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HGF and IGF-1 is Present during the Developmental Process of Murine Masseter Muscle

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Abstract: Until now the developmental process of masticatory muscle (derived from the branchial arches) is not well documented. Thus, in order to clarify the developmental process of murine masseter muscle, Hepatocyte Growth Factor (HGF), Insulin-like Growth Factor-1 (IGF-1) and Myosin Heavy Chain (MyHC) were evaluated by histological examination, western blotting and RT-PCR analyses on embryonic days 14 (E14), 16 (E16) and 18 (E18). E14 showed that the mandibular bone development is initiated by the appearance of the ossification centre in the peripheral zone of Meckel cartilage. Whereas, E16 demonstrated presence of developing masseter muscle at the lower part of the mandible composed of immature bone. On the contrary, E18 showed larger masseter muscle and more mature mandibular than E16. Interestingly, both IGF and HGF proteins and genes increased at initial stage of masseter muscle development, and decreased with the time. A significant change in composition of MyHC was detected in E18 compared with E14 and E16. This result indicates that murine masseter muscle develops in accordance with the surrounding tissue, where IGF-1 and HGF could be an important modulator of stem cell-derived myoblast proliferation and myogenic differentiation, and MyHC-2a and 2d are strongly expressed by mature muscle fibres during the developmental process of the masseter muscle.

Key words: IGF-1, HGF, MyHC, Masseter muscle, Myogenesis

Introduction

Skeletal muscle is a type of striated muscle, which is used to create movement by applying force to bones and joints via contraction. The relationship between the combination and the function of the muscle fibers has been published^{1,2)}. Skeletal muscles contain two main types of fibers: Slow-twitch or type I and fast-twitch or type II according to their different speeds of contraction³⁾.

There are two types of isoforms of muscle contractile protein known as myosin heavy chain (MyHC): the fast-type isoforms MyHC-2b, MyHC-2d, and MyHC-2a; and the slow-type isoform, MyHC-1⁴⁾. The functional roles of these four isoforms have been clarified; therefore, the functions of muscles can be elucidated by determining the composition of these four isoforms^{1,5-8)}.

Many reports have been published on the developmental processes of muscles in the trunk and the legs^{9,10)}. Lyons et al. (1990) and Sartorius et al. (1998) studied the gene expressions of MyHC isoforms at the pre and post-natal stages in murine somites and in murine hindlimbs, respectively^{11,12)}. On the other hand, there are only a few reports on masticatory muscles that are derived from the branchial arches¹³⁾. It has been reported that the masseter muscle, which is one of the masticatory muscles, expresses different compositions of MyHC isoforms compared with the tibialis anterior during the transitional phase from suckling to mastication¹⁴⁾. Contractile protein isoforms expressed only at the pre and post-natal stages were detected in masseter muscle¹⁵⁾. However, the developmental process of masseter muscle is not well documented yet.

On the other hand, it is well known that various growth factor thought to play a role in different stages of muscle regeneration. Thus, hepatocyte growth factor (HGF) affects satellite cell activation¹⁶⁾ and proliferation^{17,18)}; Insulin Growth Factor-1 and 2

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(IGF-1 and IGF-2) stimulates both proliferation and differentiation¹⁸⁾. Until now, the expression of HGF and IGF during masseter muscle development has not been reported.

For that reason in the present study, expression HGF, IGF-1 and MyHC isoforms of the masseter muscle fibers of embryonic stage were analyzed.

Materials and Methods

Specimens

A total of 25 samples of ICR mice (Sankyo Laboratory, Tokyo, Japan) were used in this study. The mice were used on embryonic days 14, 16 and 18 of pregnancy. The mice were anesthetized with pentobarbital, and sacrificed according to the Guidelines for Animal Experiments of Tokyo Dental College. Foetuses taken from the uterus were decapitated, and 5 specimens were examined by hematoxylin-eosin (H-E) staining, 10 samples for western blotting and 10 samples of RT-PCR analysis.

Morphological examination

The masseter muscle together with the mandible were removed and fixed with 4% paraformaldehyde. Then, the specimens were dehydrated, embedded in paraffin using Tissue-Tech VIP M1500, and cut at 4 µm in thickness in serial sections with a microtome (IVS-410). After deparaffin treatment, the samples were rinsed with water and stained with a routine hematoxylin-eosin (H-E).

Western blotting analysis of Insulin-like Growth Factor-1 (IGF-1) and Hepatocyte Growth Factor (HGF)

Electrophoretic separation and analysis of the protein bands by western blotting was performed. Briefly, the masseter muscle at each observation period was removed. The specimens were weighed, frozen in liquid nitrogen, and stored at -80°C. Frozen muscles were minced with scissors in nine volumes of ice-cold homogenization buffer. The minced muscle samples were subsequently sonicated in a Branson Sonifier 250D (Branson Ultrasonic Corporation, Danbury, CT, USA). The products were used for the preparation of washed myofibers, which were then boiled in sample buffer for 2 min at a final protein concentration of 0.125 mg/ml. Total proteins were determined by the Bradford technique using the Bio-Rad Protein Assay (Nippon Bio-Rad Laboratory) and a Gene Quantpro spectrophotometer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Western blotting analyses were performed for the detection of IGF-1 and HGF. Equal amount of total protein for each group (0.5 µg) was separated on 12.5% SDS-polyacrylamide gel and was transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk and incubated with each of primary antibodies of anti-IGF-1 (1:1000, LVC MS-1508-PO), anti-HGF (1:500, ADV 905-165), and detected with horseradish peroxidase-conjugated secondary anti-rabbit/mouse IgG antibody using the VECTASTAIN ABC Rabbit IgG Kit.

Table 1 The primers used for the RT-PCR analysis

IGF-1	Forward 5' - TCGTCTTCACACCTCTTCTA -3' Reverse 5' - AAGCAACACTCATCCACAAT -3'
HGF	Forward 5' - CACCCCTTGGGAGTATTGTG -3' Reverse 5' - GGGACATCAGTCTCATTACAG -3'
MyHC-1	Forward 5' - GAGTCCCAGGTCAACAAGC -3' Reverse 5' - AACCCAGAGAGGCAAGTGAC -3'
MyHC-2a	Forward 5' - CGATGATCTTGCCAGTAATG -3' Reverse 5' - ATAAGTGAAGATACCAGCG -3'
MyHC-2d	Forward 5' - GACAACTGCAATCAAAGG -3' Reverse 5' - TTGGTCACTTTCCTGCACTT -3'
MyHC-2b	Forward 5' - ACAGACTAAAGTGAAAGCC -3' Reverse 5' - CTCTCAACAGAAAGATGGAT -3'
GAPDH	Forward 5' - TGAACGGGAAGCTCACTGG -3' Reverse 5' - TCCACCACCCTGTTGCTGTA -3'

GAPDH Ct = 19, IGF-1 Ct =24, HGF Ct =24, MyHC-1 Ct =21, MyHC-2a Ct =21, MyHC-2d Ct =22, MyHC-2b Ct =23.

Reverse transcription polymerase chain reaction analysis

From mice in each age group, the muscle was removed and snap-frozen in liquid nitrogen. mRNA was extracted using a QuickPrep micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), and cDNA was prepared using a Ready-To-Go kit (Amersham Pharmacia Biotech UK Ltd.). After determination of the optimal PCR conditions for all primers, experiments were performed using a LightCycler™ (Roche Diagnostics, Mannheim, Germany), which allows RNA quantification. The two primer sets designed from the sequences of IGF-1 HGF, MyHC-1, MyHC-2a, MyHC-2d and MyHC-2b genes were used. Experiments were performed according to the standard protocol for the LightCycler™. A ready-to-use LC FastStart DNA Master SYBR Green I (Roche) was used as a hot-start PCR reaction mixture for the LightCycler™. A series of cDNA dilutions (4.0 ng/µl) including 1/10⁵, 1/10⁶, 1/10⁷, 1/10⁸, and 1/10⁹ were prepared. PCR reaction for the diluted standards contained 10.2 µl of sterile water, 5 µl of diluted control cDNA product, 1.6 µl of 25 mM MgCl₂, and 2 µl of LC FastStart DNA Master SYBR Green I containing SYBR Green I (1/60,000 dilution). In addition, 0.6 µl of each of forward and reverse primers designed by the software (Biogene Ltd.) were added to reach a final reaction volume of 20 µl for each tube. The primers based on sequences of IGF-1 HGF, MyHC-1, MyHC-2a, MyHC-2d and MyHC-2b genes were designed from specific segments of the entire DNA sequence (Table 1).

Each PCR mixture (final reaction volume, 20 µl) contained 14.2 µl of sterile water, 1.6 µl of 25 mM MgCl₂, SYBR Green I (1/60,000 dilution), 2 µl of (DNA (5 pg/µl)- containing LC FastStart DNA Master SYBR Green I, 0.6 µl of forward primer (10 pmol/

Figure 1. Histological examination of surgical section of the mandible on embryonic days 14 (E14), 16 (E16) and 18 (E18). In E14 there was no formation neither of masseter muscle nor mandible. On the contrary, presence of developing masseter muscle at the lower part of the mandible was detected in E16 and E18. H-E staining, T: tongue, C: cartilage, MB: mandible, MM: masseter muscle.

µl), 0.6 µl of reverse primer (10 pmol/µl) and 1 µl of target DNA. The PCR mixtures (20 µl each) prepared for IGF-1 HGF, MyHC-1, MyHC-2a, MyHC-2d and MyHC-2b were added to the glass portion of capillary. Initial denaturation at 95°C for 10 min was followed by 45 cycles of a denaturation step at 95°C for 10 s, an annealing step at 60°C for 10s, and an extension step at 72°C for 80 s. Gene amplification was performed according to a melting program of 70°C for 15s, and fluorescence was continuously monitored at a rate of 0.1°C /s.

Amount of each gene final expression was obtained by dividing each of the gene expression level with housekeeping gene GAPDH level. The cycle threshold values (Ct values) of the real time data for each gene were found to be: GAPDH Ct = 19, IGF-1 Ct =24, HGF Ct =24, MyHC-1 Ct =21, MyHC-2a Ct =21, MyHC-2d Ct =22 and MyHC-2b Ct =23. The primers for GAPDH had the following sequences shown in the table 1.

Statistical analysis

Student's t-test was used for statistical analysis in this study, and the *p* value of < 0.05 was designated as significant.

Results

Histological examination

In order to evaluate the histopathological findings, the samples

Figure 2. Western blot analysis of IGF-1 and HGF in masseter muscle on embryonic days 14 (E14), 16 (E16) and 18 (E18). Note strong IGF-1 and HGF protein expression in E14, which were gradually decreased in E16 and E18.

Figure 3. Expression of IGF-1 mRNA in masseter muscle on embryonic days 14 (E14), 16 (E16) and 18 (E18). A maximum IGF-1 mRNA expression was detected in E14, with a marked decrease in E16 and E18. Interestingly, E14 demonstrated a significant increase in IGF-1 expression compared to E16 and E18 (n= 10, *p*<0.05).

Figure 4. Expression of HGF mRNA in masseter muscle on embryonic days 14 (E14), 16 (E16) and 18 (E18). A maximum HGF mRNA expression was detected in E14, with a marked decrease in E16 and E18. Interestingly, E14 demonstrated a significant increase in HGF expression compared to E16 and E18 (n= 10, *p*<0.05).

were stained with H-E (Fig.1). At early stage, E14 did not show any evidence of neither masseter muscle nor mandible. The

Figure 5. Expression of four MyHC isoforms in masseter muscle at various embryonic stages. MyHC-1 did not show any significant difference in E14, E16 and E18. However, MyHC-2a and MyHC-2d demonstrated a gradually increase from E14 to E18. A maximum expression was detected in E18, with significant increase in MyHC-2a and MyHC-2d expression compared to E14 and E16 ($n = 10$, $p < 0.05$). While, MyHC-2b showed a maximum expression in E16, with statistically difference compared to E14 and E18 ($n = 10$, $p < 0.05$).

mandibular bone development was initiated by the appearance of the ossification centre in the peripheral zone of Meckel cartilage during this initial stage. Whereas, E16 demonstrated presence of developing masseter muscle at the lower part of the mandible, which was composed of immature bone. On the contrary, mandibular bone observed in E18 is actively calcified and more mature than E16.

Expression of IGF-1 and HGF proteins

IGF-1 and HGF proteins were identified in the masseter muscle at various embryonic stages (Fig.2). Results indicated that IGF-1 and HGF were detected in all period of time. The bands immunoreactive to these proteins were strongly expressed at 14 days. However, both proteins expression were weakly expressed at 16 and 18 days.

Quantitative analysis of IGF-1, HGF and MyHC genes

To determine whether IGF-1, HGF and MyHC mRNA are expressed in the masseter muscle at 14, 16 and 18 days during embryonic stage, total RNA was isolated from the masseter muscle and evaluated by RT-PCR analysis. Figure 3 and 4 summarize IGF-1 and HGF mRNA quantification of masseter muscle on embryonic days 14, 16 and 18. Both genes gradually decreased from 14 to 18 days. Figure 5 summarize expression of four MyHC

isoforms in masseter muscle at various embryonic stages. MyHC-1 did not show any significant difference in E14, E16 and E18 groups. However, MyHC-2a and MyHC-2d demonstrated a gradually increase from E14 to E18. A maximum expression was detected in E18, with a significant increase in MyHC-2a and MyHC-2d expression compared to E14 and E16 ($n = 10$, $p < 0.05$). While, MyHC-2b showed a maximum expression in E16, with statistically difference compared to E14 and E18 ($n = 10$, $p < 0.05$).

Discussion

Until now, the developmental process of masticatory muscle (derived from the branchial arches) is not well documented. Thus, in order to clarify the developmental process of murine masseter muscle, HGF, IGF-1 and MyGF were examined by western blotting and RT-PCR analyses.

It is well known that skeletal muscle cell differentiation is a process whereby mesodermal stem cell-derived myoblasts cease proliferation and acquire the biochemical and morphologic phenotype of myocytes and fused myotubes¹⁹. The decision of myoblasts to proliferate or differentiate is strongly linked to the cellular environment, influenced by cell-extracellular matrix interactions²⁰, direct cell-cell interactions²¹ and by soluble growth factors²². Although growth factors, in general, inhibit myogenic differentiation²², IGFs are distinctive in that they are the only

known mitogens that, when free of other serum components, stimulate both the proliferation and differentiation of skeletal muscle cells^{18,22}. HGF is expressed by activating satellite cells not only in regenerating muscle^{16,23} but also in normal adult skeletal muscle¹⁶. At initial stages after injury, HGF serves to activate quiescent satellite cells¹⁷. By blocking the differentiation of these activated satellite cells until there are sufficient cell numbers, HGF could serve to increase the pool of proliferating myoblasts to some optimal cell density, whereupon fusion could commence¹⁷. Such a model would predict that the levels of HGF should decrease within regenerating muscles. A similar result showed the current study during muscle development, both IGF-1 and HGF proteins and genes increase at initial stage, and decrease with the time (Fig.2, 3, and 4). The present experiment strongly suggests that IGF-1 and HGF could be considered as an important modulator of stem cell-derived myoblast proliferation and myogenic differentiation.

Four isoforms of myosin, which belongs to contractile proteins, have been reported: MyHC-2b, MyHC-2d, MyHC-2a and MyHC-1. MyHC-2b, MyHC-2d and MyHC-2a are fast-type isoforms, and MyHC-1 is a slow-type isoform^{12,24}.

The adult mouse masseter muscle consists only of fast muscle fibers^{25,26}. Many studies reported time-dependent changes in muscle fiber properties in the fetal masseter muscle⁵ and the postnatal tongue⁷. Gojo et al. (2002) attempted to elucidate detailed masseter muscle functional changes by examining the relative MyHC isoform composition (MyHC-2a and MyHC-2b), and found that the muscle characteristics changed markedly after weaning, and that these weaning-associated characteristic changes were not observed in extremity muscles¹⁴. They speculated that, coincident with the weaning period, MyHC-2a protein, which was considered to contract quickly and strongly, appeared abundantly, and that the transition to mastication promoted the development of the masseter muscle function. Furthermore, Shida et al. (2005) showed that the superficial and deep layers of the masseter muscle, with fibers running in different directions, differed with regard to the muscle fiber characteristics, suggesting that the superficial layer of the masseter muscle is more closely involved in masticatory function²⁷. On the other hand, Usami et al. (2003) reported that a significant change in composition of MyHC was identified between embryonic days 14 and 16, a stage when murine facial development is conspicuous suggesting that the development of murine masseter muscle is closely associated with facial development⁵. A similar result was observed in this study with a significant change in composition of MyHC detected in E18 compared with E14 and E16 groups (Fig.5). The histological analysis showed that the mandibular bone development is initiated by the appearance of the ossification centre in the peripheral zone of Meckel cartilage in E14. Whereas, E16 demonstrated presence of developing masseter muscle at the lower part of the mandible, which is composed of immature bone.

On the contrary, mandibular bone observed in E18 is actively calcified and more mature than E16.

Conclusion

In summary, these results indicate that murine masseter muscle develops in accordance with the surrounding tissue, where, IGF and HGF could be an important modulator of stem cell-derived myoblast proliferation and myogenic differentiation, and MyHC-2a and 2d are strongly expressed by mature muscle fibres during the developmental process of the masseter muscle. Further complementary research will be useful for future clinical application of muscle regeneration and transplantation.

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