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Ubiquitin gene expression is increased in skeletal muscle of tumour-bearing rats

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Abstract

Rats bearing the fast-growing AH-130 Yoshida ascites hepatoma showed a marked cachectic response which has been previously reported [Tessitore et al (1987) Biochem J 241, 153–159] Thus tumour-bearing animals showed significant decreases in body and muscle weight (soleus and gastrocnemius) as compared to both pair-fed and ad libitum-fed animals These decreases were related to an enhanced proteolytic rate in the muscles of the tumour-bearing animals as measured by the tyrosine released in in vitro assays In an attempt to elucidate which proteolytic system is directly responsible for the decrease in muscle mass, we have studied both lysosomal and non-lysosomal (ATP-dependent) proteolytic systems in this animal model While the enzymatic activities of the main cathepsin (B and B + L) systems were actually decreased in gastrocnemius muscles of tumour-bearing rats, thus indicating that lysosomal proteolysis was not involved, the ubiquitin pools (both free and conjugated) were markedly altered as a result of tumour burden These were associated with an increased ubiquitin gene expression in muscle of tumour-bearing rats, over 500% in relation to non-tumour bearers, thus suggesting that the ATP-dependent proteolytic system may be responsible for the muscle proteolysis and wastage observed in this animal tumour model The fact that we have previously shown that TNF enhances the ubiquitinization of muscle proteins [García-Martínez et al (1993) FEBS Lett 323, 211–214], together with the high circulating levels of TNF detected in rats bearing the Yoshida hepatoma allows us to suggest that the cytokine may be responsible, most probably indirectly, for the activation of the referred proteolytic system in tumour-bearing rats

Key words Muscle wasting, Tumour growth, TNF, Protein metabolism, Ubiquitin, Caquexia, Cathepsins

1. Introduction

The loss of body weight and development of cachexia are common signs associated with neoplastic diseases Cachexia is a poorly understood syndrome characterized by anorexia, weight loss, profound metabolic abnormalities and progressive host wasting which may result in death [32,47,51]. Tissue wasting involves mainly adipose tissue and skeletal muscle Numerous reports attribute the cachectic state of the host to cytokines released as a result of invasive stimuli (see [15] for review). However, while the role of cytokines, particularly tumour necrosis factor-α (TNF), on adipose tissue dissolution seems to be clear [14,42,45], the effects of the cytokine on muscle metabolism lead to more confusing results (see [5] for review). TNF is a molecule belonging to a polypeptide network made up of several cytokines and growth factors that have wide and varied effects on the growth, differentiation, and functions of immune system and normal cells [6,41] It is produced primarily by activated macrophages in response to invasive stimuli

Muscle wasting is generally accepted to be caused by an increase in protein breakdown Chronic treatment of rats with recombinant TNF resulted in depletion of body protein compared with pair-fed control animals [52] Indeed, chronic treatment with either recombinant TNF or interleukin-1-β (IL-1) resulted in a body protein redistribution and a significant decrease in muscle protein content associated with coordinate decreases in muscle mRNA levels for myofibrillar proteins [19] Studies involving administration of recombinant TNF in vivo have shown an increase in nitrogen efflux from skeletal muscle of non-weight losing humans with disseminated cancer [54] Flores et al [18], by infusing [^{14}C]-leucine into rats, showed that chronic recombinant TNF administration significantly enhanced muscle protein breakdown Conversely, the administration of an acute TNF dose to rats did not induce an enhanced proteolysis in soleus or EDL muscles subsequently incubated [30] Moldawer et al [40] administered recombinant IL-1 to mice and observed, after incubating their EDL muscles, that there was an increase in PGE_2 production although the rates of both protein synthesis and degradation were unaffected, thus concluding that the rise in PGE_2 was not

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associated with changes in protein turnover. Recently, Goodman [25], measuring both tyrosine and 3-methylhistidine release by incubated rat muscles of animals acutely treated with the cytokine, concluded that TNF was involved in activating muscle proteolysis The reasons for these conflicting results are not clear We have recently demonstrated that TNF treatment results in activated muscle proteolysis [20,33,34] and that the proteolytic system involved in this response seems to be associated with ubiquitinization of muscle proteins [21]

In fact, the precise mechanism by which intracellular proteins are degraded is largely unknown, although it is accepted that proteolysis can occur both inside and outside of lysosomes.In particular lysosomic proteinases, mainly cathepsins do not seem to be involved in the degradation of myofibrillar proteins in rat skeletal muscle [35] Ubiquitin, a 8600 d peptide, is involved in the targeting of proteins undergoing cytosolic ATP-dependent proteolysis In the cell, ubiquitin can be found free or conjugated in an isopeptide linkage to other cellular proteins Proteins with multiple ubiquitins are the ones targeted for degradation by an ATP-dependent protease [11,16,17,28] The ubiquitin system is postulated to account for the turnover of 'short-lived' normal proteins [12], and abnormal proteins formed during stress such as heat-shock [8] There are two kinds of ubiquitin-encoding genes one has multiple ubiquitin sequences in the form of contiguous repeats of the coding sequence and the other encode ubiquitin fusion proteins These two kinds of genes are differentially expressed whereas the ubiquitin fusion transcripts are most abundant in normal dividing cells, high levels of polyubiquitin transcripts are found in cells that undergo sporulation, differentiation or, in general, are under stress [7]

Growth of the ascites hepatoma AH-130 in rats elicits an early and conspicuous loss of body weight and skeletal muscle mass, associated with a protein hypercatabolic state in host tissues [50] as well as profound perturbations in hormonal homeostasis, an elevation of plasma prostaglandin E_2 and the presence of circulating TNF [13]. Bearing in mind the fact that TNF seems to enhance muscle proteolysis by increasing the ubiquitinization of proteins, we decided to investigate the role of the ubiquitin system in this cachectic tumour model where the endogenous production of the cytokine is high as a result of tumour burden

2. Experimental

2 1 Animals

All animals (female Wistar rats) were fed on a chow diet consisting (by weight) of 54% carbohydrate, 17% protein and 5% fat (the residue was non-digestible material) with free access to drinking water, and were maintained at an ambient temperature of 22 ± 2°C with a 12 h light/12 h dark cycle (lights on from 08 00 h) Food intake and body weight were measured daily after tumour inoculation Pair-fed controls were non-tumour bearing animals which were offered the same amount of food as that eaten by the tumour bearers the previous day For the estimation of proteolytic rates in isolated soleus muscles, smaller animals were used (60–70 g) so that their muscles could be incubated whole, without being under anoxia

2 2 Biochemicals

All enzymes and coenzymes were either obtained from Boehringer Mannheim, S A (Barcelona, Spain) or from Sigma Chemical (St Louis, USA) Cathepsin substrates were purchased from Bachem (Bubendorf, Switzerland) Recombinant-derived TNF was generously given by BASF-Knoll A G (Ludwigshafen, Germany) Polyclonal rabbit anti-rat-ubiquitin was provided by Dr Arthur L Haas, Department of Biochemistry, Medical College of Wisconsin, Milwaukee (Wisconsin, USA)

2 3 Tumour implantation

A Yoshida AH-130 ascites hepatoma cell suspension (approx 10^8 cells in 2 ml) was injected intraperitoneally, the control rats being injected with 2 ml of 0 9% (w/v) NaCl solution The Yoshida AH-130 is a rapidly growing tumour with a volume doubling time of 1 day [50] Total cell content was estimated using Trypan blue staining Food intake and body weight were measured daily after tumour inoculation

2 4 Muscle preparations and incubations

The dissection and isolation of the soleus muscles was carried out under pentobarbital anaesthesia as previously described [26] The isolated muscles were fixed to a stainless-steel clip in order to maintain the muscle under slight tension (making it comparable to resting length) during the incubation Such muscles are able to maintain normal ATP and phosphocreatine concentrations during a 3 h incubation period The muscles were incubated in a shaking-thermostatized water bath at 37°C for 3 h in 3 ml of Krebs Henseleit physiological saline pH 7 4, containing 5 mM glucose, bovine serum albumin (1 mg/ml) and 20 mM HEPES After the addition of the muscles to the vials, these were stoppered and the incubation started at a shaking rate of 45 cycles/min Vials were gassed with O_2/CO_2 19 1 during the whole incubation period The incubation medium was kept for no longer than 90 min and was renewed thereafter with fresh medium with the same composition as described above

2 5 Proteolytic rate in isolated muscles

Total protein degradation by the isolated muscles was calculated as the rate of tyrosine released into the medium in the presence of 0 5 mM cycloheximide to block the reincorporation of tyrosine into tissue protein Tyrosine was measured fluorimetrically as previously described [53]

2 6 Isolation and preparation of muscle tissue for quantification of free and conjugated ubiquitin

The soleus and gastrocnemius were removed under pentobarbital anaesthesia Excised muscles were quickly weighed and rapidly frozen in liquid N_2 Ubiquitin pools in muscle extracts were determined by immunochemical analysis Individual muscles were finely homogenized using a Polytron homogenizer in 2 ml of ice-cold 50 mM Tris-HCl buffer containing 0 25 mM sucrose, 5 mM EDTA, 1% (w/v) sodium dodecyl sulphate, 0 1 trypsin inhibitor unit/ml aprotinin, 1 μM leupeptin, 1 μM pepstatin, 5 mM *N*-ethylmaleimide and 1 mM PMSF, pH 7 4 The resulting homogenates were centrifuged at 4°C for 20 min at 15 000 × *g* to remove cell debris Aliquots of the supernatants were stored at −80°C Protein content was measured using the Bradford assay [9] using bovine serum albumin as a standard

2 7 Western blots

Aliquots of the supernatants were diluted with 0 5 volumes of 30 mM phosphate buffer pH 7 0 containing 7 5% (w/v) sodium dodecyl sulphate and 0 15% (w/v) dithiotreitol, 0 05% (w/v) Bromophenol blue and 30% glycerol Samples were boiled for 5 min, 50 μg of protein-samples being assayed for ubiquitin conjugates Sodium dodecyl sulphate (SDS)-15% polyacrylamide gel electrophoresis was performed on muscle samples Proteins were transferred to Immobilon in 25 mM Tris buffer containing 144 mM glycine, 25% methanol, 0 02% SDS for 12 h The filter was blocked for 1 h at room temperature in buffer A (25 mM Tris-HCl pH 7 5, 150 mM NaCl, 25 mg/ml BSA and 0 02% sodium azide) and incubated for 90 min with a anti-ubiquitin polyclonal antibody which recognized free and conjugated ubiquitin at a concentration of 10 μg/ml in buffer A Detection of antibody-antigen com-

plexes was carried out using [^{125}I]protein A according to standard procedures Quantitation of the different autoradiographs was done by densitometry using a scanning microdensitometer Data for both free and conjugated ubiquitin were normalized to extract protein concentration determined by the Bradford assay [9]

2 8 RNA isolation and Northern blot analysis

Total RNA from gastrocnemius muscles was extracted using the acid guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi [10] and quantified by absorbance at 260 nm

RNA samples (40 μg) were denatured, subject to electrophoresis in 1 2% agarose gels containing 6 3% formaldehyde and transferred to Hybond H membranes (Amersham) RNA was fixed to membranes by illuminating with UV light for 4 min The RNA in gels and in filters was visualized with ethidium bromide and photographed by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of RNA and to confirm proper transfer RNA was transferred in 10 × standard saline citrate (SSC, 0 15 M NaCl and 15 mM sodium citrate, pH 7 0)

Prehybridization was done in 50% formamide/5 × SCC (0 3 M NaCl, 65 mM sodium citrate)/5 × Denhart's solution (1 × Denhart's solution is 0 1% polyvinylpyrolidone, 0 1% Ficoll, 0 1% BSA)/20 mM sodium phosphate pH 6 8/0 1% SDS/100 μg/ml denatured salmon sperm DNA overnight at 42°C Hybridization was done also at 42°C overnight in the same buffer, denatured labelled probes (10^6–10^7 cpm/ml) being added Radiolabelled probes were prepared by the random primer method (Boehringer) The ubiquitin probe used was a cDNA clone containing 12 base pairs of the second ubiquitin coding sequence plus a complete third and fourth ubiquitin coding sequence and 120 base pairs of the 3'-untranslated region of the chicken polyubiquitin gene UBI [7] An actin probe was used as a control of loading Filters were exposed to X Omat AR-5 films (Kodak) at 70°C for 2–4 days and the films quantified by laser densitometry

2 9 Cathepsin activities

The stored muscle samples were homogenized in 4 vols of 50 mM sodium acetate pH 5 0 containing 0 2% Triton X-100, 100 mM NaCl, 1 mM EDTA, with a Polytron homogenizer The soluble extracts were obtained by centrifugation (32 000 × *g*, 4 min, 4°C) Portions of the extracts were used for assaying enzyme activity Protein concentration of the extracts was assayed using the Bradford [9] method Cathepsins B was assayed with 5 mM Z-Arg-Arg-AMC at pH 6 0 Cathepsin H was assayed with 5 mM H-Arg-AMC at pH 6 8 Cathepsin L activity was assayed with 2 mM Z-Phe-Arg-AMC at pH 5 5 Since this artificial substrate is not only hydrolyzed by cathepsin L but also cathepsin B at pH 5 5, its hydrolysis is expressed as the activity of cathepsin B + L In all cases, quantitation of enzyme activity was performed by measuring fluorimetrically AMC liberation The fluorescence of liberated AMC was read in a Kontron SFM-25 fluorimeter at 350 nm (excitation wavelength) and 445 nm (emission wavelength)

2 10 Statistical analysis

Statistical analysis of the data was performed by means of the Student's *t*-test

3. Results and discussion

Many previous studies have described important changes in protein and amino acid metabolism in skeletal muscle of both human subjects and tumour-bearing animals The main changes involve enhanced proteolysis and decreased protein synthesis both of which explain the profound negative nitrogen balance encountered in neoplasic states This phenomenon is linked to the muscle wasting present in cachectic cancer patients Our main objective in this investigation was to assess which proteolytic system (either lysosomal or non lysosomal) was involved in the referred muscle wastage. Bearing this in mind, we choose the Yoshida AH-130 ascites hepatoma which is a highly cachectic rat tumour of rapid growth and poorly differentiated cells. The tumour is characterized by a relatively short doubling time of one day [50], and is widely used in experimental studies

3 1 Body and tissue weights

The implantation of the ascites tumour inflicted im-

Table 1
Body and tissue weights in rats bearing the Yoshida AH-130 ascites hepatoma

	Days after tumour transplantation					
	Day 4			Day 7		
	Pair-fed (6)	Tumour (6)	Ad libitum-fed (5)	Pair-fed (6)	Tumour (7)	Ad libitum-fed (6)
Initial body weight	172 ± 2	173 ± 3	166 ± 4	178 ± 2	182 ± 2	174 ± 1
Final body weight	181 ± 2	180 ± 3	185 ± 5	174 ± 3***	169 ± 3***,†	198 ± 2
Weight increase (%)	5 0 ± 1 1	4 2 ± 0 9	10 3 ± 0 7	−3 5 ± 0 7***	−7 1 ± 1 6***,†††	13 4 ± 1 5
Tissue weights						
Gastrocnemius	583 ± 11	563 ± 25	590 ± 32	600 ± 9*	547 ± 15***,†	625 ± 6
Soleus	50 ± 1	47 ± 2**,†††	50 ± 2	54 ± 3	43 ± 1***,††	53 ± 1
Liver	4 1 ± 0 1*	5 0 ± 0 2††	4 7 ± 0 2	3 3 ± 0 1***	4 2 ± 0 2††	4 6 ± 0 2
Tumour volume		15 ± 1			51 ± 2	
Cell number (×10^6)		2192 ± 145			4659 ± 159	

For more details see the Experimental section Body and liver weights are expressed in g Muscle weights are given in mg Final body weight in tumour-bearing animals represents the weight of the animal excluding the tumour and ascitic fluid Weight increase (%) and tissue weights are corrected per 100 g of initial body weight Tumour volume is expressed in ml Results are mean ± S E M for the number of animals indicated in parentheses Statistical significance of the results (by Students *t*-test), any group versus ad libitum-fed *P < 0 05, **P < 0 01, ***P < 0 001, tumour-bearing versus pair-fed †P < 0 05, ††P < 0 01, †††P < 0 001

portant changes in body weight, as determined without the tumour, 7 days after inoculation (Table 1). At this time point, both pair-fed and tumour-bearing animals weights were significantly lower than ad libitum-fed animals although the tumour-bearing rats showed a significantly lower body weight than the pair-fed ones (Table 1) The increase in body weight was also significantly lower in tumour-bearing and in pair-fed as compared to ad libitum-fed rats, 4 and 7 days after tumor transplantation (Table 1) These data clearly demonstrate that, although the decrease of food intake associated with tumour burden is responsible for a clear decrease in body weight, the presence of the tumour results in a further decrease in body weight, particularly noticeable when the tumour has reached its stationary growth phase [38]

Muscle weights were also severely decreased by tumour growth as can be seen in Table 1. Gastrocnemius weights were decreased in all groups as compared with the ad libitum-fed rats, the decrease being the greatest in the tumour-bearing animals (12%), 7 days after tumour transplantation Soleus weights were also significantly decreased both 4 and 7 days after transplantation (Table 1). The decrease in muscle weights as a result of the Yoshida AH-130 ascites hepatoma have previously been reported [50] and it is clearly associated with an enhanced proteolytic rate while no changes are observed in protein synthesis, as measured in vivo [50] These changes in protein turnover seem to be triggered by TNF since anti-TNF treatment results in normal protein turnover in AH-130 Yoshida-bearing rats [13] In addition, the cytokine does not seem to cause these effects directly [20] nor via interleukin-1 (Costelli, P, Llovera, M., Carbó, N., López-Soriano, F J & Argilés, J.M , unpublished results) nor via glucocorticoids (Costelli, P, Llovera, M , García-Martínez, C , López-Soriano, F J & Argilés, J M , unpublished results) The molecule(s) that mediate the actions of TNF on muscle protein turnover in this experimental tumour model is thus unknown Liver weights were significantly elevated in the tumour-bearing animals in relation to the pair-fed controls Indeed, tumour growth has been reported to result in enlarged liver size together with enhanced amino acid uptake [2–4] for both liver protein synthesis and gluconeogenesis [1,22] The livers of the pair-fed animals are smaller than those found in ad libitum-fed animals (Table 1) Important changes in organ weight were also seen, 7 days after tumour transplantation, in the heart weight, tumour growth resulted in a decreased heart weight in relation with the pair-fed controls (319 ± 7 mg versus 283 ± 3 mg ($P < 0.001$) for pair-fed and tumour-bearing, respectively)

3.2 *Proteolytic rate in isolated muscles*

As previously mentioned, an enhanced proteolysis (increased fractional protein degradation rate) in gastrocnemius muscles of AH-130 Yoshida-bearing rats has been reported [50]. Our intention here was to see whether the increased proteolysis was also observed in isolated muscles of these animals since this would indicate that their proteolytic machinery was constitually elevated, independently of any hormonal or humoral changes associated with tumour burden The results presented in Table 2 show that this was indeed the case since the muscles from the tumour-bearing animals showed increased proteolytic rates both 4 (29%) and 7 (26%) days after tumour transplantation

3.3 *Cathepsin activities*

It has been proposed that lysosomal proteolytic systems may be involved in protein turnover in skeletal muscle in pathological situations [23] while others have evidenced that myofibrillar proteins are not the substrate of lysosomal proteinases [35]. Takeda et al [48] have demonstrated an active participation of the cysteine proteinases cathepsins B, H and L in xenografts of Duchenne-muscular dystrophic muscles Although many studies concern the participation of lysosomal proteinases in tumour invasion or metastasis (see Schmitt et al. [44] for review), few studies concern the role of cathepsins in muscle wastage associated with cancer Lundholm et al [36] clearly demonstrated the involvement of lysosomal proteinases in the skeletal muscle of patients with a malignant tumour. The same group also showed an increase in cathepsin D activity in liver and muscle of sarcoma-bearing mice [37] indicating a direct role of cathepsins in muscle wastage associated with cancer cachexia Bearing these observations in mind, we evaluated the effects of the major cathepsins systems in our tumour model The results presented in Table 3 show that only cathepsin H activity seems to be increased (16%) in gastrocnemius muscles of tumour-bearing animals 4 days after tumour transplantation in relation to the pair-fed animals, while

Table 2
Proteolytic rate in isolated soleus muscles from tumour-bearing rats

Experimental group	Proteolytic rate
Day 4 after transplantation	
Pair-fed	194 ± 6 5 (7)
Tumour	251 ± 11*** (6)
Day 7 after transplantation	
Pair-fed	192 ± 5 (5)
Tumour	242 ± 13*** (5)

For more details see section 2 All the incubation media contained 500 nM cycloheximide The results are expressed as nmoles of tyrosine/g h and are mean ± S E M with the number of animals used in the different groups indicated in parentheses Statistical significance of the results ***$P < 0.001$

Table 3
Cathepsins enzymatic activities in gastrocnemius muscles of AH-130 Yoshida-bearing rats

Experimental group	Cathepsin		
	B	B + L	H
Ad libitum fed	7 00 ± 0 31 (6)	10 6 ± 0 53 (6)	4 90 ± 0 17 (6)
Day 4 after transplantation			
Pair-fed	5 50 ± 0 46* (6)	11 1 ± 0 49 (6)	5 23 ± 0 10 (6)
Tumour	6 41 ± 0 41 (6)	10 9 ± 0 16 (6)	6 07 ± 0 18***,††† (6)
Day 7 after transplantation			
Pair-fed	6 02 ± 0 28* (7)	10 8 ± 0 64 (7)	4 60 ± 0 17 (7)
Tumour	4 11 ± 0 40***,†† (6)	7 74 ± 0 24***,††† (6)	4 71 ± 0 21 (6)

For more details see section 2 Enzymatic activities are expressed as nmoles of AMC/min mg of muscle protein Results are mean ± S E M for the number of animals indicated in parentheses Statistical significance of the results (by Student's t test), any group versus ad libitum-fed $^{*}P < 0\ 05$, $^{***}P < 0\ 001$, tumour-bearing versus pair-fed $^{††}P < 0\ 01$, $^{†††}P < 0\ 001$

no changes are observed 7 days after tumour growth (Table 3). The activities of cathepsins B and B + L were actually decreased in the tumour-bearing animals, 7 days after tumour inoculation as compared to both pair-fed (32% for B and 28% for B + L) and ad libitum-fed (41% for B and 27% for B + L) animals (Table 3). Thus, it does not seem from the results presented that the main cathepsin activities, cathepsin H only seems to represent a minor activity in skeletal muscle [31], are directly involved in muscle wastage observed in this tumour model It has to be pointed out that both cathepsins B and B + L activities are decreased in the pair-fed controls in relation with ad libitum-fed animals and this is probably the result of protein malnutrition as has been pointed out by Tawa et al [49].

3 4 *Western blots*

Since the lysosomal proteolytic systems did not seem to be responsible for the enhanced muscle proteolysis observed in the Yoshida AH-130-bearing rats, we decided to assess the participation of other non-lysosomal protein degradation pathways such as the ATP and ubiquitin-dependent protease system Extracts of skeletal muscle, like those from other mammalian cells, contain the soluble ubiquitin-dependent proteolytic system which is normally believed to catalize the rapid breakdown of abnormal proteins and various short-lived normal proteins [8,11,12] In this process, the carboxyl terminal of ubiquitin is conjugated to protein substrates and this modification marks them for rapid degradation by a 1300 kDa complex [55] The role of ubiquitin-dependent proteolysis can be tested by exploiting the ubiquitin moiety as an immunological probe [27] of substrate specificity. The results presented in Table 4 show the Western blot quantitations of the free and conjugated ubiquitin pools in both soleus and gastrocnemius muscles We used muscles from 7-day tumour-bearing rats since the largest changes in muscle weights were observed in this experimental group. In gastrocnemius there was a significant increase for both free (53%) and

Table 4
Free and conjugated ubiquitin in skeletal muscles of tumour-bearing rats 7 days after tumour inoculation

Experimental group	Type of muscle			
	Gastrocnemius		Soleus	
	F	C	F	C
Ad libitum-fed	0 86 ± 0 16	2 84 ± 0 15	1 17 ± 0 18	3 44 ± 0 40
Pair-fed	0 81 ± 0 03	4 28 ± 0 60*	0 81 ± 0 09	4 37 ± 0 26*
Tumour	1 32 ± 0 12*,††	4 18 ± 0 60*	0 84 ± 0 07	7 45 ± 0 09***,†††

For further details, see section 2 All data are expressed in arbitrary units/50 mg of protein and represent the mean ± S E M of five different animals F = free ubiquitin, C = conjugated ubiquitin Statistical significance of the results (by Student's t-test), any group versus ad libitum-fed $^{*}P < 0\ 05$, $^{***}P < 0\ 001$, tumor-bearing versus pair-fed $^{††}P < 0\ 01$, $^{†††}P < 0\ 001$

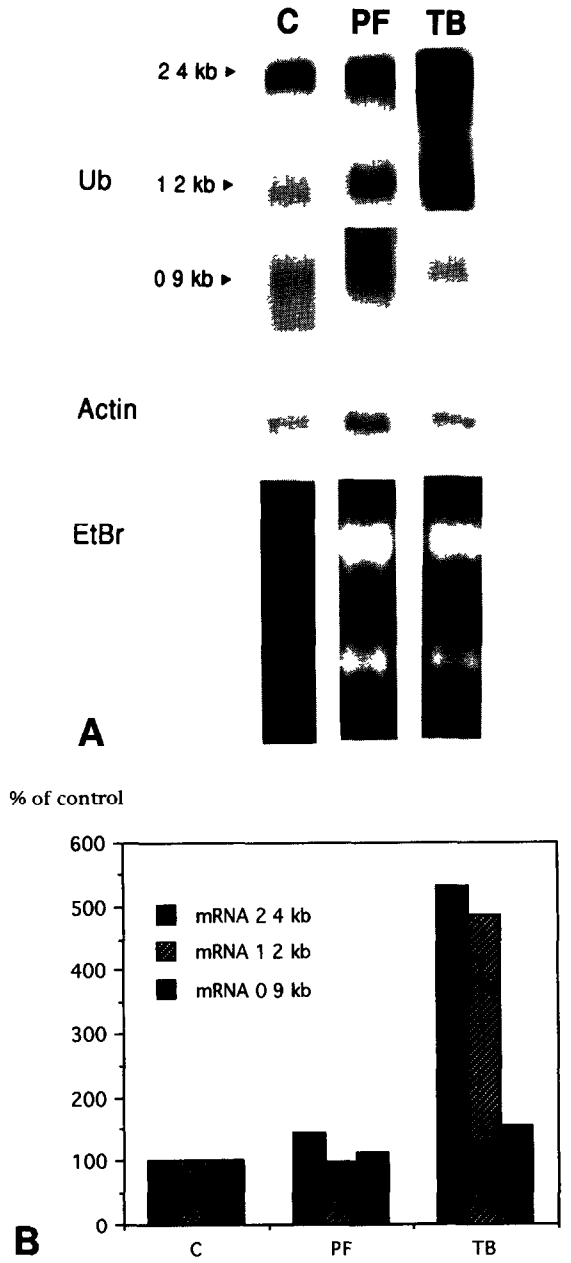

Fig 1 Northern blots of gastrocnemius muscle extracts from tumour-bearing rats Expression of ubiquitin mRNA in skeletal gastrocnemius muscles from ad libitum-fed (C), pair-fed (PF) and tumour-bearing rats (TB) at day 7 after tumour implantation It was detected after hibridization with a cDNA probe containing a region of the chicken polyubiquitin gene UBI (see section 2) Autoradiographs were subject to scanning densitometry. The results of 3 separate experiments are shown and expressed as a percentage of ad libitum-fed controls

conjugated (42%) ubiquitin pools in relation with the ad libitum-fed animals, while an important increase (63%) in free ubiquitin was also observed in relation to the fair-ped group (Table 4) In this muscle, pair-fed animals also showed an increased conjugation in relation with the ad libitum-fed animals In soleus muscles, there was a marked tendency for lower values for both pair-fed ($P = 0\ 11$) and tumour-bearing ($P = 0\ 12$) in relation with the ad libitum-fed animals, although the differences did not reach statistical significance In relation with the conjugated ubiquitin pools, both pair-fed (27%) and tumour-bearing (116%) showed higher values than the ad-libitum-fed animals The presence of the tumour resulted in significantly elevated values in comparison with the pair-fed controls (Table 4). The changes observed with pair-feeding can be interpreted as an activation of non-lysosomal proteolysis induced by food restriction as has been previously reported [49]

3 5 Ubiquitin gene expression

Bearing in mind the fact that very important changes in both free and conjugated ubiquitin were observed in the tumour-bearing animals, we decided to assess the expression of the ubiquitin gene in gastrocnemius muscles As shown in Fig 1, two polyubiquitin mRNA species (2.4 kb and 1 2 kb) and one ubiquitin-fusion protein mRNA (0 9 kb) were found in gastrocnemius muscle Tumour-bearing animals showed an increased expression (over a 500%) of the polyubiquitin genes in relation to both pair-fed and ad libitum-fed animals Conversely, no changes in the expression of ubiquitin-fusion protein and mRNA were observed in tumour-bearing animals (Figure 1) Since polyubiquitin genes have been shown to be increased in processes where an increase intracellular proteolysis is observed, these data, together with the enhanced ubiquitin conjugation of muscle proteins present in the tumour-bearing rats seem to indicate a role for the ATP-dependent proteolytic system in this tumour model In addition, our study, together with other observations concerning the role of the ubiquitin-dependent proteolytic system in different pathological situations such as atrophy [39], contributes to alter the idea that this proteolytic system is a constituent system which merely degrades short-lived or abnormal proteins The system also appears to be responsible for the breakdown of most proteins in matured reticulocytes [46] and in growing fibroblasts [24] The present data thus suggest a more general role for this system in the degradation of normal muscle proteins, including, most likely, the long-lived myofibrillar components

3 6 Concluding remarks

Tumour growth results in important changes in body weight mainly associated with decreases in adipose and muscular tissues leading to cachexia In the cachectic rat tumour model Yoshida AH-130 ascites hepatoma, muscle wastage is associated with an important increase in proteolysis while no changes are observed in the rate of protein synthesis [50] In this model we have previously reported that tumour necrosis factor-α (TNF) seems to be responsible for the enhanced proteolytic process [13] This cytokine is also able to increase the ubiquitinization of skeletal muscle proteins in the rat [21]

The results presented suggest that the enhanced pro-

tein degradation in the skeletal muscle of Yoshida-bearing rats may to some extent be related to an increased ubiquitin-dependent proteolytic system. In these animals the concentration of TNF is very elevated [13] and thus it may be suggested that the cytokine could be triggering the enhanced expression of the ubiquitin gene Further investigations are being undertaken to find out which is the mediator of the cytokine action on skeletal muscle, since TNF does not seem to have any direct effects on proteolysis in isolated muscles [20]

The ATP-dependent proteolytic system involves multiple components including ubiquitin, ubiquitin-conjugating enzymes and very large proteolytic complexes that require ATP, most of which are potential sides of regulation However, our knowledge of the regulation of this system is very primitive since only studies concerning the effects of fasting and denervation atrophy [30] have been performed, both indicating increases in gene expression, most likely in response to hormonal mediators such as glucocorticoids [56] This is the first study reporting that the ubiquitin system is activated in response to tumour growth and that it may be involved in muscle wastage associated with cancer cachexia. This may lead to interesting clinical results since cachexia is responsible for at least 30% of deaths in cancer patients and, therefore, opens a field for future promising research

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