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MUSCLE UNDERGOES ATROPHY IN ASSOCIATION WITH INCREASE OF LYSOSOMAL CATHEPSIN ACTIVITY IN INTERLEUKIN-6 TRANSGENIC MOUSE

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SUMMARY: Interleukin(IL)-6 transgenic mice were produced by microinjection of human IL-6 cDNA fused with H-2L^d promotor into the pronucleus of fertilized eggs from C57BL/6J mice. At 16 weeks old, the gastrocnemius muscles of the IL-6 transgenic mice became atrophic as compared to those of the normal mice, while the body weights increased significantly. The activities and mRNA levels of lysosomal cathepsins B and L were increased in the muscles of the transgenic mice. Immunohistochemical study on the muscles showed increased staining of both cathepsins B and L in the transgenic mice. IL-6 is responsible for enhanced muscle catabolism by activating the lysosomal cathepsin (B and L) system. © 1995 Academic Press, Inc.

The mRNA for interleukin-6 is constitutively expressed in numerous tissues, including monocytes, fibroblasts and certain types of malignant cells. Transcription and production of this protein in fibroblasts, monocytes, macrophages, keratinocytes and endothelial cells are enhanced in response to other cytokines (TNF, IL-1, platelet-derived growth factor) and lipopolysaccharide (1). IL-6 is a multifunctional cytokine which acts as a cytotoxic T cell differentiation factor as well as a stimulatory factor for T and B cells, and enhances the cytotoxic activity of NK cells via IL-2 (2). Besides these immunological actions, it induces variety of metabolic responses. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins *in vivo* (3), down-regulates albumin synthesis (4) and reduces lipoprotein lipase activity in adipose tissue of mice and in 3T3-L1 adipocytes (5). Evidence has been accumulated to believe that IL-6 is involved in cancer cachexia (6). IL-6 producing colon-26 adenocarcinoma cells cause cachexia in mice (7) and IL-6 cDNA transfected Lewis lung carcinoma cells cause weight loss and shorten survival in syngenic mice (8). Repeated injection

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of IL-6 increases proteolytic rate in isolated soleus muscles (9). Since TNF stimulates the production of IL-6, cachectic changes induced by TNF (10,11) may be overlapped and complemented by IL-6.

We established an *in vitro* myotube culture system to examine proteolytic effects of inflammatory cytokines (IL-6, TNF, IL-1α and β). Only IL-6 shortened the half-life of long-lived proteins in association with activation of intracellular proteolytic systems, including lysosomal cathepsins and proteasomes (unpublished data). IL-6 may be responsible for protein degradation in severe catabolic states, such as cancer cachexia, sepsis and severe injury. This study was therefore designed to investigate *in vivo* effects of IL-6 on muscles and muscle cathepsins B and L. In order to eliminate potential influences from tumor-bearing state, we adopted the transgenic mice of C57BL/6 origin which develop a massive plasmacytosis but not transplantable plasmacytomas (12) instead of transplantation of tumor cells. The results of the present study clearly demonstrated that IL-6 induces muscle atrophy and activates lysosomal cathepsins in muscles.

MATERIALS AND METHODS

Animals. C57BL/6J L^d-IL-6 transgenic mice were produced by micro-injection of the 3.3-kilobase-pair SphI-XhoI fragment (L^d-IL-6) containing human IL-6 cDNA fused with the H-2L^d promotor into the pronucleus of fertilized eggs from C57BL/6J mice from Nippon Clea, Tokyo, Japan, as described elsewhere (13). Integration of the transgene was screened by the Southern blot analysis of EcoRI digested tail DNA using the ^{32}P-labeled Taq I-Ban II fragment of human IL-6 cDNA as a probe. The transgenic mice were identified by PCR analysis of tail DNA to detect the transgene, using Taq DNA polymerase and two primers specific for human IL-6 cDNA:CHIL6P5 (5'-ACCTCTTCAGAACGAATTGACAAA-3') and CHIL6P7i(5'-AGCTGCGCAGAATGAGATGAGTTGT-3'). In these transgenic mice, serum IL-6 concentrations were more than 600 pg/ml after 12-week old determined by the human IL-6-specific ELISA as described (14). The transgenic mice, all of which were male, were housed in individual cages in an air-conditioned room with a 12h-light/12h-dark cycle and fed standard laboratory chow, CE-2, from Nippon Clea, Tokyo, Japan, ad libitum. As a control, normal C57BL/6J male mice were grown and housed in the same condition. At 16-week old, body weights were measured after overnight fasting, and gastrocnemius muscles were weighed after dissection and rapidly frozen in liquid N2. In one series of experiments, cathepsins (B, B+L) activities in the muscles were measured in 5 control and 7 transgenic mice. In another series of experiments, mRNA levels of cathepsins (B and L) in the muscles were measured in 10 control and 10 transgenic mice, and immunohistochemical studies were also conducted.

Measurement of cathepsin activity. The stored muscles were washed twice with the homogenization solution (250mM sucrose, 2mM EGTA, 2mM EDTA, 20mM Tris-HCl, pH7.4), homogenized in 1ml of the homogenization solution containing 0.2% Triton-X 100, and lysed by sonication. The homogenate was centrifuged at 18,000 g for 15 min. The supernatant was dialyzed against the same amount of glycerol and stored at -40°C until analysis. Cathepsin B activity was assayed with 10μM Z-Arg-Arg-MCA at pH6.0 following the method of Barrett *et al* (15). To obtain a blank sample, the extract was preincubated with 1μM of E-64 (L-3-carboxy-trans-2,3-epoxypropionyl-leucylamide-(4-quanidino) butane), Protein Research Foundation, Osaka, Japan, at 37°C for 5 min to inhibit cathepsin B activity. Cathepsin B+L activity was assayed by the same method of cathepsin B with Z-Phe-Arg-MCA. Since this synthetic substrate is not only hydrolyzed by cathepsin L but also cathepsin B, its hydrolysis is expressed as the activity of cathepsin B+L. Protein concentration of the extracts was assayed using the method of Bradford (16).

RNA isolation and Northern blot analysis. Total RNA from gastrocnemius muscles was extracted using guanidium thiocyanate as described (17) and quantified by absorbance at 260 nm. RNA samples (10μg) were subjected to electrophoresis in 1.0% agarose gels and blotted overnight to Hybond-N^+ nylon membrane with 20 x standard saline citrate (SSC;0.15M NaCl

and 15mM sodium citrate, pH7.0). The RNA in gels and filters was visualized with ethidium bromide and photographed by UV transillumination to ensure the integrity of RNA and the loading of equivalent amounts of RNA. The membrane was prehybridized for 1h and hybridized overnight with the Church buffer. The cDNAs encoding rat cathepsins B and L (18) were used and radiolabelled probes were prepared by the random primer method. A glyceraldehyde-3-phosphate dehydrogenase probe, gifted from Keiji Tanaka, Institute for Enzyme Research, University of Tokushima, was used as a control of loading. Filters were exposed to Kodak X-Omat AR films with an intensifying screen for 1-3 days at -80°C, and quantitation of the membranes was made by densitometry using a MCID system (Imaging Research Inc., Ontario, Canada).

Immunohistochemical study. Frozen transverse sections in 4μM thick of the gastrocnemius muscles were sliced and mounted on poly-L-lysine-coated glass slides. One slide was stained with hematoxylin and eosin. In the other slides, after quenching endogenous peroxidase activity for 20 min in 0.1% (W/V) sodium azide containing 0.3% (V/V) hydrogen peroxidase, nonspecific binding was blocked by the treatment with 3% (V/V) normal goat serum for 20 min. The sections were incubated with rabbit antibodies against purified rat cathepsin B (19) (2μg/ml) and L (20) (10μg/ml) at 4°C in a moist chamber for overnight . After washing in PBS, biotinylated mouse anti-rabbit immunoglobulin (Histofine SAB-PO Kit, Nichirei Co., Tokyo, Japan) was applied and the slides were incubated for 20 min at room temparature. After thorough washing in PBS, peroxidase-conjugated streptavidin was applied and the slides were again incubated for 20 min. Immunostaining was visualized with 0.02% (W/V) 3, 3'diaminobenzidine and 0.03% (V/V) hydrogen peroxidase in 0.05M Tris-HCl, pH7.6, for 3 min. A negative control employing normal goat serum was included in each staining procedure.

Statistical analysis. Values were expressed as mean ± SD. Statistical significance of the results was examinated by Student's *t* -test.

RESULTS

The weights of gastrocnemius muscles decreased significantly in the transgenic mice in comparison with those in the control mice, while the body weights were heavier in the transgenic mice. In relation to the decrease of muscle weight, both cathepsins B and B+L activities of the muscles markedly increased in the transgenic mice (Table 1). The decrease of gastrocnemius muscle weights in the transgenic mice was confirmed in another series of experiments (Table 2). Expression of cathepsins (B and L) genes was augmented in the transgenic mice (Fig.1 and Table 2). The possibility that infiltrated cells into the muscle of the

Table 1. Body and gastrocnemius muscle weights and enzymatic activities of cahtepsins in gastrocnemius muscles of control and IL-6 transgenic mice

Experimental group	Body weight (g)	Muscle weight (mg)	Cathepsin activity (nmolAMC/h/mg protein)	
			B	B+L
Control mice (n=5)	19.62±0.91	131.08±10.19	4.33±0.65	91.2±10.4
Transgenic mice (n=7)	21.26±0.58*	94.71±6.44*	57.77±15.6*	386.6±80.0*

Data are expressed as mean ± SD. Statistical significance of the difference was calculated by Student's *t*-test; *p<0.01 (control vs. transgenic mice).

Table 2. Body and gastrocnemius muscle weights and Northern blots of gastrocnemius muscle extracts from control and IL-6 transgenic mice

Experimental group	Body weight (g)	Muscle weight (mg)	Relative intensity of mRNA expression	
			Cathepsin B	*Cathepsin L*
Control mice (n=10)	25.11±1.20	170.0±11.05	100%	100%
Transgenic mice (n=10)	27.42±1.91*	135.5±18.47*	250%	160%

Data are expressed as mean ± SD. Statistical comparisons between control and transgenic mice were made for body and muscle weights by Student's *t*-test; *$p<0.01$ (control vs. transgenic mice). Values were means of 3 separate densitometry experiments.

transgenic mice may be responsible for increase of cathepsins activities was examined by the pathohistrological study. Small nodules consisted of mononuclear cells and plasmacytes scattered around the muscle in the transgenic mice, but infiltration of the cells into the muscle was not identified. Immunohistochemical study revealed strong positive reactions for cathepsins B and L in myofibers as fine granules and in subsarcolemmal perinuclear portions in the transgenic mice. No positive reactions were seen in the control mice (Fig.2). Staining positive cells were scarcely found in the perimuscular nodules.

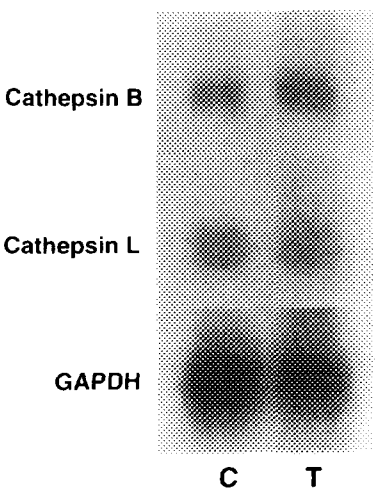

Fig.1. Expression of cathepsins B and L mRNA in the gastrocnemius muscles of the transgenic mice. Ten μg of total RNA extracted from the gastrocnemius muscles from the control(C) and the transgenic(T) mice was examined by Nothern blot analysis. The probes for rat cathepsins B and L were used. The integrity and the equivalent loading of ribosomal RNA were confirmed by visualization with ethidium bromide. Abundance of mRNA was assessed by correcting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA abundance.

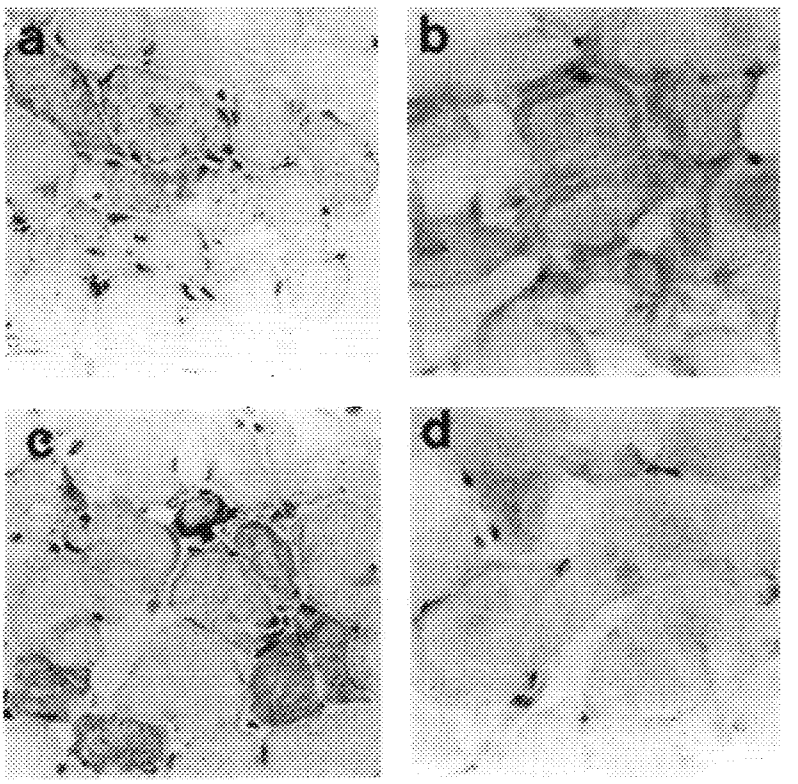

Fig.2. Immunohistochemistry of cathepsins B and L in the gastrocnemius muscles. Positive stainings for cathepsins B and L are seen in myofibers and in the perinuclear portion in the IL-6 transgenic mice(a,c), but no positive reaction for cathepsin B is observed in the control mice(b,d), respectively. Infiltration of macrophages or plasmacytes into the muscle is not identified. (Original magnification, x400)

DISCUSSION

The IL-6 transgenic mice of C57BL/6 origin show a massive plasmacytosis in lymph node, spleen and thymus, and an infiltration of plasmacytes in lung, liver and kidney (21). Serum concentration of human IL-6 in the transgenic mice was around 200 to 400 pg/ml at 4-8 weeks from the birth, began to increase at 10 week-old, and was beyond 600pg/ml after 12 weeks (unpublished data). The body weights were significantly larger due to hepato-splenomegaly than the control mice, whereas the muscles underwent severe atrophy. The results of the present study provided a clue that over-production of IL-6 *in vivo* induces muscle atrophy. In the IL-6 transgenic mice, the activities of cathepsins B and L of the gastrocnemius muscles were strikingly increased in association with progression of atrophy. Expression of cathepsins mRNA also increased, but their abundance was limited in comparison with the increase of enzymatic activities, indicating that a posttranscriptional process may be mainly responsible for the activation of cathepsins. The pattern of immunohistochemical staining of cathepsins in the muscle of the IL-6 transgenic mice was similar with that in the early stage of plasmocid-induced myopathy (22), where

immunostaining of cathepsins L and B was seen in myofibers surrounding the injection site, although infiltration of macrophages was not yet apparent. In Duchenne muscular dystrophy, positive staining for cathepsins was observed in intramyofibral portion of atrophic fibers in the presence of marked infiltration of macrophages (22). Activation of the intramyofibral lysosomal system was also observed in distal myopathy with rimmed vacuoles without macrophage infiltration (22). IL-6 may be involved in the pathogenesis of these degenerative muscle diseases, and it may play an important role in autodigestion by intramyofibral lysosomal proteinases for progression of muscle atrophy.

TNF has been considered as a mediator for protein catabolism in muscle. A single dose of recombinant TNF to rats results in increased muscle proteolysis (10). TNF administration augments the amino acid release from peripheral tissues in cancer patients (23), and muscle protein degradation was accelerated by TNF (24). TNF mediates chages in muscle protein turnover (25) and increases ubiquitin gene expression in a rat cancer cachexia model (26). Although many *in vivo* studies implicate TNF enhances muscle protein degradation, its direct action has been questionable. TNF can induce fever in rats without activating protein breakdown in muscle and TNF addition *in vitro* does not enhance proteolysis in soleus muscles (27). It was also demonstrated that activation of protein breakdown and prostaglandin E2 production in rat skeletal muscle in fever is signaled by a macrophage product distinct from IL-1 or TNF (28). Moldawer *et al* (29) concluded that TNF-α or IL-1 does not cause protein degradation. Muscle proteolytic activity is therefore attributed to other humoral factors derived from activated macrophages. Considering that the production of IL-6 is enhanced by TNF-α and IL-1 (30) and together with the present results, IL-6 may be a candidate for a muscle proteolysis inducing factor. We are now planning to reduce the changes of muscles in the transgenic mice by the treatment of anti-mouse IL-6 receptor antibody and also are planning to examine another important proteolytic system, namely the activities of proteasomes and ubiquitin gene expression.

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