Original

Characteristics and Effects of Muscle Fibers surrounding Lingual Carcinoma

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Abstract: It has been suggested that dysphagia caused by excision of carcinoma of the tongue may be affected by the hypofunction of the surrounding muscle tissues, as well as by tissue defects. However, there have been no reports concerning how muscle fiber characteristics change in the regions surrounding the lesion after excision of the carcinoma. In this study, we investigated myosin heavy chain (MyHC) isoforms at genetic levels in order to demonstrate the muscle fiber characteristics of the muscle tissues surrounding lingual carcinoma (the experimental group). High mobility group box 1 (HMGB1) was used as a marker to examine whether tongue cancer actually developed. MyHC-2a expression was high in the experimental group. Thus, the marked changes of muscle fiber characteristics, as seen in this study, were considered to be a biological reaction to changing muscle characteristics for regenerating or maintaining normal muscle tissues against the muscle atrophy caused by carcinoma.

Key words: Myosin heavy chain, Carcinoma of tongue, HMGB1, SCC VII, Mouse

Introduction

Myosin, one of the muscle-contracting proteins, is a major protein constituting muscle. Among others, myosin heavy chain (MyHC) is known to reflect muscle functions most clearly¹⁾. Recently, MyHC isoforms that are majorly classified into fast muscle types (MyHC-2b, -2d, and -2a) and slow muscle types (MyHC-1) according to their contractile rates²⁻⁴⁾. In addition, it has been suggested that composition ratio of these MyHC isoforms may characterize the muscle type. Based on these reports, investigation on the composition of MyHC isoforms is considered to reveal the muscle fiber characteristics.

Reports concerning the expressions of each MyHC isoform in the course of muscle growth and development were mainly about extremities, but recently, reports about head and neck muscles such as masticatory muscle can be seen⁵⁻¹³⁾. Gojo et al. and Shida et al. noted the phenomenon “weaning” seen in the course of growth, and observed the changes of muscle fiber

characteristics of the mouse masseter at the protein level^{7,14)}. There, they demonstrated that MyHC-2b, which produces high contractile rate and force, was highly expressed along with weaning, and thus considered that this resulted from functional changes of the masseter by chewing movement after weaning.

Maejima et al. and Yanagisawa et al. examined the changes of the muscle fiber characteristics of the upper and lower halves of the adult mouse tongue muscle before and after weaning, and confirmed marked changes of isoforms^{15,16)}. Thus, marked functional changes before and after weaning, i.e., from suckling to chewing, were demonstrated to influence the characteristics of muscle fibers in the oral cavity. It has recently been speculated that hypofunction of the normal muscle as well as tissue loss affects the dysphagia after resective surgery of the lesion due to the development of the carcinoma of tongue¹⁷⁾. However, no investigation on the muscle fiber characteristics of the surrounding muscle tissue of the carcinoma of tongue has been reported.

In this study, squamous cell carcinoma (SCC) VII were injected into the tongue muscle to artificially develop the carcinoma of tongue, and their effects on the muscle fiber characteristics of the surrounding normal tongue muscle of the

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carcinoma of tongue were evaluated by genetic analyses for comparison with the normal tongue muscle.

The tongue muscle consists of fast muscle type^{5,15,16}; thus, MyHC-2b and MyHC-2a, which is considered to have the highest and lowest contractile rates among fast muscle type MyHC isoforms, respectively, were selected to investigate the muscle fiber characteristics⁴). HMGB1 was used as a marker to examine whether the carcinoma of tongue actually developed¹⁸). HMGB1 is a DNA binding nonhistone protein that maintains chromosome structure and regulates gene transcription, and there have been many reports on it until now¹⁹⁻²²).

Materials and Methods

Cell culture

SCC VII cells, which are mouse oral SCC cell line, were used in this study. SCC VII cells are an established cell line originating from the oral floor division of the mouse. SCC VII cell is the poorly differentiated carcinoma.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO) containing Penicillin-Streptomycin Solution (Sigma Aldrich Co.) and 10% fetal bovine serum (FBS) (ICN Biomedicals Inc., Aurora, OH) in a humidified atmosphere of 5% CO₂ at 37°C.

Injection of SCC VII cells into BALB/cAJcl mice

500µl SCC VII cells (1.0×10⁶/ml) with serum-free DMEM were injected into the site at 5 mm from the tongue tip of nude male mice (BALB/cAJcl, 20g bodyweight). The mice were injected once a week, total 4 times, and were kept for another week. DMEM (Sigma-Aldrich Co) (FBS (-)) was injected to the control mice. All mice were fed powder diets.

Five mice each, total 20 mice, were used for mRNA expression analyses and immunohistochemical analysis after five weeks, we established carcinoma with tongue. According to the Experimental Animal Guideline of Meikai University School of Dentistry, the mice were deeply anesthetized with pentobarbital and killed. After SCC model mice were prepared, the tongues were removed at the root of tongue.

Hematoxylin-Eosin (H-E) staining

Following the conventional methods, serial sections were stained with H-E, then examined and photographed using a universal microscope (BX50; Olympus, Tokyo, Japan).

Immunofluorescent staining

Using a cryostat, 8 mm-thick serial frozen sections were prepared in a direction perpendicular to the long axis of the tongue. In particular, central regions where the carcinoma of tongue was completely developed were observed. Immunostaining was performed in the following manner:

First, HMGB1 was used as a marker for the carcinoma of tongue. The blocking reagent was used 10% normal goat serum (Nichirei, Tokyo, Japan). As primary antibodies, the serial sections were treated with rabbit anti-HMGB1 antibody (Abcam, Cambridge, UK). As secondary antibodies, biotin-labeled anti-rabbit IgG antibody was used for HMGB1, then the sections were colored by 0.01% 3,3'-Diaminobenzidine tetrahydrochloride (Wako, Kumamoto, Japan).

Next, MyHC-2a and 2b were used to confirm the characteristics of muscle. As primary antibodies, SC-71 (anti-MyHC-2a) and BF-F3 (anti-MyHC-2b) (American Type Tissue Culture), anti-mouse monoclonal antibodies extracted from hybridoma cells, were used^{10,14-16,23}). More specifically, after culturing the hybridoma cells in DMEM (10% FBS) medium at 37°C and 5% CO₂ for 72 hours, they were centrifuged, and the supernatants were used as antibodies.

As secondary antibodies, FITC-labeled goat anti-mouse IgG antibody (Novocastra Laboratories, Newcastle, UK) was used for SC-71, while Rhodamine-Conjugated GOAT F(AB')₂ FRAGMENT TO MOUSE IgM (MP Biomedicals, Aurora, Ohio) was used for BF-F3. Muscle fibers were examined using a confocal laser scanning microscope (MRC-1024/2P; Nippon Bio-Rad Lab. Tokyo, Japan). MyHC-2a-positive muscle fibers were defined as those labeled with FITC, while MyHC-2b-positive muscle fibers were defined as those labeled with RITC.

RNA extraction and mRNA expression analysis using the LightCycler®

MyHC-2b and MyHC-2a mRNAs were quantified using the LightCycler® (Roche Diagnostics, Mannheim, Germany). mRNA was extracted using the Quick Prep micro mRNA Purification Kit (GE Healthcare, Amersham, UK), then cDNA was synthesized by the Ready-To-Go (GE Healthcare). After setting optimal conditions for all primers, the experiment was conducted according to the standard protocol of the LightCycler®. Premix of LightCycler® Fast Start DNA Master SYBR Green I (Roche) was used as PCR reaction solution of hot-start PCR by the LightCycler®. Dilution series were prepared using 4.0 ng/ml of PCR product as a concentrate solution, of which solutions diluted at 1:10⁶, 10⁷, 10⁸, and 10⁹ were used. Primers used were MyHC-2b, 2a, prepared by the Oligo 5primer design (Nihon Gene Research Laboratories Incse., Sendai, Japan) from each full-length DNA.

Primer sequences of MyHC-2b were as follows: 5'-ACAGACTAAAGTGAAAGCC-3' (forward) and 5'-CTCTCAACAGAAAGATGGAT-3' (reverse) (Accession: XM_126119). Primer sequences of MyHC-2a were as follows: 5'-CGATGATCTTGCCAGTAATG-3' (forward) and 5'-TGATAACTGAGATACCAGCG-3' (reverse) (Accession: NM_144961). Two kinds of PCR mixtures (20 µl) of MyHC-2b, 2a, thus prepared, were added to the glass part of a capillary. Cycle

Table1. Comparison of body weights between mice that developed the experimental group and the control group (n=5).
A unit of weight: g Marked reduction of body weight was confirmed due to the development of the carcinoma of tongue.

	Start	End	Weight gain
Control	19.5	26.5	7.0
Experimental	20.8	17.6	▲ 3.2

conditions were as follows: after 95°C 10 min, 50 cycles of 95°C 10 sec, 62°C 10 sec, and 72°C 7 sec. Gene amplification was performed according to the melting program of 70°C for 15 sec, and during the transition phase from 70°C to 95°C, fluorescence was sequentially monitored at a rate of 0.1°C/sec. F1 (530 nm) was used for the fluorescence channel, and the gains indicated 89.9°C and 88.2°C for MyHC-2b and MyHC-2a, respectively. The quantities of each MyHC isoforms, calculated by the above method, were divided by the quantity of GAPDH that was used as a house keeping gene to obtain final mRNA expression levels. Primer sequences of GAPDH used are as follows: 5'-TGAACGGGAAGCTCACTGG-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse); Accession: NM_008084^{8,11)}.

Student's tukey's HSD test was used for statistical analysis in this study and a p value of <0.05 was considered significant.

Results

Body weights

Weight changes were compared between the mice that developed experimental group and the control group (Table1). In the control group, the weights had increased from 19.5g to 26.5g. However, in the experimental group, the weights had decreased about 3.2g.

H-E staining

In the control group, the muscle fibers and the loose connective tissues were observed in the section. However, in the experimental group, the poorly differentiated carcinoma was observed in the deeply tongue. Furthermore, the muscle fibers were observed in the epithelium and the injected on artificial carcinoma tissue (Fig.1).

Immunohistochemical analysis

In the experimental group, HMGB1 was stained in and around the carcinoma of tongue region where cellular dysplasia was observed (Fig.2). Meanwhile, regions without cellular dysplasia, where HMGB1 was not stained, were determined as normal tongue muscle.

In the control group, many muscle fibers positive for MyHC-2b were observed in the superior longitudinal muscle, while muscle

Figure 1. Histological observations from control group (A) and experimental group(B)

Scale bars: 100µm, H-E staining. a: normal muscle fiber; b:the carcinoma tissue infiltrated into muscle fibers; c: carcinoma tissue area.

Figure 2. HMGB1 immunostaining image Scale bar: 100µm

Arrows A: The border line of the carcinoma and muscle fibers. The carcinoma of tongue was stained positive by anti-HMGB1 antibody.

Arrow B: This is a nuclear of the muscle fiber. It was stained by anti- HMGB1 antibody.

Figure 3. MyHC-2b and MyHC-2a immunostaining images

Scale bars: 100µm; A and B: control group; C and D: experimental group

A and C: anti-MyHC-2a antibodies; B and D: anti-MyHC-2b antibodies. a: vertical muscle; b: superior longitudinal muscle; c: normal muscle; d: carcinoma.

fibers positive for MyHC-2a were observed in the vertical muscle of tongue, which enters the superior longitudinal muscle (Fig.3). In the control group, the superior longitudinal muscles that have

Figure 4. Expression level of MyHC-2b and MyHC-2a mRNA
The amount of MyHC-2b and MyHC-2a isoform calculated by the described in the text was divided by the amount of GAPDH, which was one of house keeping genes, to determine the mRNA expression. (mean: $\pm$ S.D.) Statistical analysis: * $p < 0.05$ ($n = 5$).

masticatory function are characterized by MyHC-2b, suggesting that this function is decreased in mice with the carcinoma of tongue (Fig.3A and B).

In the experimental group, MyHC-2a was stained also in muscle cells surrounding the lesion to some extent, and was observed also in the superior longitudinal muscle, usually characterized by staining of MyHC-2b. In contrast, MyHC-2b was scarcely stained (Fig.3C and D).

Namely, in the experimental muscle tissues of mouse tongue, MyHC-2a expression was confirmed in the surrounding muscle fibers of the carcinoma of tongue, but MyHC-2b expression was scarcely observed. These findings differed from those of the control group.

Analysis by the Light Cycler®

MyHC-2a and MyHC-2b mRNAs were confirmed in both the experimental and control groups. MyHC-2a expression was higher in the experimental group than control group. In contrast, MyHC-2b expression was higher in the control group than in the experimental group ($p < 0.05$) (Fig.4). These results were significant difference.

Discussion

Growth of carcinoma is known to reduce body weight and fat^{24,25}, and this weight reduction has been reported to result from consumption of skeletal muscles, before that of fat tissues²⁶. Also in our study, weight reduction was confirmed in the experimental group.

HMGB1 has been reported to be expressed usually during muscle regeneration and development²⁷. In addition, the possibility that HMGB1 may be a key marker in other organs has been suggested¹⁸. There has been no report on tongue cancer, but in this experiment, HMGB1 expression was high in the regions where tongue cancer, determined by cellular dysplasia, developed. In addition, surrounding muscle tissues without cellular dysplasia were also stained. Also, some signaling from cancer cells was considered to forecast the transition to cancer. The aim of the

present study was to investigate the muscle fiber characteristics of the completely normal muscle tissues that surround cancer lesions; thus, HMGB1-stained regions were also determined to be abnormal tissues.

Cytokines and tumor factors have been reported to suppress the expression of myosin, a muscle gene products²⁸⁻³¹. It has been suggested that selective regulation of muscle gene expression rather than consumption of muscles, induced by a tumor, plays a central role in reducing muscle proteins²⁸.

In fact, MyHC-2b expression was observed in the normal tongue muscle, while little MyHC-2b expression and high MyHC-2a expression were observed in the surrounding muscle fibers of the carcinoma of tongue. Analysis of MyHC-2b gene was expressed in the control group, but was very low in the experimental group. It is known that MyHC-2b usually appears at inactive muscle. However, in the experimental group, it might be that expression in MyHC-2b decreased because the regeneration of muscles became active for the carcinoma of tongue.

On the other hand, MyHC-2a expression was high in the experimental group, but low in the control group. MyHC-2a a common protein observed in the immature or regenerating muscles⁶; thus, the marked changes of muscle fiber characteristics found in this study were considered to be a biological reaction to change muscle characteristics to regenerate or maintain residual muscle tissues against the muscle consumption caused by tumor.

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