Annals of Anatomy 196 (2014) 410–415

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.de/aanat

Research article

Tenomodulin regulated the compartments of embryonic and early postnatal mouse masseter muscle

Iwao Sato[a,*], Yoko Miwa[a], Setsuhiro Hara[b], Yutaka Fukuyama[a], Masataka Sunohara[a]

[a] Department of Anatomy, School of Life Dentistry at Tokyo, Tokyo, Japan
[b] TMD Clinic, The Nippon Dental University Hospital, The Nippon Dental University, Tokyo, Japan

ARTICLE INFO

Article history:
Received 10 April 2014
Received in revised form 9 June 2014
Accepted 6 July 2014

Keywords:
Tenomodulin
VEGF
CD31
Masseter muscle
Development

ABSTRACT

The masseter muscle (MM) is a complex tendinous laminar structure during development; however, the stage of the laminar structure formation is unknown. Tenomodulin (TeM) is a useful marker of tendons and has an anti-angiogenic cysteine-rich C-terminal domain. Therefore, we analyzed mRNA of TeM and angiogenesis markers (CD31 and vascular endothelial growth factor (VEGF)) and performed in situ hybridization for the TeM genes in MM from on embryonic day 12.5 (E12.5) to postnatal day 5 (P5). The TeM expression is at first detectable in the middle region of the mesenchymal connective tissue in the MM at E 12.5. The expression domains of the TeM during development typically include the middle region of the MM, particularly surrounding the vascular regions. The level of TeM mRNA in the MM increased from E12.5 to E17.5 and decreased after birth. In contrast, the levels of CD31 and VEGF mRNAs were almost constant from E12.5 to E18.5 and then low from birth onward. Therefore, the development of the laminar tendinous structure in the middle region between superficial and deeper regions of the MM first occurs during the process of tendon formation at embryonic day 12.5. In our study of MM development, the laminar structure regulating TeM also prevents vascular invasion during the formation of compartment of the MM. The tendon may relate to the components of muscle mass of MM.

1. Introduction

The masseter muscle (MM) is composed of a laminated muscle mass with superficial, intermediate and deep layers that have different functions (Toldt, 1905; Schumacher, 1961; Yoshikawa et al., 1961; Heinze, 1963a,b; Sato et al., 1992; Sasaki et al., 2001). The complex masseter movement is associated with mastication with biomechanical actions of the individual masseteric compartments (English et al., 1999). This masticatory compartment may provide information for phenotypic comparison in mouse models for mechanical modeling of the mastication (Baverstock et al., 2013). The laminar structure includes collagenous components of both elastic fibers and other collagen fibers in human MM (Sato et al., 1992). The MM includes tendinous laminar structure composed of tenomodulin (TeM) determined muscle components superficial and deep layers. However, it has not been determined whether the development of the MM includes a stage of tendinous laminar structure. TeM is specifically expressed in dense connective tissues including tendons, ligaments, the epimysium of skeletal muscle and other organs (Brandau et al., 2001; Oshima et al., 2004; Pisani et al., 2004; Shukunami and Hiraki, 2001; Yamana et al., 2001). The TeM is a type II transmembrane glycoprotein and it contains an angiogenesis inhibitor with an anti-angiogenic cysteine-rich C-terminal domain. Northern blots with mouse RNAs have demonstrated the strong expression of a .5-kb TeM transcript in the skeletal muscle, and there is also a signal in tendons of the skeletal muscle at the bone insertion point (Brandau et al., 2001). TeM, which functions as an anti-angiogenic molecule, is expressed primarily in hypovascular tissues such as tendons and ligaments. TeM participates in the interaction between tissues and the vasculature during development (Shukunami et al., 1999) and is a highly specific tendon marker (Shukunami et al., 2008). Therefore, TeM might be an important element of muscle component formation because of the tendinous laminar structure of the MM and connections to the bone site appearing during development. Levels of vascular endothelial growth factor (VEGF) steadily decrease during postnatal development in sheep tendon (Meller et al., 2009). VEGF is increased in

* Corresponding author at: Department of Anatomy, School of Life Dentistry at Tokyo, The Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102-8159, Japan. Tel.: +81 3 3261 8531; fax: +81 3 3261 8531.
E-mail addresses: iwaoa1@tokyo.ndu.ac.jp (I. Sato), sthara@tokyo.ndu.ac.jp (S. Hara).

http://dx.doi.org/10.1016/j.aanat.2014.07.001
0940-9602/

tendons (flexor digitorum profundus tendon) experiencing cyclical loading and may play a role in the early vascular changes in the progression to tendinosis (Nakama et al., 2006). In human fetal Achilles and posterior tibial tendons, avascular zones in tendons are caused by a mechanically induced downregulation of VEGF expression (Petersen et al., 2002). Therefore the relationship between the RNA transcript levels of the TeM and angiogenic factors (VEGF) must also be understood in the developing mouse MM. We analyzed the expression of TeM over time via in situ hybridization and compared it to the angiogenesis markers VEGF-A and CD31 from E12.5 to P5.

2. Materials and methods

2.1. Sample preparation

C57/BL6 mice were sacrificed on E 12.5, 14.5, 17.5 and 18.5 of gestation and on P 1 and 5 by cervical dislocation after an overdose of pentobarbital. Fresh samples of the right MM were prepared from each group. Animals from each stage ($n = 4$) were analyzed for mRNA expression by real-time RT-PCR, in situ hybridization and morphology was assessed by light microscopic studies. The study followed the regulations of Nippon Dental University.

2.2. Analysis of mRNA by real-time RT-PCR

Immediately after sacrifice, the MM was removed from each mouse by scraping, then stored at −80 °C. After the muscles were cut into small pieces, the samples (0.1 g) were used for RNA isolation. Total RNA was extracted with a Quick Prep Total RNA Extraction kit (Amersham Biosciences, UK) according to the manufacturer's protocol. Contaminating DNA was removed using an RNase-free DNase (DNA-free, Ambion, Austin, TX, USA), and total RNA was quantified by spectrophotometry. The samples were stored at −80 °C until further use. Total RNA was converted to cDNA using 0.4 μM random hexamers (N808-0127; Applied Biosystems, CA, USA) in a mixture containing 1 mM of each dNTP, 20 units of RNase inhibitor (2311A; TaKaRa, Tokyo, Japan), 5 units of AMV reverse transcriptase XL (2620A; TaKaRa), 25 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM DTT, and 5 mM $MgCl_2$. The following thermal PCR cycling conditions were used: 30 °C for 10 min, 42 °C for 30 min, 90 °C for 5 min, and 5 °C for 5 min.

Quantitative real-time RT-PCR was performed using an Applied Biosystems 7300 Fast Real-Time PCR System, following the protocol recommended by the manufacturer. Each amplification mixture (50 μl) contained 100 ng of cDNA, 900 nM of forward primer, 900 nM of reverse primer, 250 of nM fluorogenic probe, and 25 μl of Universal PCR Master Mix (Applied Biosystems). The cycling parameters for the PCR were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min with primers for TeM (Applied Biosystems, Mm00491594_m1), VEGF-A (Applied Biosystems, Mm01281449_m1) and CD31 (Applied Biosystems, Mm01242584_m1). The levels of the amplified mouse cDNAs were normalized to those of GAPDH (rodent GAPDH primers and probes were obtained from Applied Biosystems, 'Assays-On-Demand'). The threshold cycle (*Ct*), defined as the cycle at which the amplification of the PCR product enters the exponential phase, was determined for each gene by plotting the fluorescence level vs. the cycle number on a logarithmic scale. The relative expression levels of the genes (TeM, VEGF-A and CD31) were estimated by calculating the ΔCt value, defined as the difference in the *Ct* values of the targets and the reference gene (GAPDH), as recommended by the supplier. The ΔCt was inversely proportional to the level of each mRNA transcript present in the muscle samples of the mice; a high *Ct* value corresponded to a lower mRNA level.

2.3. In situ hybridization

A 438 bp DNA fragment corresponding to the nucleotide positions 671-1108 of mouse TeM (GenBank accession number NM_022322.2) was subcloned into the pGEMT-Easy vector (Promega) and used for the generation of sense or anti-sense RNA probes. Paraffin-embedded blocks and sections of mouse whole body embryo for in situ hybridization (ISH) were obtained from Genostaff Co., Ltd (Tokyo, Japan). The mice were dissected, fixed with Tissue Fixative (Genostaff Co., Ltd. STF-01), and then embedded in paraffin according to their proprietary procedures, and sectioned at 6 μm. For ISH, tissue sections were de-paraffinized with xylene and rehydrated through an ethanol series and PBS. The sections were fixed in 4% paraformaldehyde in PBS for 15 min and then washed with PBS. The sections were treated with 8 μg/ml Proteinase K in PBS for 30 min at 37 °C, washed with PBS, re-fixed with 4% paraformaldehyde in PBS, again washed with PBS, and placed in 0.2 N HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1 M tri-ethanolamine–HCl, pH 8.0, 0.25% acetic anhydride for 10 min. After washing with PBS, the sections were dehydrated through an ethanol series. Hybridization was performed with probes at concentrations of 300 ng/ml in Probe Diluent-1 (Genostaff Co., Ltd. RPD-01) at 60 °C for 16 h. After hybridization, the sections were washed in 5× HybriWash (Genostaff Co., Ltd. SHW-01), equal to 5× SSC, at 60 °C for 20 min and then in 50% formamide, 2× HybriWash at 60 °C for 20 min, followed by RNase treatment in 50 μg/ml RNase A in 10 mM Tris–HCl, pH 8.0, 1 M NaCl and 1 mM EDTA for 30 min at 37 °C. Then, the sections were washed twice with 2× HybriWash at 60 °C for 20 min, twice with 0.2× HybriWash at 60 °C for 20 min, and once with TBST (0.1% Tween-20 in TBS). After treatment with G-block (Genostaff Co., Ltd. GB-01) for 30 min, the sections were incubated with anti-DIG AP conjugate (Roche, Mannheim, Germany) diluted 1:1000 with TBST for 2 h at RT. The sections were washed twice with TBST and then incubated in 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, 100 mM Tris–HCl, pH 9.5. Coloring reactions were performed with NBT/BCIP solution (Sigma–Aldrich, MO, USA) overnight and then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan), dehydrated, and then mounted with malinol (Muto Pure Chemicals). Serial sections were also stained with hematoxylin and eosin.

2.4. Statistics

The differences in the quantitative real-time RT-PCR from 12.5 of gestation to postnatal 5-day were assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test with one categorical independent variable and one continuous variable (the independent variable can consist of a number of groups). The level of significance was set at $p < 0.001$. The results are reported as the mean ± SD.

2.5. Ethics

All procedures involving mice were reviewed and approved by the Nippon Medical School Animal Resource Laboratory Committee of the Nippon Dental University (Rules for the Care and Use of Laboratory Animals, no. 03-34).

3. Results

3.1. mRNA expression using real-time RT-PCR

The mRNA expression patterns of the TeM, CD31, and VEGF in E12.5 to P5 mice are shown in Fig. 1. The expression pattern of TeM mRNA was different from the other markers.