated measures analysis of variance or paired Student t-tests. A p value of < 0.05 was considered statistically significant.

RESULTS

Echocardiographic changes secondary to pressure overload. In response to progressive pressure overload, hypertrophy of the LV initially developed in the sheep (LVMI 90.8 ± 4.9 vs. 44.0 ± 3.0 g/m², p < 0.01) (Fig. 2A), followed by gradual LV dilatation (LVIDd 4.23 ± 0.08 vs. 3.39 ± 0.07 cm, p < 0.01) (Fig. 2B), and finally deterioration in LV function (FS 18.3 ± 2.4 vs. 46.9 ± 2.6%, p < 0.01) (Fig. 2C). Associated with the deterioration in myocardial contractility, the animals displayed reduced mobility, anorexia, and were tachypneic. Biopsy samples taken from the 10 sheep at these echocardiographically distinct stages (LV hypertrophy, LV dilatation, and LV failure) during the transition to heart failure were then compared with biopsy samples taken before the operative procedure.

Activation of caspase cascade. Activities of caspase-3, -8, and -9 were investigated using specific fluorogenic substrates during the transition to heart failure. On proteolytic cleavage by caspases, these substrates release amino-4-trifluoromethyl coumarin (AFC), which can be detected by a spectrofluorometer. In the initial stages of LV hypertrophy and LV dilatation, biopsies showed significant increases in caspase-3 activity (1.62 ± 0.12 and 1.82 ± 0.13 vs. 1.00 ± 0.15 relative fluorescent units, p < 0.01, respectively) (Fig. 3). In the final stage of LV failure, substantially increased activity of caspase-3 was shown (7.92 ± 1.19 vs. 1.00 ± 0.15 relative fluorescent units, p < 0.01). Regarding caspase-8, no increase in activity was observed associated with the development of LV hypertrophy and dilatation (Fig. 3). Biopsy samples taken from sheep showing LV failure, however, had significantly increased caspase-8 activity (1.94 ± 0.21 vs. 1.00 ± 0.04 relative fluorescent units, p < 0.05). Similarly for caspase-9, there was no significant increase in activity initially (Fig. 3). In the final stages of LV dilation and LV failure, however, there were significant increases (3.34 ± 0.27 and 5.87 ± 0.97 vs. 1.00 ± 0.18 relative fluorescent units, p < 0.01, respectively). Activation of all 3 caspases increased in the transition to heart failure (analysis of variance, p < 0.01).

Immunohistochemical analysis of caspase-3. To localize increased activity of caspase-3, as determined by the caspase activity assays, immunohistochemical analysis of the ovine biopsy samples was performed using an antibody specific for the active p11 fragment of caspase-3. In the positive control sections of sheep lymph node tissue, dark brown cytoplasmic staining for active caspase-3 was observed (Fig. 4A). No staining could be detected when the primary antibody was
omitted from the reaction (Fig. 4B). No cytoplasmic staining for caspase-3 was observed in the biopsy samples taken from the sheep before the aortic banding procedure (Fig. 4C). As the sheep progressed toward pressure overload-induced heart failure, increasing levels of activated caspase-3 were observed, with weak staining in the LV hypertrophy and LV dilation stages (Figs. 4D and 4E). In the final stage of LV failure, moderate staining for active caspase-3 was identified, associated with deterioration of LV function (Fig. 4F). Immunohistochemical analysis confirmed the presence of activated caspase-3 in the cytoplasm of the cardiomyocytes, as opposed to the fibroblasts and other nonmyocytes.

Absence of apoptosis by TUNEL or ISOL. To assess whether this up-regulation in the caspase cascade was accompanied by completion of apoptosis and DNA fragmentation, TUNEL and ISOL assays were performed. In the positive control sections of sheep lymph node tissue, dark brown nuclei staining of the cells within the germinal center was observed by TUNEL and ISOL (Figs. 5A and 6A, respectively). No TUNEL-positive or ISOL-positive cardiomyocytes, however, were observed in any of the animal stages, indicating an absence of apoptotic nuclei (Figs. 5 and 6).

DISCUSSION
Executioner caspases are central to the apoptotic cascade because they mediate both the mitochondrial and the death receptor apoptotic pathways (18). Although their activation has been shown in end-stage heart failure, their exact role in the evolution of hypertrophy to failure remains unknown. In

Figure 3. Caspase activity during the transition to heart failure. Biopsy samples (50 mg, n = 10 from each stage) were taken at echocardiographically distinct stages of normal left ventricle (control), left ventricular hypertrophy (LVH), left ventricular dilation (LVD), and left ventricular failure (LVF). Activities of caspase-3, -8, and -9 were measured using specific fluorogenic substrates (DEVD-AFC for caspase-3, LETD-AFC for caspase-8, and LEHD-AFC for caspase-9) and expressed relative to control samples (mean ± SEM). *p < 0.05; †p < 0.01 comparing stages with control (paired Student t test).

Figure 4. Immunohistochemical staining for active caspase-3 during the transition to heart failure. Sheep lymph node tissue used as (a) positive control and (b) negative control with primary antibody omitted. Biopsy samples (n = 4 from each stage) were taken at echocardiographically distinct stages of (c) normal left ventricle (control), (d) left ventricular hypertrophy, (e) left ventricular dilation, and (f) left ventricular failure and probed with mouse monoclonal antibody specific for the active p11 fragment of caspase-3. Positivity for active caspase-3 is shown by brown cytoplasmic staining (magnification ×20).
this study, a functional assay, cleavage of specific fluorogenic substrates, was used to quantify activity of caspase-3, -8, and -9 during the progression to heart failure.

Previous studies have shown that stretching of cardiomyocytes and increasing LV wall stress can trigger the death receptor pathway (19,20). In an ex vivo model, overstretching rat papillary muscles produced a 21-fold increase in Fas protein expression by immunohistochemical analysis (19). In a subsequent study, increasing wall stress produced an increase in Fas and caspase-8 protein expression by Western blot analysis in an ovine model of coronary microembolization (20). Because caspase-8 activation occurs through death receptor-mediated mechanisms, it is likely that dilatation of the LV is a trigger of this initiator caspase. In this study, no increase in caspase-8 activity was observed in the initial transition to LV hypertrophy. This may be related to the initial hypertrophic response normalizing LV wall stress and preventing stretch-mediated activation of caspase-8. In the final transition from LV dilation to LV failure, however, activity of caspase-8 significantly increased.

Previous studies have also described activation of factors that control the mitochondrial apoptotic pathway being up-regulated by stretch-mediated mechanisms. When subjected to stretching, corresponding to 9% of sarcomere length, rat cardiomyocytes showed an 11-fold increase in p53 protein expression by Western blotting (21). In this study, as with caspase-8, there was minimal increase in the mitochondrial-activated caspase-9, associated with the initial hypertrophic response. In the later stages, however, the myocytes were exposed to increased diastolic load with the onset of LV dilation and failure, resulting in a marked increase in activity of caspase-9.

The death receptor-mediated and mitochondrial apoptotic pathways, through their initiator caspase-8 and -9, respectively, trigger the self-amplifying caspase cascade resulting in activation of the downstream effector caspase-3 (22). In this pressure overload model, caspase-3 activity was significantly increased in all of the stages in the transition to heart failure. This increase was particularly marked in the final transition to heart failure when the myocytes were exposed to maximal stretch, and accompanied a significant

Figure 5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) in the transition to heart failure. Sheep lymph node tissue used as (a) positive control and (b) negative control with TdT enzyme omitted. Biopsy samples (n = 4 from each stage) were taken at echocardiographically distinct stages of (c) normal left ventricle (control), (d) left ventricular hypertrophy, (e) left ventricular dilation, and (f) left ventricular failure and subjected to peroxidase labeled TUNEL assay. The TUNEL-positivity is shown by dark brown staining nuclei (magnification ×20).

Figure 6. In situ oligoligation (ISOL) in the transition to heart failure. Sheep lymph node tissue used as (a) positive control and (b) negative control with T4 DNA ligase enzyme omitted. Biopsy samples (n = 4 from each stage) were taken at echocardiographically distinct stages of (c) normal left ventricle (control), (d) left ventricular hypertrophy, (e) left ventricular dilation, and (f) left ventricular failure and subjected to peroxidase-labeled ISOL assay. The ISOL positivity is shown by dark brown staining nuclei (magnification ×20).
deterioration in myocardial contractility and ventricular function. The immunohistochemical analyses confirmed that increased activity detected by fluorogenic assays represented caspase-3 as opposed to other effector caspases that cleave DEVD-AFC (17), and that the up-regulation of caspase-3 activity originated from cardiomyocytes.

It has previously been thought that caspases represent the point of no return in the apoptotic process and that once activated, the system will progress inexorably toward cell death (18). Despite increased activity of caspase-3, -8, and -9, however, no apoptotic nuclei were detected by either TUNEL or ISOL in the ovine model of heart failure. The majority of studies that have proposed apoptosis as a cause of ongoing myocyte loss in heart failure have also used the TUNEL assay to define this mode of cell death (23,24). The specificity of TUNEL, however, has been called into question with TUNEL-positive nuclei also obtained from cardiomyocytes undergoing DNA repair (25). Furthermore, several studies more recently have failed to show TUNEL positivity when assessing for cardiomyocyte apoptosis in patients with end-stage heart failure (26,27), consistent with the results of this study. Variations in the staining procedure can cause differing results because access of the terminal deoxynucleotidyl transferase to the strand breaks depends on reagent concentration, efficiency of proteolytic pretreatment, and tissue fixation (28,29). Careful standardization of the conditions is therefore very important. Previous studies that have shown the presence of TUNEL- and ISOL-positive nuclei in human heart failure have often used explanted hearts, with a variable duration between explantation and tissue processing (23,24). In this study, fresh endomyocardial biopsy samples were obtained and immediately snap frozen in liquid nitrogen. In view of this, it has therefore been proposed that either cytochrome c release from the mitochondria or activation of caspases is considered to be the hallmark of apoptosis in terminally differentiated cells.

It is possible that the apoptotic process is initiated in the transition to heart failure because of the altering balance of proapoptotic and antiapoptotic factors, induced by the stimulus of pressure overload. Subsequent damage to the nucleus, however, may not be complete because these terminally differentiated cells try to resist fragmentation in spite of continued upstream activation of apoptotic regulators (30). It is thought that these cells can exist in a state of “apoptosis interruptus” because of upregulation of endogenous protective factors such as XIAP (8). This association of LV dysfunction with caspase activation, in the absence of apoptotic cell death, has been further substantiated by recent studies. In the first, a rat ischemia model of stunned myocardium was used to show specific caspase-3 inhibitors producing contractile recovery, independent of any apoptosis-inhibitory effects (31). In a subsequent study, transgenic mice with overexpression of caspase-3 developed depression of cardiac function associated with myofibrillar ultrastructural damage but without triggering a full apoptotic response in the cardiomyocyte (32). In the absence of completed apoptotic DNA fragmentation, caspase-3–mediated destruction of cardiac myofibrillar proteins, including alpha-actin, alpha-actinin, troponin T, and myosin light chain, is thought to be responsible for the progressive deterioration in ventricular function (33,34). What is intriguing in the present study is the activated caspase-3 and -9 (unlike in the human study [9]) but lack of DNA fragmentation (similar to the human study [9]). It seems that different rates of evolution of disease may account for such differences. It may be of interest to see whether DNAses are wiped out during chronic manifestations of heart failure (8). Furthermore, it is possible that the upregulation of caspases, not accompanied by end-stage apoptotic nuclear morphology, may indicate that an alternative form of programmed cell death, autophagy, is involved in the transition to heart failure (35). Autophagic cell death can be activated by caspase-independent or -dependent mechanisms (36). In the absence of completed apoptotic cell death, myocyte cell loss by autophagy or even oncosis may account for the persistence of LV dilatation and marked decrease in LVMI, secondary to thinning of the ventricular walls (decreased PWTd and IVSd), which accompanied the development of LV failure (35).

**Study limitations.** Although this study is limited by its small numbers, detectable changes in caspase activity were shown. Furthermore, although the ovine model was designed to simulate changes associated with human heart failure, it is recognized that this occurred in a much shorter time period compared with that experienced in human pressure overload–induced heart failure.

In conclusion, this study has shown that activation of caspase-3, -8, and -9 is associated with chronic pressure overload of the LV and in particular with decompensation of myocardial function. This initiation of the caspase cascade, however, was not associated with completion of apoptotic DNA fragmentation. Further studies of caspase-mediated myocardial dysfunction may allow the development of novel therapeutic interventions to attenuate the progression of heart failure.

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