Expression of muscle-specific integrins in masseter muscle fibers during malocclusion disease

GIUSEPPINA CUTRONEO[1,2], MARIA GRAZIA PIANCINO[3], GUGLIELMO RAMIERI[4], PIETRO BRACCO[3], GIUSEPPE VITA[5], GAETANO ISOLA[3], GIOVANNA VERMIGLIO[1,2], ANGELO FAVALORO[1,2], GIUSEPPE ANASTASI[1,2] and FABIO TRIMARCHI[1,2]

[1]Department of Biomorphology and Biotechnologies, Messina University, I-98125 Messina; [2]IRCCS Centro Neurolesi 'Bonino-Pulejo', I-98125 Messina; [3]Department of Orthodontics and Gnathology-Masticatory Function, Dental School, Turin University, I-10126 Turin; [4]Maxillofacial Surgery Division, Turin University, I-10126 Turin; [5]Department of Pathology and Experimental Microbiology, Messina University, I-98125 Messina, Italy

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Abstract. Integrins are heterodimeric cell surface membrane proteins linking the extracellular matrix to actin. α7B integrin is detected in proliferating and adult myofibers, whereas α7A plays a role in regenerating muscle fibers with a minor function in mature muscle fibers. The expression levels of β1A appear to be very low, whereas β1D appears to be the predominant integrin form in mature muscle. Considering the important features of masseter muscle we have studied integrin expression in masseter muscle specimens of surgical patients with posterior right crossbite and comparing them to left side masseter muscle specimens. Our results showed that the expression of integrins was significantly lower in the crossbite side muscle. Furthermore, the most important finding is that β1A is clearly detectable in adult masseter muscle. This behavior could be due to the particular composition of masseter, since it contains hybrid fibers showing the capacity to modify the contractile properties to optimize the energy efficiency or the action of the muscle during contraction. Moreover, masseter is characterized by a high turnover of muscle fibers producing a regeneration process. This may indicate a longer time to heal, justifying the loss of β1D and the consequential increase of β1A. Thus, our data provide the first suggestion that integrins in masseter muscle play a key role regulating the functional activity of muscle and allowing the optimization of contractile forces.

Introduction

In muscle fibers, interactions between the cell and the extracellular matrix (ECM) are considered to be important for muscular development during somitogenesis, cell migration from somites, correct innervation, and muscle patterning (1,2). Cell-ECM interactions play a key role in mechanotransduction transmitting forces across the plasma membrane (3,4).

Experimental evidence for the importance of cell-ECM interactions during muscle formation can be found in transgenic mice lacking fibronectin, which have defective somites (5). Myoblast migration from the somite can be inhibited by anti-β1-integrin antibodies (6), and during *in vitro* differentiation in which myotube formation can be inhibited with antibodies to integrins (7). In the adult muscle, an intact basement membrane-cytoskeletal linkage is important for skeletal muscle stability and integrity (8).

Integrins are a family of heterodimeric cell surface membrane proteins that mediate the interaction of cells with each other, with ECM proteins, and with additional molecules in their environment (9,10). These proteins also link ECM to cytoskeletal actin providing bidirectional signaling between the ECM and the cytoplasm (11). Thus, integrins also play a key role in cell adhesion including cell-matrix and intercellular interactions and therefore, they are involved in various biological phenomena, such as cell migration, differentiation, tissues repair and programmed cell death (12).

Each integrin is composed of a noncovalently-linked pair of α and β subunits. In particular, the α7β1-integrin is concentrated at neuromuscular and myotendinous junctions and it is located along the sarcolemma at costameres with an important role in the functions of the skeletal muscle (13). It was demonstrated that congenital myopathies may be caused by mutations in the human integrin α7 gene (ITGA7) confirming the importance of the α7β1-integrin in maintaining normal skeletal muscle physiology (14).

Regarding the α7 subunit, the α7B isoform is detected in proliferating and adult myofibres and is exclusively localized in the neuromuscular and myotendinous junctions (13,15). Instead, the α7A isoform, detected in differentiating myofibres, plays a key role in muscle regeneration during the dynamic adhesion stage, whereas it appears to have a minor role in mature skeletal muscle (16).

The β1 chain cytoplasmic domain also undergoes developmentally regulated alternative splicing (17). β1A is the most

Correspondence to: Dr Angelo Favaloro, Department of Biomorphology and Biotechnologies, Polyclinic G. Martino, Torre Biologica, Messina University, Via Consolare Valeria 1, I-98125 Messina, Italy
E-mail: angelo.favaloro@unime.it

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common isoform of the β1 chain and its expression levels appear to be very low or negligible in mature skeletal muscle, whereas the β1D isoform is the predominant β1 isoform in adult striated muscle (18). Thus, β1D-integrin plays a crucial role in linking the subsarcolemmal cytoskeleton to the surrounding extracellular matrix in adult muscle tissues (12,19).

The role of integrins was also studied in Duchenne muscular dystrophy evidencing the detection of high levels of α7β1-integrin in order to compensate for the absence of dystrophin; these results reinforced the role played by integrins in the integrity and stability of skeletal muscle fibers (20).

In agreement with several reports (21-23), we offered support for the hypothesis that integrins have a role in the function of human adult skeletal muscle (24) and demonstrated a bidirectional signaling between sarcoglycans and integrins (25). This reciprocal control may determine the prevalence of one system over another with a consequent transmission of different messages to the sarcolemma-associated cytoskeleton (24,25).

Thus, we studied α7B and β1D integrin during muscular inactivity and we showed that these isoforms were displaced by the relative isoforms α7A and β1A due to loss of regulatory effects on gene expression of these proteins (26). Moreover, we studied muscle-specific integrins and their relative isoforms, α7A and β1A in chimpanzee's masseter muscle, analyzing biopsies of alpha male and non-alpha male subjects, since this particular muscle plays a key role in many behavioral functions in respect to tasks of subjects. This study demonstrated high levels of α7A and β1A integrin in alpha males in respect to non-alpha male subjects in which only α7B and β1D showed normal staining patterns (27).

The masseter muscle shows a very high ATPase activity for contracting very quickly and forcefully participating in a wide variety of functional activities of the stomatognathic system including mastication, swallowing and speech (28,29). This diversity of functions requires coordination of motor output elements of the neuromuscular system with appropriate activation of tongue, facial and oropharyngeal muscles (23). About this, mastication is one of the most complex and co-ordinated functional movements involving diverse and accurate mandibular patterns to incise and grind food suitable for swallowing. The pattern of mandibular movement during chewing is influenced by factors such as the bolus type and the type of occlusion (30,31). The relative position of the upper and lower teeth determines occlusal stability, which is related to muscular performance (31).

Subjects with unilateral posterior crossbite exhibit different kinematics of the mandible during mastication when chewing on the affected side, resulting in an increased frequency of reverse chewing cycles (32,33). The masseter of the crossbite side is less active than the counterpart, and the co-ordination of the masticatory muscles on the two sides is altered with respect to controls (33,34).

Although the significance of masticatory muscle function has been illustrated in previous experimental studies on animals (35,36), there are insufficient data on the influence of proteins about crossbite malocclusion. In our opinion, integrins could play an important role during malocclusion diseases in masticatory muscle and in particular in masseter, in which all networks of proteins could be modified.

Thus, considering the important function of masseter muscle, and its particular role in chewing cycles, we aim to study this muscle in order to verify its composition in integrin network. Then, by immunohistochemical and molecular technique, we analyzed human masseter muscles of surgical patients affected by severe class III malocclusion to comprehend the role of integrins in this masticatory muscle.

Materials and methods

Patients and ethics. Five surgical patients, 3 men and 2 women, age 31.3±5.5 years (mean ± standard deviation), with unilateral posterior crossbite, all on the right side, were selected for the study. All the patients gave informed consent.

The inclusion criteria for the crossbite patient group were: i) severe class III malocclusion with right posterior crossbite of two or more posterior teeth, ii) complete permanent dentition, iii) no erupting teeth, iv) no caries, and v) no temporomandibular disorders. The exclusion criteria were: no history of connective tissue disorders, myopathies, endocrine disorders, autoimmune disease, bone disease, bleeding disorders.

The investigation conformed with guidelines established by the University Internal Review Board for use of Human Subjects and with the principles outlined in the Helsinki Declaration of 1975.

Muscle biopsies. Biopsies were obtained under general anesthesia from the superficial and anterior portion of both masseter muscles of patients undergoing orthognathic surgery to reposition one or both jaws in conjunction with orthodontic treatment following the protocol suggested by Boyd *et al* (37). The supero-inferior level of the biopsy was determined by the mandibular occlusal plane and was excised from the anterior, deep surface of the masseter adjacent to the anterior aspect of the mandibular ramus (38). All biopsies were obtained by the same surgeon via an intraoral incision through the mucosa and buccinator muscle, approximately 3x3x3 mm. The biopsy specimens of both masseter muscles were analyzed using immunohistochemical analysis.

Immunohistochemical analysis. The biopsies were fixed in 3% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4, for 2 h at room temperature. They were then washed extensively with 0.2 M phosphate buffer, pH 7.4, and then with phosphate-buffered saline (PBS), containing 12 and 18% sucrose. The samples were snap-frozen in liquid nitrogen and 20-μm sections were prepared in a cryostat for use in a protocol to perform immunofluorescence. The sections were placed on glass slides that were coated with 0.5% gelatin and 0.005% chromium potassium sulphate.

To block non-specific binding sites and to permeabilize the membranes, the sections were preincubated with 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS at room temperature for 15 min. Finally, the sections were incubated with primary antibodies. The following primary antibodies were used: anti-α7B integrin diluted 1:50, anti-β1D integrin diluted 1:50, anti-α7A integrin diluted 1:100, and anti-β1A integrin diluted 1:50 (synthetic peptides from the COOH terminal region; kindly provided by the laboratory of Professor Tarone, University of Torino). Primary antibodies

were detected using Texas Red-conjugated IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Slides were finally washed in PBS and sealed with mounting medium.

The sections were then analyzed and images acquired using a Zeiss LSM 5 DUO (Carl Zeiss, Jena, Germany) confocal laser scanning microscope. All images were digitalized at a resolution of 8 bits into an array of 2,048x2,048 pixels. Optical sections of fluorescent specimens were obtained using a helium-neon (HeNe) laser (wavelength, 543 nm) at a 62 sec scanning speed with up to eight averages; 1.50 μm sections were obtained using a pinhole of 250. For each reaction, at least 100 individual fibers were examined. Contrast and brightness were established by examining the most brightly labeled pixels and choosing the settings that allowed clear visualization of the structural details while keeping the pixel intensity at its highest (~200). Each image was acquired within 62 sec, in order to minimize photo-degradation.

The 'display profile' function of the laser scanning microscope was used to show the intensity profile across an image; the intensity curves are shown in graphs. Digital images were cropped and figure montages prepared using Adobe Photoshop 7.0 (Adobe Systems; Palo Alto, CA, USA).

Statistical analysis. All our observations were analyzed by an internal software for image analysis, included in the CLSM software and named 'Histo', measuring the distribution of pixel intensity of all areas corresponding to each fiber; pixel intensities were converted in a data table indicating values of single pixel intensity. By this software, we analyzed 100 fibers for each reaction, using for all reactions the same confocal parameters, and a mean and standard deviation for single fibers were obtained.

The box-and-whisker plots with the median values of fluorescence intensity in both muscle fibers crossbite (right side) and left side are shown in Fig. 4. Data analysis and graphs were plotted on GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA) and inter-integrin differences among the isoforms in masseter muscle fibers, were assessed by the Wilcoxon rank test (w); two tailed p-values <0.05 were considered statistically significant.

Total-RNA isolation. Samples containing 50-100 mg of tissue were homogenized using a power homogenizer (Ultra Turrax, IKA-Werke GmbH, Staufen, Germany). Total-RNA was isolated by a single-step RNA isolation procedure (TRIzol®, Invitrogen) (39) that uses a monophasic solution of phenol and guanidine isothiocyanate.

RT-PCR analysis. In the present study, we collected muscle biopsies of the human subjects all affected by right posterior crossbite taking muscle tissue both from the right and left side (control), in order to evaluate the expression of α7A, α7B, β1A and β1D integrin by RT-PCR. RT-PCR was carried out using the GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA, USA) in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems). In the first step, an initial reverse transcription (RT) reaction was carried out in a volume of 20 μl containing 3 μg of total-RNA, 10 units RNase inhibitor, 10 mM DTT, 15 units MultiScribe reverse transcriptase and 1.25 μM oligo(dt)16 using the following

Table I. Oligonucleotide primer sequences of the integrin isoforms and of the internal control GAPDH used for RT-PCR.

Primers	Forward	Reverse	Length (bp)	Exons	Nucleotides	Accession NCBI
α7A	5'-CGGGCCAACATCACAGTGAA-3'	5'-TCCGATGGAAGAAGCCACACT-3'	208	24-26	3342-3553	ENST00000257880
α7B	5'-CGGGCCAACATCACAGTGAA-3'	5'-GTTTGAAGAATCCCATCTTCCACAG-3'	205	23-25	3206-3410	NM_002206
β1A	5'-TGCCGTAACAACTGTGGTCA-3'	5'-TAACCATCCTGTCTCAAGTC-3'	255	16	2567-2821	NM_002211
β1D	5'-TGGAGAATCCAGAGTGTCCC-3'	5'-AGAGACCAGCTTTACGTCCG-3'	252	13-15	2144-2395	NM_033668
GAPDH	5'-AACCTGCCAAATATGATGAC-3'	5'-ACTGAGTGTGGCAGGGACTC-3'	340	8-9	854-1192	NM_002046

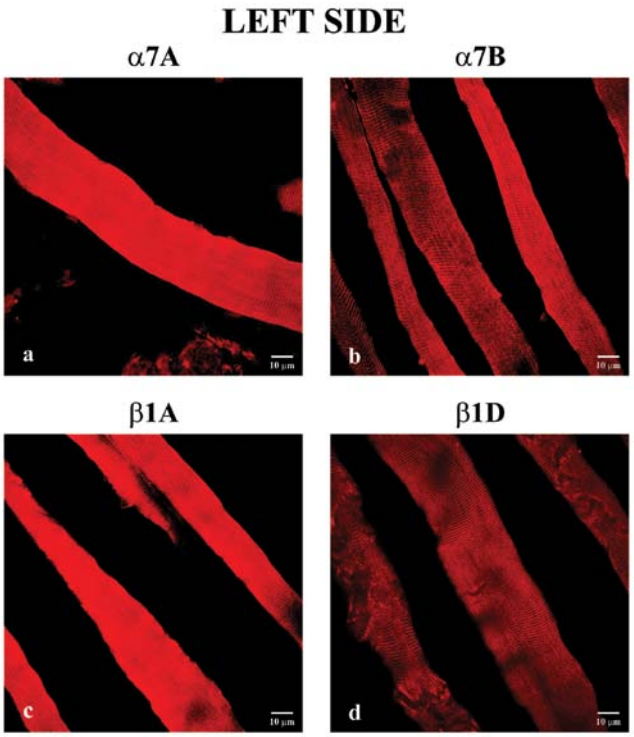

Figure 1. Immunohistochemical findings in human left masseter muscle (control side). Longitudinal skeletal muscle fibers were immunolabelled with antibodies against (a) α7A, (b) α7B, (c) β1A and (d) β1D integrin. Immunostaining of α7A integrin was increased in respect to the α7B isoform, whereas β1A integrin immunofluorescence was increased in respect to the β1D isoform.

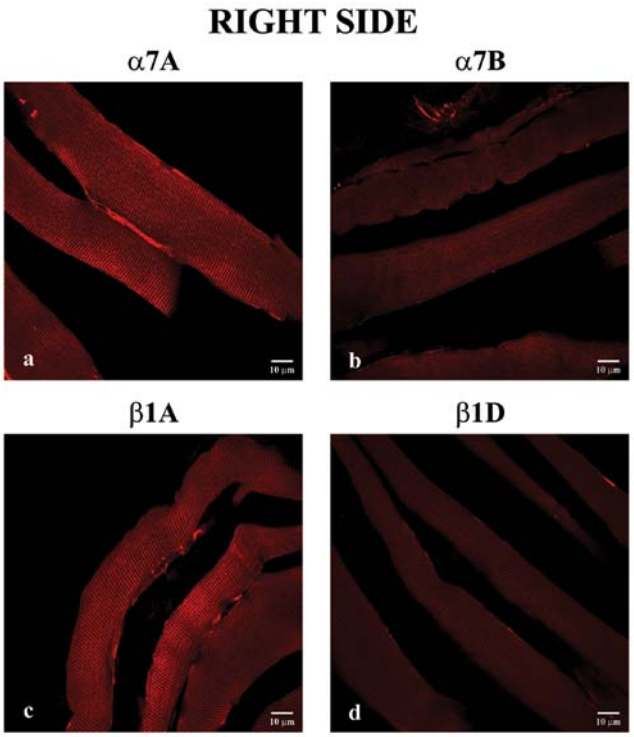

Figure 2. Immunohistochemical findings in human right masseter muscle (crossbite side). Longitudinal skeletal muscle fibers were immunolabelled with antibodies against (a) α7A, (b) α7B, (c) β1A and (d) β1D integrin. All of the integrins showed a decreased immunostaining pattern in respect to integrins of the left side. Moreover, α7A and β1A integrin showed an increased staining pattern in respect to the α7B and β1D isoforms, respectively.

thermal cycler conditions: 10 min at 25˚C, followed by 12 min at 42˚C. In the second step, a PCR was performed in a volume of 50 μl containing 5 μl of cDNA from the first step (RT) as a template, 2.5 units AmpliTaq Gold DNA polymerase and each primer at a concentration of 0.2 μM. The primer pairs used are shown in Table I.

PCR conditions were as follows: an initial 10 min denaturation step at 95˚C, followed by 35 cycles of denaturation at 94˚C for 40 sec, annealing at 62˚C for 40 sec and extension at 72˚C for 45 sec with a final extension at 72˚C for 10 min. For each protein, human GAPDH cDNA was used as an internal control and the primers are shown in Table I. Digital images were cropped and figure montages prepared using Adobe Photoshop 7.0.

Results

Immunohistochemical analysis. For immunohistochemical analysis, first, we analyzed longitudinal sections of biopsy samples from left masseter muscle without crossbite. These reactions showed that all of the integrins were detected to varying degrees along the sarcolemma (Fig. 1). In particular, immunostaining of α7A integrin (Fig. 1a) was increased in respect to the corresponding α7B isoform (Fig. 1b). Moreover, β1A integrin (Fig. 1c) immunofluorescence was increased in respect to the β1D isoform (Fig. 1d).

Secondly, we performed immunostaining on longitudinal sections of masseter muscle fibers from the right masseter. All of the integrins were detected to varying degrees along the sarcolemma, and, generally, all showed decreased immunofluorescence compared to integrins of the left side. In particular, these reactions showed an increased staining pattern for α7A (Fig. 2a) and β1A integrin (Fig. 2c) compared to the α7B (Fig. 2b) and β1D (Fig. 2d) isoforms, respectively.

To confirm the protein staining patterns, we used the 'display profile' software function of the laser scanning microscope for selected samples. This additional analysis, which reveals the fluorescence intensity profile across the image, converted the immunofluorescence signal into a graph (Fig. 3) in order to quantitate the differences between the antibodies. The display profile of the left masseter showed increased fluorescence peaks for α7A (Fig. 3a) and β1A integrin (Fig. 3c), whereas peaks of the α7B (Fig. 3b) and β1D isoform (Fig. 3d) revealed decreased intensity values. By applying this analysis to the samples of muscle fibers taken from right masseter, it was possible to show that the fluorescence peaks of all integrins showed a general decrease compared with those of all integrins observed in the left masseter, and in particular α7B (Fig. 3f) and β1D integrin (Fig. 3h) fluorescence intensity showed never reached 50, whereas the fluorescence intensity of α7A (Fig. 3e) and β1A (Fig. 3g) isoforms values were between 50 and 100.

Statistical analysis. Expression of α7A and β1A integrin in muscle fibers on the right side (crossbite) was higher compared with that of α7B and β1D isoforms of same side ($w=2.655$, $p<0.007$; $w=2.258$, $p<0.02$, respectively). Compared to isoforms of controlateral muscle fibers, the α7A, α7B and β1A

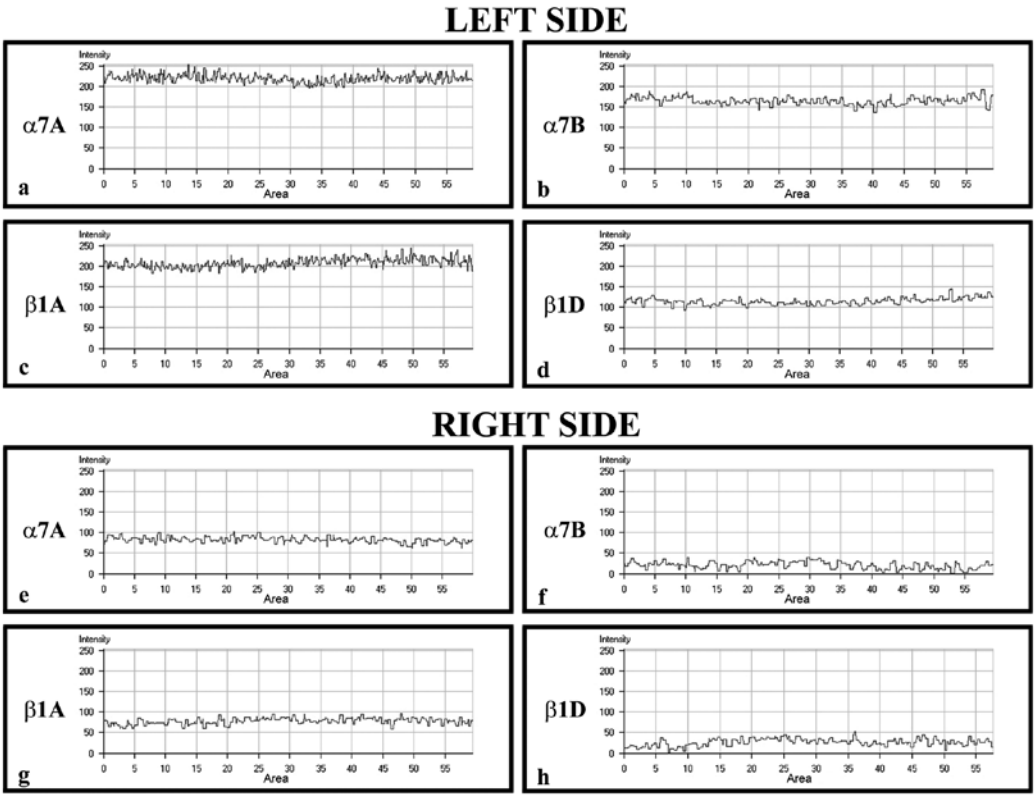

Figure 3. Display profiles in human left and right masseter of (a and e) α7A, (b and f) α7B, (c and g) β1A and (d and h) β1D integrin. In the left side, fluorescence peaks were clearly increased for α7A and β1A integrin, whereas peaks of α7B and β1D integrin revealed decreased values. In the right side, the peaks of all integrins showed a general decrease in respect to left masseter; moreover, fluorescence peaks of α7B and β1D integrin were severely decreased in respect to peaks of α7A and β1D integrin.

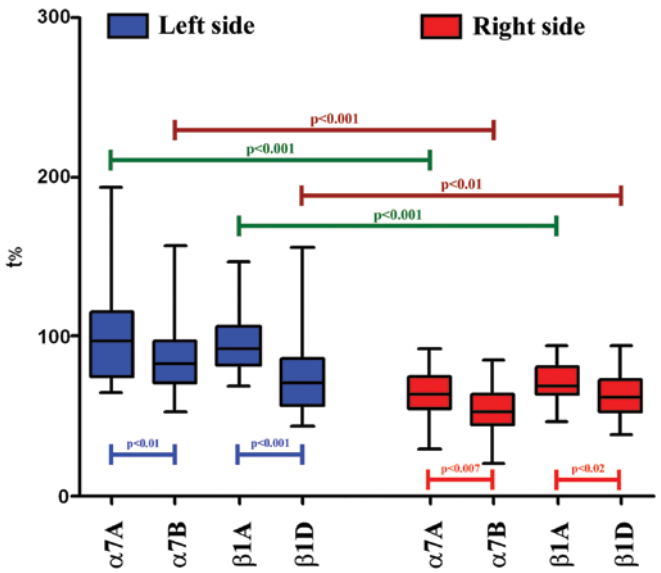

Figure 4. Immunofluorescence quantitative image analysis of α7A, α7B, β1A and β1D integrin. The immunofluorescence image of integrins distribution in right posterior crossbite and left masseter fibers, were analyzed for fluorescence intensity (t%) as described in Materials and methods. Values represent median with min to max value in the box and whisker graphs. p<0.05 was considered to indicate significant differences.

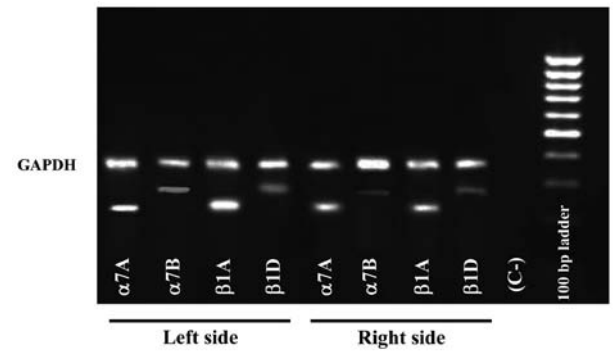

Figure 5. Agarose gel (2%) electropherogram of RT-PCR products amplified using human RNA as template, primer pairs of α7A, α7B, β1A and β1D integrin, in the left and right side. In each lane we ran an aliquot of internal control and α7A, α7B, β1A and β1D integrin coamplified RT-PCR products of left (lane 1-4) and right masseter (lane 5-8). Lane 9, negative control. Lane 10, 100-bp ladder.

isoforms of masseter fibers crossbite were markedly decreased (w=5.403, p<0.001; w=5.303, p<0.001; and w=4.886, p<0.001, respectively) whereas β1D integrin, in crossbite, showed, a significant decrease of distribution compared with muscle fibers β1D integrin, in left side (w=2.353, p<0.01). In the left side the α7A and β1A integrin showed a remarkable increase when compared to the α7B and β1D isoforms, respectively, (w=2.574, p<0.01; w=4.180, p<0.001) (Fig. 4).

RT-PCR analysis. In order to determine whether differences in the expression of integrins are due to the presence of different integrin mRNA levels, we performed RT-PCR with primers specific for muscle integrins. Using RNA samples isolated

from masseter muscle biopsies, we confirmed that levels of integrins appeared significantly lower, in the right side, in comparison with those of left side. Furthermore, α7A and β1A, compared to the α7B and β1D isoform, respectively, were predominant in both masseter muscle specimens (Fig. 5)

Discussion

In this report, for the first time, we analyzed muscle-specific integrins, using human samples of muscle fibers on both masseters obtained by patients affected by right posterior crossbite.

Our results showed that the amount of integrins appeared to be significantly lower, in the crossbite side, than that detected in their left counterpart. Furthermore, the α7A and β1A isoforms, compared to the α7B and β1D isoforms, respectively, were predominant in both masseters.

Our previous report, evaluating the muscular activation during chewing in unilateral posterior crossbite patients, showed that the kinematics and electromyography characteristics of the masseters of the non-affected side were similar to those of controls, whereas the masseters of the crossbite side were less active (34) meaning that patients with unilateral posterior crossbite show a serious functional asymmetry during chewing (33).

We thus hypothesized that the decreased functional activity of the masseter of the crossbite side can be strongly related to the behavior of integrins. It is well established that integrins play a crucial role in cell adhesion, differentiation, remodelling and programmed cell death (10,40). In particular, it was demonstrated that the β1D isoform can be associated with α7A and α7B in adult skeletal muscle, appearing immediately after myoblast fusion and continuing to rise during myotube growth and maturation (12). In this way, β1D integrin plays a key role in linking the sarcolemmal cytoskeleton to the surrounding extracellular matrix in muscle tissue (12,19). The β1A isoform is involved in signaling (41-44) and it not detected in adult skeletal muscle fibers by immunofluorescence (45). Our preliminary results demonstrate that the β1A isoform is clearly detectable in adult masseter muscle and this could be due to the particular composition of masseter.

Indeed, masseter muscle, compared with limb and trunk muscles, is highly unusual; in fact, in addition to normal slow and fast fibers, type I and type II respectively, this muscle contains fiber types which are typical for developing or cardiac muscle.

Moreover, many fibers of the masseter are hybrid; these fiber types, in limb and trunk muscles, are thought to be those that are in transition from one fiber type into another, since they are predominantly found during disuse or during extreme usage of the muscles (46) or in regenerating fibers (47). Hybrid fibers are abundantly present in normal jaw-closing muscle, both in rabbit (48), and in humans (49-51). The important presence of hybrid fibers in considerable numbers, may be due to specific functional demands of the masseter muscle; these fiber types probably increase the capacity of the masseter muscle to generate a large variety of motor tasks, since they have contractile features which lie between those of pure fibers.

In this way, the presence of hybrid fibers could reflect the adaptive ability of masseter muscle fibers, showing the capacity to modify their contractile properties to optimize the efficiency during contraction. Then, the masseter muscle could continuously switch from one fiber type to another. Based on its functional demand, in our opinion, these continuous changes in phenotypic structures could also influence normal arrangement of the entire muscle, and in masseter muscle this could provoke a rearrangement of integrin network with consequential increase of the β1A isoform.

Another possible explanation for the different arrangement of the integrin network in masseter muscle, observed in the present study, also could be due to evidence that, the membranes of normal muscle fibers are ruptured by stretch and relaxation (52), with stimulation of satellite cells to repair the damage. Thus, since masseter muscle is subject to continuous mechanical stress, as a result of continuous control of the position and motion of the mandible and creation of forces at the teeth and temporomandibular joint (53), this muscle is characterized by a particular high turnover, which produces a regeneration process initiating by fibers typical for developing muscle.

A further remarkable feature is the relationship between fiber size and fiber type in masseter muscle; fast type fibers of masseter muscle have a smaller cross-sectional area than the slow type fibers whereas in the limb and trunk muscles, the reverse is true (54). This characteristic facilitates an increase in the exchange of O_2, improving the resistance to fatigue so that it is most advantageous for mastication (55). The continued expression of these fiber types in adult masseter muscle might indicate a longer time to heal, and this could justify the loss of β1D and the consequential increased amount of β1A integrin showed by the present data.

Furthermore, interesting features of integrins allow us to propose an intriguing hypothesis on masseter muscle in patients with unilateral posterior crossbite. Previous reports have found that α-actinin, a focal adhesion component that interacts with β1A (56) reinforcing links between actin filaments, binds β1D less strongly than β1A (12). This feature correlates with the absence of α-actinin at the myotendinous junction, the major sites of force transmission in muscle (57), suggesting that β1D may be less effective than β1A with regard to integrin-mediated signaling (45). Therefore, based on our results, we could hypothesize that the lower activity of masseter on the crossbite side may cause a loss of β1D integrin and that the β1A isoform may have a role in reinforcing the arrangement of the muscle fibers and in recovering signaling role of the entire membrane in order to restore force transmission.

Previously, a similar behavior of the integrin network was demonstrated analyzing human gastrocnemius muscle of subjects affected by sensitive-motor polyneuropathy (26,58). During this muscular inactivity it is possible to hypothesize that a reorganization of the transmembrane occurred, maintaining the viability of the skeletal muscle fibers (58).

The present data provide the first suggestion that integrins in masseter muscle play a key role in regulating muscular functional activity and allowing the optimization of the contractile forces of this muscle. Therefore, these results reveal a new venue of research, which will have the aim to understand the differences of the protein composition and structural arrangement of masseter muscle fibers in respect to other muscle fibers. Thus, it is intriguing to examine whether other proteins,

such as sarcoglycans, are involved in this different protein arrangement of masseter muscle fibers.

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