A loss of cardiomyocytes by overload-induced apoptosis in end-stage heart failure is an attractive hypothesis, explaining the progressive character of the disease. (1) Apoptotic myocyte loss has been postulated as a mechanism for the transition from cardiac overload hypertrophy to cardiac failure (2). In agreement with these hypotheses, the occurrence of myocyte nuclei positive for probes detecting apoptotic deoxyribonucleic acid (DNA) damage has been documented repeatedly in nonischemic areas of explanted human hearts with terminal heart failure (1). However, the specificity of these probes for apoptotic DNA damage is under debate (3–5).

Proteolytic activation of the cascade of caspases is considered as the central element of the apoptotic machinery. The cascade is started by autoactivation of initiator caspases, triggered either by release of apoptotic signals from the mitochondria or by ligand binding to death domain receptors (6). The mitochondrially activated initiator caspase-9, can be inhibited by its short isoform, caspase-9S (7), and the death receptor-activated initiator caspase-8, is inhibited by the flice-like inhibitory proteins (FLIP) FLIP\(_{L}\) and FLIP\(_{S}\) (8).

Another group of inhibitory proteins, the family of human inhibitor of apoptosis proteins (hIAP), hIAP-1, hIAP-2 and X-linked inhibitor of apoptosis protein (XIAP), are potent suppressors of apoptosis by preventing the activation of initiator, as well as effector, caspases and by direct inhibition of activated (cleaved) caspases (9).

In view of the open debate on the specificity of DNA damage indicators, we decided to analyze the expression and activation of caspases and their endogenous inhibitors in the terminally failing myocardium. The data demonstrate a fragile balance, without measurable activation, of effector caspase-3, on the one hand, but with depressed expression of caspase inhibitors and substantial activation of two initiator caspase pathways, on the other hand.

**METHODS**

Myocardial specimens from cardiomyopathic and donor hearts. Samples from 21 donor hearts not transplanted for clinical reasons were obtained in cardioplegic arrest and from 36 explanted hearts of patients undergoing transplantation due to dilated cardiomyopathy (DCM) (n = 24), coronary artery disease (CAD) (n = 9) or a mixed etiology (n = 3), with a mean ejection fraction of 25 ± 3%, were

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immediately snap-frozen in liquid nitrogen. Eighteen of the patients with failing hearts had beta-blocker therapy, in combination with angiotensin-converting enzyme inhibitor therapy in 14 of these patients, whereas 16 of the 18 patients without beta-blocker therapy received an angiotensin-converting enzyme inhibitor. Probes from all 57 hearts were used for reverse-transcription polymerase chain reaction (RT-PCR), whereas nine failing and six nonfailing donor hearts, as examples, were used for immunoblot analysis.

The local ethics committee approved the study of these human cardiac tissues, and the patients gave written informed consent.

**Ribonucleic acid (RNA) extraction and semiquantitative RT-PCR.** Total RNA was isolated from ventricular specimens, and semiquantitative RT-PCR was performed in accordance with the protocol described previously (10,11). The primer sequences and specific characteristics of semiquantitative messenger ribonucleic acid (mRNA) analyses of the genes are available from Dr. Scheubel. The PCR products were quantified either relatively per 18S ribosomal RNA or absolutely per competitive RT-PCR (10), with internal standard complementary RNA molecules for hIAP-1 and XIAP.

**Isolation of the cytosolic fraction and immunoblot analysis.** The human myocardium was minced at 4°C in buffer A (in mmol/l: 10 N-[2-hydroxyethyl] piperazine-N’-[4-butanemethane sulfonic acid], 80 potassium chloride, 1 sodium-ethylenediamine–tetracetic acid, 1 sodium-ethyleneglycoltetraacetic acid, 4 Dithiothreitol and 250 sucrose; 50 μg/ml saponin and 5 μl protease inhibitor [Sigma, Deisenhofen, Germany] cocktail per 100 mg tissue; pH 7.4), and after 30 min, it was homogenized with DSTROY-S pestles (Biozym, Oldendorf, Germany) and centrifuged for 10 min at 500 g. The supernatant was centrifuged at 10,000 g for 30 min, and the final supernatant was used as the cytosolic fraction. The protein concentration was determined by the bicinchonic acid protein assay (Sigma). Fifty micrograms of protein of the cytosolic fraction were run on a 10% or 15% sodium dodecyl sulfate polyacrylamide gel. Protein extracts of cleaved caspase-3-positive cells (Jurkat and National Institute of Health 3T3) were purchased from Cell Signaling Technology (Frankfurt, Germany). Proteins were electroblotted onto nitrocellulose membrane (BioRad, München, Germany), blocked with 6% nonfat dry milk in Tris-buffered saline-Tween 20 (200 mmol/l Tris [hydroxy-methyl] aminomethane, 300 mmol/l sodium chloride, 0.1% Tween 20; pH 7.5) and incubated with the respective anti-human primary antibody. Antibodies against caspase-3 (pc rabbit, Pharmingen, Heidelberg, Germany; polyclonal [pc] rabbit and goat, Santa Cruz Biotechnology, Heidelberg, Germany), cleaved caspase-3 (pc rabbit, Cell Signaling Technology), caspase-9/-9S (pc rabbit, Pharmingen), caspase-8 (pc rabbit, Pharmingen), FLIPS (monoclonal [mc] mouse, Santa Cruz Biotechnology), hIAP-1 (pc rabbit, R&D Systems), hIAP-2 (pc rabbit, Santa Cruz Biotechnology), XIAP (mc mouse, Becton Dickinson, Heidelberg, Germany), gelsolin (mc mouse, Sigma), fodrin (mc mouse, Chemicon, Hoheim, Germany), cytochrome c (mc mouse, Pharmingen) and manganese superoxide dismutase (provided by Dr. H. Noak, University Halle-Wittenberg) were applied. Blots were subsequently washed in TBST and incubated with specific peroxidase-coupled secondary antibodies (anti-goat immunoglobulin G [IgG]-horseradish peroxidase [HRP], Dianova, Hamburg, Germany; anti-rabbit and antimouse IgG-HRP, Amersham Pharmacia Biotech, Freiburg, Germany). Bound antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech) and finally quantified using a laser-densitometer with imaging system (Molecular Dynamics, Sunnyvale, California).

**Immunohistochemistry.** Samples were immediately fixed in buffered 4% formalin and paraffin-embedded. Sections of 8 μm were cut, dewaxed and rehydrated. Antigen retrieval was performed in 0.1 mol/l citric acid (pH 6.0) for 4 times 5 min at 100°C. Unspecific protein bindings were blocked with bovine serum (1:5, DAKO, Hamburg, Germany). For immunostaining of XIAP, a mc mouse antibody (as described in the previous text) was applied in a humidified chamber at 37°C for 2 h, followed by incubation with a fluorescein isothiocyanate-labeled anti-mouse IgG antibody (Sigma). Slides were embedded in Mowiol (embedding reagent) (Hoechst, Frankfurt, Germany). Omission of the primary antibody served as a negative control study. Bound antibodies were detected by fluorescence microscopy.

**Cell culture of adult rat ventricular cardiomyocytes.** Ventricular cardiomyocytes of adult rats were prepared as described (12). Cultured cardiomyocytes were incubated with 10 μmol/l of epinephrine for 24 h and then washed with phosphate-buffered saline (pH 7.2), scraped off the dishes and incubated in buffer A (as previously described) for extraction of cytosolic proteins.
Data analysis. Western blotting and RT-PCR analyses were evaluated by scanning the negatives of the gel images using a computer-based imaging system (AIDA evaluation software, Raytest, Straubenhardt, Germany). The optical density units of RT-PCR products and Western blots are given as the mean value ± SEM. The significance of comparison of mean values was determined by the unpaired Student t test, using a significance level of p < 0.05.

RESULTS

Mitochondrially activated caspase pathway. We quantified cytochrome c release from the mitochondria, expression of procaspase-9 and its anti-apoptotic isofrom, caspase-9S, and cleavage of procaspase-9 protein. Procaspase-9 mRNA expression in failing ventricles (1.41 ± 0.06) was not different from that in nonfailing ventricles (1.34 ± 0.06). However, mRNA of the inhibitory splice variant caspase-9S was downregulated by 50% (Fig. 1a). In immunoblot analysis, uncleaved procaspase-9 protein tended to be elevated, whereas the activated form of caspase-9 was three times higher in failing myocardium. Conversely, the inhibitory isofrom caspase-9S protein could only be detected in donor hearts, but not in failing hearts (Fig. 1b and 1c).

Cytochrome c immunoreactivity in the mitochondria-depleted cytosol of failing myocardium was higher than that of donor hearts (Fig. 2). The mRNA expression of cytochrome c in failing hearts was 0.25 ± 0.02, as compared with 0.41 ± 0.04 in donor hearts (p < 0.001), and for apoptosis-inducing factor (AIF) (13), another apoptogenic protein released from mitochondria, it was 0.15 ± 0.01 and 0.23 ± 0.02, respectively (p < 0.01). However, mRNA of the mitochondrial matrix protein adenylate kinase-3 was similar in failing ventricles (0.15 ± 0.01) and donor hearts (0.16 ± 0.01, p = NS).

Thus, the enhanced cytochrome c content in the cytosol suggested enhanced mitochondrial release and availability for activation of procaspase-9, but the mRNA values of cytochrome c and AIF did not indicate enhanced resynthesis of these releasable mitochondrial apoptogenes in the failing myocardium.

Receptor-activated caspase pathway. This pathway includes the initiator procaspase-8 and the specific FLIP...
inhibitors. Compared with nonfailing ventricles, the myocardium of patients with heart failure was characterized by decreased mRNA expression of FLIPL (−25%), as well as FLIPS (−58%) (Fig. 3a), but no significant alteration of procaspase-8 mRNA expression (0.96 ± 0.05 in failing myocardium vs. 1.04 ± 0.9 in donor hearts). On immunoblot analysis, uncleaved procaspase-8 protein levels were similar in both groups, whereas activated caspase-8 tended to be higher (p = 0.065), and FLIPS was significantly downregulated in the failing myocardium (Fig. 3). Similarly as for mitochondrially activated procaspase-9, the receptor-activated procaspase-8 was partially activated and its inhibitors were downregulated in the failing myocardium.

Figure 2. Immunoblot analysis of cytochrome c and manganese superoxide dismutase (MnSOD; as a marker of the mitochondrial matrix) in the cytosol (a) and total cell lysate (b) of left ventricular specimens from donor hearts and failing hearts. In the failing myocardium, cytosolic cytochrome c was 53% higher (c), whereas MnSOD was not different between the two groups in terms of the cytosolic fraction and total lysate. In cultured rat cardiomyocytes, cytochrome c release could only be detected after treatment with 10 μmol/l of norepinephrine for 24 h (d). *p < 0.05.

Figure 3. Analysis of left ventricular protein expression of procaspase-8, activated caspase-8, fllice-like inhibitory protein (FLIP)S and FLIPL in the failing human myocardium, as compared with donor organs. The messenger ribonucleic acid (mRNA) expression of FLIPs and FLIPL is significantly downregulated in the failing myocardium (a). For activated caspase-8, there was an increase of 62% in the failing myocardium, as compared with donor hearts (b), whereas procaspase-8 was not different between the two groups. The FLIPS protein decreased significantly in the failing myocardium (b). (*)p < 0.1; *p < 0.05; ***p < 0.001.
Downregulation of IAP family proteins. In failing left ventricles, mRNA expression of IAPs was downregulated by 54% for hIAP-1 and by 34% for hIAP-2 and tended to be downregulated by 23% for XIAP (p<0.08). However, immunoblot analysis showed significantly lower protein levels only for hIAP-1 and XIAP in failing ventricles (Fig. 4), whereas hIAP-2 was not altered. By fluorescence immunohistochemistry, we confirmed the expression of XIAP in cardiomyocytes, but no preferential XIAP immunoreactivity in the noncardiomyocytes of failing or donor hearts.

Activation of effector caspases and substrate cleavage. Caspase-3 is the central death protease of the cascade, catalyzing the specific cleavage of many key cellular proteins. However, we found neither an alteration in procaspase-3 mRNA expression in failing hearts (0.8 ± 0.05 vs. 0.9 ± 0.07 in donor hearts, p = NS), nor any activated form of caspase-3 or any alteration in the uncleaved procaspase-3 protein by immunoblot analysis, using various antibodies against cleaved caspase-3 alone (Fig. 5a), as well as against cleaved and uncleaved caspase-3 (Fig. 5b). The caspase-3 substrate called gelsolin was also not cleaved in the failing myocardium (Fig. 5c). However, the cytoskeletal protein called fodrin, which is a substrate of several caspases, was partially cleaved in the failing myocardium (Fig. 5d), and 0.05 ± 0.01% of cardiomyocyte nuclei were terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive in failing ventricles.

Effects of beta-adrenoceptor antagonists on apoptosis-related gene expression. Despite increased pro-atrial natriuretic peptide (ANP) expression in patients with DCM, as compared with CAD (1.8 ± 0.2 vs. 1.1 ± 0.3; p < 0.05), the retrospective analysis of patients with DCM, CAD or a mixed etiology revealed no differences in expression of the apoptotic genes analyzed. However, subclassification of the terminally failing explanted hearts into those from patients receiving beta-blocker therapy (n = 18) before explantation and those from patients without this therapy (n = 18) showed that both groups had a similar degree of cardiac overload, as indicated by the six- to sevenfold elevation of pro-ANP mRNA expression (Fig. 6), as well as similar age, gender, etiology, ejection fraction and standard therapy of heart failure. In contrast, patients without beta-blocker therapy revealed a more pronounced downregulation of mRNA expression of endogenous apoptosis inhibitors, as compared with patients receiving beta-blocker therapy (Fig. 6). However, for the mRNA expression of caspases, the opposite trend became apparent: mRNA expression was elevated under beta-blockade for caspase-9 and caspase-3 (Fig. 6). The downregulation of mRNA expression of the mitochondrial factors cytochrome c, AIF and adenylate kinase-3 was significantly more pronounced in patients without beta-blockade than in patients with beta-blockade (Fig. 6).

**DISCUSSION**

Our data show that the apoptotic process is indeed activated in the terminally failing left ventricular myocardium of humans: the receptor-regulated caspase-8 and the mitochondrially regulated caspase-9 are substantially activated; extramitochondrial cytochrome c is elevated; and most endogenous caspase inhibitors are downregulated. These...
cytosolic alterations must have occurred mainly in cardiomyocytes, which yield 90% of ventricular cytosolic volume. The process of proteolytic activation does not encompass the entire cascade equally: in contrast to the strong activation of initiator caspases-8 and -9, activation of the terminal effector caspase-3 and cleavage of the caspase-3 substrate gelsolin are below the limit of detection. Although the actin modulator gelsolin in its caspase-cleaved form promotes apoptosis (14), uncleaved gelsolin still acts as an inhibitor of caspase activation (15). Furthermore, other unidentified inhibitors and/or the residual function of the downregulated caspase inhibitors might have contributed to this incompleteness of the cascade activation. The cleavage of fodrin, as observed in the failing myocardium (Fig. 5d), is not a good indicator for caspase-3 activation, because fodrin can be cleaved independently from caspase-3 activation (16).

These findings, partially comparable to previous observations (17), instigate several questions: first, is there a functional relevance of cytochrome c release and initiator caspase activation in the failing myocardium? Second, what are the mechanisms of this partial activation of the cascade? Finally, what causes TUNEL-positive nuclear changes in only a small fraction of cardiomyocytes, although caspase-3 is not measurably activated in failing myocardial tissue extracts?

**Functional relevance of mitochondrial caspase cascade activation.** We observed enhanced extramitochondrial cytochrome c, but no signs of enhanced cytochrome c resynthesis. In fact, mRNA expressions of cytochrome c and AIF, another releasable mitochondrial apoptogene, are downregulated in the failing myocardium, most pronounced in those hearts without beta-blockade before explantation (Fig. 6). In view of depressed cytochrome c expression and enhanced mitochondrial release, there must be a cytochrome c deficit in the oxidative phosphorylation in the respiratory chain of the mitochondria in the failing myocardium.

Lowered concentrations of adenosine triphosphate (ATP) and creatine phosphate have been reported in the failing human myocardium (18) and were ascribed to a primary attenuation in mitochondrial ATP synthesis (19). Our data, suggesting a mitochondrial cytochrome c deficit in the explanted hearts, yield an explanation for this postulated primary attenuation of ATP synthesis in the failing myocardium. Furthermore, this deficit must result in enhanced mitochondrial radical formation, as occurs upstream of any defect in the respiratory chain (20). Indeed, increased mitochondrial radical formation has been shown in the failing human myocardium (21).

The release of cytochrome c in the presence of low ATP availability should result in more necrotic forms of cell death (22). This is exactly what is observed in the failing myocardium: histochemical characterization of cardiomyocyte nuclei identified a more than fivefold higher occurrence of necrotic cardiomyocyte nuclei, as compared with apoptotic nuclei (3). Thus, we conclude that mitochondrial activation of the starting program of cell death in the failing myocardium is relevant for the enhanced mitochondrial radical formation, for the substantial incidence of necrotic processes under this condition and probably for the enhanced susceptibility of the failing myocardium to ischemic damage (23).
Mechanisms of partial caspase cascade activation. The mechanisms for activation of initiator caspases in the failing myocardium include two aspects: the triggering event (at mitochondria and/or death receptors), on the one hand, and the phenotype alterations with downregulated endogenous caspase inhibitors, on the other hand.

In the mitochondria, one possible mechanism for apoptogen release is the cytosolic calcium overload of the failing myocytes, which causes mitochondrial calcium overload, an established factor for cytochrome c release (24). Tumor necrosis factor (TNF) receptor-1 stimulation should be considered as a major factor for caspase-8 activation, because the TNF system is involved in the deterioration of cardiac function in severe heart failure (25). However, an activated myocardial TNF system also confers some anti-apoptotic protection (26,27), which is certainly of relevance for maintenance of the fragile anti-apoptotic balance in the failing myocardium, as demonstrated in our report.

Phenotype shifts in the failing myocardium are generally ascribed to three factors: enhanced mechanical load, exaggerated neuroendocrine activity and inflammatory activation. Enhanced mechanical load is probably not the decisive factor for these pro-apoptotic phenotype changes, because we could not obtain a re-normalization of the depressed expression of most caspase inhibitors in failing hearts under hemodynamic unloading by ventricular assist device (VAD) (11). Consistent with a role of neuroendocrine overactivation for depressed inhibitor expression, is our observation that this depression was less pronounced in patients treated with beta-blockers (Fig. 6). We assume that inflammatory activation is a major reason for the depressed expression of apoptosis inhibitors in heart failure, because inflammatory activation often remains elevated under VAD application (28), and caspase inhibitor expression remains depressed under VAD, despite hemodynamic unloading (11).

Apoptotic cardiomyocyte nuclei without caspase-3 activation? Although 0.05% of cardiomyocytes demonstrated DNA changes, as detectable by the TUNEL technique, we could not demonstrate cleavage of caspase-3 or its substrate gelsolin in extracts from these hearts, by using various antibodies that detected cleaved caspase-3 in apoptotic Jurkat and NIH 3T3 cell extracts (Fig. 5a and 5b). Probably, these biochemical analyses are not sensitive enough to detect cleavage processes in tissue extracts with only 0.05% apoptotic myocytes. Other reports on caspase-3 activation in the failing myocardium are conflicting, with minimal (29) or massive (17) caspase-3 activation, but without detectable cleavage of polyadenosine diphosphate-ribose-polymerase.
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release from cardiomyocyte mitochondria contributes to

Conclusions. Our data demonstrate that cytochrome c

from cardiomypocyte mitochondria contributes to partial

activation of the apoptotic caspase cascade in the

terminally failing myocardium of humans. Although endog-
enous caspase inhibitors are downregulated, the cascade is

still kept in a fragile balance, because the effector caspase-3

is not yet activated, and the gelsolin switch is not yet cleaved

into a pro-apoptotic mode. Activation of TNF signaling, as

indicated by caspase-8 cleavage, may contribute to main-
taining survival. Although only a tiny fraction of cardiomyc-

tocyte nuclei shows TUNEL-positive DNA alterations, the

cytochrome c release in the failing myocardium is of

functional relevance for impaired ATP production and enhanced

radical formation, and probably also in cardiomycocytes that

escape necrotic or apoptotic cell death.

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