

Interleukin 6 Receptor Antibody Inhibits Muscle Atrophy and Modulates Proteolytic Systems in Interleukin 6 Transgenic Mice

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Abstract

The muscles of IL-6 transgenic mice suffer from atrophy. Experiments were carried out on these transgenic mice to elucidate activation of proteolytic systems in the gastrocnemius muscles and blockage of this activation by treatment with the anti-mouse IL-6 receptor (mIL-6R) antibody. Muscle atrophy observed in 16-wk-old transgenic mice was completely blocked by treatment with the mIL-6R antibody. In association with muscle atrophy, enzymatic activities and mRNA levels of cathepsins (B and L) and mRNA levels of ubiquitins (poly- and mono-ubiquitins) increased, whereas the mRNA level of muscle-specific calpain (calpain 3) decreased. All these changes were completely eliminated by treatment with the mIL-6R antibody. This IL-6 receptor antibody could, therefore, be effective against muscle wasting in sepsis and cancer cachexia, where IL-6 plays an important role. (*J. Clin. Invest.* 1996. 97:244–249.) Key words: protein degradation • cathepsin • proteasomes • ubiquitin • calpain

Introduction

Muscle wasting is a common feature of systemic infections and most malignant diseases. If progression of muscle wasting in septic and cancer cachexic patients could be prevented, it would be of clinical importance in terms of shortening the period of convalescence and improving the quality of life. The most effective strategy for this type of treatment is to identify the mediator most responsible for muscle proteolysis and neutralize its effect as a result of understanding the mechanism of accelerated proteolysis.

IL-6 is a candidate for muscle proteolysis-inducing factor. It is involved in cancer cachexia where muscle atrophy is evident (1, 2) and induces skeletal muscle protein breakdown (3).

We proved that IL-6 shortened the half-life of long-lived proteins in C2C12 myotubes (4) and muscles suffer atrophy in IL-6 transgenic mice (5). Elevated levels of IL-6 have been detected in the blood of patients with microbial infections, autoimmune diseases, and neoplasia (6, 7). TNF- α has long been considered as having a proteolysis-inducing effect in vivo since long-term treatment with recombinant TNF- α results in a significant decrease in muscle protein (8) and administration of recombinant TNF- α causes an increase in the nitrogen efflux from the skeletal muscles of non-weight losing humans (9). However, no studies have been able to prove the direct action of TNF- α in muscle proteolysis in vitro (10, 11). Since TNF induces the production of IL-6 (12), some of the metabolic effects of TNF are thought to be mediated by IL-6.

Skeletal muscle contains multiple proteolytic systems, while the lysosomal pathway in muscle involves four major proteases (cathepsins B, H, L, and D). Enhanced lysosomal proteolysis has been reported in muscle from cancer patients (13) and in skeletal muscle of septic rats (14), though lysosomes are presumed to play a minor role in the increase in skeletal muscle proteolysis in many catabolic states and are not involved in the breakdown of myofibrillar proteins (15, 16). Skeletal muscle contains two major cytosolic proteolytic pathways, Ca²⁺-dependent (17) and ATP-ubiquitin (Ub)¹-dependent (18). Furthermore, there are three forms of Ca²⁺-dependent proteases, namely μ , m, and muscle-specific calpains (19), and the muscle-specific calpain is now called calpain 3. However, μ and m calpains have not been considered to play predominant roles in muscle catabolism (15, 16).

The most important system responsible for muscle protein degradation is the ATP-Ub-dependent proteolytic system, which degrades not only abnormal and short-lived proteins (20, 21) but also the long-lived myofibrillar proteins (15, 16, 22). Ub covalently binds to protein substrates, and ubiquitinated proteins are then degraded by 26S proteasome, an active form of the multicatalytic ATP-dependent proteinase complex, which requires ATP for activation and substrate hydrolysis (20). The 26S proteasome itself is composed of the 20S proteasome and associated regulatory subunits (23). The present study was designed to examine the reasons for the success of treatment with IL-6 receptor antibody of muscle atrophy in the IL-6 transgenic mice and to confirm the proteolytic pathways responsible for IL-6-induced muscle atrophy.

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1. Abbreviations used in this paper: MCA, methylcoumaryl-7-amide; mIL-6R, mouse IL-6 receptor; s, soluble; Ub, ubiquitin.

Methods

IL-6 transgenic mice. C57BL/6J L^d-IL-6 transgenic mice were produced by microinjection of the 3.3-Kbp SphI-XhoI fragment (L^d-IL-6) containing human IL-6 cDNA fused with the H-2L^d promoter (24), donated by Professor Tadimitsu Kishimoto, Osaka University, into the pronucleus of fertilized eggs from C57BL/6J mice as described elsewhere (25). Integration of the transgene was screened by Southern blot analysis of EcoRI-digested tail DNA using the ³²P-labeled TaqI-BanII fragment of human IL-6 cDNA as a probe. In these transgenic mice, serum IL-6 concentration was > 600 pg/ml when they were 12 wk old as determined by human IL-6-specific ELISA as described elsewhere (26). The transgenic mice, all of which were female, were housed in individual cages in an air-conditioned room with a 12-h light/12-h dark cycle and fed standard laboratory chow ad lib. (CE-2; Nippon Clea, Tokyo, Japan).

Preparation of rat anti-mouse IL-6 receptor (mIL-6R) mAb, MR16-1. Rat anti-mIL-6R mAb (MR-16) was prepared as described previously (26, 27). Briefly, Wistar rats were immunized with 50 µg of purified soluble (s) mIL-6R in CFA (subcutaneously), followed 2 wk later by four 50-µg boosts of smIL-6R in Freund's incomplete adjuvant once a wk (subcutaneously). 1 wk after the last boost, the rats were injected with 50 µg of smIL-6R in 100 µl of PBS (intravenously). The rats were killed 3 d later, and spleen cells were fused with mouse P3U1 myeloma cells at a ratio of 10:1 with polyethylene glycol 1500 (Boehringer Mannheim Biochemicals, Indianapolis, IN). After overnight incubation at 37°C in the wells of a 96-well plate (3075; Falcon Labware, Becton Dickinson & Co., Oxnard, CA) with 100 µl of complete RPMI 1640 medium containing 10% FBS, 100 µl of hypoxanthine/aminopterin/thymidine-containing human IL-6 (28) was added to each well. Half of the culture medium was replaced with hypoxanthine/aminopterin/thymidine medium daily for 4 d. 7 d later, hybridomas producing anti-smIL-6R were selected with a smIL-6R-binding assay (ELISA). Hybridomas recognizing smIL-6R were cloned twice by limiting dilution. For ascites production, BALB/c nu/nu mice were injected twice with 0.5 ml of pristane, and 3 d later, 3 × 10⁶ established hybridoma cells were injected intraperitoneally. Ascites fluid was collected 10–20 d later, and the mAb (MR16-1) was purified from the fluid with protein G column (Oncogene Science Inc., Mineola, NY).

Experimental design. IL-6 transgenic mice were injected with 2 mg/body of MR16-1 i.v. once at 5 wk old and then with 100 µg/body s.c. twice weekly from 6 to 14 wk old (Ab treated group, *n* = 4), and with the same volume of PBS (PBS treated group, *n* = 4). At 16 wk old, and after overnight fasting, the mice were weighed, and their bilateral gastrocnemius muscles were dissected under pentobarbital anesthesia, weighed, and rapidly frozen in liquid nitrogen. As controls, normal littermate female C57BL/6J mice (*n* = 6) were used.

Measurement of cathepsin activities. The stored muscles were washed twice with a homogenization solution (250 mM sucrose, 2 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl, pH 7.4), homogenized with a homogenizer (Polytron; Brinkman Instruments, Co., Westbury, NY) in 1 ml of the homogenization solution containing 0.2% Triton-X100, and lysed by sonication. The homogenate was centrifuged at 18,000 *g* for 15 min. The supernatant was then dialyzed against the same amount of glycerol and stored at -40°C until analysis. Cathepsin B activity was assayed using 10 µM Z-Arg-Arg-4-methylcoumaryl-7-amide (MCA) (Peptide Institute, Osaka, Japan) as a substrate at pH 6.0 according to the method of Barrett et al. (29) with some modifications. To obtain a blank sample, the extract was preincubated with 1 µM of L-3-carboxy-trans-2,3-epoxypropionyl-leucylamide-(4-guanidino) butane (E-64) (Peptide Institute) at 37°C for 5 min to inhibit cathepsin B activity. Cathepsin B+L activity was assayed by the same method as for cathepsin B, but with Z-Phe-Arg-MCA (Peptide Institute). Since this synthetic substrate is hydrolyzed by not only cathepsin L but also cathepsin B (29), its hydrolysis is expressed as the activity of cathepsin B+L. The fluorescence of liberated 7-amido-4-methylcoumarin was measured with a fluorometer (F-3000; Hitachi Ltd., Tokyo, Japan) at excitation wavelength 380 nm

and emission wavelength 460 nm. Protein concentrations of the extracts were determined by the method of Bradford (30) using a reagent of Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) and BSA as a standard.

RNA isolation and Northern blot analysis. Total RNA from the pooled muscle samples obtained from each of the mice in the same groups was extracted using guanidium thiocyanate as described elsewhere (31) and quantified by absorbance at 260 nm. RNA samples (20 µg) were subjected to electrophoresis in 1.0% agarose gels and blotted overnight on a Hybond-N+ nylon membrane (Amersham International, Little Chalfont, UK) with 20 × SSC (0.15 M NaCl and 15 mM sodium citrate, pH 7.0). The RNA in gels and filters was visualized with ethidium bromide and photographed by ultraviolet transillumination to ensure the integrity of RNA and the loading of equal amounts of RNA. The membrane was prehybridized for 1 h and hybridized overnight with Church buffer. The cDNAs encoding rat cathepsin B and L (32), m and muscle-specific calpain (33), mono- and poly-ubiquitins (34), subunits C2 and C8 of rat 20S proteasomes (35), and subunits S4 and S7 of human 26S proteasomes (36, 37), were used. Radiolabeled probes were prepared by the random primer method. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as a control for loading. Filters were exposed to X-Omat AR films (Eastman Kodak Co., Rochester, NY) with an intensifying screen for 1–3 d at -80°C, and quantification of the membranes was done by densitometry using an MCID system (Imaging Research Inc., St. Catherines, Canada).

Immunohistochemical study. Frozen transverse sections, 4-µm thick, of the gastrocnemius muscle were sliced and mounted on poly-L-lysine-coated glass slides. After endogenous peroxidase activity had been quenched and nonspecific binding was blocked, the sections were incubated with rabbit Ab against purified rat cathepsin B (38), L (39) at 4°C in a moist chamber overnight as described before (5). After washing in PBS, biotinylated mouse anti-rabbit Ig (Nichirei Co., Tokyo, Japan) was applied for 20 min at room temperature. After thorough washing in PBS, peroxidase-conjugated streptavidin was applied for 20 min and immunostaining was then visualized for 3 min with 0.02% (wt/vol) 3,3'-diaminobenzidine and 0.03% (vol/vol) hydrogen peroxidase in 0.05 M Tris-HCl, pH 7.6.

Statistical analysis. Values were expressed as mean ± SD, and statistical analysis of the data was performed by ANOVA using the Schaffe test.

Results

In the PBS-treated IL-6 transgenic mice, the weight of the gastrocnemius muscles was significantly lower in comparison to the control group, even though overall body weight showed no change. Treatment with mIL-6R mAb (MR16-1), however, completely prevented atrophy of the muscle (Table I). The weights of the livers were 862.8 ± 77.4 mg in the control mice, and 1,242 ± 292 mg in the PBS-treated IL-6 transgenic mice. By the treatment with MR16-1, the wt decreased to 888.5 ± 33.2 mg. IL-6 caused redistribution of body proteins. The activities of cathepsins B and B+L in the muscles were significantly higher in the PBS-treated IL-6 transgenic mice than those of the control group. Again, in the MR16-1-treated group, these activities returned to the same levels as those of control group, indicating that the activation of cathepsins observed in the IL-6 transgenic mice was completely inhibited by the treatment with MR16-1 (Table I). The expression of the mRNA levels of cathepsin B and L was also examined. As shown in Fig. 1, in the PBS-treated IL-6 transgenic mice, the levels of cathepsin B and L mRNAs as measured by densitometry were 277 and 257%, respectively, compared to corresponding levels in the control mice. The treatment with MR16-1 resulted in a

Table I. Body and Gastrocnemius Muscle Weights and Enzymatic Activities of Cathepsins in Gastrocnemius Muscles of Control Mice, and PBS-treated or Anti-mouse IL-6 Receptor Antibody (MR16-1)-treated IL-6 Transgenic Mice

Experimental group	Body weight g	Muscle weight mg	Cathepsin activity	
			B	B+L
<i>nmol AMC/h per mg protein</i>				
Control mice (n = 6)	20.5±1.2	119.3±9.1	3.3±0.4	67.3±5.9
IL-6 transgenic mice + PBS (n = 4)	21.7±2.7	91.0±15.2*	66.6±30.6‡	419.3±171‡
IL-6 transgenic mice + MR16-1 (n = 4)	19.7±1.2	117.3±10.8	4.1±0.6	58.0±14.9

IL-6 transgenic mice were randomized to receive PBS or MR16-1 with injection of 2 mg/body intravenously once at 5 wk old and 100 mg/body intraperitoneally twice weekly from 6 to 14 wk old. At 16 wk old the body weights and gastrocnemius muscle weights were measured. The activities of cathepsins B and B+L in the gastrocnemius muscle were measured as described in Methods using Z-Arg-Arg-4-MCA and Z-Phe-Arg-MCA as respective substrates. Data expressed are mean±SD. * $P < 0.05$, ‡ $P < 0.01$ (vs. control mice and IL-6 transgenic mice treated with MR-16). AMC, 7-amido-4-methylcoumarin.

decrease in the levels of cathepsin B and L mRNAs to 122 and 170%, respectively. Since the mRNA levels of GAPDH were consistently lower in the PBS-treated IL-6 transgenic mice than in the control mice and were reversed by the MR16-1 treatment, the amount of GAPDH mRNA could not have been used for normalization. IL-6 thus not only increased the enzymatic activities of the cathepsins, but also increased their mRNA levels. The effect of IL-6 on lysosomal cathepsins was further confirmed by the immunohistochemical study. As shown in Fig. 2, strong stainings of both cathepsins were identified in the muscle fibers of the PBS-treated IL-6 transgenic mice. Stainings were not uniform in the muscles. Strong stainings showing a fine granular appearance were observed exclusively in the atrophying muscle fibers. The percentage of the numbers of atrophying fibers with strong stainings of cathepsins B and L were counted in the separate 100 fibers in the PBS-treated transgenic mice. The ratios were 59.7±11.1% for cathepsin B and 60.6±17.5% for cathepsin L. Treatment with MR16-1 resulted in no strong staining of cathepsins as observed in the control mice. The ubiquitin-proteasomes system is one of the major intracellular proteolytic systems. The potential for participation of this system was examined in the IL-6 transgenic mice. As shown in Fig. 3, the mRNA levels of mono- and poly-ubiquitins in the PBS-treated transgenic mice increased to 193 and 169%, respectively, compared to corresponding levels in the control mice. In the Ab-treated transgenic mice, however, they decreased to 92.5, and 142%, respectively. On the other hand, the mRNA levels of subunits of 26S proteasome (S4, S7) and 20S proteasome (C2, C8) were not significantly different in the three groups. Calpains account for another intracellular proteolytic system, and the effect of IL-6 on these enzymes was also examined. The mRNA level of muscle-specific calpain (p-94), calpain 3, in the PBS-treated IL-6 transgenic mice was reduced to 50% of that in the control mice. In the antibody-treated transgenic mice, this reduction did not occur indicating that IL-6 mediates down-regulation of muscle-specific calpain.

Discussion

Chronic overproduction of human IL-6 in the transgenic mice causes IgG1 plasma cytosis, hepato-splenomegaly (40), and

muscle weight loss (5). Enzymatic activities and mRNA levels of the lysosomal cathepsins (B, L) in the muscle of the transgenic mice were observed to increase in association with muscle atrophy. Treatment with anti-mouse IL-6 receptor Ab (MR16-1) completely blocked these phenomena, suggesting that it prevented the binding of human IL-6 to the mouse IL-6 receptor from triggering the association of a non-ligand-binding 130-kD signal-transducing molecule, gp130 (41). A previous study of ours demonstrated that immunohistochemical staining of cathepsins (B, L) was strongly positive and showed granular patterns in the muscle fiber, especially in the atrophying muscle (5). Staining patterns similar to that of cathepsins (B, L) have been observed in various dystrophic muscles, and fine granules with positive staining have been identified as autolysosomes (42). Signaling processes induced by IL-6 may thus be closely linked to the production of cathepsins and the formation of autophagosomes. Therefore, it is conceivable that other cytokines such as oncostatin M, leukemia inhibitory factor,

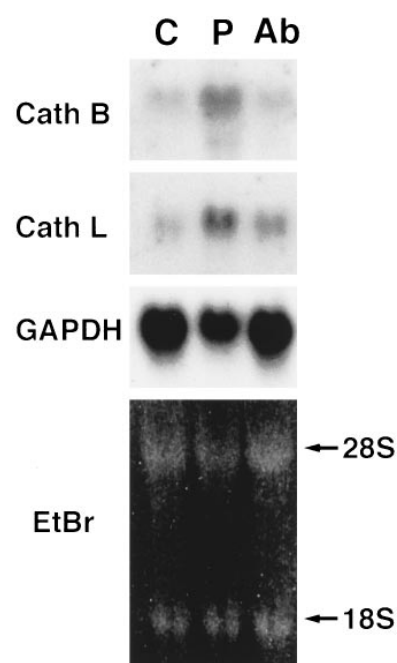


Figure 1. Expression of mRNA for cathepsins B and L extracted from the gastrocnemius muscles of the control mice (C), IL-6 transgenic mice treated with PBS (P) and treated with MR-16 (Ab). 20 µg of total RNA was examined by Northern blot analysis as described in Methods. The probes for rat cathepsins B and L cDNA were used. The integrity and the equivalent loading of ribosomal RNA were confirmed by visualization with ethidium bromide, and a GAPDH probe was used for control. *Cath*, cathepsin; *EtBr*, ethidium bromide.

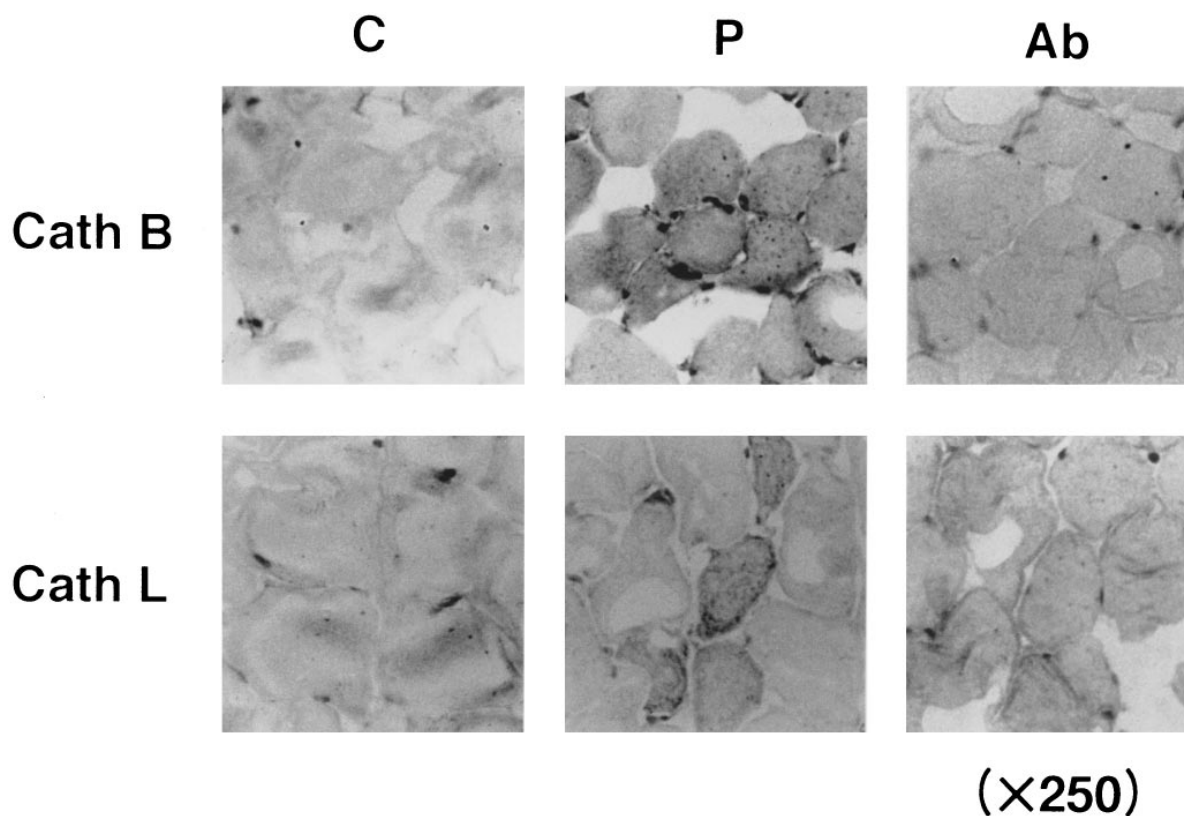


Figure 2. Immunohistochemistry of cathepsins B and L in the gastrocnemius muscles of the control mice (C), IL-6 transgenic mice treated with PBS (P) and treated with MR-16 (Ab). Positive stainings for cathepsins B and L were seen in the myofibers in the IL-6 transgenic mice treated with PBS, but very few positive reactions of both cathepsins were observed in the control mice and the IL-6 transgenic mice treated with MR-16. (Original magnification, $\times 250$.) *Cath*, cathepsin.

and ciliary neurotrophic factor, whose receptor complexes share a signal-transducing component, gp130, also activate lysosomal cathepsins. The present study indicated that IL-6 enhanced the capacity for lysosomal proteolysis via an increase of cathepsins activities, which may enhance the breakdown of endogenous proteins (specific soluble or membranous components) or alternatively endocytosed proteins (43). The functional importance of lysosomal proteolysis in muscle degradation has been, however, questioned. Inhibition of cathepsin activity does not affect overall protein degradation or myofibrillar proteolysis in fasting (22), denervation atrophy (16), and sepsis (14), though cathepsin B activity increases in these conditions. Therefore, the role of cathepsins for muscle atrophy observed in the IL-6 transgenic mice is uncertain.

Activation of the ATP-Ub-dependent proteolytic pathway has been reported in a variety of pathologic conditions. In Yoshida sarcoma-bearing rats, the increase in mRNA levels for Ub, Ub carrier protein (E₂), and proteasome subunits (C8, C9) is found correlated with the enhancement of energy-dependent proteolysis (44). It was also reported in rats implanted with Yoshida ascites hepatoma that both lysosomal and ATP-dependent processes are activated in epitrochlearis muscles (45). In coordination with activation of the ATP-dependent proteolysis, levels of ubiquitin-conjugated proteins and a 27-kD proteasome subunit increases in association with an increase in mRNA levels of ubiquitin and multiple subunits of the proteasome. ATP-dependent proteolysis is also enhanced in denervation (16), fasting (22), and acidosis (46). In

addition, mRNA levels of poly-ubiquitin and proteasomes increase in skeletal muscle during starvation and denervation atrophy, though mRNA levels of cathepsins (L and D) and calpain I show no change (47). TNF has been reported to cause an increase in the ubiquitination (48) and ubiquitin gene expression (49) in skeletal muscle. Furthermore, TNF is responsible for the decrease in muscle weight in Yoshida AH-130 ascites hepatoma-bearing rats associated with an increase in ubiquitination and Ub-gene expression (48), but TNF does not seem to cause these effects directly (50). Considering that TNF induces the production of IL-6 (12) and that the results of the present study show that Ub-gene expression was enhanced in IL-6 transgenic mice, IL-6 could be a candidate for direct mediator of Ub-gene expression.

Calpains have not been considered to play an important role in muscle degradation in denervation atrophy (16), fasting (22), and tumor-bearing rats (44, 45), since they are committed to limited proteolysis of targeted proteins (17). Therefore, they have been proposed as having regulatory rather than degradative roles. We found in this study that the mRNA level of muscle-specific calpain mRNA was decreased in the IL-6 transgenic mice. It was recently reported that mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A (51). Though the pathogenesis of how a defect of calpain 3 causes muscle dystrophy is yet uncertain, their results, which are compatible with our present study, demonstrated that a decrease of calpain 3 mRNA level was associated with muscle atrophy. The following possibility was drawn that IL-6 or some

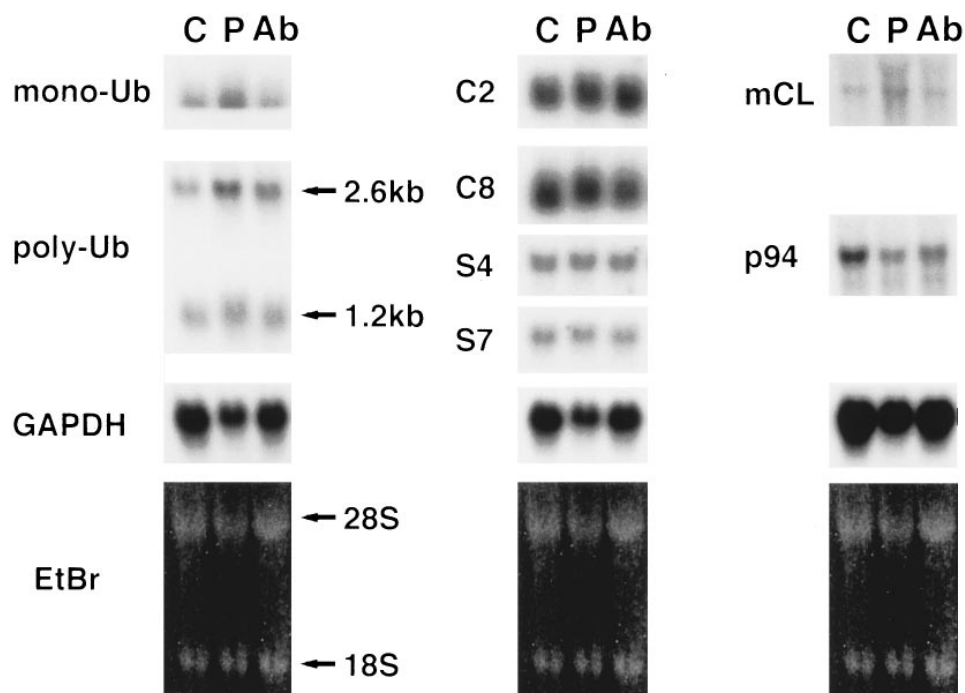


Figure 3. Expression of mRNA for mono- and poly-ubiquitin, subunits for proteasomes (subunits C2 and C8 of rat 20S proteasomes, and subunits S4 and S7 of human 26S proteasomes) and m calpain (*mCL*) and muscle-specific calpain (*p94*) extracted from the gastrocnemius muscles of the control mice (C), IL-6 transgenic mice treated with PBS (P) and treated with MR-16 (Ab). 20 μ g of total RNA was examined by Northern blot analysis, and the respective cDNA probes were used. The integrity and the equivalent loading of ribosomal RNA were confirmed by visualization with ethidium bromide, and a GAPDH probe was used for control. *EtBr*, ethidium bromide.

transcriptional factors induced by IL-6 may be involved in negative regulation of calpain 3 gene transcription. Our present results implicate a counterregulatory role of calpain 3 in muscle proteolysis observed in many pathologic conditions, such as sepsis and cancer cachexia.

A mouse Ab against human IL-6 receptor has been isolated (52). This antibody inhibits IL-6 functions (52) and shows a strong anti-tumor cell activity against multiple myeloma cells in vivo (53). To be effective as therapeutic agents for human patients, mouse Ab can be engineered to resemble human Ab (54). The present study indicates a potential treatment with humanized IL-6 receptor Ab of muscle wasting in patients with various pathologic conditions where IL-6 plays a predominant role.

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