our laboratory were in strict accordance with the guidelines for animal and recombinant DNA experiments put forth by Kumamoto University and The University of Tokyo.

RT-PCR

Total RNA was isolated from the skeletal muscle using ISOGEN (Nippon gene). 5 μg of each sample was reverse-transcribed using superscript II (Gibco-BRL). To detect the expression of transgene, we designed a forward primer from the mBD-6 first exon (5'-ACCATGAAGATCCATTACCTG-3') and the reverse primer from the rabbit β-globin (5'-ATTTGTGAGCCAGGGCATTG-3'), and we performed real-time PCR reaction using Fluorescent Quantitative Detection System Version 3.02 (LineGene).

Antiserum preparation

The putative mature peptide composed of the carboxyl-terminal 40 amino acids of mBD-6 was chemically synthesized at Peptide institute (Minoh, Japan), as previously described (20). The anti-mBD-6 rabbit serum was prepared at Peptide institute (Minoh, Japan) using this synthetic mBD-6 peptide, conjugated to keyhole limpet hemocyanin and injected into rabbits.

Isolation of mBD-6 peptide

We followed the procedure described by Valore *et. al.* (30). Frozen skeletal muscle was homogenized in ISOGEN (Nippon gene) and the protein was extracted according to the manufacturer's protocol. Protein pellets were incubated overnight in 5% acetic acid at 4 ℃ and the dissolved proteins were neutralized with 10% NH_3. These protein solutions were separated on Tris-Glycine SDS-PAGE, transferred onto polyvinylidene difluoride membrane. The membrane was probed with anti-mBD-6 rabbit serum followed by a peroxidase-conjugated anti-rabbit IgG antibody (ICN), visualized using the enhanced chemiluminescence plus system (Amersham Pharmacia Biotech).

Evaluation of the muscle strength

We evaluated muscle strength by measuring the time during which mice could hang down from a stainless lattice. The procedure was repeated twice for each mouse and the better record was indicated.

Evans Blue Dye staining and measurement of serum creatine kinase activity

We performed the intraperitoneal injection of 10 mg/ml Evans Blue Dye solution of phosphate-buffered saline (PBS) (0.1 ml/10g body) on 2-month-old Tg1 mice and

wild-type mice. The skeletal muscle samples were removed 16 hours after the injection. The frozen 10 μm sections were fixed in acetone for 1 minute and observed under a fluorescence microscope. Serum creatine kinase (CK) activity was measured at SRL (Tachikawa, Japan)

Tissue preparation and immunohistochemistry

Skeletal muscle samples were removed and frozen in isopentane chilled in liquid nitrogen. The frozen 10-μm sections were processed for hematoxylin and eosin (H&E) staining or immunohistochemical analysis. For the immunohistochemistry, the sections were fixed in acetone for 5 minutes and probed with the following primary antibodies: anti-dystrophin for C-teminal (C-20) (Santa Cruz), anti-α-dystroglycan for internal core part (E-21) (Santa Cruz), anti-laminin-alpha 2 chain (LSL), anti-neural-cell-adhesion-molecule (Chemicon), anti-IκBα (Santa Cruz), anti-cleaved-caspase-3 (Trevigen), and anti-calpain-3 antibody (gift from H. Sorimachi).

Statistics

Comparison of the body weights or serum CK activity was made with Student's *t* test. Values of $p<0.05$ were considered significant.

Results

Generation of the transgenic mice overexpressing mBD-6

To achieve broad and high expression of mBD-6, a cDNA fragment encoding mBD-6 was connected to the 3'-end of the chicken β-actin promoter flanked with a cytomegalovirus immediate early enhancer (Figure 1A). While 6 founder transgenic mice were identified using PCR and Southern blot analysis, the transgene was transmitted to germline in two lines. On Southern blot analysis, the one line, Tg(CAG-mBD6)1 was estimated to harbor several copies of the transgenes because of multiple extra-bands including 2.0-kB DNA fragment conrresponding to the full-length transgene size, while the wild-type genomic DNA showed two copies of 1.4-kb intrinsic mBD-6 gene and more faint 3.2-kb band maybe composed of mBD-6 pseudogene. The other line, Tg(CAG-mBD6)2 was estimated to harbor a single copy because of single 3.4-kb extra-band (Figure 1B). Tg(CAG-mBD6)1 and Tg(CAG-mBD6)2 mice were referred to as Tg1 mice and Tg2 mice here, in sequence.

In both lines, the transgene expression was detected in the skeletal muscle by RT-PCR using the primers from mBD-6 cDNA and rabbit β-globin cDNA (Figure 1A, B). Tg1 mice showed 3.4 times higher–level expression of the transgene than Tg2 mice..

Western blot analysis could detect mBD-6 peptide in the extracts from Tg1 and Tg2 skeletal muscle, and the expression level was also low in Tg2 mice (Figure 1C). In wild-type mice, the mBD-6 signal was not detected in the Western blot analysis under the same experimental condition. The immunohistochemical analysis of mBD-6 peptide showed the mBD-6 peptide stored in the cytoplasmic garanules in some skeletal muscle myofibers of Tg1 mice.

Tg(CAG-mBD6) mice develop muscle degeneration

At birth, the Tg1 mice or Tg2 mice were indistinguishable from their wild-type littermates. By 6 weeks of age, poor growth of Tg1 mice became evident, and at 8 weeks of age, both the male and female body weights of Tg1 mice were significantly lower than those of their wild-type littermates ($p<0.01$) (Figure 2A). The mean body weight of Tg1 mice was about 80% that of their wild-type littermates. They showed contracted stiff limbs and progressive kyphosis by 6 months of age (Figure 2B). Most of Tg1 mice died before 11 months of age. The short life-span of Tg1 mice was more evident after backcrossed to C57BL6/J strain. Many of Tg1 mice died before 8 months and no mice lived for more than 1 year on C57BL6/J strain. We could not clarify the specific cause of Tg1 mice death except severe loss of body weight. Although another transgenic line Tg2

did not reveal so prominent poor growth or kyphosis, 6-month-old Tg2 male mice also showed significantly lower body weights than the wild-type littermates. Tg2 mice lived for more than 12 months.

About the decreased body weight, we evaluated the food intake of 5-week-old Tg1 mice. The food intake of Tg1 mice was 3.31±0.12 g/day which was significantly lower than the food intake of the wild-type littermates which was 4.00±0.29 g/day (p<0.05). Because the progressive kyphosis is a prominent feature caused by the functional impairment of skeletal muscle, we evaluated muscle strength of Tg1 mice by hanging them from a stainless lattice. 2-month-old Tg1 mice dropped significantly shorter time from the lattice, indicating muscle weakness in Tg1 mice. While most of the wild-type littermates hang down for more than 120 seconds, significantly more Tg1 mice dropped before 60 seconds (p<0.01) (Figure 3).

H&E staining of skeletal muscle revealed degenerative myofibers, infiltration of mononuclear cells, and some centronucleated myofibers in 4-week-old Tg1 mice. After 8 weeks of age, centronucleated myofibers became much more predominant, and faint-stained necrotic myofibers, basophilic regenerating myofibers and fiber splitting were frequently encountered. The myofibers also showed prominent difference in size

(Figure 3). These features were observed in all the skeletal muscles examined, including the gastrocnemius muscles, the anterior tibial muscles, soleus muscles, diaphragm, and muscles of the back. Meanwhile, no histological abnormalities were noted in 20-day-old Tg1 mice (Figure 4). Another transgenic line Tg2 revealed no histological abnormalities in the skeletal muscle till about 6 months of age. However, 1-year-old Tg2 mice showed a few faint-stained degenerative myofibers in various muscles. The number of centronucleated myofibers in 1-year-old Tg2 mice was also significantly increased in comparison with wild-type mice of the same age (2.5+/-0.5% vs.0 2+/-0.1 %).

We measured serum creatine kinase (CK) activity in 3-month-old Tg-1 mice and their wild-type littermates because the measurement of serum CK activity is used clinically to ascertain muscular damage. Tg1 mice showed significantly higher serum CK activity than the wild-type littermates (WT) (p<0.01) (Figure 4A). Evans Blue Dye labelling also detected more clearly the damaged myofibers with increased membrane permeability in Tg1 mice and aged 1-year-old Tg2 mice (Figure 4B).

Evaluation of causative proteins of muscular dystrophy

These skeletal muscle phenotypes caused by mBD-6 overexpression are reminiscent of muscular dystrophies, characterized by the progressive myofiber degeneration. In

mBD-6 transgenic mice, the organization of dystrophin-glycoprotein-complex was not different from their wild-type littermates on the immunohistochemistry of dystrophin, α-dystroglycan and laminin (Figure 6). Likewise, the expression of calpain 3 showed no abnormality in mBD-6 transgenic mice (Figure 6).

Immunohistochemical abnormalities of the transgenic myofibers

To investigate the molecular mechanisms of myofiber degeneration, we evaluated the conserved immunohistochemical features of young Tg1 mice and aged Tg2 mice. In 1-month-old Tg1 mice, many myofibers showed high-level expression of neural cell adhesion molecule (NCAM). Also in Tg2 mice, many NCAM-positive myofibers were detected at 11-12 months of age, while their wild-type littermates of the same age showed only a few NCAM-positive myofibers (Figure. 7A). Although denervated myofibers up-regulate the NCAM expression, the number and morphology of motor neurons were not different between the Tg1 mice and their wild-type littermates (Figure. 8). We also examined the distribution of IκBα in Tg1 and Tg2 mice. We detected the accumulation of IκBα in many myofibers of 1-month-old Tg1 mice and 12-month-old Tg2 mice (Figure. 7B), as reported in LGMD2A patients (31). We also evaluated the apoptotic features of the IκBα-positive myofibers in transgenic mice. In the staining of

serial sections, some of the IκBα-positive myofibers showed the signal of cleaved caspase 3, the active form of caspase 3, indicating the activation of apoptotic pathway (Figure 9).

Other pathological changes and food intake in the mBD-6 over-expressing mouse

The mBD-6 overexpression decreases body weights, and increases centronuclated myofibers and degenerative myofibers in mice. Kyphosis of vertebra, short of height, and slow movement were observed in the mouse. The food intake of Tg1 mice was significantly lower than the wild-type littermates. The short life-span of Tg1 mice is evident especially after backcrossed to C57Bl6/J strain. Many Tg1 mice died before 8 months and no mice of more than 10 Tg1 mice lived for more than 1 year on C57Bl6/J strain. There is no evidence of cancer in the dead Tg1 mice

Discussion

Our data first demonstrated the pathogenic effects of dysregulated β-defensin expression *in vivo*. Western blot analysis of the muscle extracts ascertained the overproduction of mature mBD-6 peptide in the Tg1 and Tg2 mice. The dysregulated

β-defensin expression induced poor growth, short life-span and functional muscle impairment. Pathologically, the skeletal muscle of Tg1 mice showed progressive degeneration and regeneration of myofibers, consistent with the histology of muscular dystrophy. The elevated serum CK activity and positive evans blue dye labelling in Tg1 mice indicate the disruption of myofiber plasma membrane, also consistent with muscular dystrophy.

Despite the recent identification of causative genes, the clinical course of muscular dystrophy is miserable without established therapy. Although the gene therapy could be curative replacing the ultimate defect, many obstacles exist to technical progress. So, current important therapy targets are the factors modulating the state of muscle degeneration. Clinically, glucocorticoids are utilized to delay the progression of Duchene muscular dystrophy (32-34), and actually, the invasion of lymphoid and myeloid cells is an early-stage feature of Duchene muscular dystrophy (28). β-defensin would be the first reported component of inflammation which induced alone the typical phenotype of muscular dystrophy. Because mBD-6 and human defensin-3 showed the intrinsic expression in skeletal muscle (7, 20) and the invaded myeloid cells and lymphocytes would secrete abundant α-defensin in human muscular dystrophy (35), our findings suggest the significance of defensin family in the pathogenesis of muscular dystrophy.

In aged Tg2 mice, mBD-6 overexpression induced NCAM-positive myofibers and IκBα accumulation with mild histological abnormality. Interestingly, aging alone induced the slight increase in NCAM-positive myofibers and IκBα–positive myofibers. The augmentation of these aging phenomena in Tg2 mice suggested that the defensin-mediated muscle degeneration would not be limited to distinct muscular dystrophy, but would be associated with much more common late-onset muscular wasting degeneration like as a sarcopenia, cachexia or senescence acceleration.

Previous transgenic mice overexpressing antimicrobial proteins (e.g. lysozyme) or peptides (e.g. human defensin-5) have indicated the beneficial roles of these substances *in vivo* situation (36, 37). Contrasting with these animal models, Tg1 mice succumbed to muscular degeneration and short life-span, showing completely novel aspect of antimicrobial peptides. The various mediators like reactive oxygen metabolites, complement cascades and some proteases are involved in the known immune-mediated tissue injury in inflammatory conditions. Our investigation established defensin family as a novel effector of immune-mediated tissue injury.

The molecular mechanism of defensin-mediated tissue injury remained to be clarified. Generally, pore-formation and permeabilization of target membranes are common mechanisms of defensin effects (3, 14, 15). Because dystrophin, dystroglycan

and laminin distributions were normal, mBD-6 would induce muscle degeneration independent of dystrophin-glycoprotein complex.

Although the up-regulation of NCAM had been shown in denervated myofibers, the histology of motor neurons of Tg1 mice showed no abnormality that could be causative of massive myofiber degeneration. So, these NCAM-positive myofibers would indicate the regenerative process and/or association of motor endplate degeneration in Tg1 and Tg2 mice, as indicated in some types of muscular dystrophy (38-40). At the same time, the accumulation of IκBα in Tg1 and Tg2 mice with apoptotic features share the features of LGMD2A patients and their animal models (31, 41). In LGMD2A patients, the defect of IκBα turnover inhibited the activity of NF-κB, associated with myofiber apoptosis. Because the perturbation of NF-κB/IκB pathway could cause subsequent perturbation of many survival genes, it could be common pathways in various mechanisms of myofiber degeneration.

The next question is what down-stream events of beta-defensin-6 pathway might be involved in the mechanisms of myofiber degeneration. Actually, both CCR6 and TLR4 are expressed in skeletal muscle. The other investigators have reported that the TLR4 expression is identified in skeletal muscles (42). We also confirmed the expression of TLR4 using RT-PCR. However, we do not think the down-stream events of

beta-defensin-6 pathway are important in the pathogenesis of muscle degeneration phenotype. Until now, there are no data about the pathological relationship between muscle degeneration and TLRs. While various functions of defensin family on mammalian cells had been reported including the cytotoxicity, most of down-stream events had not been clarified and the contribution of CCR6 or TLR4 on the pathogenesis of muscle degeneration would be limited, if existed. Certainly, CCR6 receptors and TLR4 are expressed in skeletal muscle and NF-κB pathway is directly associated with TLR4. However, it is reasonable to speculate that continuous stimulation of these receptors could not primarily contribute to induce degeneration of myofibers in vivo. If such phenomenon was happened, the muscles could be totally abolished and severe muscle damage occurred in earlier life.

In the study, body weights of Tg1 mice were significantly lower than wild type mice. The food intake of Tg1 mice was significantly lower than the wild-type littermates. The decreased food intake could explain some portions of decreased body weights. However, the prominent myofiber degeneration could be never induced by the decreased food intake and it is more likely that the decreased food intake resulted from the decreased muscular mass and strength. Further, Tg1 mice had a short life span. The most of Tg1 mice died before 11 months of age. This short life-span of Tg1 mice was

evident especially after backcrossed to C57Bl6/J strain. Many Tg1 mice died before 8 months and no mice of more than 10 Tg1 mice lived for more than 1 year on C57Bl6/J strain. We could not clarify the specific cause of Tg1 mice death except severe loss of body weight. There is no evidence of cancer in the dead Tg1 mice, then we speculate the impaired immune function in the mice may cause systemic inflammation and decreased food intake in relatively younger age, resulting in the malnutrition related short-life span.

In conclusion, our study demonstrated that defensin family could contribute to the pathogenic immune response in animal models, especially in the pathogenesis of myofiber degeneration.

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Disclosures

The authors have no conflicting financial interests.

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