Desmin and Vimentin Expression during Embryonic Development of Tensor Veli Palatini Muscle in Mice

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Abstract: During embryonic palatogenesis, development of the secondary palate represents a particularly complex process involving the formation, elevation and midline fusion of the palatal shelves. In recent years, it has been shown that the specific expression of type I collagen and periostin in the palatal aponeurosis results in formation of the soft palate. However, few reports have examined soft palate development in relation to the skeletal muscles connected to the palatal aponeurosis. Thus, in the present study, focusing on the tensor veli palatine muscle and surrounding tissues in embryonic mice, we performed immunohistochemical examination and real-time PCR (RT-PCR) in order to investigate the expression of desmin and vimentin. Low levels of desmin expression were observed in the tensor veli palatine muscle on embryonic day (ED) 12.5, with the levels of expression increasing from ED 13.5 to 15.5. In addition at ED 12.5, desmin was observed to accumulate in the area of the myotendinous junction, and this accumulation remained unchanged up to ED 15.5. Meanwhile, vimentin expression was observed in the tensor veli palatine muscle and surrounding tissue at ED 12.5, and this level of expression did not change up to ED 15.5. Strong expression of vimentin was observed at ED 14.5 only in the medial edge epithelium (MEE). Both immunohistochemical examination and RT-PCR yielded consistent results. In the present study, we found that desmin accumulates in the vicinity of the myotendinous junction at ED 12.5, which is prior to initiation of swallowing movement. From this, we are able to conclude that this accumulation of desmin is caused by factors other than mechanical stress resulting from initial muscle contraction. Furthermore, the elevated expression of vimentin during embryogenesis, and that of desmin after differentiation, suggest that there may be some interaction between the two intermediate filaments in determining muscle cell differentiation.

Key words: Secondary palate, Desmin, Vimentin, Embryo, Mouse

Introduction

Development of the mammalian secondary palate represents a complex process, involving the formation, elevation and midline fusion of palatal shelves1). To date, numerous studies have used mouse models to investigate secondary palate development. In mice, palatal shelves are thought to start as outgrowths of the medial maxillary prominence at ED 11. These palatal shelves then grow vertically, flanking the developing tongue between ED 12.5 and 13.5. At ED 14, the elevated left and right palatal shelves reorient horizontally and, at ED 14.5, meet at the midline and begin fusing2,3). Finally, at ED 15.5, the midline edge epithelial seam (MES) formed by fusion of the palatal shelves disappears from the secondary palate, marking completion of palate fusion4).

Research utilizing knock-out mouse models to study the processes involved in secondary palate development in greater detail has elucidated the role of numerous genes contributing to palatogenesis5,6). For example, it has been demonstrated that mesenchymal cells of the developing secondary palate express not only growth factors such as BMP and FGF, but also transcription factors including MSX1, Shox2 and Tbx22,3)1). It is further known that the TGF-β signaling pathway contributes to fusion of palatal shelves12,13). This TGF-β signal has been shown to stimulate expression of type I collagen and periostin in the extracellular matrix14-17). It has also been elucidated that expression of type I collagen and periostin in the palatal aponeurosis promotes formation of the soft palate18). However, there are few reports and, consequently, substantial gaps in knowledge regarding the skeletal muscles and surrounding tissue connected to the palatal aponeurosis during soft palate formation.
In recent years, it has been shown that expression of desmin, an intermediate filament, plays a significant role during the maturation of skeletal muscles. Desmin is a muscle-specific intermediate filament that putatively links microfibrils at the Z-band level and connects microfibrils to the sarcolemma. As such, desmin is currently used as a specific marker for muscle development. It has also been shown that the level of desmin expression increases with muscle development.

In addition, it has been reported that, in embryonic stages, desmin accumulates earlier in areas of skeletal and cardiac muscle that are subjected to mechanical stress. Vimentin, which is an intermediate filament-like desmin, coexists with desmin in skeletal muscles. It has been reported that vimentin is important in maintaining the stability of mesenchymal cells. As such, desmin is currently used as a specific marker for muscle development. It has also been shown that the level of desmin expression increases with muscle development.

In the present study, we performed morphological observations in embryonic mice focusing on the tensor veli palatini muscle and surrounding tissues connected to the palatal aponeurosis. At the same time, we investigated the expression of desmin, which is a specific marker for muscle development, and vimentin, which is necessary for the stabilization of mesenchymal cells.

Materials and Methods

ICR mice at ED 12.5, 13.5, 14, 14.5 and 15.5 were used in this study. Tests were conducted in accordance with the Guidelines for Animal Experiments at Tokyo Dental College (No. 250109). A female mouse was housed with a male overnight, and noon of the day when the vaginal plug was observed was designated as ED 0.5. Ten fetuses at each embryonic stage were utilized, giving a total of 50 specimens. Fetal mice were obtained from mother mice that had been euthanatized using diethyl ether. Fetal tissues were then fixed in 4% phosphate-buffered paraformaldehyde. Paraffin blocks were prepared using standard methods, and a series of 5- to 10-µm tissue sections were cut using a sliding microtome. Frontal sections were prepared, and were then stained with hematoxylin and eosin (H&E) to enable morphological observation of the tensor veli palatini muscle and surrounding tissue.

Immunohistochemical analysis

Table 1. Base Sequence of the Primers used (Desmin, Vimentin, GAPDH)

<table>
<thead>
<tr>
<th>Gene (accession no.)</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>Desmin (NM_010043.1)</td>
<td>Forward 5'-GCCGTGACAACCTGATAGACG -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGTGATTTCTCCCTGAGTATTTTGA -3'</td>
</tr>
<tr>
<td>Vimentin (NM_009115.3)</td>
<td>Forward 5'-AAACAGGCTCTCTCCTCCTC -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTCCATCACTTTGTCACCACCA -3'</td>
</tr>
<tr>
<td>GAPDH (NM_008085.1)</td>
<td>Forward 5'-CCTTGAGATCAACACGTACCAG -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CGCCTGTACACTCCACCAA -3'</td>
</tr>
</tbody>
</table>

Figure 1. Cells collected from the tensor veli palatini muscle on ED 15.5 by laser microdissection. Before laser microdissection (Panel A). After laser microdissection (Panel B). Panels A and B show samples stained with eosin, and all are frontal sections. The left-hand sides of the images represent the exterior of the head, and the tops of the images represent the front of the head (Panels A and B). MPP: medial pterygoid plate; OC: oral cavity; SP: sphenoid bone; TVP: tensor veli palatini muscle. Bar=200 µm
Figure 2. Hematoxylin and eosin-stained sections showing development of tensor veli palatini muscle (TVP). All panels show frontal sections. The left-hand sides of the images represent the exterior of the head, and the tops of the images represent the front of the head. Panels A, B, C, D and E show samples on ED 12.5, 13.5, 14, 14.5 and 15.5, respectively.

On ED 12.5, the developing tensor veli palatine muscle could be seen medially from the medial pterygoid muscle. On embryonic day 13.5, the medial pterygoid plate appears as an undifferentiated mass of mesenchymal cells, and the tensor veli palatine muscle can be seen between the medial pterygoid muscle and the medial pterygoid plate. On ED 14, the palatal shelves had reached the height at which horizontal outgrowth is initiated. On ED 14.5, the palatal shelf has risen to the horizontal outgrowth point, and the tensor veli palatine muscle has moved inward. On ED 15.5, the medial pterygoid plate begins full-fledged calcification, and the inferiorly located tensor veli palatine muscle begins developing medially.

M: medial pterigoid muscle; MPP: medial pterygoid plate; MC: Meckel cartilage; OC: oral cavity; PS: palatal shelf; T: tongue; TGG: trigeminal ganglion; TVP: tensor veli palatini muscle. Bar=200 µm

In order to analyze the expression of protein in the samples, immunolocalization of desmin and vimentin was investigated. Antigen retrieval was performed by immersing the slides in citric acid buffer (pH 6.0) and placing them in a pressure chamber (SP1 125 °C, 30 s, SP2 10 s; Dako, Glostrup, Denmark). Sections were treated with 4 % normal goat serum in order to prevent nonspecific reactions for 30 s. Sections were treated with primary antibody against vimentin (dilution, 1:1000; Abcam, Cambridge, UK) or desmin (dilution, 1:1000; EPITOMICS, Burlingame, CA), and were incubated at 4 °C overnight. Secondary antibody was then applied using Alexa Fluor 488 (dilution, 1:1000; Invitrogen, Carlsbad, CA) for 60 s at room temperature. Sections were examined using an Axiohot 2 polarizing light microscope (Carl Zeiss Micromaging GmbH, Oberkochen, Germany).

**Laser microdissection (LMD)**
We selectively harvested the tensor veli palatini muscle tissue by LMD and quantified its mRNA expression. Fetal mice were obtained from mother mice that had been euthanatized using diethyl ether. Fetal mice were perpendicularly embedded in SCEM (Leica Microsystems Japan, Tokyo, Japan) and then immediately fast-frozen in isopentane cooled in liquid nitrogen. Specimens were then made into frozen blocks and stored at -80 °C. Frozen samples prepared for LMD were sliced using a Leica CM 3000 cryostat (Leica Microsystems, Wetzlar, Germany) at 8-μm thickness. Samples were mounted onto membrane slides (1 mm PEN; PALM) for LMD, immersed in eosin for 30 to 40 s at room temperature, and rinsed for 30 s with RNas-free water at 4°C. Thereafter, slides were dried for 5 to 10 min at 4°C. We selectively removed tensor veli palatini muscle tissue using a PALM MB-III LMD system (Microlaser Technologies, Bemried, Germany) (Fig. 1).

Following LMD, total RNA was extracted from the harvested samples using an RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA quality and size distribution were analyzed using an Agilent 2100 Bioanalyzer with the RNA 6000 Pico LabChip kit (Agilent Technologies, Carlsbad, CA). Finally, cDNA was synthesized from mRNA using a QuantiTect Reverse Transcription kit (Qiagen).

After determination of optimal PCR conditions for all primers, RNA quantification was performed using a LightCycler™ (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer’s instructions. Ready-to-use Light Cycler Taqman
Figure 4. Expression of vimentin protein in the tensor veli palatini muscle. Panel A represents a hematoxylin and eosin-stained sample on ED 14.5. Panels B, C, D and E show vimentin protein expression on ED 12.5, 13.5, 14.5 and 15.5, respectively. All panels show frontal sections. The left-hand sides of the images represent the exterior of the head, and the tops of the images represent the head. On ED 12.5, vimentin is expressed not only in the tensor veli palatine muscle but in all surrounding tissues, including the palatal shelf. This distribution remained unchanged until ED 15.5.

M: medial pterigoid muscle; MPP: medial pterygoid plate; OC: oral cavity; PS: palatal shelf; TVP: tensor veli palatini muscle.

Master (Roche Diagnostics) was used as a hot-start PCR mixture. Aliquots of cDNA (4.0 ng/µl) at dilutions of 1:10, 1:10², 1:10³, 1:10⁴ and 1:10⁵ were used. Each sample was added to 10.0 µl of sterile water, 5.0 µl of diluted control cDNA product, 4.0 µl of LightCycler Taqman Master, 0.2 µl of Universal Probe Library probe and 0.4 µl of forward and reverse primer designed using Probe Finder software (Roche Diagnostics) to give a final reaction volume of 20 µl. Primers based on sequences of the desmin and vimentin genes were designed from specific segments of the entire DNA sequence. Specific primers used in this analysis are shown in Table 1. PCR mixtures (20.0 µl each) prepared for the desmin and vimentin genes were added to glass capillaries. PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, an annealing step at 60°C for 10 s, and an extension step at 72 C for 8 s. Gene amplification was performed according to a melting program of 70°C for 15 s, and fluorescence was monitored continuously at a rate of 0.1°C/s. Final expression levels of desmin and vimentin genes were obtained by normalization against the expression of a housekeeping gene, GAPDH. Quantitative PCR results for desmin and vimentin are shown at ED 12.5, 13.5, 14.5 and 15.5.

**Statistical comparison**

Statistical comparisons were made using one-way analysis of variance (ANOVA). Tukey’s multiple comparison test was used for further comparisons between occlusal areas (p<0.05), using...
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the SPSS® software program (SPSS Japan, Inc., Tokyo, Japan).

Results

Morphological examination of the tensor veli palatine muscle and surrounding tissue

At ED 12.5, the immature tensor veli palatine muscle could be seen just medially to the medial pterygoid muscle (Fig. 2A). By ED 13.5, the tensor veli palatine muscle had moved between the medial pterygoid muscle and the medial pterygoid plate, which appeared as an undifferentiated mass of mesenchymal cells (Fig. 2B). By ED 14, the palatal shelves had reached the height at which horizontal outgrowth is initiated (Fig. 2C). By ED 14.5, when the palatal shelves had reached the horizontal growth height, the tensor veli palatine muscle had moved further inward (Fig. 2D). By ED 15.5, the medial pterygoid plate had begun to undergo full-fledged calcification (Fig. 2E) and the inferiorly located tensor veli palatine muscle had developed medially when compared to ED 14.5.

Immunohistochemistry

On ED 12.5, desmin was weakly expressed across the entire tensor veli palatine muscle (Fig. 3B). Desmin expression spread to all muscle fibers at ED 13.5, 14.5 and 15.5, as the tensor veli palatine muscle continued to develop (Fig. 3C, D, E). In addition, at ED 12.5, desmin was observed to accumulate in the area of the tensor veli palatine muscle destined to become the myotendinous junction. This accumulation did not change at ED 13.5, 14.5 and 15.5 (Fig. 3C, D, E). Meanwhile, on ED 12.5, vimentin was expressed not only in the tensor veli palatine muscle but in all surrounding tissues, including the palatal shelf (Fig. 4B). This distribution did not appear to change on ED 13.5, 14.5 and 15.5. Furthermore, vimentin was observed to be strongly expressed in the MEE, immediately prior to palatal shelf fusion on ED 14.5 (Fig. 5B).

Quantitative polymerase chain reaction

Quantitative PCR data provided greater quantitative estimation than immunohistochemical data. On ED 12.5, mRNA coding for desmin was weakly expressed. Levels of expression of this desmin-coding mRNA increased significantly between ED 12.5 and 15.5 and between ED 13.5 and 15.5 and between ED 14.5 and 15.5 (Fig. 6). Meanwhile, levels of mRNA expression for vimentin did not change between ED 12.5 and 15.5 (Fig. 7).

Discussion

Development of the secondary palate entails complex processes related to the formation, elevation, and midline fusion of palatal shelves. In recent years, a variety of genes contributing to development of the secondary palate have been elucidated. Yet, much remains uncertain regarding the tensor veli palatine muscle, which is known to contribute to the formation of the soft palate.

While there have been a small number of reports related to the embryonic tensor veli palatine muscle to date, the majority of these are morphological investigations. The tensor veli palatine muscle has been observed in human fetuses with a crown-rump length (CRL) of 20 to 22 mm, prior to the development of the medial pterygoid plate, as an agglomeration of undifferentiated mesenchymal cells. Furthermore, it has been reported that, in embryos with CRL of 14.5 mm (6-week-old fetus), the undifferentiated tensor veli palatine muscle lies proximal to the medial pterygoid muscle and that both the tensor veli palatine and the medial pterygoid muscles differentiate from the same blastema. It has also been confirmed that the tensor veli palatine muscle is attached to the primordium of the palatal aponeurosis...
in 8-week-old fetuses, prior to fusion of the palatal shelves\(^{13}\). In the present study, we observed that the mouse tensor veli palatine muscle is located medially to the medial pterygoid muscle during the initial stages of development. At that point, however, the medial pterygoid plate was not yet visible. These results suggest that, as in the case of humans, the tensor veli palatine and medial pterygoid muscles in mice differentiate from the same blastema. Furthermore, our observation that the tensor veli palatine muscle is present prior to development of the medial pterygoid plate is consistent with the results of research on human embryos. However, in the present study, we were unable to see the mouse palatal aponeurosis, even immediately after fusion of the palatal shelves. As such, it was found that palatal aponeurosis development relative to palate fusion occurs later in mice than humans.

The mesenchyme of the craniofacial region contains two cell types originating from different lineages: myogenic cells derived from paraxial mesoderm and osteogenic or fibrogenic cells derived from neural crest cells. In the soft palate region, two different cell lineages make up the mesenchyme structure: Mesoderm-derived cells differentiate into palatine muscles such as the tensor and levator veli palatini and compose the skeletal muscle structures along with connective tissues derived from cranial neural crest (CNC) cells. In recent years, It has also been elucidated that expression of type I collagen and periostin in the palatal aponeurosis promotes formation of the soft palate on ED 16.5 in mice. Furthermore, TGF-β signals induced the expression of both periostin and type I collagen, while CNC cells induced further expression of periostin and type I collagen to form palatine aponeurosis\(^{19}\). In the present study, we observed the tensor veli palatine muscle move inward as the palatal shelves elevated. This suggests that development of the secondary palate creates a fusion of separately formed tensor veli palatine muscle and palatine aponeurosis. Moreover, in our present investigation at ED 12.5 desmin was observed to accumulate in the area of the myotendinous junction, and this accumulation remained unchanged up to ED 15.5. That is to say, it appears that desmin accumulation in the area of the myotendinous junction is involved in the fusion of separately formed tensor veli palatine muscle and palatine aponeurosis.

It is believed that the mechanism of palatal shelf elevation differs between the anterior and posterior parts of the secondary palate. The anterior palatal shelves are reported to elevate themselves by ‘flipping-up,’ while the posterior palatal shelves are elevated as a result of remodeling, which involves the swelling of the medial wall and regression of the distal edge of palatal shelves\(^{34,2}\). In the present study, we observed the tensor veli palatine muscle move inward as the palatal shelves elevated. Thus, we speculate that the remodeling of the palatal shelves of the posterior secondary palate resulting from the swelling of medial walls causes the tensor veli palatine muscle to move inward.

In addition, in the present immunohistochemical examination, an accumulation of desmin in the area of the tensor veli palatine muscle destined to become the myotendinous junction was observed at ED 12.5. Previously, it was believed that the increased expression of desmin and other intermediate filaments was a pathological or physiological response to mechanical or osmotic stress\(^{35}\). It has been suggested that desmin accumulates in the area of the myotendinous junction as a result of mechanical stress occurring during initial muscle contraction\(^{24}\). However, based on the recent report that jaw movements can clearly be seen in embryonic mice on ED 15.5\(^{36}\), it would be reasonable to assume that jaw movements had not yet begun at the point when we observed desmin accumulation on ED 12.5. It would be similarly unlikely that swallowing movement had begun at this point. Furthermore, given that the tensor veli palatine muscle has been shown to assist swallowing movement\(^{37}\), we consider that the
tensor veli palatine muscle had not started functioning at this point. In other words, these observations suggest that the accumulation of desmin at the myotendinous junction is due to something other than mechanical stress.

It has recently been reported that vimentin is strongly expressed in the MEE immediately prior to the fusion of palatal shelves\(^7\). Conversely, Montenegro et al.\(^6\) demonstrated that the level of vimentin expression in the palatal shelves does not change substantially immediately prior to fusion. In the present study, we observed substantial accumulation of vimentin in the MEE immediately prior to fusion. However, given the extreme rapidity of the palatal fusion process, it was difficult to observe the change in vimentin expression. Lacking the ability to monitor this rapid change in vimentin expression in the MEE, it appears as if vimentin expression does not change before and after fusion.

In the early stages of development, the surrounding mesenchymal tissues expressing vimentin form a scaffold for the developing tensor veli palatine muscle. Muscle attachment at ED12 showed steady desmin expression, which was maintained until maturity. The elevated expression of vimentin during embryogenesis, and that of desmin after differentiation, suggest that there may be some interaction between the two intermediate filaments in determining muscle cell differentiation. Furthermore, the diverse expression of both vimentin and desmin in terms of location and time reflects the maturation of muscle fiber attachments.\(^6\)

Acknowledgments

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References