Functional Histology of the Macula Flava in the Human Vocal Fold – Part 2: Its Role in the Growth and Development of the Vocal Fold

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Abstract

Objective: This study aims to clarify the role of the maculae flavae (MFe) during growth and development of the human vocal fold mucosa (VFM).

Methods: Our current results concerning the MFe in the human newborn, infant, and child VFM are summarized.

Results: Newborns already had immature MFe at the same sites as adults. They were composed of dense masses of vocal fold stellate cells (VFSCs), whereas extracellular matrix components were sparse. VFSCs in the newborn MFe had already started synthesizing extracellular matrices (EM). During infancy, the EM synthesized in the MFe appeared in the VFM to initiate the formation of the three-dimensional extracellular matrix structure of the human VFM. During childhood, MFe including VFSCs continued to synthesize EM such as collagenous, reticular, and elastic fibers, and hyaluronic acid (glycosaminoglycan), which are essential for the human VFM as a vibrating tissue. The MFe in newborns, infants and children were related to the growth and development of the human VFM.

Conclusion: Human MFe including VFSCs were inferred to be involved in the metabolism of EM, essential for the viscoelasticity of the human VFM, and are considered to be an important structure in the growth and development of the human VFM.

Introduction

In adults, the vocal fold (VF) has a layered structure consisting of epithelium, lamina propria (superficial, intermediate and deep layer) and the vocalis muscle [1, 2]. The superficial layer is referred to as Reinke’s space. The structure consisting of the intermediate and deep layers of the lamina propria is called the vocal ligament (fig. 1). It runs between the anterior and posterior maculae flavae (MFe, fig. 1). The mucosal portion of the layered structure is based on the differences of extracellular matrix distribution; it is essential for VF vibration and phonation [1].

The anterior MFe of the VF have already been described as the nodulus elasticus in the anatomic textbook by Lanz and Wachsmuth [3]. They said that the MFe were elastic nodules located at the anterior end of the VF with numerous elastic fibers [3]. Hirano [1] noted the MFe to be masses of dense elastic fibers at the anterior and posterior ends of the VF, referring to the former as the anterior macula flava (nodulus elasticus) and the latter as the posterior macula flava. The histological structure of the MFe in the human vocal fold mucosa (VFM) is unique, and their role in the VF as a vibrating tissue is interesting. However, their role in the human VFM has not been clarified until now [1–7].

During the past decade, we have investigated the morphology of the MFe in the human VFM [8–22]. Human adult MFe were found to be composed of dense masses of...
vocal fold stellate cells (VFSCs) and extracellular matrices (EM) such as collagenous, reticular, and elastic fibers, glycoprotein, and hyaluronic acid (glycosaminoglycan). These EM are essential for the human VFM as a vibrating tissue. The VFSCs in the human adult MFe form an independent cell category that should be considered a new category of cells in the human VF.

On the basis of the results of our current study, human MFe located at both ends of the VFM are inferred to be involved in the metabolism of EM, essential for the viscoelastic properties of the lamina propria of the human adult VFM [23]. Human adult MFe are inferred to be responsible for maintaining the characteristic layered structure of the human VFM [23]. Human adult MFe are also considered to be an important structure in the aging of the human VFM [23].

Human VF grows and develops, and its layered structure matures during adolescence [24, 25]. The purpose of the present paper is to summarize the results of our current morphological investigations into the MFe during growth and development of the human VF.

Fine Structure of Newborn MFe and VFM (fig. 2a) [18–20]

The structures of the newborn VFM are immature and differ from those of adults. In newborn VF, the entire lamina propria appears as a uniform structure with no vocal ligament, no Reinke’s space or no layered structure [2, 18–20]. The lamina propria of the newborn VFM is a loose structure composed of ground substances and sparse fibers. Ground substance is present and glycoproteins (fibronectin) are abundant in the lamina propria [19]. The EM structure of the newborn VFM is incomplete.

Newborns have MFe at the same sites as adult VFM, but they are immature. The MFe of the newborn VFM are formed by dense masses of VFSCs (fig. 3, 4) and are situated bilaterally at the anterior and posterior ends of the newborn VFM. They are round in shape and measure approximately 1 × 1 × 1 mm. In comparison with adult MFe, the relative size is almost the same in both age groups. The density of cells in the MFe is about 5 times that in the adult MFe (fig. 5). On the other hand, fibroblasts are sparse in the lamina propria of the newborn VFM. The density of fibroblasts in the newborn lamina propria is about one eighth that of VFSCs in the newborn MFe (fig. 5). The MFe are composed of VFSCs, elastic fibers, reticular fibers, collagenous fibers, and ground substances. Cellular components are markedly abundant and fibrous components are sparse. More collagenous fibers are present than elastic fibers.

VFSCs in the newborn MFe are stellate or oval in shape, and possess cytoplasmic processes (fig. 6a). The newborn VFSCs are smaller in size than those of the adult, and some cells form gap junctions with each other (fig. 6a). A few lipid droplets are present in the cytoplasm but they are
much fewer than those found in adults. The lipid droplets are 0.6–0.7 μm in diameter, and are thus smaller than those of adults. The nucleus-cytoplasm ratio is high, and intracellular organelles, such as rough endoplasmic reticulum and Golgi apparatus, are not very well developed. Free ribosomes are well developed in the cytoplasm. Along the periphery of the cytoplasm of the newborn VFSCs that have developed intracellular organelles, a number of vesicles are present. Occasionally, a basal body (fetal rudiments) is noted in the cytoplasm. These findings indicate that the VFSCs in the newborn MFe are immature and possess some features of mesenchymal cells. The morphological findings of the newborn VFSCs mentioned above are recognized to various degrees.

In many types of tissue and cultured cells, the interiors of adjacent cells communicate with each other through cell-to-cell channels [26]. The fine structure of the cell-to-cell channel has been well studied and defined as a gap junction [26]. Cell communication is proposed to play an important role in cell growth and differentiation [26]. The VFSCs in the newborn MFe may communicate with each other for their growth and differentiation.

Fig. 3. Posterior macula flava (arrows) on coronal section of the human newborn VF. Cellular components are markedly abundant and fibrous proteins are sparse. TAM = Thyroarytenoid muscle. a Hematoxylin and eosin stain. b Elastica-van Gieson stain. Original ×40.

Fig. 4. Light microscopy of the macula flava of the human newborn VF. Arrows: VFSCs. a Hematoxylin and eosin stain. b Elastica-van Gieson stain. Original ×400.
There are many collagenous fibers around the VFSCs in the adult MFe in contrast with the newborn MFe (fig. 4b). Few reticular fibers can be detected around the newborn VFSCs. Electron microscopy indicates that the newborn VFSCs have started to synthesize collagenous and reticular fibers. Synthesis of collagenous and reticular fibers occurs in the same way as in the adult MFe.

There are some vesicles along the periphery of the cytoplasm, and newly released amorphous materials are present on the cell surface of newborn VFSCs (fig. 6b). Microfibrils 10–15 nm in width are observed around the amorphous material. Collagen fibrils are detected near the microfibrils (fig. 6b). Collagenous fibers are made up of several collagen fibrils. Thus, the VFSCs have started to
synthesize not only collagenous fibers, but also reticular fibers, in the human newborn MFe.

There are many elastic fibers around the VFSCs in adult MFe, whereas not very many elastic fibers are present around the VFSCs in the newborn MFe (fig. 4b). Electron microscopic study indicates that newborn VFSCs start to synthesize elastic fibers. Synthesis of elastic fibers is the same as in the adult MFe. There are some vesicles at the periphery of the cytoplasm, and newly released amorphous materials are seen on the cell surface of the VFSCs. Microfibrils 10–15 nm wide are situated around the amorphous material. There are microfibril assemblies on which elastin is deposited (fig. 6b). The amorphous substance of the elastic fibers is produced by fusion of microfibrils. The elastic fibers are composed of microfibrils and amorphous substances. The former are abundant, and the latter are sparse and reticular in shape, indicating that the elastic fibers are immature. The number of elastic fibers is small compared to that of collagenous fibers in the newborn MFe.

The ground substance around the newborn VFSCs is slightly stained light blue with Alcian blue at pH 2.5 and at pH 1. Glycosaminoglycan is situated around the VFSCs, but not abundant, in the human newborn MFe. The percentage of CD44-positive cells in the newborn MFe is not very high, and 34.5 ± 4.4% VFSCs are stained with CD44 [12]. About 55% of the fibroblasts in the newborn lamina propria are stained with CD44 [12]. In the newborn VFM, before the appearance and distribution of hyaluronic acid, CD44 is expressed in one third of the VFSCs in the MF and one half of the fibroblasts in Reinke’s space. This finding may be due to the fact that cells and EM of the newborn VFM are immature.

To summarize, newborns have MFe at the same sites of the VF as adults. Newborn MFe are formed by dense masses of VFSCs, whereas extracellular matrix components are sparse. The morphological characteristics of the newborn MFe are not completely the same as those of adults, but they are immature. The newborn VFSCs possess some features of mesenchymal cells. VFSCs in the newborn MFe have already started synthesizing EM, such as collagenous fibers, reticular fibers, elastic fibers, and glycosaminoglycan, essential for the viscoelastic properties of the human VFM, even though their structure is immature.

Fine Structure of Infant MFe and VFM (fig. 2b) [19]

In infancy, many reticular and collagenous fibers extend from the MFe toward the middle of the lamina propria, in which glycoprotein (fibronectin) is abundant, and reticular and collagenous fibers increase throughout the entire lamina propria of the infant VFM as compared to that of newborns, and run roughly parallel to the VF edge. Elastic fibers can be seen in the lamina propria of the infant VFM at low density, and are composed of abundant microfibrils and sparse and reticular amorphous substances. The elastic fibers are immature, but increase in amount over time in the lamina propria after collagenous and reticular fibers appear. No structure corresponding to the vocal ligament can be found.

Fibronectin is a glycoprotein that serves as a template for the oriented deposition of collagen [27]. It acts as an interfibrillar stabilizing factor between collagen fibrils and as a skeleton for elastic tissue formation and is involved in the aggregation of proteoglycans [27]. Reticular and collagenous fibers synthesized in infant MFe extend toward the middle of the membranous portion of the VF, in which fibronectin (glycoprotein) is abundant. Fibronectin in the lamina propria appears to direct the oriented deposition of reticular and collagenous fibers. Reticular and collagenous fiber formation may be induced in the lamina propria of infant VFM with growth. It decreases with the increase in fibrous components in the lamina propria of the VFM. Fibronectin acts as an interfibrillar stabilizing factor between collagen fibrils and is involved in the aggregation of elastic fibers and glycosaminoglycan. Fibronectin acts as a skeleton for elastic tissue formation in the human infant VFM.

The MFe of infant VFM are also dense masses of VFSCs. The density of VFSCs in the infant MFe is about 3 times that in the adult MFe and about two third that in the newborn MFe (fig. 5). On the other hand, fibroblasts are sparse in the lamina propria of infant VFM. The density of fibroblasts is about one sixth that of VFSCs in the infant MFe (fig. 5).

Infant MFe are composed of VFSCs, collagenous fibers, reticular fibers, elastic fibers, and ground substances. Cellular components are more abundant than fibrous components. However, fibrous components have increased by comparison with those of the newborn MFe. More reticular and collagenous fibers are present than elastic fibers.

Many VFSCs in the infant MFe are stellate in shape, possess cytoplasmic processes, and have a small nucleus-cytoplasm ratio. The rough endoplasmic reticulum and Golgi apparatus in the cytoplasm are developed. Newly released amorphous material is present on the cell surfaces. Collagenous, reticular, and elastic fibers are seen close to VFSCs.

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Role of Human Newborn, Infant, and Child Vocal Fold Macula Flava
The infant membranous portion of the VFM is stained light blue with Alcian blue at pH 2.5; in particular, the MFe are strongly stained. The material that stains with Alcian blue (pH 2.5) is digested by hyaluronidase. Hyaluronic acid appears in the VFM and much hyaluronic acid is produced around the VFSCs in the infant MFe. Most of the VFSCs in the infant MFe are stained with CD44. The percentage of CD44-positive cells in the infant macula flava becomes larger, and 87.2 ± 3.0% of the stellate cells are stained with CD44 [12]. However, CD44-positive fibroblasts become sparse (1.9 ± 2.0%) in the infant lamina propria of the VFM [12]. The expression of CD44 and the distribution of hyaluronic acid are the same as in the adult VFM. VFSCs and CD44 cooperatively start to play important roles in the maintenance of hyaluronic acid in the human VFM during infancy.

To summarize, during infancy, EM synthesized in the MFe appear in the lamina propria of the VFM between the anterior and posterior MFe at different times to initiate the formation of three-dimensional extracellular matrix structure of the VFM that has the viscoelastic properties of a vibrating structure.

**Fine Structure of Child MFe and VFM (fig. 2C) [21]**

There are collagenous, reticular and elastic fibers in the lamina propria of the child VFM. Vocal ligament and layered structure are not present in the lamina propria of the child VFM. The ground substance throughout the VFM is stained light blue with Alcian blue at pH 2.5. The material that stains in the lamina propria of the mucosa with Alcian blue (pH 2.5) is digested by hyaluronidase. Hyaluronic acid is present in the lamina propria of the child VFM.

MFe are located bilaterally at the anterior and posterior ends of the child VFM. They are approximately 1 × 1 × 1 mm in size and consist of dense masses of VFSCs. Many more VFSCs are distributed in the child MFe than in those of adults. The density of VFSCs in child MFe is about twice that in adult MFe, and about half that in newborn MFe (fig. 5). On the other hand, fibroblasts are sparse in the lamina propria of child VFM. The density of fibroblasts is about one fourth that of VFSCs in child MFe. The child’s MFe are composed of VFSCs, collagenous fibers, reticular fibers, elastic fibers, and ground substances. The fibrous components have increased by comparison with those of the infant MFe.

The VFSCs are stellate and possess cytoplasmic processes. A few lipid droplets are present in the cytoplasm, but they are much fewer and smaller (0.6–1 μm in diameter) than those found in adults. Vitamin A is stored in their cytoplasm. The nucleus-cytoplasm ratio is relatively small, and intracellular organelles, such as rough endoplasmic reticulum, are not very well developed. Free ribosomes are present in the cytoplasm. Along the periphery of the cytoplasm of the child’s VFSCs, vesicles are present.

There are many collagenous, reticular, elastic fibers around the VFSCs in the child MFe. Synthesis of these fibers occurs in the same way as in adult MFe. The electron microscopic study indicates that the VFSCs in the child MFe continue to constantly synthesize collagenous, reticular, and elastic fibers.

Hyaluronic acid is present in the lamina propria, in particular, in the MFe of the child VFM. Most of the VFSCs (94.7 ± 1.9%) in the child MFe are stained with CD44 [12]. Almost all of the VFSCs in the child MFe show CD44 expression and a large amount of hyaluronic acid is present immediately adjacent to VFSCs in the child MFe. On the other hand, CD44-positive fibroblasts are sparse (5.6 ± 3.0%) in the lamina propria of the child VFM [12]. These findings are the same as those in adults. The VFSCs in the MFe and CD44 both continue to play roles in the metabolism of hyaluronic acid in the human VFM during childhood.

To summarize, the child MFe are composed of dense VFSCs and EM. The morphological characteristics of the VFSCs in the child MFe are not completely the same as those of adults. However, the VFSCs in the child MFe have features characteristic of adult VFSCs and continue to constantly synthesize EM, essential for viscoelastic properties of the lamina propria of the human VFM as a vibrating tissue. The child MFe including VFSCs continue to synthesize EM and EM have increased. MFe have a role to play in the metabolism of EM of the VFM in the stage of VF growth and development. The VFSCs in the MFe are inferred to be involved in the growth and development of human VFM.

**Growth and Development of the Human VFM [22]**

Among mammals, only humans can speak and only human adult VF has a vocal ligament, Reinke’s space, and a layered structure [28]. Why do only human adults have such a characteristic VF structure? Why and how does newborn VFM grow, develop and mature? What are the factors for initiating and continuing the growth of the human VFM?

Tension is the most important factor which influences the synthesis of collagenous fibers by fibroblasts [29, 30]. The bending stresses on the VF associated with phonation are greatest in the region of the MFe [31]. We hypoth-
esize that the tension caused by phonation (VF vibration) after birth stimulates VFSCs in the anterior and posterior MFe to accelerate production of EM and form the vocal ligament, Reinke's space, and the layered structure [18, 19].

Human adult VFM that remains unphonated (nonvibrated) after birth is hypoplastic and rudimentary, and does not have a vocal ligament, Reinke's space or a layered structure [22]. The MFe are atrophic and VFSCs have decreased activity [22]. These results support the hypothesis that the tensions caused by phonation (VF vibration) after birth stimulate the VFSCs in the anterior and posterior MFe to accelerate production of EM and form the vocal ligament, Reinke's space and layered structure.

To summarize, VF vibration (phonation) after birth is one of the important factors in the growth and development of the human VFM. The tension caused by VF vibration stimulates VFSCs in the MFe, and it is inferred that the MFe are responsible for forming the characteristic layered structure of the human VFM.

**Conclusion**

Newborns already have immature MFe which are composed of dense masses of VFSCs at the same sites as in the adult. Their cellular components are markedly abundant and extracellular matrix components are sparse. VFSCs in the newborn MFe have already started the synthesis of EM.

During infancy, the EM synthesized in the MFe appear in the VFM to initiate the formation of three-dimensional EM structure of the human VFM.

In the child, MFe including VFSCs continue to synthesize EM such as collagenous, reticular, and elastic fibers, glycoprotein, and hyaluronic acid (glycosaminoglycan), which are essential for the human VFM as a vibrating tissue. The MFe in newborns, infants and children are connected with the growth and development of the human VFM.

On the basis of the results of our current study, human MFe located at both ends of the VFM are inferred to be involved in the metabolism of EM, which is essential for the viscoelastic properties of the lamina propria of the human VFM. Human newborn, infant, and child MFe are inferred to be responsible for forming the characteristic layered structure of the human VFM. Human MFe before adolescence are also considered to be an important structure in the growth and development of the human VFM.

Our current morphological results concerning the MFe during growth and development of the human VFM are summarized. More researches, especially using various methods, are needed to validate the significance and roles of the human MFe.

**References**


