J. Biochem. 122, 595-600 (1997)

Involvement of Interleukin-6 in Activation of Lysosomal Cathepsin and Atrophy of Muscle Fibers Induced by Intramuscular Injection of Turpentine Oil in Mice

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Received for publication, April 15, 1997

Serum IL-6 level increased after the injection of turpentine oil into the right gastrocnemius muscle in mice. The mRNA level of IL-6 was highest in the injected muscle at 12 h after injection, but was not identified in the opposite muscle. The activities of cathepsins B and B+L started to elevate after 12 h in the injected muscle and markedly increased after day 3. Likewise, the mRNA levels of cathepsins B and L markedly increased from day 1 to day 5 in the injected muscle. However, a very mild increase was also observed in the opposite muscle. Immunohistochemical staining of cathepsins B and L exhibited positive reactions as fine granules in myofibers at 12 h and strong positive reactions in the infiltrating macrophages at 3 days. Atrophy of myofibers type 1 and 2 was evident in a time-dependent manner in the injected muscle. Treatment with rat anti-mouse IL-6 receptor monoclonal antibody inhibited the increase in cathepsin activities in the injected muscle. We conclude that IL-6 produced in the inflamed muscle is involved in the process of muscle degeneration, especially through the activation of lysosomal cathepsins.

Key words: cathepsin, degenerative myopathy, interleukin-6, interleukin-6 receptor antibody, turpentine oil.

Intramuscular injection of certain agents, such as bupivacaine and plasmocid, causes acute myofiber necrosis and consequently induces muscle atrophy, as observed in muscle dystrophic diseases (*1*). Activation of the proteolytic systems in muscles is known to be involved in the pathogenesis of degenerative myopathies (*2-5*). Kominami *et al.* (*2*) reported that the activities of cathepsins B and L and thiol proteinase inhibitor were markedly increased in muscles of dystrophic hamsters, and the dystrophic muscles were infiltrated by phagocytes that were stained with anti-cathepsin B and -thiol proteinase inhibitor antibodies. Moreover, at a very early stage of plasmocid-induced myopathy, myofibers were stained with anti-cathepsin L antibody in the absence of the infiltration of macrophages (*5*). In Duchenne muscular dystrophy, positive staining for cathepsins was observed in the intramyofibral portion of atrophic fibers in the presence of marked infiltration of macrophages (*5*). It was thus suggested that lysosomal cathepsins may play an important role in autophagocytosis and heterophagocytosis of the muscle fiber under these pathologic conditions.

IL-6 is a multifunctional cytokine, which not only induces a variety of immunological actions (*6*), but also induces metabolic responses, such as modulation of the synthesis of acute phase proteins (*7*). Furthermore, IL-6 may be a proteolysis-inducing factor for muscles. Repeated injection of IL-6 increases the proteolytic rate in isolated soleus muscles (*8*). We established an *in vitro* myotube culture system and demonstrated that IL-6 shortened the half-life of long-lived proteins by activating intracellular proteolytic systems, including lysosomal cathepsins and proteasomes (*9*). Furthermore, we found that the activities of cathepsins B and B+L in the gastrocnemius muscles strikingly increased in IL-6 transgenic mice in association with the progression of muscle atrophy (*10*) and that the increase in muscle cathepsin activity was completely abolished by treatment with anti-mouse IL-6 receptor antibody (*11*). Since we noticed that the pattern of immunohistochemical staining of cathepsins in the muscle of IL-6 transgenic mice shared many similarities with those in the early stage of plasmocid-induced myopathy (*5*) and in distal myopathy with rimmed vacuoles (*12*), it was hypothesized that IL-6 may be involved in the pathogenesis of muscle degeneration induced by injection of plasmocid or that observed in distal myopathy. IL-6 may play an important role in the process of muscle degeneration in degenerative myopathies by activating intramyofibral lysosomal proteinases.

It is well-known that turpentine oil induces acute inflammation at the injection site and causes aseptic abscess formation. In the present study, we investigated the hypothesis that intramuscular injection of turpentine oil induces muscle degeneration and atrophy as does bupivacaine or plasmocid. In addition to examining the lysosomal cathepsin system, we investigated the potential role of IL-6 in the process of muscle degradation in turpentine oil-induced muscle degeneration.

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MATERIALS AND METHODS

Materials—Polyclonal antibodies and cDNA of cathepsins B and L were produced as described previously (*13-15*). The cDNA of mouse IL-6 was a gift from Dr. Taga (Institute for Molecular and Cellular Biology, Osaka University, Osaka). All other materials were of reagent grade. Rat anti-mouse IL-6 receptor monoclonal antibody, MR-16 was provided from Dr. Katsume (Fuji-Gotemba Research Laboratories, Chugai Pharmaceutical, Gotemba).

Animals—Eight-week-old male C57BL/6J mice (8 weeks old, 20 g body weight) were purchased from Clea Japan (Tokyo). The mice were housed in individual cages in an air-conditioned room with a 12-h light/12-h dark cycle and given free access to water and a nonpurified laboratory diet (Oriental Yeast, Tokyo) until they were used in the experiment.

Examination of Lysosomal Cathepsin System and IL-6 after Turpentine Oil Injection—Experimental design: The mice received an injection of 0.1 ml of turpentine oil (Nacalai-Tesque, Kyoto) or 0.1 ml of saline in the right gastrocnemius muscle under ether anesthesia. After recovery from the anesthesia, the mice were returned to individual cages and fed *ad libitum*. After 12 h, 1, 3, 5, and 7 days ($n=6$, each point), bilateral gastocnemius muscles were obtained under Nembutal anesthesia.

The activities of cathepsins (B, B+L), mRNA levels of IL-6 and cathepsins (B, L), and immunohistochemical staining with anti-cathepsin (B, L) antibodies were examined in both muscles. Serum was collected by cardiac puncture and serum IL-6 concentrations at 12 h and day 3 were assayed by mouse IL-6 specific ELISA standardized with mouse IL-6 (EM-IL-6 kit, Endogen).

Cathepsin Activity—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 Polytron homogenizer 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 −20°C until analysis. Cathepsin B activity was assayed using 10 μM Z-Arg-Arg-4-methylcoumaryl-7-amide (MCA) (Peptide Institute, Osaka) as a substrate at pH 6.0 as described before (*11*). Cathepsin B+L activity was assayed using Z-Phe-Arg-MCA (Peptide Institute) as described before (*11*). This synthetic substrate is hydrolyzed by not only cathepsin L, but also cathepsin B (*16*), and its hydrolysis is expressed as the activity of cathepsin B+L. The fluorescence of liberated 7-amido-4-methylcoumarin (AMC) was measured with a Hitachi F-3000 fluorometer at the excitation wavelength of 380 nm and the emission wavelength of 460 nm. Protein concentrations of the extracts were determined using a Bio-Rad reagent and bovine serum albumin as a standard.

Northern Blot Analysis—Total RNA from the pooled muscle samples obtained at the each time point from the mixture of the six mice was extracted using guanidium thiocyanate and quantified by measuring the absorbance at 260 nm. RNA samples (20 μg) were subjected to electrophoresis and Northern blotting was carried out as described before (*11*). The cDNAs encoding rat cathepsins B and L and mouse IL-6 were used. Radiolabeled probes were prepared by the random primer method. Filters were exposed to Kodak X-Omat AR films with an intensifying screen for 1-3 days at −80°C, and quantification of the membranes was conducted by densitometry using an MCID system (Imaging Research, Ontario).

Immunohistochemical Study—Frozen transverse sections, 4 μm thick, of the bilateral gastrocnemius muscles were sliced, mounted on poly-L-lysine-coated glass slides and fixed with ethanol. After endogenous peroxidase activity had been quenched and nonspecific binding was blocked, the sections were incubated with rabbit antibodies against purified rat cathepsins B and L at 4°C in a moist chamber overnight as described before (*11*). They were then washed in PBS, and biotinylated mouse anti-rabbit immunoglobulin (Nichirei, Tokyo) was applied for 20 min at room temperature. After thorough washing of the sections in PBS, peroxidase-conjugated streptavidin was applied for 20 min and immunostaining was then visualized for 3 min with 0.02% (w/v) 3,3′-diaminobenzidine and 0.03% (v/v) hydrogen peroxide in 0.05 M Tris-HCl, pH 7.6.

Differentiation of Muscle Fibers and Measurement of Muscle Atrophy—Experimental design: The mice received an injection of 0.1 ml of turpentine oil (Nacalai-Tesque) or 0.1 ml of saline in the right gastrocnemius muscle under ether anesthesia. After recovery from the anesthesia, the mice were returned to individual cages and fed *ad libitum*. After 1, 3, 5, and 7 days ($n=6$, each point), bilateral gastocnemius muscles were obtained under Nembutal anesthesia. These muscles were also obtained from control mice without any treatment ($n=6$). Eight-micrometer-thick frozen sections of the bilateral gastrocnemius muscles were stained with hematoxylin and eosin, and nictonamide-tetrazolium reductase (NADH-TR) (*17*). The three cross sections were produced from each gastocnemius muscle and investigated. Type 1 muscle fibers were strongly stained with NADH-TR, and type 2 muscle fibers were stained negatively. Photographs of each section were taken at 100× magnification. The sizes of type 1 and 2 muscle fibers were calculated in cross section by measuring the largest diameter and its right angular diameter. After measuring more than 100 muscle fibers of each type in each mouse, the mean sizes were computed.

Treatment of Rat Anti-Mouse IL-6 Receptor Monoclonal Antibody, MR.-16—The mice received an intraperitoneal injection of either rat IgG (1 mg, $n=6$) or MR-16 (1 mg, $n=6$) 30 min before the injection of turpentine oil (0.1 ml) into the right gastrocnemius muscle. The mice were then given free access to water and a regular diet. Three days after the turpentine oil injection, the gastrocnemius muscles were harvested under Nembutal anesthesia for analysis of cathepsin B and B+L activities.

Statistical Analysis—Values were expressed as mean± SD. Differences were tested using Student's unpaired t test and the Kruskal-Wallis X test. A p value of less than 0.05 was considered significant.

RESULTS

Serum IL-6 Concentration—Serum IL-6 concentrations in the mice receiving the injection of turpentine oil were significantly higher at 12 h and day 3 after injection than those in the mice receiving the saline injection (Table I).

The serum concentration of IL-6 was higher at 12 h than that at day 3 in the mice receiving the turpentine oil injection.

Northern Blot Analysis of IL-6 mRNA Level in the Muscle—The mRNA level of IL-6 in the gastrocnemius muscle was assessed by Northern blot analysis (Fig. 1). The mRNA level was highest at 12 h in the muscle receiving turpentine oil injection; it decreased at day 1 and was almost undetectable at day 3. In contrast, the levels in the opposite site were undetectable at all time points. When saline was injected into the muscle, the mRNA levels were undetectable either in the injected muscle or in the contralateral muscle (data not shown).

Cathepsins B and B+L Activities—The activities of cathepsins B and B+L in muscle receiving the turpentine oil injection significantly increased after 12 h as compared with the opposite muscle in the turpentine oil-injected mice and the bilateral muscles in the saline-injected mice (Fig. 2). Thereafter, the activities of cathepsins in the turpentine oil-injected muscle markedly increased and reached the peak from day 3 to day 5. On the other hand, the activities of cathepsins both in the opposite muscle and in the bilateral muscles in mice receiving the saline injection did not show any time-dependent changes: these activities stayed within the control range obtained in normal gastrocnemius muscles from untreated control mice.

Northern Blot Analysis of mRNA Levels of Cathepsins B and L—The mRNA levels of cathepsins in the gastrocnemius muscle were assessed by Northern blot analysis (Fig. 3). The mRNA levels of cathepins B and L increased in the muscle that received the turpentine oil injection and reached the maximum level from day 1 to day 5. The mRNA levels of both cathepsins showed the same profile. In the opposite muscle, the mRNA levels of cathepsins B and L slightly increased from day 1 to day 5, though the intensities were much weaker than those in the injected site. However, in the mice receiving the saline injection, the

TABLE I. **Changes of serum IL-6 levels after intramuscular turpentine oil injection in mice.**

Treatment	Serum IL-6 level (pg/ml)	
	At 12 h	At day 3
Turpentine oil	1,213±118*,#	421±219*
Saline	48.3±24.6	20.7±10.9

Data are means±SD. $n=6$ at each time point. Mice received either turpentine oil (0.1 ml) or saline (0.1 ml) injection into the right gastrocnemius muscle and blood was sampled by cardiac puncture at designated time points. Serum IL-6 concentration was measured by mouse IL-6 specific ELISA. *$p<0.01$, turpentine oil *vs.* saline, #$p<0.01$, at 12 h *vs.* at day 3.

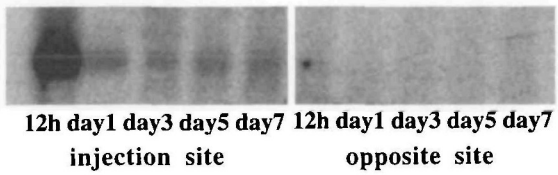

Fig. 1. **Changes in IL-6 mRNA levels in the gastrocnemius muscles after intramuscular turpentine oil injection in mice.** Turpentine oil (0.1 ml) was injected into the right gastrocnemius muscle and sampling was conducted for Northern blot analysis at the designated time points from the muscle receiving turpentine oil injection (injection site) and the opposite muscle (opposite site).

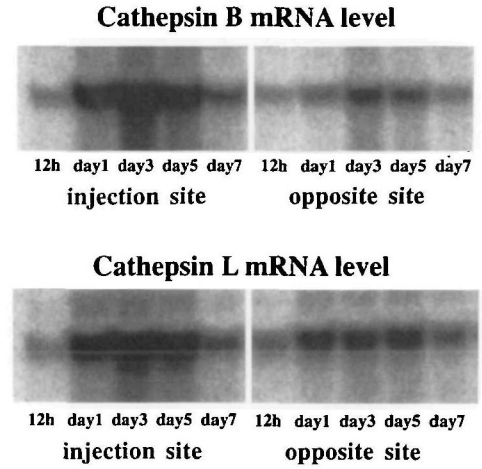

Fig. 3. **Changes in the mRNA levels of cathepsins B and L in gastrocnemius muscles after intramuscular turpentine oil injection in mice.** Northern blot analysis was performed on the samples obtained from the turpentine oil-injected muscle (injection site) and the opposite muscle (opposite site) at the designated time points.

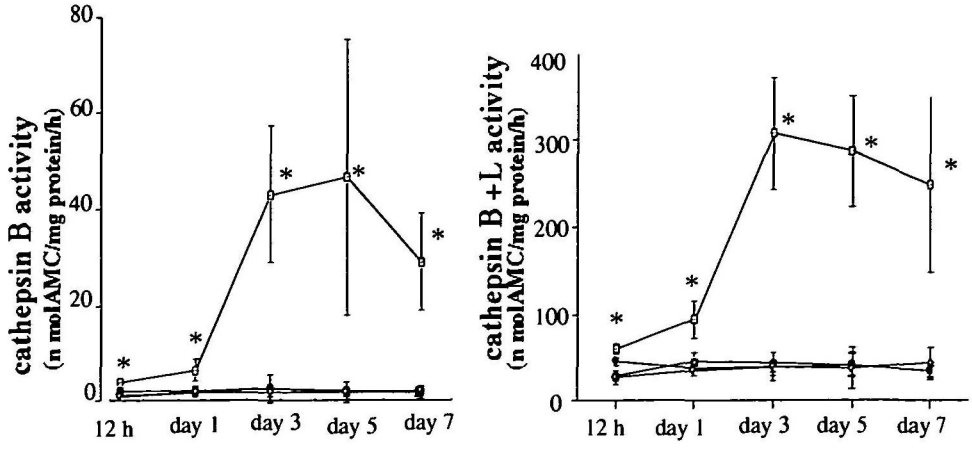

Fig. 2. **Serial changes in the activities of cathepsins (B, B+L) in gastrocnemius muscles after intramuscular turpentine oil injection in mice.** The activities of cathepsins B and B+L were assayed using Z-Arg-Arg-4-methylcoumaryl-7-amide (MCA) and Z-Phe-Arg-MCA as substrates, respectively. Muscle samples were obtained at the designated time points from the injected muscle (⊡) and the opposite muscle (■) in the mice given an intramuscular turpentine oil injection (0.1 ml) into the right gastrocnemius muscle and from the injected muscle (○) and the opposite muscle (●) in the mice given an intramuscular saline (0.1 ml) injection. *$p<0.05$, the turpentine oil-injected muscle *vs.* the other three muscles.

mRNA levels of both cathepsins did not show any time-dependent changes in either of the muscle sites (data not shown).

Immunohistochemical Staining of Cathepsins B and L—Immunohistochemical study revealed moderate positive reactions for cathepsins B and L after 12 h in the muscle that had received turpentine oil injection, in the form of fine granules in myofibers; there were no infiltrating cells around the myofibers. After 3 days, strong positive reactions in the myofibers and the infiltrating macrophages were seen at the injection site (Fig. 4). No positive reactions were seen after injection of the opposite muscle, or in either of the muscles in the mice receiving the saline injection (data not shown).

Muscle Atrophy—We assessed muscle atrophy by measuring the sizes of the type 1 and 2 myofibers in the cross section of the muscle. Firstly, the differentiation of muscle types was achieved by NADH-TR staining. As shown in Fig. 5, type 1 fibers were stained positively with NADH-TR and type 2 fibers were stained negatively. The sizes of both fibers in the turpentine oil-injected muscle were apparently smaller than those in the opposite muscle. Sequential changes in the sizes of both fibers were monitored and the difference between the injection and the opposite sites was compared (Fig. 6). The size of type 1 myofibers at the injection site decreased time-dependently and the difference in size compared to the opposite site and the control muscle (day 0) was significant after day 3. The size

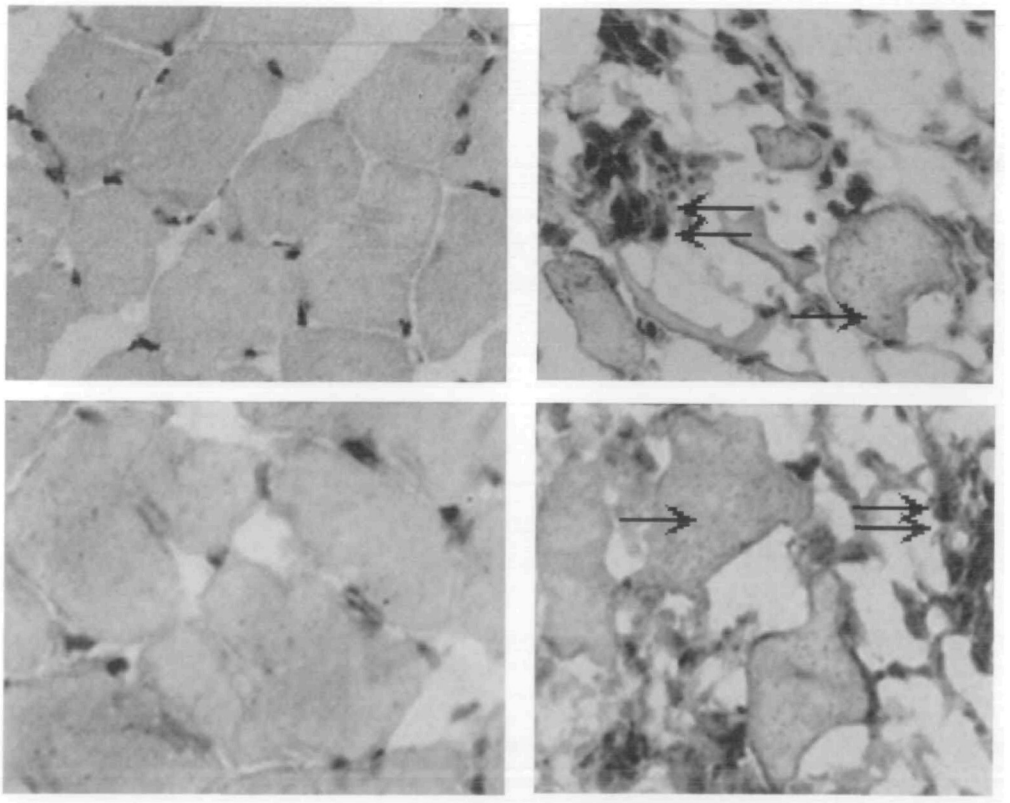

Fig. 4. **Immunohistochemical staining of cathepsins B and L in gastrocnemius muscles after intramuscular turpentine oil injection in mice.** Immunohistochemical staining of cathepsins B and L was carried out on muscle samples obtained from the injected muscle at designated time points after turpentine oil injection. Strong positive reactions were observed at day 3 in both myofibers (single arrow) and infiltrating macrophages (double arrow) in the injected muscle as showen here. (cathepsin B; upper panel, the opposite muscle; left hand side, the turpentine oil-injected muscle; right hand side, cathepsin L; upper panel, the opposite muscle; left hand side, the turpentine oil-injected muscle; right hand side). Original magnification, ×200.

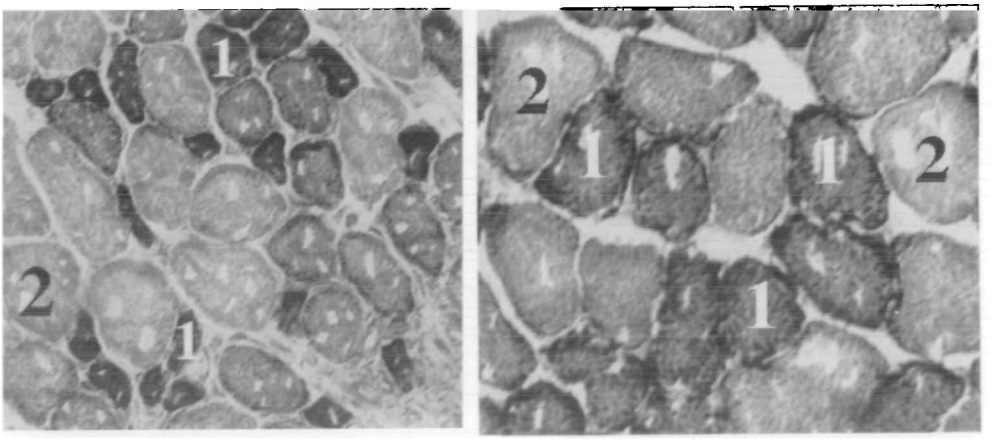

Fig. 5. **Differentiation of myofibers type 1 and type 2 in the gastrocnemius muscles after intramuscular turpentine oil injection in mice.** Immunohistochemical staining of NADH-TR was carried out on muscle samples obtained from the injected muscle and the opposite muscle at designated time points after turpentine oil injection. The staining of the injected muscle (left hand side) and the opposite muscle (right hand side) obtained 3 days after turpentine oil injection is shown. Type 1 fibers (1) were stained dark and type 2 (2) fibers were stained negative. The size of the respective fibers was then measured. Original magnification, ×200.

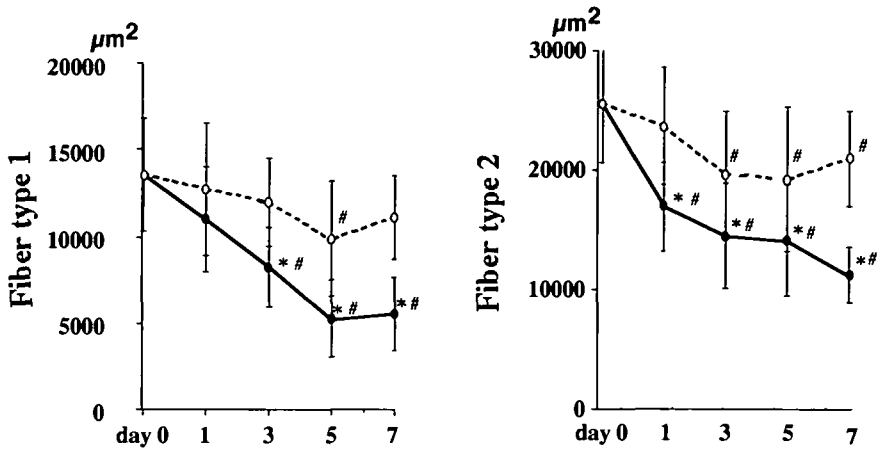

Fig. 6. **Serial changes in the size of myofibers type 1 and type 2 in gastrocnemius muscles after intramuscular turpentine oil injection in mice.** The size of the myofibers in the turpentine oil-injected muscle is indicated by open circles (○) and the size in the opposite muscle, by closed circles (●). Data are expressed as mean ± SD. $^{\#}p<0.05$ (*vs.* day 0, control muscle obtained from normal mice without any treatment), $^{*}p<0.05$ (the injection site *vs.* the opposite site).

TABLE II. **Effect of rat anti-mouse IL-6 receptor monoclonal antibody, MR-16, on cathepsin activities in the gastrocnemius muscle after turpentine oil injection.**

Muscles	Cathepsins	Treatment	
		MR-16	Rat IgG
		(nmol AMC/mg protein/h)	
Turpentine oil injected site	B	23.09±5.64$^{*,\#}$	58.77±15.53$^{\#}$
	B+L	232.72±79.94$^{*,\#}$	403.00±103.58$^{\#}$
Opposite site	B	3.89±1.49	4.10±1.34
	B+L	12.67±5.80	13.29±4.52

Data are means ± SD. Mice received intraperitoneal injection of either rat IgG (1 mg, $n=6$) or MR-16 (1 mg, $n=6$) 30 min before the injection of turpentine oil into the right gastrocnemius muscle. Three days after the turpentine oil injection, both muscles were harvested for analysis of cathepsin B and B+L activities. $^{*}p<0.05$, MR-16 *vs.* rat IgG, $^{\#}p<0.01$, turpentine oil-injected site *vs.* opposite site.

of type 2 myofibers decreased in a time-dependent manner in both the injection site and the opposite site. Compared to the size in the control muscle, a significant difference was observed after day 1 in the injection site and after day 3 in the opposite site. Type 2 fibers were more susceptible to the systemic changes caused by turpentine oil injection, such as increase in serum IL-6 level (Table I). However, the size of type 2 fibers at the injection site was significantly smaller than that on the opposite site after day 3.

Effect of Rat Anti-Mouse IL-6 Receptor Monoclonal Antibody MR-16 on the Activities of Cathepsins B and B+L in Mice Receiving Intramuscular Injection of Turpentine Oil—Involvement of IL-6 in the pathogenesis of muscle degeneration induced by turpentine oil injection was further confirmed by treatment with MR-16. MR-16 modified the activities of cathepsins B and B+L (Table II). The activities of cathepsins in mice treated with MR-16 measured at day 3 in the turpentine oil-injected muscle were significantly lower than those in mice treated with rat IgG. The activities of cathepsins in the opposite site did not differ between the two treatments. When the differences between the turpentine oil-injected and opposite muscles were compared in mice treated with MR-16, the activities of cathepsins in the injected site were significantly higher than those in the opposite site. These results suggested that treatment with MR-16 did not completely abolish the change in the cathepsin activity induced by turpentine oil injection.

DISCUSSION

In plasmocid-induced muscle degeneration, breakdown of myofibers is seen at the early stage of 2 h after plasmocid injection, and extensive digestion of degenerated fibers by infiltrating macrophages takes place after 6 h (*5*). The activities of cathepsins B and B+L in the muscle increase markedly after 3 days. Lysosomal cathepsins may be committed to the initial disruption of myofibers and formation of autolysosomes, while cathepsins derived from infiltrating macrophages may play an important role in the extensive muscle necrosis. After the intramuscular injection of bupivacaine, muscle fibers rapidly become necrotic and the muscle is invaded by phagocytic cells (*18*); the increase in cathepsin B and L activities in the muscle in this case may be derived from macrophages (*19*).

In turpentine oil-induced muscle degeneration, as shown in the present study, the activities of cathepsins B and B+L in the muscle began to elevate at 12 h after injection when infiltration of macrophages had not yet become evident. Furthermore, the activities markedly increased after 1 day in association with massive infiltration of macrophages, and atrophy of myofibers was observed. These macrophages were stained strongly positive for cathepsins.

A common process of muscle degradation seems to exist in these drug-induced myopathies, and lysosomal cathepsins may play a central role in muscle degradation. This raises the question of whether there is some common mediator that activates the cathepsin system after injection of inflammatory agents. Previously, we found that the activities of cathepsins B and B+L are very high in the muscle of IL-6 transgenic mice in association with muscle atrophy (*10*) and that IL-6 increases the activities of cathepsins B and B+L in cultured myotube cells (*9*). It was thus hypothesized that IL-6 may be produced locally in the muscle by the inflammatory stimulus and trigger activation of the cathepsin system. As expected, strong expression of IL-6 mRNA was found at 12 h in the muscle injected with turpentine oil and serum IL-6 increased, staying high until day 3. The level of IL-6 in the injected muscle might have been much higher than the serum IL-6 level. A high concentration of IL-6 in the injected muscle may induce the activation of lysosomal cathepsins and consequently enhance autodigestion of myofibrils. These degenerated

myofibers are ultimately phagocytosed by infiltrated macrophages. Involvement of IL-6 in the activation of cathepsins was confirmed by the experiment using rat anti-mouse IL-6 receptor antibody, MR-16. The MR-16 treatment significantly suppressed the activities of cathepsins B and B+L at day 3 after the injection of turpentine oil. However, the levels in the injected muscle treated with MR-16 were still significantly higher than those in the opposite muscle. Inhibition of the action of IL-6 by the receptor antibody may not completely abolish the autodigestion of myofibrils and consequently may fail to block infiltration of macrophages. In addition to IL-6, there may be another factor responsible for inducing infiltration of macrophages rich in lysosomal cathepsins. The details of the mechanism, however, require further investigation.

Serum IL-6 level was elevated by turpentine oil injection. The systemic effect of IL-6 may be observed in the opposite muscle. Though the activities of cathepsin were not affected, the mRNA levels of cathepsins B and L were increased in the opposite muscle after injection. This finding was compatible with our previous reports (*9-11*) showing that IL-6 enhances transcription of cathepsins (B, L). A much higher concentration of IL-6 may be necessary to increase the enzymatic activity of cathepsins as expected in the turpentine oil-injected muscle. Possibly there is a threshold concentration of IL-6 required to induce translation of cathepsins.

Type 1 fiber is called red muscle and type 2 fiber is called white muscle. The energy metabolism in the individual muscles is reported to be different (*20*), and the different types of skeletal muscle may respond differently in acute inflammation. Protein breakdown in the extensor digitorum longus (EDL) muscle, rich in white muscle, occurs more readily than in the soleus (SOL) muscle, rich in red muscle, in sepsis (*21*). We found that the activities of cathepsins B and L in the EDL muscle are significantly higher than those in the SOL muscle in rats (*22*). Therefore, it was of interest to examine the atrophy of type 1 and type 2 fibers in mice given a turpentine oil injection. Atrophy of type 2 (white muscle) proceeded earlier than that of type 1 fibers (red muscle) both in the turpentine oil-injected muscle and in the opposite muscle. This observation is compatible with the report that white muscle becomes more atrophic than red muscle in sepsis (*21*). Further investigation is, however, necessary to elucidate the precise role and interrelationship of IL-6 and lysosomal cathepsin in turpentine oil-induced muscle atrophy.

In conclusion, turpentine oil injection induces acute muscle degradation and the lysosomal cathepsin system may play a central role in this process. IL-6 is produced locally in the turpentine oil-injected muscle and may trigger the activation of cathepsins. Administration of the IL-6 receptor antibody inhibits activation of cathepsins and may modulate the muscle degradation induced by an inflammatory agent.

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