Molecular Medicine

Myocytes Die by Multiple Mechanisms in Failing Human Hearts

Sawa Kostin, Lieven Pool, Albrecht Elsässer, Stefan Hein, Hannes C.A. Drexler, Eyal Arnon, Yukihiro Hayakawa, René Zimmermann, Erwin Bauer, Wolf-Peter Klövekorn, Jutta Schaper

Abstract—We tested the hypothesis that myocyte loss in failing human hearts occurs by different mechanisms: apoptosis, oncosis, and autophagic cell death. Explanted hearts from 19 patients with idiopathic dilated cardiomyopathy (EF=20%) and 7 control hearts were analyzed. Myocyte apoptosis revealed by caspase-3 activation and TUNEL staining occurred at a rate of 0.002±0.0005% (P<0.05 versus control) and oncosis assessed by complement 9 labeling at 0.06±0.001% (P<0.05). Cellular degeneration including appearance of ubiquitin containing autophagic vacuoles and nuclear disintegration was present at the ultrastructural level. Nuclear and cytosolic ubiquitin/protein accumulations occurred at 0.08±0.004% (P<0.05). The ubiquitin-activating enzyme E1 and the ligase E3 were not different from control. In contrast, ubiquitin mRNA levels were 1.8-fold (P<0.02) elevated, and the conjugating enzyme E2 was 2.3-fold upregulated (P<0.005). The most important finding, however, is the 2.3-fold downregulation of the deubiquitination enzyme isopeptidase-T and the 1.5-fold reduction of the ubiquitin-fusion degradation system-1, which in conjunction with unchanged proteasomal subunit levels and proteasomal activity results in massive storage of ubiquitin/protein complexes and in autophagic cell death. A 2-fold decrease of cathepsin D might be an additional factor responsible for the accumulation of ubiquitin/protein conjugates. It is concluded that in human failing hearts apoptosis, oncosis, and autophagy act in parallel to varying degrees. A disturbed balance between a high rate of ubiquitination and inadequate degradation of ubiquitin/protein conjugates may contribute to autophagic cell death. Together, these different types of cell death play a significant role for myocyte disappearance and the development of contractile dysfunction in failing hearts. (Circ Res. 2003;92:715-724.)

Key Words: cell death □ oncosis □ apoptosis □ autophagy □ ubiquitin

During recent years, it has been suggested that loss of cardiomyocytes and increased replacement and reactive fibrosis resulting in scar formation are important causative factors in the development of heart failure.1,2 However, by which mechanism cardiomyocytes die and eventually disappear from the tissue is not entirely clear.

The concept that myocyte death in heart failure is primarily due to apoptosis has been put forward by several groups during the last decade,3–6 but this has been questioned by others.7–10 In addition to apoptosis, cells may die by acute ischemic “accidental” cell death, ie, oncosis.11 The introduction of complement 9 (C9), a part of the membrane attacking complex C5b-9, as a tool for the detection of early oncosis represents a major advantage.12,13 This method was used in the present study to establish a systematic and quantitative description of oncosis in failing hearts.

As research in this area progresses, there seems to be no doubt that there exist more than two types of cell death.14 Lockshin discussed this in a broader cell biological sense and stated: “The term ‘apoptosis’ is used as a shibboleth to cover many issues,” (page 4) and that autophagic cell death may be encountered more commonly than previously acknowledged.15 There are many lines of evidence that connect the autophagic machinery with the ubiquitin/protein degradation system.16 Autophagic cell death plays an important role in organ development17 and in neurogenerative disorders such as Parkinson’s or Alzheimer’s disease.18,19 It represents a programmed and dynamic process that proceeds by sequestration of cellular material into double membrane vacuoles that dock to and fuse with lysosomes forming autophagic vacuoles. These are degraded by lysosomal proteases such as cathepsin D and removed from the cell by exocytosis.20

Ubiquitination of proteins involves activation of ubiquitin by the ubiquitin-activating enzyme E1 and the subsequent action of ubiquitin-conjugating enzymes E2 and ubiquitin-ligases E3. Ubiquitination, however, is not an irreversible process as ubiquitin moieties can be removed from ubiquitin/protein conjugates by a large group of enzymes, of which...
Isopeptidase T and the ubiquitin-fusion degradation system (UFD1) are prominent members. Proteins are subsequently channeled into the proteasome and degraded, whereas monomeric ubiquitin is recycled (see review21). The proteasome is a major intracellular proteolytic system involved in the removal of ubiquinated proteins. Proteasomes eliminate not only unwanted, ie, mutated or misfolded proteins, but they are responsible for turnover of almost all cellular proteins.22 Proteasomal protein degradation is a “highly complex, temporally controlled, and tightly regulated process” (page 373).21 It is functionally analogous to autophagy, which ensures turnover of cellular organelles.23 The contribution of the ubiquitin/proteasome pathway, and autophagic cell death to heart muscle degeneration has largely remained obscure, with the exception of recent reports investigating failing human hearts24 and Danon’s cardiomyopathy.25 Evidence is growing that myocyte cellular degeneration is one of the most prominent phenomena in failing human myocardium.2,26 We hypothesized that myocytes may not always die of apoptosis but also as a consequence of slow degenerative processes involving the ubiquitin/proteasomal pathway. The present work is the first to analyze molecules involved in the ubiquitin conjugation/proteasomal degradation cascade in human myocardium and to identify functional defects in this system responsible for autophagy that ultimately contribute to cardiomyocyte loss. Furthermore, we have investigated the differential contribution to myocyte loss of apoptosis, oncosis, and autophagic cell death to heart failure and can demonstrate that all 3 killing mechanisms act in parallel to varying degrees.

Materials and Methods
Cardiac tissue from 19 patients with end stage heart failure (EF≤20%) due to idiopathic dilated cardiomyopathy was studied (described in Heling et al26). All patients underwent cardiac transplantation. Three donor hearts not used for transplantation and intraoperative biopsies from 4 patients with mitral valve stenosis but with preserved EF served as controls. The institutional Ethical Committee approved the study.

Tissue Sampling
At the time of transplantation, tissue was removed from the left ventricular free wall. Up to 3 samples from each heart were used for either Western or Northern blot and 5 to 8 samples for confocal microscopy. Samples were immediately frozen in liquid nitrogen and stored at −80°C. Smaller samples were immediately fixed in buffered glutaraldehyde for electron microscopy.

Electron Microscopy
The samples were embedded in Epon using a standard protocol. Ultrathin sections were double-stained with uranyl acetate and lead citrate before examination in a Philips CM 10 electron microscope.

Immunogold Electron Microscopy
Immunogold electron microscopy was performed using a preembedding procedure. In brief, myocardial specimens were fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde for 2 hours. After being washed in PBS, the blocks were immersed in 20% sucrose overnight and then quickly frozen in methylbutane at −130°C. Cryosections (5 μm thick) were placed on precooled positively charged slides and incubated with 5% bovine serum albumin (BSA, Sigma) and 10 mmol glycine to block nonspecific labeling. The sections were then incubated with rabbit or mouse anti-ubiquitin antibodies, followed by incubation with 10-nm gold-conjugated donkey anti-rabbit or anti-mouse IgG (Aurion). Omission of primary anti-ubiquitin antibodies served as negative control (online Figure 6, available in the online data supplement at http://www.circresaha.org). After incubation and washing, the sections were fixed in 4% glutaraldehyde and embedded in Epon following routine methods. Ultrathin sections were examined in a Philips CM 10 electron microscope.

Immunolabeling and Confocal Microscopy
Frozen sections 10 μm thick were fixed for 10 minutes with 4% paraformaldehyde and then exposed for 10 minutes in 1% BSA, followed by incubation with the corresponding antibodies in single, double, or triple staining procedures. All primary antibodies used are listed in online Table 2. The secondary detection system was biotinylated anti-mouse or anti-rabbit IgG (Biotrend) conjugated with Cy-2, Cy-3, or Cy-5. Autophagic vacuoles were visualized with monodansylcadaverine (Sigma) as described.27 Counterstaining for myocyte identification was done with TRITC-phalloidin (Sigma). Nuclei were stained with TOTO-3 (Molecular Probes). Omission of primary antibodies served as negative control. The samples were examined with a confocal scanning laser microscope Leica TCSNT, equipped with argon/krypton and helium/neon lasers.

Quantification of TUNEL-, C9-, and Ubiquitin-Positive Myocytes
In situ labeling of fragmented DNA (TUNEL) was performed in 19 patients and controls using a commercially available kit (Boehringer-Mannheim). From each tissue block, 3 sections 5 μm thick, cut at a distance of 100 μm, were stained and quantitatively evaluated. Counterstaining for identification of myocytes was done with TRITC-phalloidin (Sigma). TUNEL-positive nuclei were counted for the entire section. The number of myocyte nuclei per 5 randomly chosen fields of vision (×400) was counted and calculated per mm2: control myocardium 462±5 myocytes/mm2, diseased myocardium 330±9 myocytes/mm2. From these data and the area of the tissue section, the total number of myocytes was determined, and TUNEL-positive cells were expressed as percentage of the total number of cardiomyocyte nuclei. The same counting procedure was performed for C9 and ubiquitin labeling.

Northern Blot
A human ubiquitin cDNA probe (No. 9806-1, CLONTECH) and a murine 18S ribosomal RNA cDNA probe (Dr Oberbäumer, München, Germany) were used in samples from 8 patients and 3 controls. Standard methods for Northern blots were used. Each gel was done at least in duplicate. For each well, 15 μg of total RNA were loaded. Quantification was done using a PhosphorImager and ImageQuant Software (Amersham Pharmacia Biotech). To normalize each blot for loading differences, the data of each ubiquitin hybridization was divided by the values for the matching 18S signal. Data from control patients were set as 100%.

Western Blot
SDS-PAGE (separating gels 12%) and immunoblotting were performed in samples from 13 patients and 7 controls following routine protocols. At least 3 different gels were prepared from each heart. For each lane, 30 μg protein was loaded. All antibodies used are listed in online Table 2. Quantification was done by scanning of the immunoblots on a STORM 860 (Amersham Pharmacia Biotech) using ImageQuant software. In order to exclude the influence of fibrosis on myocyte proteins, immunoblotting for sarcomeric α-actin was performed and all specific values of proteins evaluated here were standardized to sarcomeric actin.

Determination of Proteasome Activity in Heart Tissue Lysates
Heart tissue samples from 13 patients and 3 controls were homogenized by sonication and boiled, and the tissue was snap frozen in liquid nitrogen, in ice-cold M-PER buffer (Pierce) supplemented
with 150 mmol NaCl, and subsequent grinding using an Ultra-Turrax T8 homogenizer (IKA Labortechnik). After a further incubation for 10 minutes on ice, insoluble material was removed by centrifugation at 14 000 rpm for 10 minutes at 4°C. The protein concentration in the supernatant was determined by using the BCA reagent (Sigma) using BSA as standard. Extracts were stored at −20°C. To determine proteasomal activity 40 μL of extract were mixed with 160 μL of proteasome assay buffer (10 mmol Tris-HCl pH 7.5; 20% glycerol; 5 mmol CaCl2) containing 10 μmol/L Suc-Leu-Leu-Val-Tyr-AMC as substrate for the chymotrypsin-like activity of the proteasome. The amount of free AMC as a measure of proteasome activity was assessed after a 60-minute incubation of the mixture at 37°C in a microplate fluorescence reader (Lambda Fluoro 320; MWG) with excitation and emission wavelengths set at 360 nm and 460 nm, respectively, against blanks containing only proteasome assay buffer and M-PER. A 10-μmol solution of AMC in assay buffer served as standard. All samples were measured in duplicate. The amount of free AMC (in pmol/mL) was calculated according to the formula (10 nmol × ΔF%/×0.2 mL)/(1 mL × 100% × 60 minutes) and was related to the amount of protein assayed.

**Statistics**

All data are presented as mean±SEM. Differences by unpaired t test were considered significant when P<0.05.

**Results**

**Different Types of Cell Death**

The typical appearance of cells undergoing apoptosis, oncosis, and autophagic cell death is illustrated in Figure 1. All 3 types of cell death were single cell phenomena occasionally observed in close vicinity to each other (Figure 2). An example of triple labeling using immunohistochemical markers for apoptosis (TUNEL), oncosis (C9), and autophagy (ubiquitin) is provided in online Figure 1.

**Apoptosis**

Control tissue was negative for TUNEL labeling. In failing hearts, myocytes with DNA fragmentation occurred at a rate of 0.002±0.0005%; P<0.05. By immunohistochemistry, the myocytes from diseased hearts showed 3 distinct patterns of in situ activated caspase-3: (1) cross-striated, (2) nuclear/perinuclear, and (3) large amorphous cytosolic accumulations (online Figure 2). It is important to note that only the last two patterns of caspase-positive myocytes were associated with TUNEL positivity.

**Oncosis**

C9-positive myocytes (Figures 1 and 2) were found in all patients but not in the controls. The number of positive cells ranged from 1 to 20 per section with a mean value of 0.06±0.001%; P<0.05. In contrast to ubiquitin, C9 staining often appeared in the periphery of the myocyte and showed progression toward the cell center.

**Autophagy and Ubiquitin Accumulations**

Different stages of ubiquitination ranging from the deposition of small nuclear or cytosolic aggregates to large accumulations occupying most of the cellular volume were present (Figures 1E, 2C, 2E, 2H, and 3). In myocytes with punctate ubiquitin labeling, the nucleus was still present and the majority of sarcomeres were intact. In contrast, myocytes with large deposits usually contained less intact sarcomeres but still exhibited some amorphous contractile material.

Double labeling procedures provided evidence that ubiquitin colocalized with different contractile proteins (Figures 3D and 3E). Only 19% of the cardiomyocytes with large ubiquitin/protein accumulations still contained nuclear structures. The mean rate of cardiomyocytes with ubiquitin punctate labeling was 0.03±0.008% and that of large ubiquitin deposits was 0.05±0.004% (P<0.05 as compared with control where ubiquitin-positive cells were absent). Double and triple labelings showed that myocytes with ubiquitin accumulations were TUNEL negative without signs of activated caspase-3 (Figure 2E, and online Figures 1 and 3).

Western blot for ubiquitin showed labeling predominantly of high molecular weight bands (MW >100 kDa) indicating ubiquitination and/or polyubiquitination of various proteins (data not shown). The mRNA for ubiquitin was significantly (1.6-fold) upregulated in diseased heart samples as compared with controls (online Figure 4).
To investigate whether myocytes with ubiquitin accumulations are linked to increased levels of autophagy, we performed double labeling for ubiquitin with monodansylcadaverine, which is a specific marker of autophagic vacuoles. The results shown in Figures 3F and 3G demonstrate that ubiquitin is exclusively accumulated in myocytes positive for monodansylcadaverine, thus providing evidence for a link between ubiquitin accumulations and autophagy.

**Ubiquitin-Processing Enzymes**

The ubiquitin-activating enzyme E1 was localized in the myocyte nucleus (online Figure 5A). There were no differences between groups in the amount of E1A protein normalized per sarcomeric actin in Western blots (Figure 4A). The ubiquitin-conjugating enzyme E2 (UBC-2) was colocalized with large ubiquitin/protein complexes (Figures 4D through 4F), and it was significantly upregulated in diseased human myocardium (Figure 4B). The ubiquitin-ligating enzyme E3 (E6-AP) was localized in the nucleus of the myocyte (online Figure 5B). The quantity of this enzyme in Western blot was unchanged in diseased hearts when compared with control (Figure 4C).

**Deubiquitinating Enzymes**

By Western blot, isopeptidase T and UFD1 were significantly downregulated (respectively 2.3- and 1.5-fold) in diseased myocardium as compared with control (Figures 5A and 5B). By immunohistochemistry, isopeptidase T was detected in only a few ubiquitin-positive myocytes still containing a nucleus, but it was never colocalized with large ubiquitin/protein accumulations (Figure 5C).

**Proteasome**

The α1 subunit of the 20S proteasome was found in the nuclei of cardiomyocytes (Figures 6A and 6B). The protein content was unchanged in failing myocardium as compared with control (Figure 6C). Measurements of proteasomal activity showed widely ranging values with a mean for control of $10.1 \pm 5.3$ pmol/min per mg and for failing myocardium of $12.5 \pm 9.4$ pmol/min per mg ($P < 0.7$; Figure 6D). A direct correlation was found between proteasome and UBC-2 and isopeptidase protein content in controls but this was reversed in diseased myocardium (Figures 6E and 6F).

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**Figure 2.** Immunofluorescent features of different types of cell death. A, An example of double labeling for C9 (green) with TUNEL (red). TUNEL-positive myocyte is denoted by arrows. B, Same optical field shown in A after photobleaching of the FITC/TRITC fluorochromes and subsequently counterstained with F-actin (red) and TOTO-3 (blue). C, Double labeling for C9 (green, arrows) and ubiquitin (blue, asterisk). D, Identical field as in C showing F-actin and nuclear labeling (TOTO-blue). E, Double labeling for ubiquitin (asterisk) showing a TUNEL-positive myocyte (arrow) and a TUNEL-positive interstitial cell (arrowhead). F, Identical field as in E. G and H, Survey confocal micrographs showing that C9-positive myocytes (arrow in G) and myocytes with nuclear (asterisk) and cytoplasmic ubiquitin-accumulations (arrow in H) are scattered throughout the left ventricular wall tissue.
Lysosomal Enzyme Cathepsin D

By Western blot, cathepsin D was significantly (2-fold) downregulated in diseased hearts (Figure 7A). Cathepsin D was found immunohistochemically in the cytoplasm at the nuclear poles corresponding to the localization of lysosomes. It was present in varying amounts, but it was absent in cells showing large ubiquitin/protein complexes (Figures 7B through 7D).

Ultrastructure of Autophagy in Cardiomyocytes

Ultrastructurally, autophagic myocytes displayed depletion of contractile material and large numbers of cytoplasmic vacuoles associated with severe disintegration of the nuclear structures (Figure 8A). These features were never observed in apoptosis or oncosis. Immunogold electron microscopy revealed that ubiquitin accumulates specifically in myocytes with autophagic vacuoles (Figures 8B and 8C and online Figure 6). These data provided another evidence for the direct link between autophagy and ubiquitin accumulations. Figures 8D and 8E show two myocytes filled with nonspecified cytoplasm and autophagic vacuoles, which completely lack contractile material and nuclear structures. Both cells still maintain cell-to-cell contacts. At later stages, autophagically dying myocytes progressively lose their connections with neighboring myocytes, are detached at the intercalated disk, lack a nucleus, and are very small in size (Figure 8F). Only high magnifications revealed myocyte-specific structures, such as sarcoplasmic reticulum, or remnants of the myofibrillar apparatus, Z-disks, and the myocyte basement membrane (Figure 8G). The cellular debris originating from cellular sequestration is most probably taken up by surrounding fibroblasts and macrophages. These data strongly suggest that autophagocytosis is profound enough to cause myocyte cell loss in the failing human myocardium.

Discussion

In this study, we present evidence that myocytes die by multiple mechanisms in the failing human heart: apoptosis, oncosis, and autophagic cell death. We also present evidence that autophagic cell death is associated with major defects in...
the ubiquitin/proteasome cascade specified by increased ubiquitin conjugation and reduction of the deubiquitinating enzymes isopeptidase T and UFD-1. Because, in addition, the lysosomal proteolytic enzyme cathepsin D was downregulated, it appears that loss of deubiquitinating enzymes accompanied by unchanged (proteasome) and reduced (cathepsin D) proteolytic activities leads to the accumulation of polyubiquitinated proteins, which play a major role in causing autophagic cell death.

Apoptosis
The issue of apoptotic cell death in failing myocardium is not yet entirely clear because values for apoptotic rates vary from very high to almost absent. Narula’s concept of interrupted apoptosis is of special interest in this context and might explain the low apoptotic rate observed in the present study. Because heart failure is a chronic situation, our values of apoptotic myocytes should be considered for a prolonged period of time. When calculated for 1 year and assuming that apoptosis takes several hours until DNA fragmentation is completed, our data will predict cell loss by apoptosis to be in the range of 2% to 4%. This calculation, however, is significantly hampered by the lack of knowledge of the time needed to remove apoptotic cells from the tissue.

Activated caspase-3 was found in single myocytes. It occasionally colocalized with TUNEL positivity but was absent in ubiquitin or C9-positive cells, indicating independence of autophagic and oncotic cell death from caspase activation. In this study, we present for the first time immunohistochemical evidence that activated caspase is localized at the Z-disc and therefore is capable to directly cleave cardiac myofilaments. Caspase activation, therefore, may be one of several causes of contractile dysfunction before loss of myocytes by apoptosis.

Cell Death by Oncosis
Oncosis was observed in single cells scattered throughout the tissue in all patients at a mean rate of 0.06%. Leakage of C9 into myocytes indicates loss of membrane integrity typical of oncotic cell death and activation of the classical complement pathway causing secondary cytolytic processes after primary injury. Oncosis most probably was caused by 2 different factors, acting either in concert or alone: (1) defects in the microvasculature resulting in focal myocyte ischemia and (2) by low-grade inflammation with complement activation and perforin release from lymphocytes causing membrane damage. Because acute ischemic cell death occurs within a rather short time span (within 1 hour), but the dead cells remain in the myocardium for at least 48 hours, our values,
when calculated for 1 year, will predict \(\approx 11\%\) cell loss by oncosis.

**Ubiquitination and Autophagic Cell Death**

Proteins undergo ubiquitination accompanied by continuous and often cotranslational proteasomal degradation, which is a recycling process necessary for cell survival.\(^{33}\) The entire process is functional through several enzymes, including E1-E3 that are hierarchically ordered.\(^{34}\) Whereas in diseased tissue the content of the activating enzyme E1 remained unchanged, levels of the conjugating E2 were significantly elevated. However, because in our patients increased levels of conjugation of ubiquitin to proteins were not associated with similarly elevated levels of the ligase E3, which is necessary for completion of the targeting process of substrate proteins, this phenomenon may represent a first defect in the ubiquitin/proteasome pathway.

The second, probably most important defect, is the significant reduction of the cleaving enzymes isopeptidase T and UFD-1. Isopeptidase-T removes polyubiquitin chains from ubiquitin/protein conjugates and it stimulates protein degradation.\(^{35}\) Recently, it has been shown in Alzheimer’s disease that the ubiquitin/proteasome system is inhibited by accumulation of polyubiquitin\(^{11}\) chains that are resistant to disassembly by isopeptidase T, which in turn inhibits the degradation of ubiquitinated substrates by the 26S proteasome.\(^{36}\) A similar mechanism might be present in cardiomyocytes as a consequence of preproteasomal overloading, resulting in storage of ubiquitin/protein conjugates that eventually might be leading to cell death.\(^{37,38}\)

UFD1, a member of the ubiquitin-fusion degradation pathway,\(^{39}\) was likewise downregulated. Because both the
overall proteasome content and proteasomal activity remained largely unchanged in the patients myocardium, it appears that the cellular armament with proteasome particles is confronted with elevated amounts of ubiquitinated proteins, which these are unable to handle. In a positive feedback loop, accumulation of ubiquitinated proteins in degenerating myocytes may then further impede proteasome function, similar to the situation in neurons.40

Furthermore, the lysosomal enzyme cathepsin D was reduced in most cells but totally absent from cells with ubiquitin/protein storage. This indicates that another proteolytic component of the cell, which exerts an important function during autophagy and could potentially act as surrogate mechanism for the defective ubiquitin/proteasome system, is no longer available and will add to the accumulation of unwanted proteins. Thus, an insufficiency of the lysosomal degradation machinery eventually contributes to the cytotoxicity of accumulated polyubiquinated protein complexes. This may be identified as a third defect in the proteolytic system.

The time necessary from the first ultrastructural signs of degeneration to the massive cytosolic presence of ubiquitin/protein complexes and overt cell death is difficult to estimate. On the provision that ATP resources are not exhausted, storage of ubiquitin/protein complexes most probably is a slow continuous process that finally results in loss of the nucleus and death of the myocyte. Because of its abundance, availability of ubiquitin does not seem to be a decisive factor for the regulation of protein degradation.41 In addition, the increase in ubiquitin mRNA observed in this study may further ensure that the supply of this protein will not be a limiting step for cellular destruction.

In analogy to studies on neuronal cell death in neurodegenerative diseases,19,37,42 it was assumed, therefore, that
cardiomyocytes with ubiquitin/protein complexes undergo cell death by autophagy, as reported earlier by Knaapen.\textsuperscript{24} The present study showed that autophagic vacuoles are positive for ubiquitin, indicating a close relationship between the ubiquitin/proteasome degradation system and degeneration followed by autophagy. In contrast, autophagic myocytes were negative for both TUNEL and C9 staining, which discriminates it from apoptosis and oncosis. Our findings demonstrating a close link between the ubiquitin/proteasomal pathway and autophagic cell death are further supported by a recent in vitro study showing that disruption of the ubiquitin/proteasomal system was accompanied by accumulation of polyubiquitinated proteins, a marked increase in autophagic vacuoles, an impairment of lysosomal proteolysis and proteasomal function, and the occurrence of autophagic cell death.\textsuperscript{43} The hypothesis that overloading of myocytes with polyubiquitinated proteins results in autophagic cell death is furthermore strengthened by the observation that numerous small cellular remnants are positive for ubiquitin representing the final stage of cellular degeneration and atrophy before the cell is removed by phagocytosis. It may be speculated that autophagic cell death may be due to stress and oxidative damage of proteins as well as to “starvation” of myocytes isolated in fibrotic tissue. It was concluded, therefore, that autophagic cell death featuring defects in ubiquitin-dependent proteolysis combined with oncosis and apoptosis plays an important role in decreasing the number of viable myocytes. At the present time, it is unknown what type of cell death is predominant in causing myocyte loss. There is no doubt, however, that the combined action of different killing mechanisms causes and further aggravates the functional deterioration observed in failing hearts.

Acknowledgments

This study was supported by grants from the Max-Planck-Gesellschaft (to S.K. and J.S.) and from the Kerschkoff Clinic, Bad Nauheim (Forschungsprojekt PFOR-371 to S.K. and R.Z.). This study was supported by grants from the Max-Planck-Gesellschaft (to S.K. and J.S.) and from the Kerckhoff Clinic, Bad Nauheim (Forschungsprojekt PFOR-371 to S.K. and R.Z.).

References

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Circ Res. 2003;92:715-724; originally published online March 20, 2003;
doi: 10.1161/01.RES.0000067471.95890.5C
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Legends for figures

Online Figure 1. Immunoconfocal features of different types of cell death identified by triple immunolabeling for C9 (arrow), ubiquitin (asterix) and TUNEL (arrowhead) in a myocardial tissue sample from a patient with DCM. The image shown in panel A was obtained using sequential scanning of the tissue section with three confocal detectors for reflected fluorescence and one confocal detector for transmitted light. When the images are superimposed, apart from the fluorescent colour signals, the underlying myocardial tissue can be easily recognized as a dark gray structure. B: Identical field as in Panel A after complete photobleaching of the FITC/TRITC/Cy-5 fluorochromes and subsequently counterstained with F-actin (red) and nuclear dye TOTO-3 (blue).

Online Figure 2. Distinct immunoconfocal patterns of activated caspase-3 in myocytes from the LV ventricular myocardium in patients with DCM. Panels A and B are examples of a cross-striated pattern of caspase-3 confined to the sarcomeric Z-disks. This immunohistochemical pattern of activated caspase-3 was not associated with TUNEL positivity (data not shown). C: Nuclear (arrow) and perinuclear immunoreactivity of the activated caspase-3. D: A comparison with the nuclear stain TOTO (blue) confirms that the fluorescent signal of the activated caspase-3 shown in C is confined not only to the nucleus (arrow) but also to the perinuclear area. Shown in panel E is a myocyte exhibiting TUNEL positivity. F: After complete photobleaching of the FITC-phalloidin signal, the cell was labeled with anti-activated
caspase-3 antibody which showed a nuclear/perinuclear localization. Panel G shows another distinct pattern of caspase-3 positivity characterized by massive, amorphous cytosolic accumulations of the immunofluorescent signal. Panels H is the same confocal field shown in panel G and depicts that the caspase-3 positive myocyte displays a TUNEL positive stained nucleus in purple colour as a result of TUNEL staining (red) with the nuclear dye TOTO (blue). Arrows in panels H and G indicate the identical myocyte.

**Online Figure 3.** Absence of colocalization between ubiquitin and cleaved caspase-3 in patients with DCM. Panel A through C: Double labeling for ubiquitin and activated caspase-3 showing prevalence of the number of myocytes with ubiquitin accumulations over those with signs of in situ cleaved caspase-3 (arrows in panels A and B) and that ubiquitin-positive myocytes lack the caspase-3 immunosignal. C: From all myocytes shown in this panel only one caspase-positive-myocyte (in the boxed region) displayed TUNEL positivity (arrow in the inset, which was made at higher magnification).

**Online Figure 4.** Northern blot for ubiquitin mRNA in 3 control and 8 DCM patients.

**Online Figure 5.** Immuconfofocal images of the ubiquitin activating enzyme E1 (panel A) and ubiquitin ligase E6-AP (Panel B). C: A comparison with the nuclear stain TOTO (blue) confirms that the fluorescent signal shown in B is confined to the nuclei. In all images F-actin is labeled in red with TRITC-phalloidin.
Online Figure 6. Immunogold electron microscopic images of ubiquitin localization in control tissue (panel A) and in apparently preserved myocytes in patients with DCM (panel B). Arrows indicate colloidal gold particles confined to the nucleus (Nuc) and cytosol. Mf - myofibrils. The anti-ubiquitin antibody was visualized using a 10-nm gold-conjugated anti-rabbit secondary antibody C: Omission of the primary anti-ubiquitin antibodies (negative control) resulted in absence of immunogold particles in a myocyte with multiple lamellar-vesicular structures. Arrow depicts a typical double-membrane autophagic vacuole containing a mitochondrion (M). D: In contrast, using primary anti-ubiquitin antibodies resulted in a massive deposition of gold particles in a similar myocyte showing an autophagic phenotype (asterix). Panel E is a higher magnification of the autophagic vacuole shown in panel D with asterix. Note abundance of gold particles within the autophagic vacuole (arrows). F: An example of a typical autophagic vacuole containing cellular organelles (mitochondrion – M) and numerous gold particles (arrow). Scale bars: 1 µm in panels A through D, and 0.5 µm in panels E and F.

Online Table 1: Rate of cell death in normal and failing human myocardium

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* p<0.05 compared to control
Online Table 2: Antibodies used for Western blotting and immunofluorescence

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Host</th>
<th>Dilution:WB/IF</th>
<th>Company</th>
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<tr>
<td>Ubiquitin</td>
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<td>1:250/1:20*#</td>
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<td>Cell Signaling</td>
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</tbody>
</table>

IF: immunofluorescence; WB: Western blot. * - antibodies used for quantitative analysis; # - antibodies used for immunogold electron microscopy
online Figure 1
online Figure 2
Northern blot for the ubiquitin mRNA

online Figure 4
online Figure 5
online Figure 6