Skeletal muscle growth defect in human growth hormone transgenic rat is accompanied by phenotypic changes in progenitor cells

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Received: 23 September 2009 / Accepted: 4 January 2010 / Published online: 23 January 2010
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Abstract Growth hormone (GH) is known to have a pivotal role in the maintenance of skeletal muscle mass. Sarcopenia, the loss of skeletal muscle mass, is a common phenomenon in aging, and it is widely accepted that sarcopenia is largely attributed to age-related decline in GH secretion. In the present study, we tested if human growth hormone transgenic rats (GH-TG rats) whose plasma GH levels are maintained relatively low could be an appropriate model for sarcopenia. Analyses of GH-TG rats revealed that they exhibit skeletal muscle growth defect as well as atrophy of myofibers. The number of myofibers in tibialis anterior muscle was comparable to that of WT rats, while the proportion of type I slow myofibers in tibialis anterior muscle was increased in GH-TG rats after 5 months. Neither increased expression of ubiquitin ligases, MuRF1 and MAFbx, nor indication

of apoptotic cell death was observed. Notably, myogenic differentiation potential of skeletal muscle progenitor cells in GH-TG rats was lower than WT rats, and this was accompanied by increased adipogenic potential. These results indicate that GH-TG rats could be a useful model to elucidate the mechanism of sarcopenia induced by reduced GH action and raised the possibility that decreased GH action may cause an alteration of differentiation potential of skeletal muscle progenitor cells.

Keywords Differentiation · Growth hormone · Sarcopenia · Skeletal muscle

Introduction

Skeletal muscle mass is maintained by a coordinated balance between anabolism and catabolism. The former includes not only protein synthesis but also an addition of new nuclei to myofibers by fusion of myogenic progenitor cells, and the latter includes a loss of nuclei due to apoptosis as well as protein degradation. Under the condition where catabolism exceeds anabolism, myofibers show atrophic response. Age-related decline in skeletal muscle mass is called sarcopenia. The common phenomena seen in sarcopenia include decreased myofiber diameter that is more severe in fast-type myofiber than in slow-type myofiber and increased percentage of slow-type myofiber (Lexell and Downham 1992).

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Among the factors involved in the maintenance of skeletal muscle mass, growth hormone (GH) is known to have a pivotal role (Florini et al. 1996). GH is a peptide hormone derived from anterior pituitary. In the human, GH levels are maximal during peripubertal period and begin to decline shortly thereafter (Bartke et al. 2000). This decline continues throughout life, and plasma GH levels in elderly individuals are much lower than in young adults (Bartke et al. 2000). The decline in GH levels with aging is not unique to the human and is well documented to occur in other species, such as dogs, rats, and mice (Bartke et al. 2000). GH secretion is pulsatile in nature, and in old animals, it has been shown that both the amplitude and duration of each pulse are reduced (Sonntag et al. 1980). Multiple actions of GH include anabolic actions in skeletal muscle and other tissues, involvement in maintenance of bone mineral density (Yamanouchi et al. 2004), and mobilization of fat from the adipocytes (Ikeda et al. 1994). Therefore, age-related declines in lean body mass and bone mineral density, as well as increase in adiposity, can be causally related to reduced GH action due to both decreased level and lack of pulsatile secretion of GH. However, the exact mechanism by which the decline of GH action leads to the loss of skeletal muscle mass is still under investigation.

We had previously produced human GH (hGH) transgenic (GH-TG) rats that exhibit unique serum GH phenotype (Ikeda et al. 1994; 1998). Serum hGH levels are relatively low in GH-TG rats, and the pulsatile secretion of their endogenous rat GH (rGH) was almost totally suppressed (Ikeda et al. 1994, 1998). The remarkable characteristic of the GH-TG rats is that, although both rGH and hGH secretions are relatively low, GH action is still maintained at some level and not completely absent like in the *little (lit/lit)* and the GH-receptor-deficient mouse (Rosen 2007; Zhou et al. 1997) that show dwarfish phenotype. In fact, they develop severe obesity, representing the reduced lipolytic action of GH, but their body length is normal (Ikeda et al. 1998). Analyses of GH-TG rats revealed that they exhibit growth defects in their hind leg skeletons as well as the early onset of osteoporosis, suggesting that the GH-TG rats could be a useful model to elucidate the age-related dysfunctions due to the reduced GH secretion (Yamanouchi et al. 2004).

Several types of progenitor cells are present in skeletal muscle (Chargé and Rudnicki 2004). Satellite

cells are one of the most widely studied cells which had been originally identified as myogenic progenitor cells (Chargé and Rudnicki 2004; Mauro 1961). Recent evidence suggests that, under some particular conditions, satellite cells acquire adipogenic potential (Asakura et al. 2001; Hosoyama et al. 2009; Shefer et al. 2004; Shefer and Yablonka-Reuveni 2007; Yamanouchi et al. 2009), sometimes with concomitant decrease of their myogenic potential. In addition to satellite cells, the presence of highly adipogenic/osteogenic progenitor cells that are identified as CD31(–)CD45(–) side population (SP) cells in mouse skeletal muscle was reported (Uezumi et al. 2006). The CD31(–)CD45(–) SP cells also give rise to myotubes when cocultured with myoblasts or transplanted into regenerating muscle. Interestingly, CD31(–)CD45(–) SP cells isolated from regenerating muscle, not from normal muscle, spontaneously differentiated to adipocytes without any stimulation, and their osteogenic response to BMP-2 was higher than those from normal muscle. Collectively, these studies indicate plasticity of differentiation phenotypes of skeletal muscle progenitor cells. Recent findings indicate that the age-related decrease of myogenic potential of skeletal muscle progenitor cells is accompanied by their acquirement of adipogenic (Taylor-Jones et al. 2002) or fibroblastic potential (Brack et al. 2007) and suggest that these phenotypic changes could relate to sarcopenia.

In the present study, we initially examined if skeletal muscle phenotype of GH-TG rats is similar to that seen in sarcopenia by morphological analyses and found that GH-TG rats show typical phenotype of sarcopenia. Then, we further investigated if the altered growth of skeletal muscle in GH-TG rats is accompanied by apoptotic cell death, increased protein degradation, and phenotypic changes in progenitor cells.

Materials and methods

Animals

Generation of the GH-TG rats and their serum concentrations of endogenous rGH and hGH were described previously (Ikeda et al. 1994). They were bred in our laboratory by artificial insemination, and the resulting transgenic and nontransgenic male

littermates were housed in a room at 23°C with a lighting condition of 12-h light and 12-h darkness (lights on at 0700 hours). Food and water were provided ad libitum. All animal experiments performed in this study were according to the Guide for the Care and Use of Laboratory Animals, the University of Tokyo, and approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Tissue preparation for histological analyses

At indicated ages (see “Results”), the rats were weighed and killed with inhalation of carbon dioxide gas. Tibialis anterior (TA), extensor digitorum longus (EDL), and soleus muscles were excised and snap-frozen in liquid-nitrogen-cooled isopentane. The weights of epididymal fat pads were also recorded at the time of sampling.

Histological analyses

Frozen sections (6–8 μm) of TA muscles were prepared transversely by cryostat. The sections were stained with hematoxylin and eosin, labeled immunohistochemically for measurement of total number and diameter of myofibers, or used for detection of apoptotic nuclei. For immunohistochemistry of slow-type myosin heavy chain (MHC), the sections were fixed with acetone for 15 min at -80°C and air-dried. The fixed sections were then blocked with 5% normal goat serum (NGS) in phosphate-buffered saline (PBS) containing 0.6% hydrogen peroxide for 30 min at room temperature (RT). After washing with PBS, the sections were incubated with antislow MHC (Sigma, MO, USA; 1:500 with 1% bovine serum albumin in PBS) for 1 h at RT. The sections were washed with PBS and incubated with horseradish-peroxidase-conjugated secondary antibody (simple stain rat MAX-PO, Nichirei, Tokyo, Japan) for 1 h at RT. The labeled antibodies were visualized by incubating the sections in diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.03%) in PBS and observed with light microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera (MP5Mc/OL, Olympus, Tokyo, Japan).

For the calculation of the total number of myofibers, nine randomly chosen different areas using a $\times 10$ objective in each section ($n=4$ per

group) were photographed. Then, the number of myofibers in the randomly chosen areas was counted. From these counts, the theoretical total number of myofibers in each section was calculated based on the entire cross-sectional area of the section. The number of myofibers stained with antislow MHC was counted in nine randomly chosen different areas using a $\times 10$ objective in each section. Then, the percentage of slow MHC-positive (type I) myofibers were calculated by dividing by the total number of myofibers. Myofibers that were not stained with antislow MHC were considered as fast-type (type II) myofibers. The diameter (minor axis) of each myofiber was measured in nine randomly chosen different areas using a $\times 10$ objective in each section, and the histograms were shown in results. The diameter of at least 200 myofibers was measured for each rat.

For the detection of apoptotic nuclei, ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) was used. As a positive control, frozen sections of rat small intestine were included. Frozen sections were fixed with 4% paraformaldehyde (PFA)/PBS and processed for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining as described in manufacturer’s protocol.

Measurement of specific forces

At the ages of 3 and 6 months, EDL muscle was removed from the rats and attached to an isometric force transducer (TB-611 T, Nihon Koden). The muscle was maintained in Tyrode solution (93 mM NaCl, 20 mM CH_3COONa , 20 mM NaHCO_3 , 1 mM Na_2HPO_4 , 1 mM MgSO_4 , 5 mM KCl, 10 mM Glucose, 2 mM CaCl_2 , 5 mM HEPES) at 37°C and gassed with 95% O_2 /5% CO_2 . The muscle was stimulated via platinum electrodes, and twitch response was elicited by a single 0.5-ms supramaximal stimulus (170 V, 50 Hz). The maximal isometric force (gF) was recorded by polygraph system (LEG-1000, Nihon Koden).

Northern blot analyses

At the ages of 3 and 6 months, TA muscle was obtained from the rats and snap-frozen in liquid nitrogen. Total RNA was isolated by Trizol reagent (Invitrogen, CA, USA) according to the manufac-

turer's protocol. Ten micrograms of total RNA was separated electrophoretically on 1% agarose/3-(*N*-morpholino)propanesulfonic acid-formaldehyde gel and transferred onto the membrane (Biodyne B, PALL, NY, USA). After prehybridization in prehybridization solution (6× saline sodium citrate (SSC), 5× Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 µg/ml sonicated salmon sperm DNA) at 68°C for 90 min, the membrane was hybridized with digoxigenin (DIG)-labeled cRNA probe diluted in hybridization solution (6× SSC, 0.5% SDS, 100 µg/ml sonicated salmon sperm DNA) at 68°C for 18 h or longer. After hybridization, the membrane was washed with 2× SSC and 0.1% SDS twice, followed with 0.1× SSC and 0.1% SDS. Signals were detected by chemiluminescent method using CSPD according to the manufacturer's protocol.

For the preparation of DIG-labeled cRNA probes, RT-PCR was performed to generate cDNA fragments of rat MAFbx and MuRF1 using reverse-transcribed cDNA from rat skeletal muscle as a template. The primer set used were as follows: MAFbx-forward, 5'-ATGCCGTTTCCTTGGTCAGGACTGGC-3', and MAFbx-reverse, 5'-ATGTTGCCACACGACACGGAC-3'; MuRF1-forward, 5'-TCCGGACGGAATGCTATGGAGAACC-3', and MuRF1-reverse, 5'-CTGGTGGCTGTTTTTCCTTGGTCACTC-3'. Obtained cDNA fragments were subcloned into pGEM-T easy vector (Promega, WI, USA), and the resultant plasmids were used for DIG-labeled cRNA synthesis using T7/SP6 RNA polymerase (DIG RNA Labeling Kit, Boehringer Mannheim).

Western blot analysis

TA muscle was obtained from wild-type (WT) and transgenic (TG) rats at 9 months and snap-frozen in liquid nitrogen. Tissues were homogenized in PBS with protease inhibitor cocktail and centrifuged at 105,000×g for 90 min. The resultant supernatant was collected as cytosolic fraction, and its protein concentration was determined. Fifteen micrograms of protein was separated on SDS-PAGE and transferred to PVDF membrane. Cleaved caspase 3 (activated form of caspase 3) was detected by rabbit monoclonal antibody (clone 5A1E, Cell Signaling, Danvers, MA, USA). Protein samples were also prepared from ventral prostate of intact and castrated adult male rats and used as negative and positive controls. To ensure

the equal loading of samples, gel was stained with Coomassie brilliant blue.

Cell culture

Procedures for isolating skeletal muscle progenitor cells were previously described (Yada et al. 2006; Yamanouchi et al. 2006, 2007). Briefly, rats were killed by inhalation of carbon dioxide gas. TA or gastrocnemius muscles were excised, trimmed of fat and connective tissue, hand-minced with scissors, and digested for 1 h at 37°C with 1.25 mg/ml pronase (protease; Sigma, MO, USA). Cells were separated from myofiber fragments and tissue debris by differential centrifugation and plated on poly-L-lysine and fibronectin-coated dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (HS), 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin (10% HS/DMEM). Cells were plated at a density based on the starting tissue weight from which the cells were isolated (0.25 g of muscle tissues per square centimeter). Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. For MyoD staining, cells from TA muscle were cultured for 2 days. For MHC staining, cells from TA muscle were cultured for 3 days and subsequently cultured in 2% HS in DMEM containing antibiotics for 5 days. For the experiments where the adipogenic potential of the cells was examined, cells from gastrocnemius muscles were cultured in adipogenic differentiation condition. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (10% FBS/DMEM) supplemented with insulin (1 µg/ml), dexamethazone (0.1 µg/ml), isobutylmethylxanthine (27.8 µg/ml), and troglitazone (10 mM; kindly donated by Sankyo Co. Ltd.) for initial 2 days and subsequently cultured in 10% FBS/DMEM supplemented with insulin and troglitazone for 4 days.

Immunocytochemistry

Cells were subjected to indirect immunofluorescence for detection of MyoD and MHC. They were fixed in 4% PFA in PBS at RT for 15 min. After fixation, the cells were washed three times with PBS. Cells were then blocked with 5% NGS/PBS containing 0.1% Triton-X 100 (Sigma, MO, USA) for 20 min at RT. After blocking, the cells were washed three times with

PBS and then reacted with primary antibodies (described below) for 2 h at RT. After three washes with PBS, the cells were incubated with AlexaFluor-conjugated secondary antibodies (Invitrogen, CA, USA) for 1 h at RT. Nuclei were counterstained with Hoechst 33258. Observations were made using a fluorescence microscope (BX50, Olympus) equipped with a digital camera (DP70, Olympus, Tokyo, Japan). For quantitative analysis of myogenic differentiation, the number of nuclei in MyoD- or MHC-positive cells and total nuclei were counted in six randomly chosen microscopic fields using a $\times 10$ objective to calculate the percentage of positive cells. This was done for triplicate wells per rat ($n=3$). The following primary antibodies were used: anti-MyoD (mouse, clone 5.8A, Novocastra, Newcastle upon Tyne, UK, 1:100 dilution) and anti-MHC (mouse, clone MF20, hybridoma supernatant, DSHB, 1:10 dilution). All primary antibodies and AlexaFluor-conjugated secondary antibodies were diluted with 5% NGS/PBS.

Oil Red-O staining

Oil Red-O staining was used for detection of accumulated cellular lipid droplets. Cells were fixed in 4% PFA in PBS for 10 min at RT, rinsed three times with PBS, stained with Oil Red-O working solution (2:3 mixture of 0.5% (w/v) Oil Red-O (Sigma) in 2-propanol and distilled water) for 7 min, and rinsed with PBS. Observations were made using a microscope (BX50, Olympus, Tokyo, Japan) equipped with a digital camera (DP70, Olympus, Tokyo, Japan). For quantification of adipogenesis, six different fields that were randomly chosen under the microscope using a $\times 10$ objective were photographed, and the areas occupied with Oil-Red-positive cells were quantified using ImageJ software (ver. 1.36b, NIH). This was done for triplicate wells per rat ($n=3$). Values representing incidence of adipogenesis were expressed as the mean pixel measurements for the triplicate culture wells.

Statistical analyses

For comparisons of the myofiber diameters, Mann–Whitney test was used to evaluate statistical differences between the two groups. For the other graphed data that are expressed as means $\pm$ standard error of the mean

(SEM), Student's *t* test was used. *P* values less than 0.05 were considered as statistically significant.

Results

The body weights of the WT and TG rats were increased with age, and the weight was significantly higher in GH-TG rats than in WT rats after 3 months (102%, 132%, 164%, and 163% of WT at 3, 5, 9, and 11 months, respectively; Fig. 1a). We had already shown that the increased body weight of GH-TG rats is due to an excess visceral fat (Ikeda et al. 1998; Yamanouchi et al. 2004), representing the reduced lipolytic action of GH. In accordance with this, epididymal fat weights were significantly higher in GH-TG rats than in WT rats from 3 months (256%, 265%, 340%, and 378% of WT at 3, 5, 9, and 11 months, respectively; Fig. 1b).

In order to know if the skeletal muscle growth is affected in GH-TG rats, age-related changes in the weights of TA and EDL (both are fast-type myofiber dominant) and soleus (slow-type myofiber dominant) muscles were examined. In TA muscles, the weights were significantly lower in GH-TG rats than WT rats at all ages examined (95%, 64%, 60%, 69%, and 53% of WT at 1, 3, 5, 9, and 11 months, respectively; Fig. 1c). This was also the case for EDL muscles except that there was no significant difference at 1 month (65%, 60%, 77%, and 61% of WT at 3, 5, 9, and 11 months, respectively; Fig. 1d). Similar tendency was seen in soleus muscles, but the statistical significances were noted only at 3 and 11 months (80% and 79% of WT at 3 and 11 months, respectively; Fig. 1e). These results indicated the occurrence of growth retardation of skeletal muscles in GH-TG rats.

To examine if the decrease of skeletal muscle weight in GH-TG rats is due to a decreased myofiber diameter or number, morphometric analyses on TA muscles were performed. As shown in Fig. 2, the distributions of myofiber diameter in WT and GH-TG rats were within a similar range at 1 month. After 3 months, the overall distributions were shifted apparently to the right, indicating the occurrence of myofiber growth in both WT and GH-TG rats. However, the degree of the growth was significantly lower in GH-TG rats than in WT rats ($P<0.05$). In addition, an appearance of myofibers whose diameter

Fig. 1 Body weights (a), epididymal fat weights (b), and weights of tibialis anterior (TA; c), extensor digitorum longus (EDL; d), and soleus (e) muscles of wild-type (WT) and GH-TG (TG) rats. Data are means $\pm$ SEM ($n=4$ at each age). * $P<0.05$ vs WT rats

is smaller than 24 μm (7 to 24 μm) was noted only in GH-TG rats from 5 months. There was no statistical difference in the number of myofibers between WT and GH-TG rats at 3, 5, and 11 months (Fig. 3a). These results indicated that both the growth retardation and atrophic response, but not the reduced number of myofiber, are involved in the decreased skeletal muscle weight seen in GH-TG rats.

It is known that, in sarcopenia, atrophic response is more severe in fast-type myofiber. In addition, it has been shown that the proportion of slow-type myofiber is increased in the muscle of fast-type myofiber dominant (Lexell and Downham 1992). To examine if such a phenotype is seen in GH-TG rats, immunohistochemical analysis of slow-type MHC was performed on TA muscles, and the percentage of slow-type MHC-positive myofibers was calculated. As shown in Fig. 3b, c, age-related increase in the percentage of slow-type MHC-

positive myofibers was evident in GH-TG rats from 5 months (234% and 375% of WT at 5 and 11 months, respectively) whereas no change was seen in WT rats. The distributions of the diameter of both fast- (slow-type MHC-negative) and slow-type MHC-positive myofibers in TA muscles of WT and GH-TG rats were shown in Fig. 3d. Both distributions of the diameters of GH-TG rats were shifted to the left compared to those of WT rats, indicating the decreased diameters of both fast- and slow-type myofibers in GH-TG rats. It should be noted that differences in the diameters were more evident in fast-type myofibers (median, 49 μm in WT

Fig. 2 Distribution of myofiber diameters of TA muscle of wild-type (WT) and GH-TG (TG) rats ($n=4$ at each age). The left panel depicts the relative distribution of myofibers with certain diameters. The right panel depicts relative cumulative plots based on the data shown in the left panel. Black and red lines in right panel are WT and TG rats, respectively

Fig. 3 Total number of myofibers (a) and percentage of type I (slow; b) myofibers in TA muscle of wild-type (*WT*) and GH-TG (*TG*) rats ($n=4$ at each age). Representative photos of entire tissue section of TA muscle (11 months) immunohistochemically stained with antislowl myosin were shown in c. Panel d depicts the relative distribution of type I and type II myofibers at 11 months old. Arrows in panel d indicate the median diameter of myofibers

vs. 38 μm in GH-TG) than in slow-type myofibers (median, 38 μm in WT vs. 31 μm in GH-TG). These results suggested that similar phenomena seen in sarcopenia is indeed occurring in GH-TG rats.

In order to know whether the decreased skeletal muscle mass and myofiber diameters in GH-TG rats affect the muscle strength, specific forces of EDL muscles were measured (Fig. 4). The weights of EDL muscle used for the measurement of specific forces were lower in GH-TG rats than in WT rats at both 3 and 6 months (74% and 54% of WT at 3 and 6 months, respectively; Fig. 4a), confirming the result shown in Fig. 1d. However, reduced specific force in GH-TG rats compared to WT rats was noted only at 6 months (73% of WT; Fig. 4b), suggesting that the retarded skeletal muscle growth does not necessarily match with the onset of decreased muscular strength.

In catabolic states where proteolysis is increased, two genes specific to muscle atrophy, MuRF1 and MAFbx, have been shown to be upregulated (Bodine et al. 2001; Gomes et al. 2001). Both MuRF1 and MAFbx genes encode ubiquitin ligases, which bind to and mediate ubiquitination of myofibrillar proteins for subsequent degradation during muscle atrophy. In order to know if the expression of these two genes is upregulated in the skeletal muscle of GH-TG rats, Northern blot analyses were performed. As shown in Fig. 5, no increase in the expression of MuRF1 and MAFbx was seen in the TA muscles of GH-TG rats compared to WT rats, suggesting that reduced skeletal muscle growth seen in GH-TG rats may not be due to the excessive proteolysis mediated by these ubiquitin ligases.

Occurrence of apoptotic cell death has also been suggested in age-related skeletal muscle atrophy

though it is still controversial (Dirks and Leeuwenburgh 2002; Dupont-Versteegden 2005). In order to know if the atrophic response seen in skeletal muscle of GH-TG rats involves the loss of nuclei due to apoptotic cell death, TUNEL assay was performed on TA muscles obtained from 4-month-old rats. As shown in Fig. 5d, no TUNEL-positive nuclei was detected in TA muscles of both WT and GH-TG rats, suggesting that involvement of apoptotic cell death is unlikely in the atrophic response in skeletal muscle of GH-TG rats. This was also confirmed by the lack of cleaved caspase 3 expression in TA muscles of both WT and GH-TG rats (Fig. 5e).

Skeletal muscle progenitor cells, including satellite cells, are indispensable for skeletal muscle growth, and age-related decrease in their myogenic potential associated with sarcopenia has been reported (Brack et al. 2007; Taylor-Jones et al. 2002). To test if the myogenic potential of skeletal muscle progenitor cells are altered in GH-TG rats, cells were isolated from TA muscles, and their MyoD expression was examined by immunocytochemistry. As shown in Fig. 6a, percentage of MyoD-positive cells was significantly lower in cells from GH-TG rats than those from WT rats at all ages tested (11% to 16% reduction vs WT), suggesting the reduced myogenic potential of skeletal muscle progenitor cells in GH-TG rats. This was also followed by the experiment where cells were cultured in reduced serum concentration to induce myotube formation. Staining of MHC revealed that myotube formation was reduced in cells from GH-TG rats compared to WT rats (27% reduction vs WT; Fig. 6b).

Age-related decline in the myogenic potential of skeletal muscle progenitor cells often correlates with

Fig. 4 Weights (a) and muscle strength (b) of EDL muscle of wild-type (WT) and GH-TG (TG) rats. Data are means $\pm$ SEM ($n=4$ at each age). * $P<0.05$ vs WT rats

Fig. 5 Northern blot analysis of MuRF1 and MAFbx (a–c). Expression levels of MuRF1 (b) and MAFbx (c) were normalized by the intensities of ethidium-bromide-stained rRNA levels. Data in b and c are mean $\pm$ SEM ($n=3$ at each age). Representative TUNEL staining on tissue sections of TA muscles and small intestines (4 months; d). Note that there was no indication of apoptotic cell death in TA muscle of wild-type (WT) and GH-TG (TG) rats, while numerous number of TUNEL-positive nuclei are present in small intestine. Western blot analysis of cleaved caspase 3 (e). No expression of cleaved caspase 3 was detected in TA muscle of WT ($n=3$) and TG ($n=3$) rats. Samples obtained from ventral prostate of intact and castrated adult male rats were used as negative and positive controls. Lower panel shows Coomassie-brilliant-blue-stained gel

Fig. 6 Myogenic potential of progenitor cells from TA muscle of wild-type (*WT*) and GH-TG (*TG*) rats. Panel **a** depicts representative immunocytochemical staining of MyoD (nuclei are counterstained with Hoechst 33258) and percentage of MyoD-positive cells. White and black arrowheads indicate MyoD-positive and MyoD-negative nuclei, respectively. Graphed data are means $\pm$ SEM ($n=3$ at each age). Panel **b** depicts representative immunocytochemical staining of myosin heavy chain (*MHC*; nuclei are counterstained with Hoechst 33258) and percentage of MHC-positive nuclei. Graphed data are means $\pm$ SEM ($n=3$ at 5 months). Bar=100 μ m. * $P<0.05$ vs WT rats

their increased adipogenic potential (Taylor-Jones et al. 2002). In order to examine if this is the case in GH-TG rats, cells were isolated from gastrocnemius muscles and cultured in adipogenic differentiation medium. The degree of the appearance of adipocytes as revealed by Oil Red-O staining was higher in the cells from GH-TG rats than from WT rats (200% of WT; Fig. 7), indicating the increased adipogenic potential of skeletal muscle progenitor cells in GH-TG rats.

Discussion

The present study demonstrated that GH-TG rats whose endogenous GH secretion is maintained at a low level

show skeletal muscle growth defect, which is accompanied by phenotypic changes in progenitor cells.

It should be noted that, in the present study, the decreased skeletal muscle mass was not correlated with the decline in muscular strength in GH-TG rats at 3 months old. It is widely considered that, in sarcopenia, the decreased skeletal mass results in a decline in muscular strength. However, the term “sarcopenia” simply means just the age-related decrease in skeletal muscle mass, and this does not necessarily involve the decline in the muscular strength. In fact, in humans, it has been reported that the loss of skeletal muscle mass begins around the age of 40, but the muscular strength does not change from 30s to 50s (Janssen et al. 2000), indicating that the

Fig. 7 Adipogenic potential of progenitor cells from gastrocnemius muscle of wild-type (*WT*) and GH-TG (*TG*) rats. *Left panel* depicts representative Oil Red-O staining of adipocytes (nuclei are counterstained with hematoxylin). *Bar*=200 μ m. *Right panel* depicts Oil-Red-O-stained area representing the number of adipocytes. Graphed data are means $\pm$ SEM ($n=3$ at 3 months). * $P<0.05$ vs *WT* rats

loss of skeletal muscle mass precedes the onset of decline in muscular strength. Another example showing the discrepancy between the skeletal mass and its strength is that, in myostatin knockout mice, whose EDL muscles are twice the weights of the wild-type mice, their muscular strength is unaffected (Amthor et al. 2007). Therefore, qualitative changes rather than quantitative changes of skeletal muscle would have more impact on the muscular strength. Alternatively, there may be some compensatory mechanism to prevent the decline in muscular strength at the beginning of growth retardation of muscle.

A number of studies had shown the age-related phenotypic changes of skeletal muscle progenitor cells (Brack et al. 2007; Shefer and Yablonka-Reuveni 2007; Taylor-Jones et al. 2002; Yada et al. 2006). Among these studies, Brack et al. (2007) showed the decreased myogenicity of skeletal muscle progenitor cells in aged mice, suggesting that this might be a cause of age-related diminished capacity for regeneration and hypertrophy. In the present study, decreased myogenicity as well as increased adipogenicity of skeletal muscle progenitor cells was observed in GH-TG rats. Taylor-Jones et al. (2002) reported an age-related increase in the adipogenicity in satellite cells (myoblasts) accompanied by decreased Wnt10B expression. Brack et al. (2007) showed that the age-related decrease in the myogenicity of satellite cells is due to the extrinsic influences such as increased Wnt signaling. They demonstrated that the myogenic satellite cells obtained from young animals acquire nonmyogenic fate when incubated in serum of aged animals, and

this effect is suppressed by the addition of Wnt inhibitor, sFRP3. Although a discrepancy does exist among these reports in terms of the involvement of Wnt signaling in age-related phenotypic changes of skeletal muscle progenitor cells, it should be pointed out that changes in the cellular niches associated with aging would have great impact on progenitor cell function (for a review, see Gopinath and Rando 2008). In this regard, we have previously demonstrated that myofibers may act as cellular niches, and alteration of their condition induces the phenotypic changes of satellite cells through the alteration of RhoA signaling pathway (Hosoyama et al. 2009). Thus, the phenotypic changes of skeletal muscle progenitor cells in GH-TG rats might be a consequence of atrophic response of the myofibers, while it is also possible that the decreased myogenicity of the cells might prevent the hypertrophy of myofibers, leading to skeletal muscle growth defect.

Skeletal muscle mass is maintained by the coordinated balance between anabolic and catabolic actions. The former includes de novo protein synthesis, increased number of myofiber nuclei due to fusion of progenitor cells, while the latter includes protein degradation, loss of nuclei due to apoptotic cell death. Atrophy of myofiber occurs if catabolic actions exceed anabolic ones. Our data obtained in the present study did not show any evidences that indicate the excess catabolic actions. Neither upregulation of MuRF1 and MAFbx, skeletal-muscle-specific ubiquitin ligases, nor incidence of apoptosis has been observed in GH-TG rats. However, our data also indicated atrophy of myofiber is indeed occurring

in GH-TG rats. Cohen et al. (2009) reported that thin, but not thick, filaments such as actin, tropomyosin, and α -actinin are degraded by MuRF1-independent mechanism during muscle atrophy. Therefore, atrophy of myofiber in GH-TG rats may be due to the degradation of these thin filaments. In this regard, Du et al. (2004) demonstrated that activation of caspase 3 is an initial step that triggers actin degradation, while Goll et al. (2008) suggested that the involvement of caspases is unlikely. In the present study, the lack of activated caspase 3 expression in skeletal muscle of GH-TG rats indicates that caspase-3-mediated proteolysis may not be involved in atrophy of myofiber.

It is widely accepted that growth-promoting effect of GH is mediated by circulating or locally produced insulin-like growth factor (IGF)-I (Le Roith et al. 2001). Circulating IGF-I is mostly derived from liver and acts in an endocrine manner, while locally produced IGF-I acts in an autocrine/paracrine fashion. GH receptor (GH-R)-deficient mice show severe growth retardation accompanied by short body length as well as reduction of organ weights, and their serum IGF-I concentrations are extremely low (approximately 10% of WT; Zhou et al. 1997). It is currently unknown if the growth retardation of skeletal muscle in GH-TG rats is due to the reduced direct GH action or indirect GH action mediated by circulating IGF-I. However, it should be emphasized that body length of GH-TG rats is not affected, and although serum IGF-I concentrations in GH-TG rats are lower than in WT rats, they are maintained at 70–80% levels of WT rats (data not shown). This indicates that some of the GH actions are still preserved in GH-TG rats and may represent the unique characteristics of GH-TG rats that lack the pulsatile GH secretion but maintain low-level GH secretions. Since not only the level but also the pulsatile secretion is important for GH to induce some of its broad biological functions (Gebert et al. 1999), it is possible that the lack of pulsatile secretion of GH greatly affects skeletal muscle (shown in the present study) and fat (Ikeda et al. 1998) but has a minor impact on IGF-I secretion from liver as far as some levels are maintained. Collectively, we suspect that skeletal muscle phenotype seen in GH-TG rats may not be due to reduced circulating IGF-I action. However, since it has been reported that locally produced IGF-I (mechano growth factor) is under the regulation of GH (Iida et al. 2004), it is still

possible that locally produced IGF-I may be altered in GH-TG rats, and thus altered local IGF-I may contribute to their the skeletal muscle phenotype.

The presence of GH-R in skeletal muscle tissue and progenitor cells has been demonstrated in several species (Florini et al. 1996; Halevy et al. 1996; Jennische and Andersson 1991; Sotiropoulos et al. 2006). In addition, numerous studies have been conducted to examine the direct GH action on muscle progenitor cells. In mammals, no effect of GH on proliferation and differentiation of myogenic cells has been shown (Allen et al. 1983, 1986; Ewton and Florini 1980), whereas in chicken satellite cells, GH has been shown to be inhibitory on their differentiation (Halevy et al. 1996). Sotiropoulos et al. (2006) demonstrated that GH promotes myotube hypertrophy independently of an IGF-I-mediated action. Thus, skeletal muscle growth defect in GH-TG rats may be partially due to the reduced GH action to promote myotube hypertrophy.

Androgens have an anabolic effect on skeletal muscle (Deschenes 2004). In addition to GH, androgen levels are also decreased in aged men (Gooren 1998), and this is also implicated as one of the causes of sarcopenia (Deschenes 2004). In our preliminary experiments, serum testosterone levels in GH-TG rats are comparable to those of WT rats for up to 6 months but decreased (approximately 50% reduction vs WT) at 11 months (data not shown). This implies that the skeletal muscle phenotypes seen for up to 6 months in the GH-TG rats are not due to the decreased androgen levels. Therefore, though GH-TG rats may not represent an entire phenotypes seen in sarcopenia, they may be useful for analyses of sarcopenic changes induced specifically by decreased GH.

While the reduced GH action has been shown to be involved in part in the occurrence of sarcopenia, there have been a few rat model that represents the reduced but not completely lost GH action. For example, in GH-deficient dwarf (dw/dw) rats, circulating GH level is reduced to 5% of WT rats that is accompanied by the reduction of circulating IGF-I level to 36% of WT rats (Stevenson et al. 2009), but the pulsatile GH secretion is still maintained (Legraverend et al. 1992). dw/dw rats exhibit atrophy of both type I and II myofibers as was seen in GH-TG rats but show no increase in the proportion of type I myofiber in fast-twitch muscle (EDL muscle). Unlike GH-TG rats, dw/dw rats do not show adiposity (Stevenson et al. 2009).

Transgenic growth-retarded (Tgr) rats are another example that represents GH deficiency (Flavell et al. 1996). In Tgr rats, circulating GH level is reduced 30% of WT rats, accompanied by the decreased circulating IGF-I level to 35% of WT rats (Stevenson et al. 2009) but the pulsatility of GH secretion is maintained (Wells et al. 1997). Currently, as far as we know, no information on the skeletal muscle phenotype, as we examined in GH-TG rats in the present study, is available on Tgr rats; however, it should be noted that, like the GH-TG model, Tgr rats also display elevated adiposity (Stevenson et al. 2009). Although there are several phenotypic differences among these GH-deficient rat models including our GH-TG rats, more detailed comparative analyses will be valuable to understand the mechanism of GH action on skeletal muscle.

Acknowledgements This work was supported by Grants-in-Aid for Scientific Research (B) from Japan Society for Promotion of Science, the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), and Mitsui Sumitomo Insurance Welfare Foundation. The technical expertise for the measurement of muscle strength by Dr. Katsumasa Goto of Toyohashi Sozo University is gratefully acknowledged. The authors also thank Dr. Kazuyuki Uchida of The University of Tokyo for the valuable discussion. The critical reading of this manuscript by Drs. Sakae and Akihiro Ikeda is greatly acknowledged. The mouse anti-myosin heavy chain monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the NICHD and maintained by The University of Iowa.

References

- Allen R, Masak K, McAllister P, Merkel R (1983) Effect of growth hormone, testosterone and serum concentration on actin synthesis in cultured satellite cells. *J Anim Sci* 56:833–837
- Allen R, Dodson M, Boxhorn L, Davis S, Hossner K (1986) Satellite cell proliferation in response to pituitary hormones. *J Anim Sci* 62:1596–1601
- Amthor H, Macharia R, Navarrete R, Schuelke M, Brown S, Otto A, Voit T, Muntoni F, Vrbóva G, Partridge T, Zammit P, Bunger L, Patel K (2007) Lack of myostatin results in excessive muscle growth but impaired force generation. *Proc Natl Acad Sci USA* 104:1835–1840
- Asakura A, Komaki M, Rudnicki M (2001) Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation* 68:245–253
- Bartke A, Brown-Borg H, Kinney B, Mattison J, Wright C, Hauck S, Coschigano K, Kopchick J (2000) Growth hormone and aging. *J Am Aging Assoc* 23:219–225
- Bodine S, Latres E, Baumhueter S, Lai V, Nunez L, Clarke B, Poueymirou W, Panaro F, Na E, Dharmarajan K, Pan Z, Valenzuela D, DeChiara T, Stitt T, Yancopoulos G, Glass D (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294:1704–1708
- Brack A, Conboy M, Roy S, Lee M, Kuo C, Keller C, Rando T (2007) Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* 317:807–810
- Chargé S, Rudnicki M (2004) Cellular and molecular regulation of muscle regeneration. *Physiol Rev* 84:209–238
- Cohen S, Brault J, Gygi S, Glass D, Valenzuela D, Gartner C, Latres E, Goldberg A (2009) During muscle atrophy, thick, but not thin, filament components are degraded by Murf1-dependent ubiquitylation. *J Cell Biol* 185:1083–1095
- Deschenes MR (2004) Effects of aging on muscle fibre type and size. *Sports Med* 34:809–824
- Dirks A, Leeuwenburgh C (2002) Apoptosis in skeletal muscle with aging. *Am J Physiol Regul Integr Comp Physiol* 282: R519–527
- Du J, Wang X, Miereles C, Bailey JL, Debigare R, Zheng B, Price SR, Mitch WE (2004) Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J Clin Invest* 113:115–123
- Dupont-Versteegden E (2005) Apoptosis in muscle atrophy: relevance to sarcopenia. *Exp Gerontol* 40:473–481
- Ewton D, Florini J (1980) Relative effects of the somatomedins, multiplication-stimulating activity, and growth hormone on myoblasts and myotubes in culture. *Endocrinology* 106:577–583
- Flavell DM, Wells T, Wells SE, Carmignac DF, Thomas GB, Robinson IC (1996) Dominant dwarfism in transgenic rats by targeting human growth hormone (GH) expression to hypothalamic GH-releasing factor neurons. *EMBO J* 15:3871–3879
- Florini J, Ewton D, Coolican S (1996) Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev* 17:481–517
- Gebert CA, Park SH, Waxman DJ (1999) Termination of growth hormone pulse-induced STAT5b signaling. *Mol Endocrinol* 13:38–56
- Goll DE, Nesti G, Mares SW, Thompson VF (2008) Myofibrillar protein turnover: the proteasome and the calpains. *J Anim Sci* 86:E19–E35
- Gomes M, Lecker S, Jagoe R, Navon A, Goldberg A (2001) Atrogin-1, a muscle-specific f-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA* 98:14440–14445
- Gooren LJ (1998) Endocrine aspects of ageing in the male. *Mol Cell Endocrinol* 145:153–159
- Gopinath S, Rando T (2008) Stem cell review series: aging of the skeletal muscle stem cell niche. *Aging Cell* 7:590–598
- Halevy O, Hodik V, Mett A (1996) The effects of growth hormone on avian skeletal muscle satellite cell proliferation and differentiation. *Gen Comp Endocrinol* 101:43–52
- Hosoyama T, Ishiguro N, Yamanouchi K, Nishihara M (2009) Degenerative muscle fiber accelerates adipogenesis of intramuscular cells via rhoa signaling pathway. *Differentiation* 77:350–359
- Iida K, Itoh E, Iida K, Itoh E, Kim DS, del Rincon JP, Coschigano KT, Kopchick JJ, Thorner MO (2004) Muscle

- mechano growth factor is preferentially induced by growth hormone in growth hormone-deficient lit/lit mice. *J Physiol* 560:341–349
- Ikeda A, Matsuyama S, Nishihara M, Tojo H, Takahashi M (1994) Changes in endogenous growth hormone secretion and onset of puberty in transgenic rats expressing human growth hormone gene. *Endocr J* 41:523–529
- Ikeda A, Chang K, Matsumoto Y, Furuhashi Y, Nishihara M, Sasaki F, Takahashi M (1998) Obesity and insulin resistance in human growth hormone transgenic rats. *Endocrinology* 139:3057–3063
- Janssen I, Heymsfield S, Wang Z, Ross R (2000) Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. *J Appl Physiol* 89:81–88
- Jennische E, Andersson G (1991) Expression of GH receptor mRNA in regenerating skeletal muscle of normal and hypophysectomized rats. An in situ hybridization study. *Acta Endocrinol (Copenh)* 125:595–602
- Le Roith D, Bondy C, Yakar S, Liu J, Butler A (2001) The somatomedin hypothesis: 2001. *Endocr Rev* 22:53–74
- Legraverend C, Mode A, Wells T, Robinson I, Gustafsson JA (1992) Hepatic steroid hydroxylating enzymes are controlled by the sexually dimorphic pattern of growth hormone secretion in normal and dwarf rats. *FASEB J* 6:711–718
- Lexell J, Downham D (1992) What is the effect of ageing on type 2 muscle fibres? *J Neurol Sci* 107:250–251
- Mauro A (1961) Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 9:493–495
- Rosen C (2007) Mouse models for understanding the growth hormone insulin-like growth factor-I axis. *Horm Res* 68 (Suppl 5):2–4
- Shefer G, Yablonka-Reuveni Z (2007) Reflections on lineage potential of skeletal muscle satellite cells: do they sometimes go mad? *Crit Rev Eukaryot Gene Expr* 17:13–29
- Shefer G, Wlekinski-Lee M, Yablonka-Reuveni Z (2004) Skeletal muscle satellite cells can spontaneously enter an alternative mesenchymal pathway. *J Cell Sci* 117:5393–5404
- Sonntag WE, Steger RW, Forman LJ, Meites J (1980) Decreased pulsatile release of growth hormone in old male rats. *Endocrinology* 107:1875–1879
- Sotiropoulos A, Ohanna M, Kedzia C, Menon R, Kopchick J, Kelly P, Pende M (2006) Growth hormone promotes skeletal muscle cell fusion independent of insulin-like growth factor I up-regulation. *Proc Natl Acad Sci USA* 103:7315–7320
- Stevenson AE, Evans BA, Gevers EF, Elford C, McLeod RW, Perry MJ, El-Kasti MM, Coschigano KT, Kopchick JJ, Evans SL, Wells T (2009) Does adiposity status influence femoral cortical strength in rodent models of growth hormone deficiency? *Am J Physiol Endocrinol Metab* 296:E147–E156
- Taylor-Jones J, McGehee R, Rando T, Lecka-Czernik B, Lipschitz D, Peterson C (2002) Activation of an adipogenic program in adult myoblasts with age. *Mech Ageing Dev* 123:649–661
- Uezumi A, Ojima K, Fukada S, Ikemoto M, Masuda S, Miyagoe-Suzuki Y, Takeda S (2006) Functional heterogeneity of side population cells in skeletal muscle. *Biochem Biophys Res Commun* 341:864–873
- Wells T, Flavell DM, Wells SE, Carmignac DF, Robinson IC (1997) Effects of growth hormone secretagogues in the transgenic growth-retarded (Tgr) rat. *Endocrinology* 138:580–587
- Yada E, Yamanouchi K, Nishihara M (2006) Adipogenic potential of satellite cells from distinct skeletal muscle origins in the rat. *J Vet Med Sci* 68:479–486
- Yamanouchi K, Yada E, Hozumi H, Ueno C, Nishihara M (2004) Analyses of hind leg skeletons in human growth hormone transgenic rats. *Exp Gerontol* 39:1179–1188
- Yamanouchi K, Yada E, Ishiguro N, Hosoyama T, Nishihara M (2006) Increased adipogenicity of cells from regenerating skeletal muscle. *Exp Cell Res* 312:2701–2711
- Yamanouchi K, Yada E, Ishiguro N, Nishihara M (2007) 18alpha-glycyrrhetic acid induces phenotypic changes of skeletal muscle cells to enter adipogenesis. *Cell Physiol Biochem* 20:781–790
- Yamanouchi K, Hosoyama T, Murakami Y, Nakano S, Nishihara M (2009) Satellite cell differentiation in goat skeletal muscle single fiber culture. *J Reprod Dev* 55:252–255
- Zhou Y, Xu B, Maheshwari H, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigano K, Wagner T, Baumann G, Kopchick J (1997) A mammalian model for laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the laron mouse). *Proc Natl Acad Sci USA* 94:13215–13220