Apoptosis in the skeletal muscle of patients with heart failure: investigation of clinical and biochemical changes

G Vescovo, M Volterrani, R Zennaro, M Sandri, C Ceconi, R Lorusso, R Ferrari, G B Ambrosio, L Dalla Libera

Abstract

Objective—To investigate the contribution of apoptosis in the development of the skeletal myopathy in chronic heart failure.

Design—The electrophoretic pattern of myosin heavy chains (MHC), fibre cross sectional area, number of in situ nick end labelling (TUNEL) positive apoptotic myocyte nuclei, and the tissue levels of caspase-3, Bcl-2, and ubiquitin were determined in biopsies taken from the vastus lateralis muscle. The study involved nine patients with severe chronic heart failure caused by ischaemic heart disease and hibernating myocardium and five controls.

Results—In chronic heart failure patients the vastus lateralis showed a significant increase of MHCa and MHCa and a greater degree of fibre atrophy, as demonstrated by the decreased cross sectional area. There was also an increased number of TUNEL positive apoptotic myocyte nuclei. Tissue concentrations of Bcl-2 were decreased, while those of caspase-3 and ubiquitin were increased. Peak oxygen consumption (Vo2) was negatively correlated with the number of TUNEL positive nuclei and the fibre cross sectional area. There was a correlation between the number of apoptotic nuclei and the fibre cross sectional area, but no correlation between myosin heavy chains and number of apoptotic nuclei.

Conclusions—Myocyte apoptosis occurs in the skeletal muscle of patients with chronic heart failure, and its magnitude is associated with the severity of exercise capacity limitation and the degree of muscle atrophy. Muscle atrophy contributes to the limitation of exercise capacity, together with the increased synthesis of fast, more fatiguable myosin heavy chains.

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Keywords: apoptosis; chronic heart failure; exercise capacity; myosin heavy chains

Chronic heart failure is a clinical syndrome characterised by decreased exercise capacity caused by the early occurrence of fatigue and dyspnoea.1–4 There are several theories about the origin of these symptoms, although the contribution of intrinsic skeletal muscle abnormalities, with shift from the “slow” fatigue resistant type I to the more fatiguable “fast” type II fibres, is widely recognised.5–9 However, the pathophysiology of this myopathy is still poorly understood. Triggers for changes in fibre type and their relation to muscle atrophy are obscure, and whether atrophy by itself contributes to the decreased exercise capacity is still debated.10–13 We have recently shown in man that the myosin heavy chain (MHC) composition of the leg skeletal muscle contributes to the limitation of exercise capacity, as peak oxygen consumption (Vo2) correlates positively with the expression of the slow fatigue resistant MHCa and the improvements in AVo2 after six months of pharmacological treatment correlate with the magnitude of changes in MHCa.10 In an experimental model of chronic heart failure we have recently shown that changes in myosin heavy chain composition are not secondary to muscle atrophy. Atrophy correlates with the magnitude of skeletal muscle apoptosis, which is likely to be triggered by circulating concentrations of tumour necrosis factor α (TNFα).11 Our aim in this study was to investigate the role of apoptosis in producing muscle atrophy and changes in fibre type in severe chronic heart failure in the human. This was done by looking at the relation between exercise capacity, changes in myosin heavy chains, degree of fibre atrophy, and myocyte and interstitial apoptosis.

Methods

The study was approved by the ethics committee of the University of Brescia, and written informed consent was obtained.

Patients

We studied nine male patients with severe chronic heart failure who underwent coronary revascularisation. Their details are given in Table 1. They were all diagnosed as having ischaemic heart disease and hibernating myocardium by coronary angiography, dobutamine echocardiography, exercise thallium201 scan, and deoxyglucose positron emission tomography. All underwent maximum symptom limited cardiopulmonary exercise testing with a modified Naughton protocol. A Schiller Cardiovit CS100 (Baar, Switzerland) with 1308 capnograph was used. Oxygen consumption at maximum exercise was expressed as peak oxygen consumption (peak Vo2) defined as the mean oxygen consumption of the last 30 seconds of an incremental exercise test. In all the patients the ejection fraction and the left ventricular end systolic and end diastolic...
volumes were measured with an apical four chamber echo approach. New York Heart Association (NYHA) functional class and diuretic consumption score were also determined.13

QUADRICEPS FEMORIS BIOPSY
A surgical biopsy was taken at the time of bypass surgery and before thoracotomy from the vastus lateralis muscle of the right leg. The size of the specimens was 6–14 mm × 4–8 mm. Biopsies, pinned to a polystyrene support to avoid shrinkage, were immediately frozen in liquid nitrogen.

MUSCLE FATIGUABILITY
The subjects were seated in a rigid frame and maximum isometric strength of the quadriceps femoris was measured with a Cybex Orthotron device (Medway, Massachusetts, USA).13 Thereafter patients were asked to carry out repeated voluntary contractions during a five minute protocol at 30–40% of the maximum. After five minutes a maximum contraction was repeated and fatigability was expressed as per cent of baseline maximum quadriceps strength.17 18

CONTROL SUBJECTS
Controls were five male patients with no history of cardiovascular disease and with a normal ECG and echocardiogram, who were undergoing orthopaedic surgery for mid-femur fracture. None had hypertension or diabetes. A biopsy of the vastus lateralis was taken at the time of surgery.

ELECTROPHORETIC SEPARATION OF MYOSIN HEAVY CHAINS
The method is an improvement of that published by Carraro and Catani,19 and is described in detail by Vescovo and colleagues.11 Biopsies were homogenised and solubilised in 2.3% sodium dodecylsulfate (SDS), 10% glycerol, 0.5% 2-mercaptoethanol, and 6.25 mM Tris- HCl, pH 6.8. Analytical SDS-page was performed on 7% polyacrylamide slabs with 37.5% (vol/vol) glycerol. Identification of individual myosin heavy chains was performed in a separate series of experiments by immunoblotting the gel bands with a panel of monoclonal antibodies against MHC (slow isoform), MHCα (fast oxidative), and MHCβ (fast glycolytic).11 16

ASSOCIATION OF APOPTOSIS
In situ DNA nick end labelling
Serial 8 µm thick cryosections from the vastus lateralis were cut and collected on polylysine precoated slides. In situ nick end labelling (TUNEL) of fragmented DNA was performed using terminal deoxynucleotidyl transferase (TdT) and fluorescein conjugated nucleotides with the in situ cell death detection kit, POD (Boehringer-Mannheim, Mannheim, Germany), as described in the manufacturer's instructions. Negative control slides were prepared by substituting distilled water from the TdT enzyme and continuing with the staining. Labelled nuclei were identified from the negative nuclei counterstained by Hoechst 33258 and counted after being photographed under a fluorescence microscope at 250× magnification as described by Vescovo and colleagues14 and Sandri and associates.21 The total number of TUNEL positive nuclei (muscle nuclei + all other nuclei present in the specimen) was determined by counting all the labelled nuclei present in the whole specimen. Specimen size varied between 22 and 45 mm². The count was performed blind. The number

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Table 1 Patient characteristics

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<th>CHF (months)</th>
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<th>NYHA</th>
<th>EF</th>
<th>LVEDV</th>
<th>LVESV</th>
<th>VO₂</th>
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CAD, coronary artery disease; CHF, duration of chronic heart failure (months); Diur score, diuretic score: 1, thiazide only; 2, 20–40 mg frusemide (furosemide); 3, 40–80 mg frusemide; 4, 80–120 mg frusemide; 5, > 120 mg frusemide; EF, ejection fraction (%); Fatig, fatigability as % of initial maximum strength after 5 min exercise; Fibre CSA, myofibre cross sectional area (µm²); Interst apopt, number of TUNEL positive interstitial nuclei/mm³; LVESV, left ventricular end diastolic volume (ml); LVESV, left ventricular end systolic volume (ml); MHCα, per cent distribution of myosin heavy chain 1 (%); MI, myocardial infarction; Myo apopt, number of TUNEL positive myocyte nuclei/mm³; VO₂, peak oxygen consumption (ml/kg/min).
of positive nuclei was then expressed as number of apoptotic nuclei/mm³, taking into account that each specimen was 8 µm thick. A separate count was carried out for myofibres and for all the other nuclei, which we called interstitial nuclei—these included fibroblasts, immune cells, and vascular endothelial cells. The myocyte nuclei were distinguished from the interstitial nuclei on the basis of their location on sections stained with laminin, which selectively reacts with the basal lamina. TUNEL positive nuclei that were clearly within the muscle fibre boundary (basal lamina) were counted as myonuclei (fig 1).22 All other nuclei were counted as interstitial. It is possible that some of the nuclei may reside in satellite cells, which lie adjacent to the muscle fibres and within the basal lamina. The presence of significant inflammatory infiltrates was excluded on serial histological section stained with haematoxylin and eosin. Separate data for total TUNEL positive nuclei, TUNEL positive myonuclei, and TUNEL positive interstitial nuclei are presented (table 2). Two separate counts were performed by two independent observers and the means reported.

**Western blot for Bcl-2, caspase-3, and ubiquitin**

Fragments of the vastus lateralis were homogenised in SDS buffer. The amount of protein was determined by the Lowry method after precipitation with 10% trichloroacetic acid.23 The homogenates were analysed by SDS-PAGE, 12.5% polyacrylamide, and western blotting by loading 45 µg protein/lane. The following conditions were used for binding the antibody: anti-caspase-3 CPP 32 (H-277) (34 kDa) and anti-Bcl-2 (29 kDa) (Santa Cruz Biotechnology, Santa Cruz, California, USA) were diluted 1:200 and 1:100, respectively; anti-ubiquitin and anti-rabbit alkaline phosphatase linked antibody (Sigma Chemical Co, St Louis, Missouri, USA) were diluted 1:100 and 1:4000, respectively. The absolute values were calculated on the blot bands with the same densitometric system described above. Values are expressed in arbitrary units (AU) (integrated area of densitometric scans). Bcl-2, caspase-3, and ubiquitin were assessed in all the biopsies.14 For caspase-3, we analysed the band derived from the reaction between the caspase-3 antibody and the activated 20 kDa subunit.

**Statistical analysis**

Mean (SD) values are given. Student’s t test for unpaired data was used where appropriate. Linear regression was also used. A 5% difference was considered significant.

**Results**

**Patient characteristics**

Patient characteristics are summarised in table 1.

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**Comparison between patients with chronic heart failure and control subjects**

**Age**

Age was not significantly different between chronic heart failure patients and controls, at 57.1 (11.9) v 56.8 (8.6) years (NS).

**Myosin heavy chain composition**

Heart failure patients had a significant decrease in the slow MHC, (58.4 (14.9)% v 76.0 (1.6)%; p < 0.0001) and an increase in the fast oxidative MHC2a, (19.8 (4.4)% v 17.8 (1.3)%; p < 0.0001) and the fast glycolytic MHC2b, (20.7 (12.8)% v 6.2 (1.5)%; p < 0.0013).

**TUNEL positive apoptotic nuclei**

In the vastus lateralis of the patients with chronic heart failure there was a significantly greater number of TUNEL positive myocyte and interstitial nuclei (fig 1). The numbers of apoptotic myocyte nuclei were 18.8 (19.6) in the chronic heart failure group versus 0 (0) in the controls (p < 0.021), while the numbers of interstitial apoptotic nuclei were 33.7 (25.9) v 4.0 (5.5) (p < 0.03). Because of their intralaminin position and their relation to the capillary wall, the majority of the positive interstitial nuclei could be identified as endothelial cell nuclei.

**Bcl-2, caspase-3, and ubiquitin**

Caspase-3 was increased in the vastus lateralis of the heart failure patients (453.6 (160.8) AU) v 151.2 (1.65) AU, p < 0.0001), while Bcl-2 was decreased (65.3 (26.7) v 101.2 (1.8) AU, p < 0.0001). Ubiquitin was slightly, though...
significantly, increased as well (157.5 (31.4) vs 133.9 (6.0) AU, p < 0.0001). An example of western blot for Bcl-2 and caspase-3 is shown in fig 2.

Fibre cross sectional area

Vastus lateralis muscle from the chronic heart failure patients showed a significantly decreased fibre size (3748 (1024) vs 4415 (129) µm², p < 0.01).

CORRELATION BETWEEN APOPTOSIS, DEGREE OF FIBRE ATROPHY, MYOSIN HEAVY CHAIN PATTERN, AND INDICES OF SEVERITY OF CHRONIC HEART FAILURE AND FATIGUABILITY

Apoptosis vs fibre cross sectional area

We found a negative correlation between number of TUNEL positive myocyte nuclei and fibre cross sectional area ($r^2 = 0.51$, $p = 0.004$). When the data were analysed only within the heart failure group this correlation could still be found ($r^2 = 0.44$, $p = 0.05$) (fig 3).

Apoptosis versus myosin heavy chain composition

Although there was a trend for MHC1 to be negatively correlated, and MHC2a and MHC2b to be positively correlated, with the number of TUNEL positive nuclei, this did not reach significance ($r^2 = 0.14$, 0.16, 0.11 for MHC1, 0.05, 0.04, 0.06 for MHC2a and 0.17, 0.19, 0.13 for MHC2b (total, myocyte, and interstitial nuclei, respectively)).

Apoptosis and myosin heavy chains versus indices of severity of chronic heart failure

There was a correlation between peak VO2 and the number of TUNEL positive nuclei ($r^2 = 0.56$, $p = 0.03$ for total, $r^2 = 0.73$, $p = 0.006$ for myocytes, and $r^2 = 0.41$, $p = 0.08$ for interstitial cells) (fig 4). No significant correlation was found between TUNEL positive nuclei, NYHA class, ejection fraction, diuretic score, and ventricular volumes.

We could not find any significant correlation between Bcl-2, ubiquitin, and caspase-3 and any of the indices of severity of chronic heart failure (peak VO2, NYHA class, diuretic score, ejection fraction, and ventricular volumes).

Although there was a trend for peak VO2 to be positively correlated with MHC1 ($r^2 = 0.16$, $p = 0.5$) and negatively with MHC2a ($r^2 = 0.11$, $p = 0.4$) and MHC2b ($r^2 = 0.13$, $p = 0.38$), this did not reach significance. The same was true for NYHA class ($r^2 = 0.2$ for MHC1, $p = 0.2$). No significant correlation was found between myosin heavy chains and ejection fraction, ventricular volumes, and diuretic score. The percentage distribution of myosin heavy chains was not correlated with fibre cross sectional area ($r^2 = 0.02$, $r^2 = 0.00$, $r^2 = 0.11$). There was, however, a significantly positive correlation between quadriceps femoris fatiguability, peak VO2 ($r^2 = 0.48$, $p = 0.038$), fibre cross sectional area ($r^2 = 0.44$, $p = 0.048$), and myocyte apoptosis ($r^2 = 0.45$, $p = 0.049$).

Discussion

It is well known that in chronic heart failure central haemodynamic variables such as cardiac output, ejection fraction, and ventricular volumes and pressures do not correlate with the reduction in exercise capacity.9

The early appearance of muscle fatigue is the result of a reduced oxidative capacity, which is closely linked to changes in fibre type and myosin heavy chain composition9,10 and to the muscle bulk loss. The relative contribution of these muscle abnormalities to the decreased exercise tolerance and muscle endurance has not been fully elucidated yet. Moreover, little is
known about the pathogenesis of muscle atrophy. In this paper we have investigated the role played by apoptosis in reducing muscle endurance and exercise capacity. Exercise capacity is expressed in terms of peak V\textsubscript{O}\textsubscript{2}, which is certainly one of the most precise indices of exercise tolerance.\textsuperscript{25, 26} At the same time we tested the hypothesis that apoptosis could produce muscle atrophy.

It has been shown by Volterrani and colleagues that the best predictors of exercise capacity in chronic heart failure are measures of skeletal muscle function and bulk.\textsuperscript{17} These investigators have reported that muscle strength and cross-sectional area explain 82% of the variation in peak V\textsubscript{O}\textsubscript{2}. Anker and colleagues also showed that there was a correlation between measures of fatigability and peak V\textsubscript{O}\textsubscript{2}.\textsuperscript{19} However, the link between changes in muscle mass, strength, endurance, and fibre type, and their relation with exercise capacity are still debated.

In this paper we have shown, in accord with Adams and colleagues,\textsuperscript{27} that apoptosis occurs in the skeletal muscle of patients with chronic heart failure to a higher degree than in subjects with no evidence of cardiovascular disease, in whom this phenomenon is almost absent. However, in the present paper, we have for the first time tried to determine whether this biological phenomenon is associated with some of the known pathophysiologic and clinical features of chronic heart failure. This is a novelty in that previous work on apoptosis in chronic heart failure has been purely observational.\textsuperscript{28, 29} We have shown that patients with severely limited exercise capacity—as demonstrated by the very low peak V\textsubscript{O}\textsubscript{2} and high fatigability—have muscle fibre atrophy and increased numbers of myocytes and interstitial nuclei undergoing apoptosis. Because of the relation between the number of apoptotic nuclei and the degree of fibre atrophy, it can be speculated that apoptosis may play a role in determining muscle bulk loss. This is in accordance with some recently published data showing that, in a rat model of heart failure, skeletal muscle apoptosis is accompanied by the occurrence of muscle atrophy.\textsuperscript{11} This is also in keeping with data showing that apoptosis plays an important role in the pathophysiology of chronic heart failure and cardiomyopathies, contributing to myocardial cell loss and therefore to the appearance or worsening of ventricular dysfunction.\textsuperscript{25, 26, 29} The only difference between the myocardium and skeletal muscle is that skeletal myocytes are multinucleated; therefore loss of nuclei produces atrophy rather than cell death.\textsuperscript{25, 30}

In our study the magnitude of apoptosis correlated negatively with peak V\textsubscript{O}\textsubscript{2}. This is not surprising as the more symptomatic the patient, the greater are the skeletal muscle abnormalities. The reduction in peak V\textsubscript{O}\textsubscript{2} and endurance correlates with an index of muscle atrophy—the fibre cross sectional area. On the other hand we confirmed in this study an observation made in an experimental model of chronic heart failure, where it was found that levels of apoptosis correlated with the severity of decompensation.\textsuperscript{14} The occurrence of apoptosis in the vastus lateralis muscle of our patients is confirmed by changes in the tissue concentrations of specific factors that can protect from or induce programmed cell death. Bcl-2, which is known to play a protective role, is in fact decreased, and caspase-3, which is a compulsory step in the formation of the apoptotic bodies, is significantly increased.\textsuperscript{25, 31} The increased ubiquitin concentrations indicate that the proteosome pathway is also activated, leading to protein waste, which may contribute to atrophy independently of apoptosis.\textsuperscript{32}

The magnitude of apoptosis is greater in interstitial cells than in myocytes. This has been shown both by our group in experimental chronic heart failure\textsuperscript{15} and by others in skeletal muscle affected by dystrophinopathies.\textsuperscript{21, 30} The magnitude of apoptosis in our observations is comparable to that found in other studies, the total number/mm\textsuperscript{3} of apoptotic nuclei being similar.\textsuperscript{14, 22, 35}

At the same time, in the vastus lateralis a significant shift toward the expression of “fast” fatigue fibres occurred, as shown by the increase of the MHC\textsubscript{a} and MHC\textsubscript{b} isoforms. Myosin heavy chain redistribution is not likely to be the result of myocyte apoptosis or selective type fibre loss by apoptosis.\textsuperscript{19} Our findings confirm previous reports in which patients with chronic heart failure were found to have greater expression of MHC\textsubscript{a} and a decreased percentage of chronic heart failure\textsuperscript{9, 15} This shift is known to contribute to the limitation of exercise capacity in patients with chronic heart failure. A correlation between peak V\textsubscript{O}\textsubscript{2} and MHC, has in fact been described. In the present study we only found a trend for peak V\textsubscript{O}2 to correlate with MHC\textsubscript{a} but our study was conducted in severely sick patients (11.1 ml/kg/min \( <V\textsubscript{O}2 < 15.6\) ml/kg/min), while previous correlations between exercise capacity and MHC\textsubscript{a} have been obtained across a wide range of chronic heart failure severity, from asymptomatic early ventricular dysfunction to patients on the waiting list for cardiac transplantation.\textsuperscript{8, 15}

In the present study exercise capacity seems to have been more influenced by skeletal muscle trophism than by myosin heavy chain composition, as peak V\textsubscript{O}\textsubscript{2} correlated with muscle fibre cross sectional area, while the correlation between peak V\textsubscript{O}2 and myosin heavy chains was weak. Moreover, it looks as though myosin heavy chain composition and muscle trophism contribute independently to the limitation of exercise capacity, as there was no statistical correlation between these two factors.

Our present data confirm previous observations made by our group in the gastrocnemius of patients with chronic heart failure, in whom the myosin heavy chain shift was neither correlated with muscle mass nor with muscle strength.\textsuperscript{33}

The influence of muscle stress and fatigability on exercise capacity remains controversial, and it has been suggested that some of the reported differences may reflect the varying disease severity in the patients investigated, who therefore presented different degrees of

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muscle atrophy. In fact, while in this study—which only included patients with severe chronic heart failure—we found a correlation between indices of muscle trophism (cross-sectional area), fatigability, and peak VO2 in a paper previously published by our group and looking at mild to moderate chronic heart failure, exercise capacity was only marginally dependent on muscle bulk. However, muscle bulk was estimated in terms of muscle cross-sectional area calculated on computed tomography scans, slices, which is less sensitive than the measurement of single fiber cross-sectional area. The findings in our present paper are partially in agreement with those of Mannini and colleagues, who found skeletal muscle atrophy in 68% of patients with chronic heart failure and a correlation between peak VO2 and measures of muscle bulk. However, our data are more in accord with those ofVolterrani et al, Harrington et al and Anker et al, who found that muscle atrophy becomes a major determinant of exercise capacity. The influence of muscle mass in limiting physical work was also shown by Shepard and colleagues in normal subjects and by Pleg and Lakatta in the elderly. In this study we therefore confirm that peak VO2 and atrophy are linked. Our data strengthen the link between apoptosis, atrophy, and muscle function, as myocyte apoptosis and fibre cross-sectional area correlated with the decreased muscle strength during repetitive exercise. However, this relation may be true for severely sick and deconditioned patients, while in earlier stages of the chronic heart failure syndrome it may not be so evident. It looks as though the patients in the present study, who had severe chronic heart failure, behaved like those with cardiac cachexia, in whom muscle atrophy further contributes to the deterioration in exercise capacity, not only because of the loss of muscle bulk but also because of the decreased strength per unit area.

CONCLUSIONS

Myocyte and interstitial apoptosis occurs in the leg skeletal muscle of patients with chronic heart failure. The limitation in functional capacity reflects two different skeletal muscle dependent factors: muscle atrophy, which accompanies skeletal myocyte apoptosis, and a shift of myosin heavy chains toward fast more fatigable isoforms that have higher oxygen and ATP consumption and reach the anaerobic threshold earlier.

We thank Dr Stefano Fregua for the collection of muscle biopsies of control subjects and Mr Valentino Gobbo for skilful technical assistance. The work has been supported in part by a grant “Progetto finalizzato” of the “Veneto Region” Italy.

Prolapse of an atrial myxoma into the apex of the left ventricle

A 31 year old scuba diving instructor presented with a history of recurrent palpitations while diving to depths of 30 m. An early diastolic sound was heard on cardiac auscultation. Transthoracic and transoesophageal echocardiography demonstrated a large left atrial mass, attached to the interatrial septum, which prolapsed into the left ventricle during diastole. Surgical exploration of the left atrium revealed a 7.5 x 1.9 x 0.9 cm polypoid, gelatinous tumour arising from the fossa ovalis via a narrow pedicle. The lesion was excised in its entirety and subsequent histology confirmed the clinical diagnosis of left atrial myxoma. Following surgery the patient remains well.