Identification of cathepsin L as a differentially expressed message associated with skeletal muscle wasting

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Alteration of skeletal muscle protein breakdown is a hallmark of a set of pathologies, including sepsis, with negative consequences for recovery. The aim of the present study was to search for muscle markers associated with protein loss, which could help in predicting and understanding pathological wasting. With the use of differential display reverse transcription-PCR, we screened differentially expressed genes in muscle from septic rats in a long-lasting catabolic state. One clone was isolated, confirmed as being overexpressed in septic skeletal muscle and identified as encoding the lysosomal cysteine endopeptidase cathepsin L. Northern- and Western-blot analysis of cathepsin L in gastrocnemius or tibialis anterior muscles of septic rats confirmed an elevation (up to 3-fold) of both mRNA and protein levels as early as 2 days post-infection, and a further increase 6 days post-infection (up to 13-fold). At the same time, the increase in mRNAs encoding other lysosomal endopeptidases or components of the ubiquitin–proteasome pathway did not exceed 4-fold. Cathepsin L mRNA was also increased in tibialis anterior muscle of rats treated with the glucocorticoid analogue, dexamethasone, or rats bearing the Yoshida Sarcoma. The increase in cathepsin L mRNA was reduced by 40% when the tumour-bearing animals were treated with pentoxifylline, an inhibitor of tumour necrosis factor-α production. In conclusion, these results demonstrate a positive and direct correlation between cathepsin L mRNA and protein level and the intensity of proteolysis, and identify cathepsin L as an appropriate early marker of muscle wasting. Cathepsin L presumably participates in the pathological response leading to muscle loss, with glucocorticoids and tumour necrosis factor-α potentially being involved in the up-regulation of cathepsin L.

Key words: cancer, lysosome, muscle loss, sepsis.

INTRODUCTION

The response to infection, injury or cancer is often associated with a loss of body weight. In particular, there is a pronounced muscle wasting manifested as an excessive nitrogen loss, that cannot solely be explained by the reduction in food intake that characterizes these pathologies [1]. Skeletal muscle wasting during infection provides amino acids used for host defence, including synthesis of acute phase proteins in the liver and activation of immune cells [2,3]. However, pronounced muscle wasting is usually a negative prognostic factor for the recovery of patients. Thus the ability to anticipate such muscle loss by having appropriate early markers would be advantageous, together with the use of an appropriate nutritional supply.

The main cause of muscle wasting is via increased protein breakdown, sometimes in association with depressed protein synthesis [4–6]. In muscle and most tissues, at least three intracellular proteolytic systems can contribute to the degradation of proteins. Cytosolic proteins can be conjugated to ubiquitin for their degradation by the 26 S proteasome [7], or they can be hydrolysed by the Ca^{2+}-activated μ- or m-calpains [8]. Intracellular proteins can also be targeted to the lysosome, which is enriched in numerous hydrolytic enzymes, including the cathepsins [9]. Although lysosomal proteolysis can be stimulated by the induction of macroautophagy in conditions of lowered amino acid supply [10], this proteolytic system is not involved in the initial degradation of myofibrillar proteins [11]. The calpains could play a role in the disassembly of sarcomeric proteins, in particular by degrading nebulin [12]. In contrast, the ubiquitin–proteasome-dependent pathway has been found to be activated in all animal models of muscle wasting [13], except in dystrophin-deficient mice [14].

In a rat model of infection, reproducing a sustained and reversible catabolic state as observed in humans, it has been shown that the ubiquitin–proteasome system has a major role in the acceleration of muscle proteolysis [5]. However, both lysosomal- and Ca^{2+}-dependent pathways also appear to be activated later on during the chronic septic phase [5]. This model was used in the present study to identify and characterize the genes whose expression was altered in catabolic muscles. The objectives were (1) to define early markers of muscle wasting and (2) to better understand the molecular mechanisms associated with protein loss. The mRNA expression pattern was compared between muscles of infected animals and their pair-fed controls. The use of pair-fed animals as controls enabled us to specifically study the role of infection without the effect of anorexia. Differential gene expression was analysed by differential display reverse transcription-PCR (DDRT-PCR). One cDNA, corresponding to cathepsin L mRNA, was confirmed as being positively regulated in muscle. The subsequent documentation of its regulation in other catabolic models suggests a correlation between muscle wasting and increased cathepsin L gene expression.

EXPERIMENTAL

Animals and treatments

Experimental catabolic animal models of sepsis or tumour-bearing animals were used as previously described [5,15,16].

Abbreviations used: DDRT-PCR, differential display reverse transcription-PCR; MMA, multiplex mRNA analysis; TNF-α, tumour necrosis factor-α; IFN-γ, interferon γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Table 1 Names and nucleotide sequences of arbitrary primers used in the PCR reaction

Name	Sequence (5′ → 3′)	Name	Sequence (5′ → 3′)
HPA1	AAGCTTGATTGCC	HPA9′	AAGCTTCACAGCG
HPA2	AAGCTTCGACTGT	HPA10′	AAGCTTGATCCCC
HPA3	AAGCTTTGGTCAG	HPA11′	AAGCTTCGACTCC
HPA4	AAGCTTCTCAACG	HPA12′	AAGCTTATCCACC
HPA5	AAGCTTAGTAGGC	HPA13′	AAGCTTATCTCCG
HPA6′	AAGCTTACATCCG	HPA14′	AAGCTTAGTACCG
HPA7′	AAGCTTATCCAGC	HPA15′	AAGCTTCGATACC
HPA8′	AAGCTTCTCGTAC	HPA16′	AAGCTTACACG

Briefly, male Sprague–Dawley rats, with an initial body weight of 300 g, received either saline or live *Escherichia coli* (serotype $0153:K^-:H^-$; 7×10^8 colony-forming units) by intravenous tail injection and were studied at day 2 or 6 after infection. Control animals were pair-fed to the intake of infected animals [5]. Young male Wistar rats weighing 50–70 g were injected via the vastus lateralis muscle with 0.3 ml of a homogenate of Yoshida Sarcoma (200 mg of solid tumour tissue/ml of sterile 0.9% NaCl) or with saline for 9 days. Half of the animals injected with the Yoshida Sarcoma were also treated daily with pentoxifylline (100 mg/kg) [16]. Studies were performed on the contralateral muscles not injected with the Yoshida Sarcoma. In a third catabolic model, male Sprague–Dawley rats, with an initial body weight of 300 g, were given the synthetic glucocorticoid analogue, dexamethasone, in the drinking water so that animals received approx. 550 μg/kg per day [17] for 5 days.

DDRT-PCR

Total RNA was extracted from gastrocnemius muscles of septic rats 6 days post-infection using guanidinium thiocyanate [18]. DDRT-PCR was performed as previously described [19] with minor modifications. Briefly, a degenerate anchored oligo-dT primer ($T_{12}MG$, $T_{12}MA$, $T_{12}MT$ or $T_{12}MC$; where M represents dA, dC or dG) was annealed to 0.3 μg of DNase I-treated total RNA. mRNA reverse transcription was then performed for 1 h at 37 °C using murine Moloney-murine-leukaemia virus reverse transcriptase (Life Technologies). Duplicates of the reverse transcription reaction product (2 μl) were amplified by PCR in the presence of the anchored primer and one arbitrary 13-oligomer primer (see Table 1) for 40 cycles (94 °C, 30 s; 40 °C, 2 min; 72 °C, 30 s) and further extended for 5 min at 72 °C. [α-^{33}P]dATP (1 μCi) was included in the PCR reaction mixture. Radiolabelled PCR products were resolved on a 6% non-denaturing polyacrylamide gel and revealed using a Phosphofluoro-Imager (Amersham Pharmacia Biotech). Each differential fragment was cut out of the gel, hydrated for 15 min in 100 μl of H_2O and eluted at 100 °C. The fragments were re-amplified by PCR using the corresponding 13-oligomer and anchored oligo-dT primer that incorporated a T7 RNA polymerase binding site at its 5′ end (5′ GAATTCTAATACGACTCACTAGGGAA-GCT_{11}MN 3′; where M represents dA, dC or dG) [20]. At this step, the presence of contaminating re-amplified bands eluted from the gel, together with the fragment of interest, is often observed [21]. Thus a secondary screening was performed to eliminate such contaminating differentially displayed fragments and also to confirm the results of the first screening. The re-amplified mixture was sub-cloned into the pGem T vector (Promega) and propagated in DH5α F' *E. coli* strain. After selection on ampicillin, amplification and DNA extraction of individual clones, two Southern blots were performed and hybridized using radiolabelled residual DDRT-PCR products from control and infected samples. After selection of the positive clones, RNA analysis was performed by ribonuclease protection assay and by Northern-blot analysis.

Northern-blot analysis

Total RNA (10–30 μg) was resolved on 1% denaturing agarose
gels, blotted on to nylon Hybond N^+ and cross-linked by UV irradiation. Probes were generated from 50–100 ng of mouse cathepsin L, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18 S cDNA inserts in the presence of [α-^{32}P]dCTP using the Ready To Go labelling kit (Amersham Pharmacia Biotech), and purified by gel filtration (Sephadex G50). Pre-hybridization for 2 h and hybridization for 20–24 h were performed at 68 °C in 5 × SSC/5 × Denhardt's reagent/0.5% SDS/100 μg/ml sonicated DNA, followed by successive washes in 2 × SSC/0.1% SDS and 0.5 × SSC/0.1% SDS. Hybridization signals were visualized with Phosphofluoro-Imager and quantified using the ImageQuant software (Amersham Pharmacia Biotech). The rat cathepsin L mRNA is 1.7 kb long [22].

DNA sequencing

DNA sequences were determined by the dideoxynucleotide chain-termination method [23] using double-stranded plasmid DNA. All sequences were compared with the non-redundant GenBank®/EMBL database using the FASTA software (GCG, Madison, WI, U.S.A.).

Multiplex mRNA assay (MMA)

Target DNAs were amplified by PCR, purified and denatured at 95 °C for 10 min before being dotted on to a Hybond N^+ membrane (50 ng for each cDNA), and cross-linked by UV irradiation. A synthetic mRNA of *Arabidopsis thaliana* cytochrome *c*554 (cDNA in pHD-1 vector; kindly provided by H. Hofte, UR 501 Unité Biologie Cellulaire, INRA Versailles, France) was synthesized from the T3 promoter using the RiboMax Large Scale production system (Promega). Following oligo(dT) annealing, 15 μg of total RNA was reverse transcribed at 42 °C for 2 h with Superscript II in the presence of [α-^{32}P]dCTP. Cytochrome *c*554 mRNA was added in the reverse transcription reaction [0.25% of the estimated (A^+)RNA] for normalization and quantification after hybridization. Following reverse transcription, total RNA was degraded by NaOH treatment (0.4 M NaOH for 30 min at 68 °C) and the ^{32}P-labelled cDNA probe purified by gel filtration (Sephadex G50). Pre-hybridization for 24 h and hybridization for 48 h were performed at 68 °C in 5 × SSC/5 × Denhardt's reagent/0.5% SDS/100 μg/ml sonicated DNA, and membranes were washed at 68 °C successively with 2 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS and finally with 0.1 × SSC/0.1% SDS. Hybridization signals were detected using a Phosphofluoro-Imager and quantified using ImageQuant software.

Western-blot analysis

A portion (200 mg) of muscle was homogenized in 20 mM phosphate buffer (pH 6.25)/0.1% Triton X-100, and frozen (−80 °C) and thawed twice. The homogenate was centrifuged at 15000 ***g*** for 15 min at 4 °C. Equal amounts of protein (30 μg) were heated at 90 °C for 10 min, loaded on to a 12% (w/v)

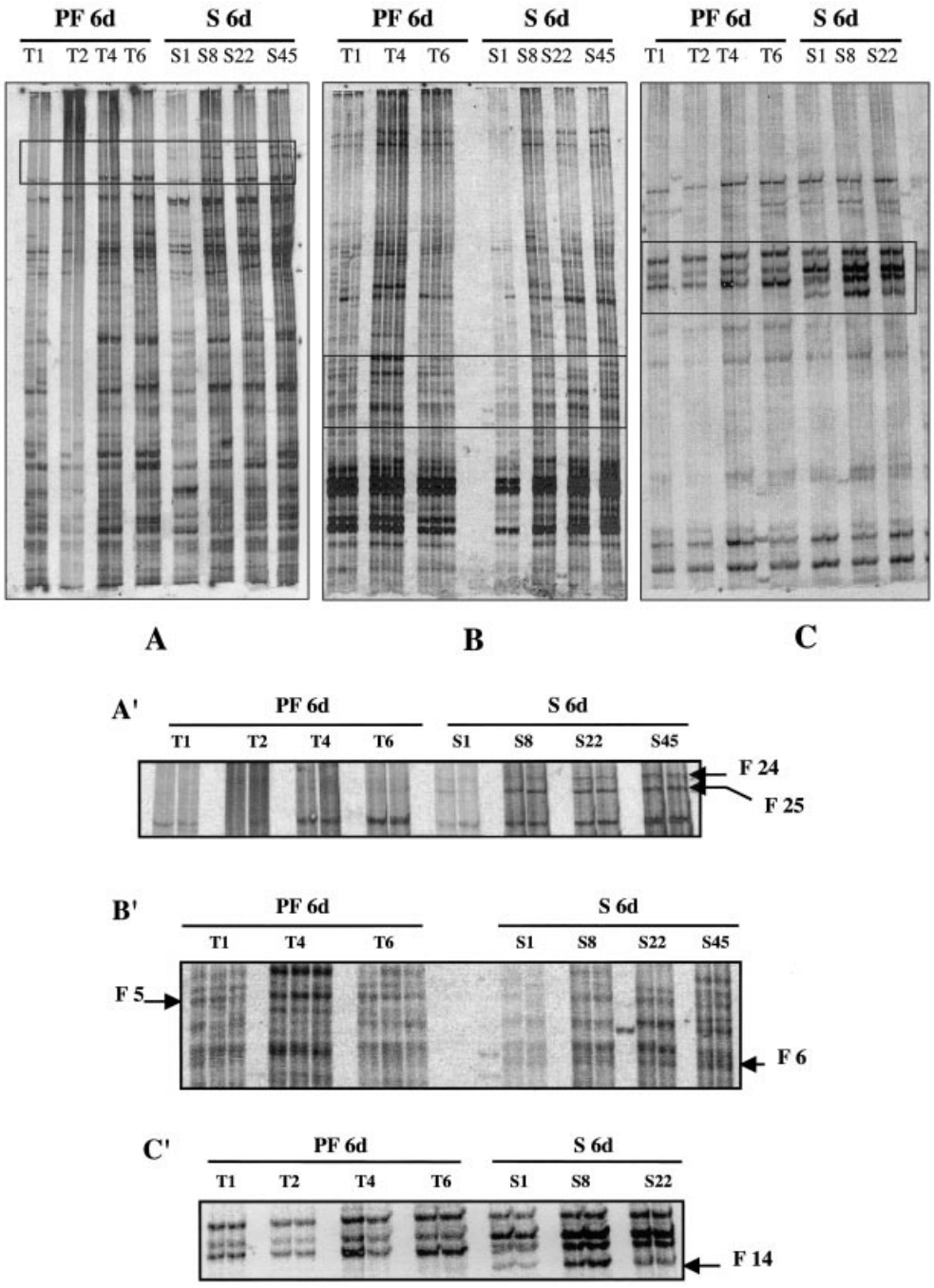

Figure 1 Screening of differentially expressed genes in muscle from septic rats

DDRT-PCR was performed as described in the Experimental section. Parts of three representative non-denaturing PAGE gels of RT-PCR-labelled products synthesized from gastrocnemius total RNA are shown (**A**, **B** and **C**). In these examples, RT was performed with T_{12}MC followed by PCR with T_{12}MC and the arbitrary 13-oligomer HPA1 (**A**), HPA12′ (**B**) or HPA6′ (**C**). The DNA pattern was compared between muscles of non-infected pair-fed control rats at 6 days (PF 6d; four animals T1, T2, T4 and T6) and septic rats 6 days post-infection (S 6d; four animals S1, S8, S22 and S45). (**A**′, **B**′ and **C**′) An enlargement of zones containing differential fragments. The arrows show some RT-PCR products differentially amplified (F) in muscle of septic and pair-fed animals.

polyacrylamide gel and resolved by SDS/PAGE under reducing conditions [24]. Proteins were transferred on to Hybond C and the membrane was blocked in PBS containing 0.2% non-fat dried milk and incubated with an antibody raised against mouse cathepsin L (kindly provided by Professor M. M. Gottesman, National Cancer Institute, Bethesda, MD, U.S.A.) for 1 h at 20 °C. After incubation, the membrane was washed with PBS and incubated with an alkaline phosphatase-conjugated anti-rabbit IgG. Antigen–antibody complexes were revealed using enhanced chemiluminescence detection reagents (ECL®; Amersham Pharmacia Biotech), detected using a Phosphofluoro-Imager and quantified using ImageQuant software.

RESULTS

Identification of cathepsin L as a differentially expressed gene in septic muscle

Using a previously characterized animal model of sepsis [5,15], we have searched for genes that are overexpressed in muscle undergoing wasting by using the DDRT-PCR technique first reported by Liang and Pardee [19]. Total RNA was extracted from the gastrocnemius muscle of rats 6 days post-infection, a time point when muscle protein loss is significant, and compared with total RNA from gastrocnemius muscles of pair-fed control animals. For the primary screening (see the Experimental section), a total of 32 reactions were performed using 16 different random 13-oligomer primers (Table 1) in combination with two anchored primers (T_{12}MC and T_{12}MG). The PCR products from a series of three independent RNA samples from infected animals, or a series of four independent RNA samples from pair-fed animals, showed virtually identical banding patterns with each primer set. Representative gels of DDRT-PCR-labelled products obtained using the T_{12}MC anchored primer and the arbitrary HPA1, HPA12′ and HPA6′ 13-oligomers (Table 1) are shown in Figure 1. Most of the random 13-oligomers amplified 41 ± 10 mRNA fragments as revealed on non-denaturing gels, corresponding to approx. 10 % of the estimated 10000 mRNA species present in a cell [25]. Figure 1 shows that five (F5, F6, F14, F24, F26) out of 29 differentially displayed bands were reproducibly observed when the pattern between septic and pair-fed animals was compared. However, among the 29 differentially expressed bands, only 15 were confirmed as positive after the secondary screening (see the Experimental section). Finally, only F14 (Figure 1C′) was confirmed as being overexpressed in muscle of septic animals as shown by ribonuclease protection assay (results not shown). The nucleotide sequence obtained for F14 was analysed using the non-redundant GenBank®/EMBL database and was identified as encoding the distal 270 nucleotides of the 3′ untranslated region of the rat lysosomal cysteine endopeptidase, cathepsin L cDNA.

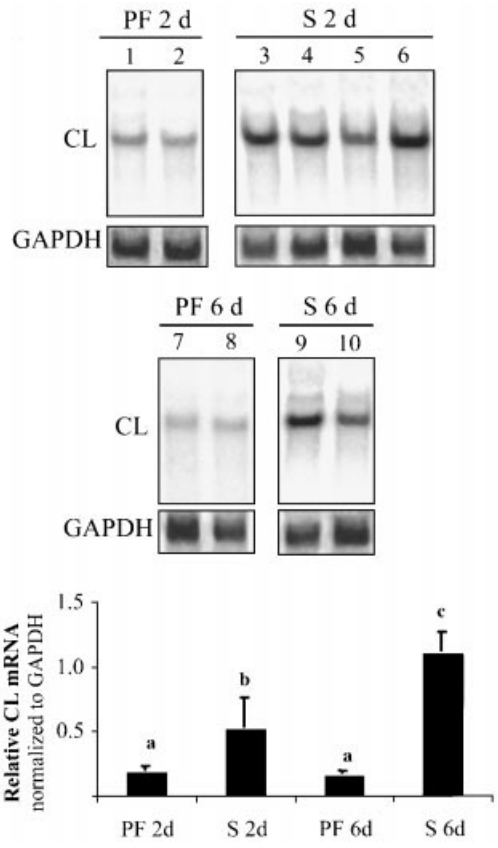

Figure 2 Cathepsin L (CL) mRNA expression in gastrocnemius muscle of septic rats

Total RNA was extracted from rat gastrocnemius muscle of septic (S) and pair-fed (PF) animals. Muscle samples obtained 2 days (2d) post-infection (upper panel: PF 2d, lanes 1 and 2 from 2 animals; S 2d, lanes 3–6 from 4 animals) were analysed together with samples used in the DDRT-PCR experiments obtained 6 days (6d) post-infection (middle panel: PF 6d, lanes 7 and 8 from 2 animals; S 6d, lanes 9 and 10 from 2 animals). A portion (20 μg) of total RNA was analysed by Northern blot using a mouse cathepsin L probe. The same membrane was re-hybridized with a GAPDH probe. Lower panel: CL mRNA content was normalized to the GAPDH signal and the data expressed in arbitrary units as means $\pm$ S.D. after ImageQuant analysis from at least three independent determinations. Values not sharing a common letter are significantly different ($P < 0.05$).

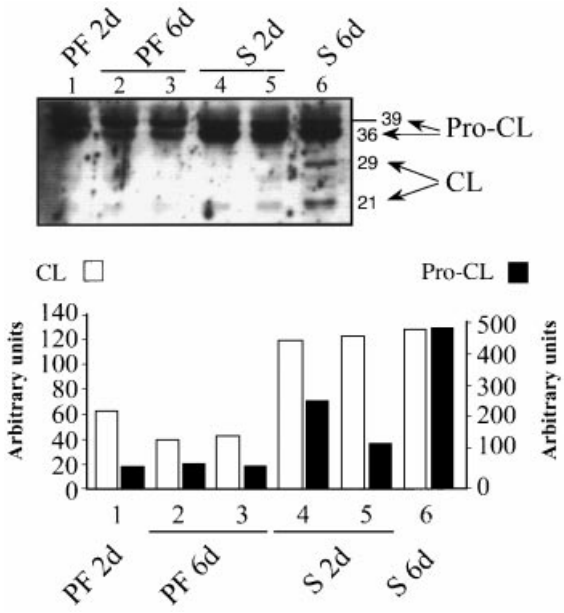

Figure 3 Immunoblot of cathepsin L in gastrocnemius muscle of septic rats

Gastrocnemius muscle proteins were extracted from septic (S) or pair-fed (PF) rats at day 2 (2d) or 6 (6d) post-infection. Proteins were resolved by SDS/PAGE and Western blotted with an antibody raised against mouse cathepsin L as described in the Experimental section. Molecular mass (in kDa) for the mature cathepsin L (CL) and pro-cathepsin L (Pro-CL) are indicated on the right. The relative amounts of CL and pro-CL in samples from pair-fed and sepsis rats at days 2 and 6 are indicated.

Both cathepsin L mRNA and protein are increased in muscle of septic rats

In order to confirm overexpression of the cathepsin L gene in septic muscle, a complete cDNA encoding mouse cathepsin L was used as a probe for Northern-blot analysis. The expression of cathepsin L in gastrocnemius muscle was tested 2 or 6 days post-infection and compared with that of pair-fed rats (Figure 2). A 2.8-fold increase in cathepsin L mRNA was detected during the acute septic phase (2 days post-infection). During the chronic septic phase (6 days post-infection), the cathepsin L mRNA level was further increased (up to 7.4-fold). To ascertain that the elevation of cathepsin L mRNA level correlated with an increase

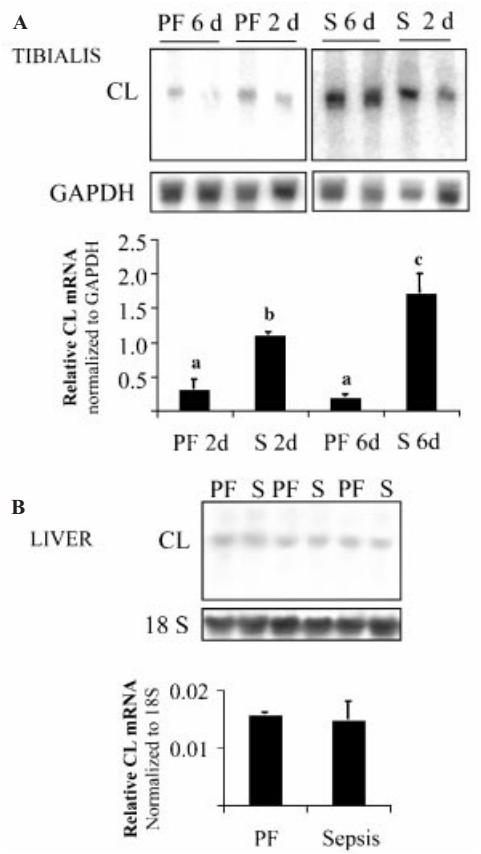

Figure 4 Cathepsin L mRNA expression during sepsis in tibialis anterior muscle and liver

A portion (20 μg) of total RNA was analysed by Northern blot using a mouse cathepsin L probe. (**A**) A representative Northern blot obtained with total RNA extracted from tibialis anterior of septic (S) and pair-fed (PF) rats, at 2 days (PF 2d; S 2d) or 6 days (PF 6d; S 6d) post-infection (two animals each). The same membrane was re-hybridized with a GAPDH probe. (**B**) Total RNA was extracted 6 days post-infection from the liver of septic (S) or pair-fed (PF) rats (three animals). The same membrane was re-hybridized with a 18 S probe. The CL mRNA content was normalized either to the GAPDH (**A**) or 18 S (**B**) signal and the data expressed in arbitrary units as means $\pm$ S.D. after ImageQuant analysis from at least three independent determinations. Values not sharing a common letter are significantly different ($P < 0.05$)

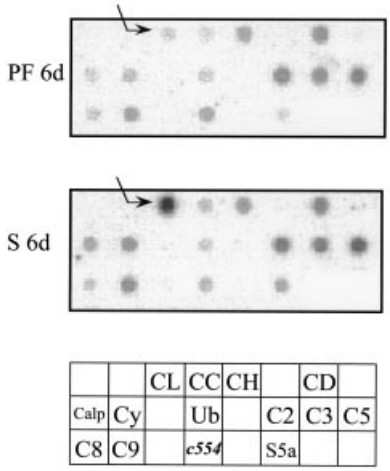

Figure 5 Effect of sepsis on expression of mRNAs encoding different proteolytic genes in muscle

cDNA for a variety of target proteolytic genes were obtained, dotted on to a Hybond N^+ membrane and hybridized with ^{32}P-labelled cDNA probes generated with 15 μg of total RNA extracted from the gastrocnemius muscle of septic (S) and pair-fed control (PF) rats 6 days post-infection, as described in the Experimental section. A representative blot of at least three independent experiments using distinct samples is shown. The arrows indicate the position of cathepsin L (CL). The positions of the dotted cDNAs used to perform the MMA are shown in the grid at the bottom. Abbreviations used are: CC, cathepsin C; CH, cathepsin H; CD, cathepsin D; Calp; calpastatin; Cy, cystatin C; Ub, ubiquitin.; *c554*, *A. thalania* cytochrome *c*554. C2, C3, C5, C8 and C9 are 20 S proteasome subunits and S5a is a subunit of the 26 S proteasome.

in protein expression, immunoblot analysis was performed using an antibody raised against mouse cathepsin L. As shown in Figure 3, two major procathepsin L species (36 and 39 kDa) were detected in gastrocnemius muscle of pair-fed animals (lanes 1–3). These forms of cathepsin L could either be attributed to differences in glycosylation [26,27], or to partial processing of the proenzyme [28]. Proteolytic processing of procathepsin L generates mature enzymically active forms of cathepsin L, which exist either as a single-chain enzyme (29 kDa) or as a two-chain enzyme [obtained by one peptide cleavage this form contains a heavy chain (21 kDa) associated with the residual peptide, the light chain] [29]. Single (29 kDa) and heavy (21 kDa) forms of mature cathepsin L were also detected in muscle of pair-fed rats, although at very low levels. In muscle from infected animals (Figure 3, lanes 4–6), the amount of pro-cathepsin L (36 or 39 kDa) was increased approx. 3-fold after 2 or 6 days post-infection. The level of mature cathepsin L (21 or 29 kDa) was increased 2–5-fold after 2 days post-infection and a further elevation was observed at 6 days post-infection (Figure 3, lane 6). Taken together these data confirm that cathepsin L mRNA is overexpressed in muscle during the early stages of infection and is maintained for at least 6 days. This increase in mRNA correlated with an elevation in the amount of pro- and mature cathepsin L.

Cathepsin L mRNA overexpression during sepsis appears to be restricted to tissues characterized by a catabolic state

To further confirm that cathepsin L mRNA elevation was a marker of muscle wasting associated with sepsis, its expression was studied in another atrophied muscle, the tibialis anterior. As shown in Figure 4(A), the expression of cathepsin L mRNA in tibialis anterior muscle paralleled that observed in the gastrocnemius with a 3.5-fold elevation during the acute response (after 2 days), and a further elevation at 6 days post-infection (8.9-fold). An increase in cathepsin L protein level was also observed in this muscle (results not shown). However, at 6 days post-infection, the expression of cathepsin L mRNA was not affected in the liver (Figure 4B), a tissue characterized by anabolic activity in this catabolic model [6]. Thus overexpression of cathepsin L at both mRNA and protein levels appears to prevail in tissues characterized by a catabolic state.

Comparison of the expression of cathepsin L mRNA and other proteolytic genes

In the same model of sepsis, increased mRNA expression for some components of the lysosome-, proteasome-, and calcium-dependent proteolytic systems in the tibialis anterior muscle has been previously reported [5]. None of these, nor additional mRNAs encoding other proteolytic enzymes, were identified when the DDRT-PCR was performed. Therefore a MMA approach (see the Experimental section) was developed to

Table 2 Summary of the data obtained by MMA analysis of gastrocnemius muscle at 6 days post-infection.

Results are expressed in arbitrary units from ImageQuant software as means ± S.D. from at least three independent determinations. $^{***}P < 0.01$; $^{**}P < 0.05$ compared with the corresponding pair-fed animals.

Targets	Expression (units)		Fold increase
	Pair-fed	Sepsis	sepsis/pair-fed
Lysosome-dependent proteolysis			
Cathepsin L	0.17 ± 0.01	2.15 ± 0.33***	13.0
Cathepsin C	0.11 ± 0.04	0.24 ± 0.12**	2.3
Cathepsin H	0.44 ± 0.18	0.73 ± 0.33	1.7
Cathepsin D	0.68 ± 0.49	1.35 ± 1.00	2.0
Proteasome-dependent proteolysis			
Ubiquitin	0.17 ± 0.07	0.33 ± 0.24	1.9
Subunit C2	0.87 ± 0.52	1.76 ± 0.43	2.0
Subunit C3	1.11 ± 0.09	1.62 ± 0.28**	1.5
Subunit C5	1.46 ± 0.06	2.71 ± 0.59**	1.9
Subunit C9	0.44 ± 0.24	1.06 ± 0.19**	2.4
Subunit S5a	0.16 ± 0.01	0.56 ± 0.32**	3.5

specifically study the expression of additional proteolytic genes in the gastrocnemius muscle of septic animals 6 days post-infection. A representative result is shown in Figure 5 and the quantification of the normalized data obtained is given in Table 2. The increase in the mRNAs encoding the cysteine endopeptidases, cathepsin C or cathepsin H, and the aspartyl endopeptidase, cathepsin D, in gastrocnemius muscle of septic animals did not exceed 2.3-fold. A slight increase was also observed for mRNAs encoding the subunits of the 20 S proteasome (C3, C5 and C9) and a 3.5-fold elevation was measured for the 19 S proteasome regulatory subunit, S5a. These data indicate a general increase in mRNA levels encoding proteolytic proteins; however, the most striking effect remains the 13-fold induction of cathepsin L mRNA (denoted by an arrow in Figure 5).

Overexpression of cathepsin L mRNA in muscle is observed in several catabolic states: role of cytokines and glucocorticoids

Muscle atrophy is observed in animal models of cancer [1] and also occurs as a consequence of elevated circulating glucocorticoid levels [30]. In rats bearing the Yoshida Sarcoma, a 3.9-fold increase in cathepsin L mRNA in the tibialis anterior muscle was observed (Figure 6A). This increase was comparable with that observed in tibialis anterior muscles of septic rats 6 days post-infection. When animals were treated for 5 days with dexamethasone, a synthetic glucocorticoid analogue, an increase in cathepsin L mRNA was also detected, but this increase was not greater than 2-fold (mean, 1.7-fold; Figure 6B).

Pro-inflammatory cytokines, and especially tumour necrosis factor-α (TNF-α), have been shown to indirectly induce muscle protein loss [31]. Pentoxifylline suppresses TNF-α production [32], and its daily administration decreases proteolysis in this cancer model [16]. As shown in Figure 6(A) (compare Y + P with Y), the daily administration of pentoxifylline decreased cathepsin L mRNA expression in three out of four animals, but did not return the level back to that found in controls.

Taken together, the data presented in this study demonstrate that the increase in muscle cathepsin L mRNA is a good marker of muscle wasting associated with various catabolic states, and that both cytokines and glucocorticoids regulate this response.

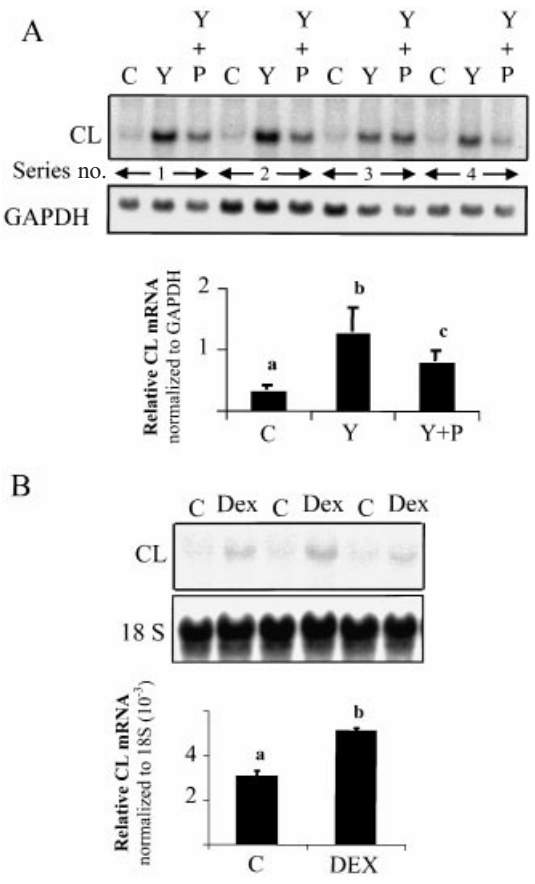

Figure 6 Muscle cathepsin L expression analysis in catabolic models distinct from sepsis

Northern blot of total RNA (20 μg/lane) was hybridized using a mouse cathepsin L probe. (**A**) Analysis was performed on muscle from cachectic tumour-bearing animals 9 days after injection of Yoshida Sarcoma homogenate. Total RNA was extracted from the contralateral tibialis muscle of rats bearing the Yoshida Sarcoma (Y), the Yoshida Sarcoma plus daily treatment with pentoxifylline (Y + P) and controls (C). The same membrane was re-hybridized with a GAPDH probe. (**B**) Total RNA was extracted from tibialis muscle of rats treated in the absence (C) or presence of approx. 500 μg/kg of dexamethasone (Dex) for 5 days. The same membrane was re-hybridized with an 18 S probe. The CL mRNA content was normalized either to the GAPDH (**A**) or 18 S (**B**) signal and the data expressed in arbitrary units as means ± S.D. after ImageQuant analysis from at least three independent determinations. Values not sharing a common letter are significantly different ($P < 0.05$).

DISCUSSION

The aim of the present study was to identify genes whose expression is altered in atrophying muscle. In addition to validating such genes as potential markers of muscle wasting, their identification in mediating the alteration of muscle function could provide a better understanding of the mechanisms leading to muscle wasting. To achieve this, DDRT-PCR has been used on RNA samples isolated from the gastrocnemius muscle of infected animals, an experimental model developed by Breuillé et al. [15]. Although the primary screening led to the identification of 29 differentially expressed mRNAs in muscle from septic animals when compared with pair-fed animals, only one product was confirmed to be differentially displayed and overexpressed in septic muscles. This product was identified as encoding the lysosomal cysteine endopeptidase, cathepsin L.

The experimental model used in the present study is characterized by an acute septic phase, lasting 2–3 days after infection,

followed by a chronic septic phase between days 3 and 8 [5]. Muscle wasting is significant after 2 days, a time at which a 4-fold increase in cathepsin L mRNA was observed. After 6 days, when muscle loss is further pronounced, cathepsin L mRNA was also further increased (up to 13-fold). The cathepsin L mRNA expression profile was similar in both gastrocnemius and tibialis anterior muscles. This indicates that cathepsin L expression is induced early during sepsis, correlates with the intensity of protein catabolism and thus is a good marker of muscle loss. Several additional findings support this conclusion. First, no regulation of cathepsin L expression has been observed in the liver, a tissue characterized by intense anabolism during sepsis [2,6]. Second, for one animal that did not lose weight, there was no elevation in cathepsin L mRNA and protein levels (results not shown). Finally, cathepsin L mRNA increased in other models of muscle wasting, including tumour-bearing animals and animals treated with dexamethasone.

It is recognized that infection, injury and cancer are characterized by the activation of the immune system and tissue infiltration by immune cells. In a rat model of local trauma induced by a single impact on one hindlimb, there is a local invasion by mononuclear cells (phagocytes) that overexpress cathepsin B mRNA (6–7-fold) and protein. This response is restricted to the injured hindlimb (M. C. Farges, D. Balcerzak, B. D. Fisher, D. Attaix, D. Béchet, M. Ferrara and V. Baracos, unpublished work). As the cathepsin B mRNA level remained low and did not change in septic muscle (results not shown), and as contralateral muscles of tumour-bearing animals were studied, this strongly suggests that muscle, and not infiltrated cells, is the site for the increased cathepsin L expression.

A comparison between the induction of mRNA encoding a variety of proteolytic proteins in muscle of septic and pair-fed animals 6 days post-infection has been determined and compared with that of cathepsin L mRNA. We observed a significant elevation of mRNAs encoding the C3, C5, C9 and S5a subunits of the proteasome in gastrocnemius muscle. For additional targets, including other lysosomal endopeptidases, the induction in septic muscle did not exceed a 3–5-fold increase. Thus with a 7–13-fold increase in mRNA level, the cathepsin L gene undergoes specific regulation and can be considered a better marker of muscle wasting than other lysosomal endopeptidases or components of the ubiquitin–proteasome-dependent pathway.

In septic muscle, the increase in cathepsin L mRNA is associated with an elevation in the active protein of up to 10-fold at 6 days post-infection. Taken together with the finding that the lysosome-dependent proteolytic pathway is elevated during the chronic septic phase [5], this observation strongly supports a role for cathepsin L in the pathological response leading to muscle wasting. The increased proteolysis observed in the muscles of both septic and tumour-bearing animals can be reduced when TNF-α production is inhibited by treatment with pentoxifylline [15,16]. Accordingly, in the present study we demonstrate that pentoxifylline treatment also reduced the observed increase in cathepsin L mRNA levels by 40% in the muscle of tumour-bearing animals. This observation further confirms a role for cathepsin L in muscle wasting, but the specific function of the peptidase in this process is unknown. These data also suggest, although indirectly, that the pro-inflammatory cytokine, TNF-α, could be involved in cathepsin L gene regulation.

The study presented here also indicates that glucocorticoids regulate cathepsin L gene expression. Indeed, the cathepsin L mRNA level is twice that of controls in muscles from animals treated with the synthetic glucocorticoid analogue, dexamethasone. It has been established that glucocorticoids directly control protein degradation in rat L8 myotubes and induce a 3.3-fold increase in cathepsin B mRNA [33]. Glucocorticoids mediate their biological effect at both the transcriptional and post-transcriptional levels by increasing mRNA half-life [34,35]. The existence of cross-talk between glucocorticoids and TNF-α signalling pathways has been demonstrated. In particular, TNF-α can enhance cell sensitivity to glucocorticoids by increasing the transcriptional activity of the glucocorticoid receptor and/or increasing glucocorticoid receptor binding to the glucocorticoid response element ('GRE') [36]. As both glucocorticoids and TNF-α are released during sepsis [37,38], it is conceivable that a synergistic effect between these two mediators could occur in muscle cells and account for the observed increase in cathepsin L mRNA. However, because TNF-α secretion is transient during sepsis, peaking 1.5–2 h post-infection [39] and lasting, at best, for 10 h in experimental intra-abdominal sepsis [40], such synergy may only be transient as well.

TNF-α has been shown to induce immune cells to produce additional pro-inflammatory cytokines, in particular interferon γ (IFN-γ) [41]. One consequence of this will be oxidative stress, especially in muscle with an increased NO production via inducible NO synthase ('iNOS') gene induction [42]. Nude mice injected with TNF-α-producing Chinese-hamster ovary cells develop muscle wasting that is correlated with the associated oxidative stress [40]. Data from our laboratory using mouse C2C12 muscle cells has shown that TNF-α treatment failed to modify basal cathepsin L expression, but a combination of cytokines that induce NO production (TNF-α, IFN-γ and interleukin-1β) increased cathepsin L mRNA levels (S. Mordier, unpublished work).

In conclusion, we have shown that muscle wasting in various catabolic models is associated with an overexpression of lysosomal cathepsin L mRNA (up to a 10-fold increase) in the muscle of septic animals. Cathepsin L appears to be a better marker of muscle protein loss than other lysosomal endopeptidases or components of the ubiquitin-proteasome-dependent pathway, whose overexpression is lower. Evidence showing that lysosomal-dependent proteolytic pathway is activated during the chronic septic phase [5], and the elevation of active cathepsin L in muscle suggest the specific involvement of this peptidase in protein loss. The molecular mechanisms of cathepsin L gene regulation and its specific function in muscle wasting remain to be elucidated, but the data show that at least two factors, glucocorticoids and TNF-α, could participate either directly or indirectly in the regulation of this gene.

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