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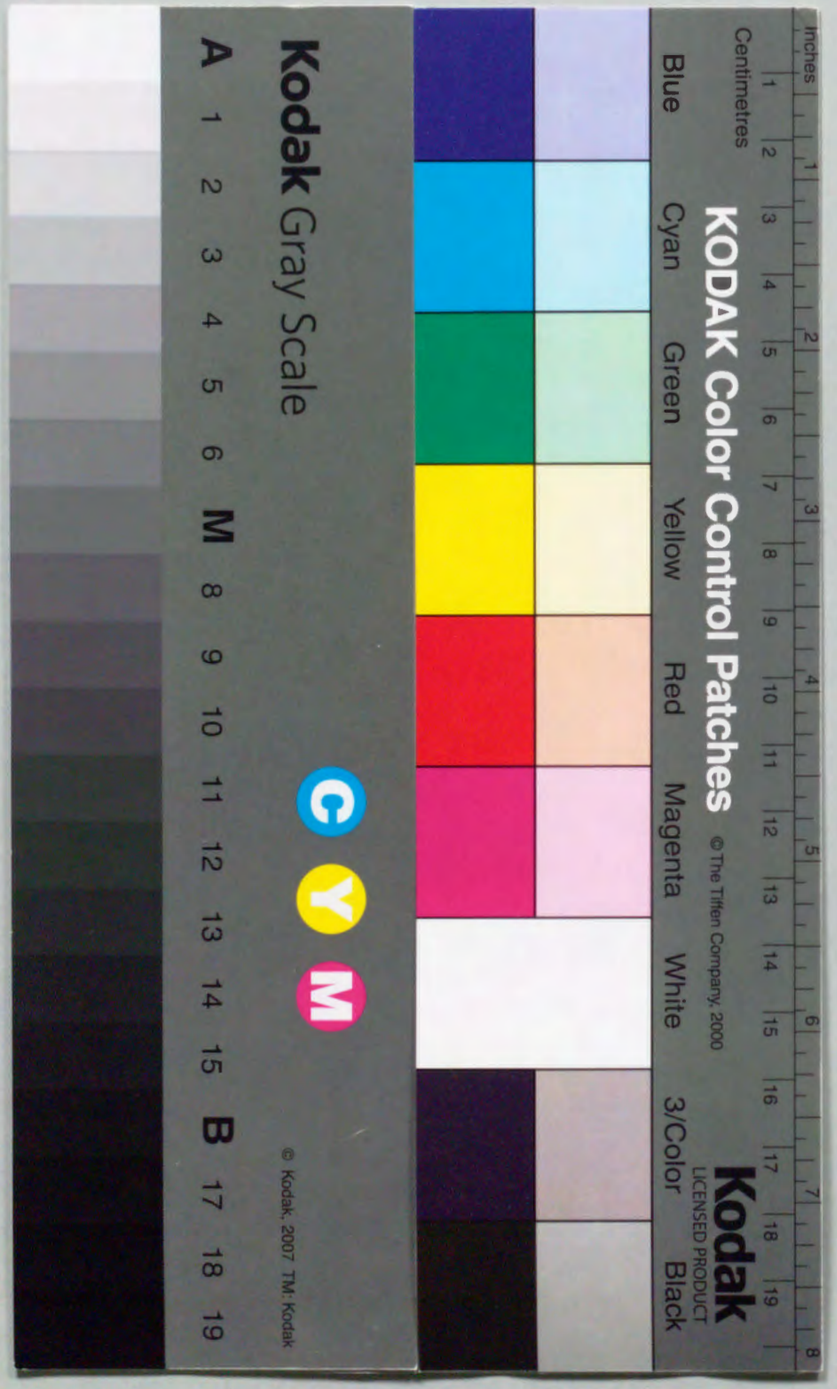

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ORIGINAL

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Abstract: The distribution of types I and III collagen and fibronectin was investigated in the antero-posterior middle portion of the palatal shelves before (day 14.0) and after (day 16.0) the shelf elevation of mouse fetuses by the electron immunogold technique using affinity purified polyclonal antibodies. In the area adjacent to the basement membrane, type I collagen was localized more densely in the nasal area than in the oral area on day 14.0 and on day 16.0. However, the distribution of type III collagen showed no significant difference between the nasal area and the oral area on either days. The ratios of type I to type III collagen varied among different areas of palatal mesenchyme both on day 14.0 and on day 16.0, namely, it was higher in the nasal area than in the oral area and, in the nasal area, it was higher on day 14.0 than on day 16.0, whereas in the oral area, there was no difference between either days. By the double immunostaining technique, it was revealed that types I and III collagen were often present in the same cross-banded fibrils. Fibronectin was localized between the surface of palatal mesenchymal cells and collagen fibrils, within collagen fibers and on filamentous structures in the extracellular space. Fibronectin was also localized within the basement membrane of the palatal epithelium, both in the lamina lucida and the lamina densa. The role of types I and III collagen and fibronectin in the palatal shelf elevation is discussed on the basis of the results obtained.

Introduction

During mammalian palatogenesis, the palatal shelves protrude from the maxillary processes and grow vertically along the tongue. In the mouse embryo, they reorient from a vertical to a horizontal position on day 14.0, adhering to each other, and the epithelial seam formed temporarily is disrupted on day 15.0, with each palatal mesenchyme being intermixed on day 16.0[1,2]. The elevation of the shelves is complex process involving the proliferation, migration and differentiation of cells and the accumulation of extracellular matrix including collagen, fibronectin and glycosaminoglycans[3-5]. It has been thought that the elevation of the palatal shelf occurs by rotation in the middle portion and by the remodeling of the tissue in the posterior portion of the shelf[6]. Although this rotation has been considered to be caused by an intrinsic shelf force[7], the origin of this force remains controversial.

The distribution of types I and III collagen in the palatal shelves has been studied using the immunofluorescence technique[2,8-10]. However, the ultrastructural relationship between these types of collagen in the palatal shelves has not yet been reported. It has been reported that in several tissues, type I collagen molecules typically form thick fibrils with a cross-banded pattern[11-14], while type III collagen molecules form thin fibrils without any cross-banded pattern[13,14]. Recently, however, type III collagen has been demonstrated to be present on cross-banded fibrils regardless of the fibril diameter in the human skin, tendon and amnion[15] and in the mouse periodontal ligament[16]. Therefore, it is significant for understanding the development of tissues including the palate to elucidate the ultrastructural interrelationship between type I and type III collagen and the ultrastructure of type III collagen fibrils.

Fibronectin is a glycoprotein which participates in a variety of interactions with other extracelular matrices or with cells. It has two major forms: a soluble dimeric form, and an insoluble dimeric or corss-linked multimeric form[17,18]. Although the immunofluorescent localization of fibronectin in the palatal shelves has been reported[7,10], the ultrastructural distribution of fibronectin in them has not been examined. There is a controversy as to the localization of fibronectin in the basement membrane[19-22]. It is possible that the distribution of fibronectin in the basement membrane differs among various tissues. It is well known that the medial edge epithelium (MEE) of the palatal shelf at which the shelves fuse to each other is different from that of the nasal or oral side of the shelf, which differentiates into ciliated columnar or stratified squamous epithelium, respectively[23-25]. The component of epithelial basement membrane participates in the epithelial-mesenchymal interaction in various tissues[26]. It is therefore interesting to localize the components of epithelial basement membrane, for example, fibronectin, in MEE and compare them with those in the nasal or oral side of the palatal shelf.

In the present study, in order to understand the role of types I and III collagen and fibronectin in shelf reorientation, we investigated the ultrastructural distribution of these components in the mesenchyme of the middle portion of mouse palatal shelves before (day 14.0) and after (day 16.0) elevation. We also investigated the localization of the fibronectin in the epithelial basement membrane on day 14.0 of the shelves in order to examine whether or not fibronectin participates in the epithelial-mesenchymal interaction in palatogenesis.

Materials and methods

Preparation of antigens and antibodies

Types I and III collagen were extracted from the skin of adult CF1 mice and were purified by DEAE-cellulose chromatography, as described by Chung and Miller[27]. Fibronectin was isolated from the blood plasma of adult mice by gelatin-Sepharose affinity chromatography[28]. Their purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The antisera for these three antigens were raised in rabbits. The antiserum for type III collagen was raised also in guinea pigs. The antibodies were purified by Sepharose affinity chromatography as described by Linsenmayer[29]. The cross-reactivity of these antibodies were excluded by the immunoadsorbent technigue[30]. For example, antibody to type I collagen was adsorbed by type III collagen and fibronectin to make it monospecific for type I collagen. The specificity of each antibody was verified by Western blotting. The affinity purified goat antibodies against rabbit IgG were purchased from Cappel Products (Westchester, PA) while those against guinea pig IgG were purchased from Chemicon International Inc. (Segundo, CA). These secondary antibodies were conjugated with colloidal gold as described by Slot and Geuze[31,32] as follows: Goat anti-rabbit IgG was labeled with ϕ6 nm or ϕ11 nm colloidal gold. Goat anti-guinea pig IgG was labeled with ϕ6 nm colloidal gold.

Preparation of tissue

Two CF1 female mice were mated in a cage with a male moues between 10:00 and 16:00. The presence of a vaginal plug was designated as day 0 of gestation at 13:00. The pregnant mice were killed by cervical dislocation on days 14.0 and 16.0 of gestation. Extirpated palatal shelves and palates were fixed at 4°C in a solution of 4% paraformaldehyde and 0.1% glutaraldehyde buffered to pH 7.4 with 0.1M sodium cacodylate buffer and dehydrated in a graded series of ethanol. They were then

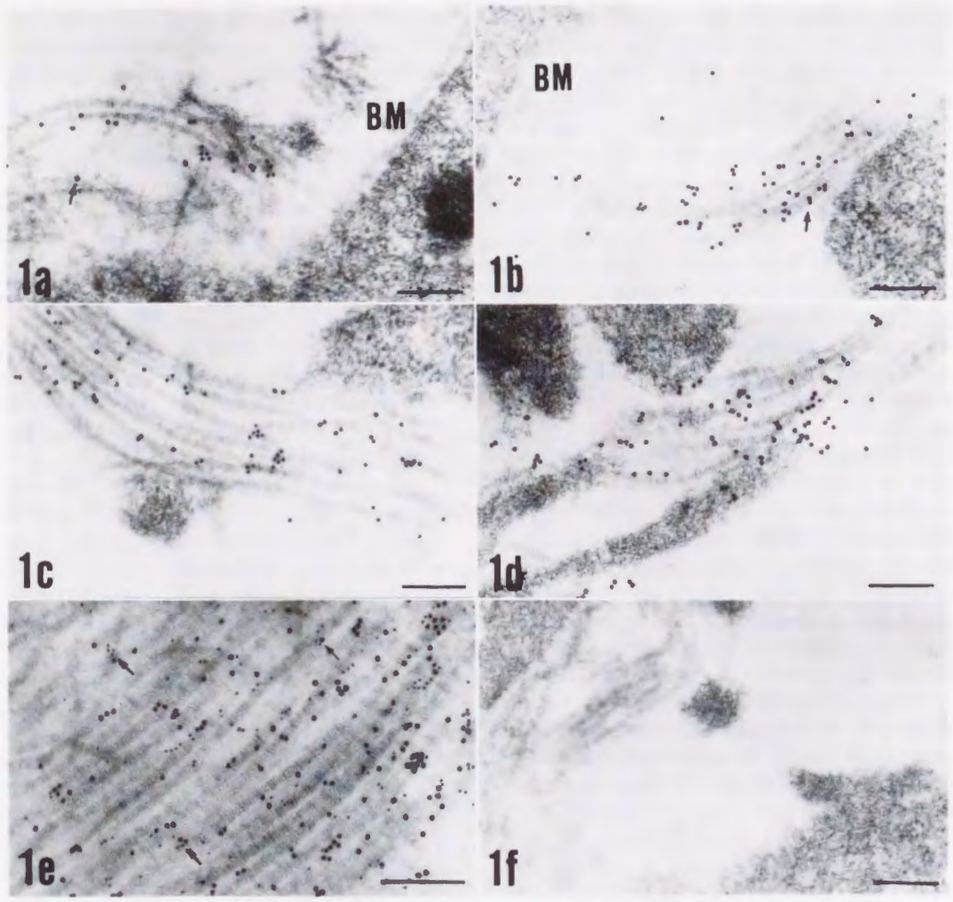


Fig. 1 a–f The distribution of type Ⅰ and type Ⅲ collagen in the palatal mesenchyme.
Nasal (a, c) and oral (b, d) areas in the palatal mesenchyme on day 14.0 are stained for type Ⅰ collagen (a, c) and type Ⅲ collagen (b, d). Cross-banded fibrils are positive both for type Ⅰ (c) and type Ⅲ (d) collagen. Arrows (a, b) show non-banded filaments. Nasal area of palatal mesenchyme on day 16.0 (e) is double-stained for both type Ⅰ collagen (ϕ 11 nm) and type Ⅲ collagen (ϕ 6 nm). Arrows show the fibrils stained simultaneously for type Ⅰ and type Ⅲ collagen. Control section (f) on day 14.0 incubated with normal rabbit serum in place of primary antibodies shows negative staining.
(a, b, c, d, f : ×55,000, e : ×70,000), BM : basement membrane, Bar = 200 nm.

embedded in Lowicryl K4M resin (Chemische Werk Lowi, Waldkraiburg, Germany) and polymerized with UV light at −20°C for 24 h and cured at 20°C for 24 h.

Immunogold staining

Ultrathin sections were cut and mounted on nickel grid mesh. The grids were floated on phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1 M lysine-HCl for 10 min, then incubated with each specific antibody for 1 h. On some sections, double immunostaining was employed as follows: The sections were treated with a mixture of rabbit anti-type Ⅰ and guinea pig anti-type Ⅲ collagen. After extensive rising with PBS, they were incubated with a mixture of colloidal gold (ϕ 11 nm)-labeled anti-rabbit IgG and colloidal gold (ϕ 6 nm)-labeled anti-guinea pig IgG. After being washed for 15 min with PBS, the sections were incubated for 30 min with a solution of secondary antibodies diluted (1 : 500) in PBS containing 1% BSA. They were finally washed with PBS followed by distilled water. All steps were carried out in a moist chamber at room temperature to prevent any drying up of the sections. After these steps, the sections were stained with uranyl acetate.

Quantitative analysis

For the quantitative evaluation of the distribution of collagen in the middle palatal shelves, gold particles were counted in the extracellular matrix of the following three areas. These were the nasal and oral areas comprising subepithelial mesenchyme of the basal two thirds of the shelf, to a depth of about 5 μm from the basement membrane of the nasal or oral epithelium, respectively, plus the core area which was mesenchyme beneath the subepithelial areas described above. The areas of extracellular matrix were measured by a Nikon Cosmozone. For the examination of the distribution of fibronectin within the epithelial basement membrane of the palatal shelves, the shortest distance between the outer leaflet of the plasma membrane and the center of each gold particle was measured in the areas where the plasma membrane was cut perpendicularly.

Table 1 The density of gold particles for collagens in different areas of the mouse palatal mesenchyme on day 14.0 and day 16.0

		observed areas			
		nasal (n)	oral (n)	core (n)	Background (n)
day 14.0	Type Ⅰ	24.6± 3.6^{a,b}(25)	15.8± 1.6^{a,c}(25)	5.8±0.6^{b,c}(25)	1.2±0.2 (10)
	Type Ⅲ	18.6± 2.2^{d,f}(16)	22.1± 3.0^{e,f}(16)	6.7±1.0^{d,e}(16)	1.4±0.2 (10)
day 16.0	Type Ⅰ	147.8±13.8^{g} (26)	96.0±12.8^{g} (21)	N.E.	1.6±0.2 (10)
	Type Ⅲ	122.0±16.5^{h} (26)	105.6±16.0^{h} (21)	N.E.	3.9±0.3 (10)

^{a,g} : $p<0.05$, ^{b,c,d,e} : $p<0.01$, ^{f,h} : $p<0.05$, N.E. : not examined (mean±S.E./10 μm^2)

Table 2 The ratio of type Ⅰ to type Ⅲ collagen in different areas of the mouse palatal mesenchyme on day 14.0 and day 16.0

	observed areas	
	nasal (n)	oral (n)
day 14.0	1.72±0.15^{a,c}(45)	1.05±0.08^{a,d}(52)
day 16.0	1.48±0.09^{b,c}(27)	1.08±0.08^{b,d}(16)

^{a} : $p<0.01$, ^{b} : $p<0.05$, ^{c,d} : $p<0.05$
(mean±S.E.)

Results

Collagen

In the palatal shelves of day 14.0 fetuses, the fibrils and filaments positive for type Ⅰ and type Ⅲ collagen were distributed much more densely in the mesenchyme adjacent to the epithelial basement membrane than in the core area. Labeling particles for type Ⅰ collagen were found on the fibrils with characteristic 64 nm cross-banded periodicity which were 25.3±3.0 nm (n=75) in average diameter and as well as on the non-periodic filaments which were 10.2±0.3 nm (n=11) in diameter (Fig. 1-a and 1-c). The labeling pattern for type Ⅲ was very similar to that for type Ⅰ collagen, namely, gold particles were present on the characteristic cross-banded fibrils and also on the non-periodic filaments. The average diameters of the fibrils and filaments were 23.4±2.6 nm (n=75) and 10.2 ±0.5 nm (n=11), respectively (Fig. 1-b and 1-d). There was no significant difference between the mean diameter of fibrils positive for type Ⅰ and that of those positive for type Ⅲ collagen. The same situation was observed in the filaments positive for type Ⅰ and in those positive for type Ⅲ collagen. In the double immunostaining technique for type Ⅰ and type Ⅲ collagen, some fibrils with a corss-banded pattern showed positive labeling for both sizes

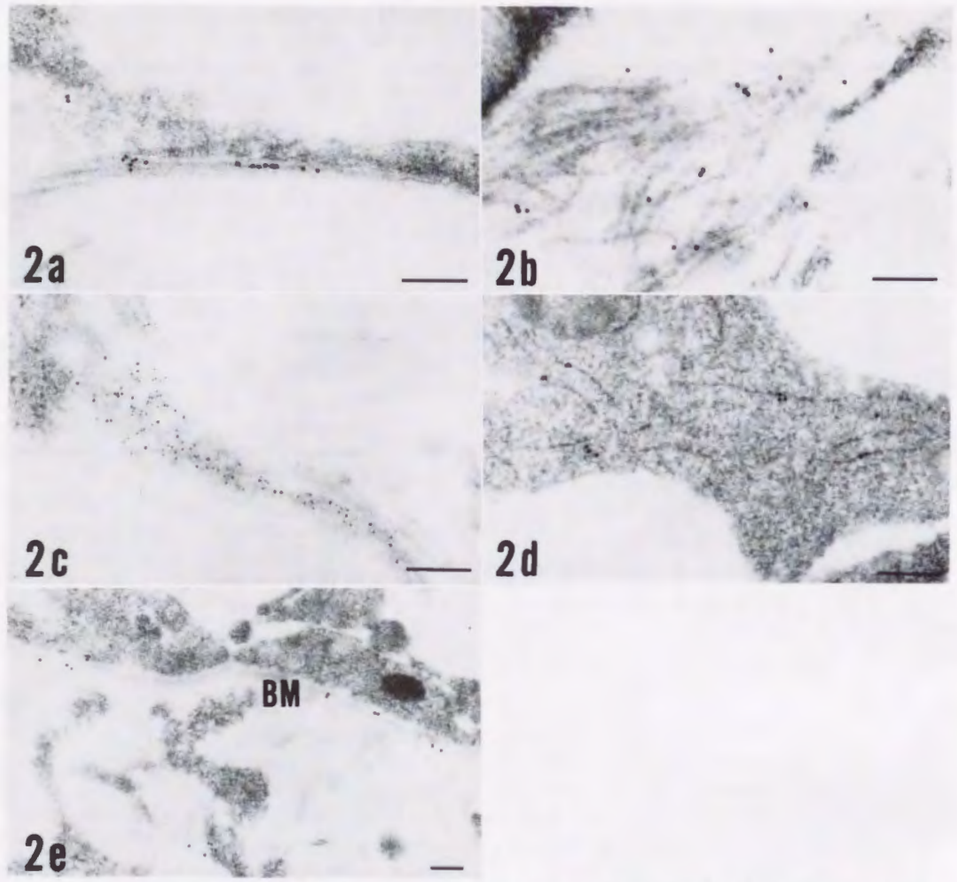


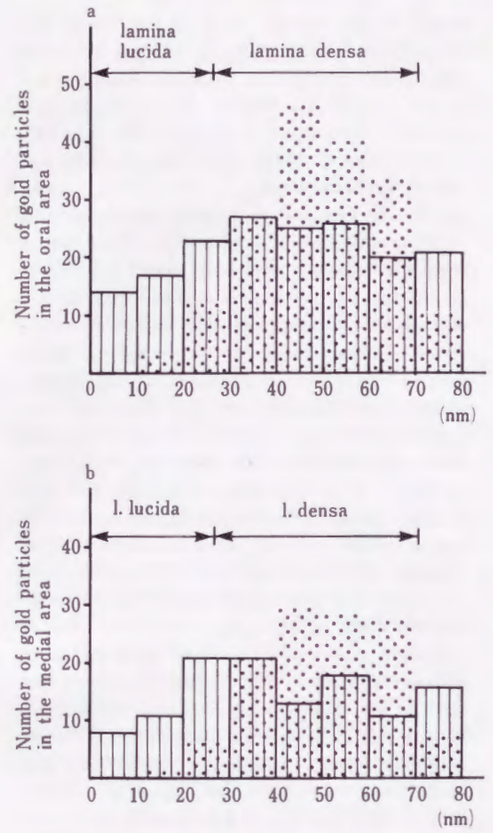
Fig. 2 a–e The distribution of fibronectin in the palatal mesenchyme on day 14.0 (a, c, d, e) and on day 16.0 (b).

Labeling particles are observed on amorphus materials localized between the mesenchymal cells and collagen fibils (a) and within the collagen fibrils (b). Labeling particles are observed on a meshwork adjacent to the mesenchymal cell (c). Labeling particles are observed on the rough endoplasmic reticulum of the mesenchymal cell (d). Positive labeling is observed both on the lamina lucida and the lamina densa of the epithelial basement membrane (e).

(a, b, c, d, ×55,000, e : 27,000) ; BM, basement membrane, Bar = 200 nm.

of gold particles simultaneously in the nasal and also in the oral mesenchyme as observed on day 16.0 (Fig. 1–e), suggesting that at least some of the fibrils consisted of type Ⅰ and type Ⅲ collagen molecules. Filaments present between collagen fibrils and the epithelial basement membrane were barely labeled by either type Ⅰ or type Ⅲ collagen (Fig. 1–a and 1–b). Almost the same situation was observed in the staining patterns for types Ⅰ and Ⅲ collagen and the relationship between them on day 16.0, except for the remarkable increase in the number of collagen fibrils when compared with that on day 14.0 (Fig. 1–e). The control sections in which normal rabbit serum were substituted for primary antibodies and reacted with the colloidal gold-labeled secondary antibodies showed negative labeling (Fig. 1–f).

In order to make a quantitative analysis on



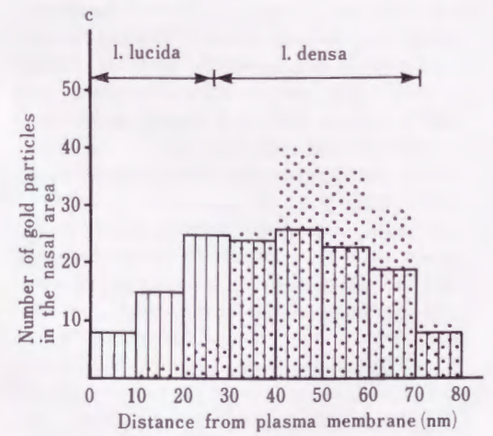


Fig. 3 Histogram showing the distributional pattern of labeling particles for fibronectin within the basement membrane of the palate epithelium in various areas on day 14.0.

Abscissas represent the ranges of distance from the plasma membrane of the epithelial cells. Perpendicular axes represent the number of gold particles within each range of distance. Total number of particles counted were 172, 122, and 148 for nasal (a), medial (b), and oral (c) areas, respectively. The thickness of the lamina lucida (28.5 ± 5.0 nm) and the lamina densa (43.5 ± 8.6 nm) are indicated. Striped bars represent the number of the gold particles actually observed. Stippled bars represent model distribution, in which it is assumed that antigens are in the lamina densa only and are distributed evenly within it. The mathematical condition is that a particle may exist at any given position on the circle, the radius of which is 10 nm (length of IgG molecule), and whose center is the true localization of the given "antigen". Total numers of particles counted were 172, 122, and 148 for nasal, medial, and oral areas, respectively in the model, as in the actual case.

the distribution of both types of collagen in the palatal mesenchyme, the density of gold particles in various areas was examined (Table 1). On day 14.0, the density of gold particles for type Ⅰ collagen was significantly higher in the nasal area than in any other area ($p < 0.05$). On the other hand, that for type Ⅲ collagen was highest in the oral area, although the difference between that of the oral area and the nasal area was not significant. In the core area, the density of gold particles for both type Ⅰ and type Ⅲ collagen was significantly lower than in the nasal or oral areas ($p < 0.01$). On day 16.0, the density of both types of collagen became remarkably increased. The density of type Ⅰ collagen was significantly higher in the nasal area than in the oral area ($p < 0.05$), while that of type Ⅲ collagen showed no difference between either area. In every case, those in the background were significantly lower than in any other area ($p < 0.01$). Because of the commencement of bone formation in the basal region on day 16.0, the densities of gold particles for types Ⅰ and Ⅲ

collagen in the core area were not measured. The ratios of type Ⅰ to type Ⅲ collagen obtained by the double immunostaining technique were different between the nasal and oral areas on both days (Table 2). They were significantly higher in the nasal area than in the oral area, both on day 14.0 ($p<0.01$) and on day 16.0 ($p<0.05$). In the nasal area, they were higher on day 14.0 than on day 16.0, although the differences between them were not significant. In the oral area, there was no significant difference on either day.

Fibronectin

There were two types of extracellular structures positive for fibronectin in the palatal mesenchyme. One was amorphous materials, and the other was a filamentous meshwork structure which was rarely observed. The distribution of fibronectin was almost the same on day 14.0 and on day 16.0. Amorphous materials with positive labeling were localized between the mesenchymal cells and collagen fibrils (Fig. 2-a), and within the collagen fibrils (Fig. 2-b). Filamentous structures positive for fibronectin (9.6±0.4 nm in diameter, n=9) formed meshwork structures adjacent to the mesenchymal cells (Fig. 2-c). Labeling particles were often observed on the rough endoplasmic reticulum of the mesenchymal cells (Fig. 2-d), suggesting that palatal mesenchymal cells synthesized fibronectin. Labeling particles were also localized within the basement membrane of the palatal epithelium. They were observed both in the lamina lucida and in the lamina densa (Fig. 2-e). Figure 3 shows the histogram of labeling particles for fibronectin in the basement membrane of the palatal epithelium. The distribution patterns of the labeling particles in the nasal, medial, and oral areas were almost the same, suggesting the absence of any difference in the distribution pattern of fibronectin among the three areas (Fig. 3-a～c).

Discussion

It has been demonstrated using the immunofluorescent technique that types Ⅰ and Ⅲ collagen and fibronectin are distributed throughout the mouse palatal mesenchyme on embryonic day 14.0[2,8–10]. However, light microscopic study cannot provide information about the supramolecular organization of these extracellular matrices. In this study, we showed the ultrastructural distribution of collagen and fibronectin using the immunogold method. Types Ⅰ and Ⅲ collagen were distributed more densely in the mesenchyme adjacent to the basement membrane of the palatal epithelium, but were less dense in the core of the palatal shelves (Table 1). Hassell and Orkin[33] reported that cross-banded collagen fibrils did not appear adjacent to the basement membrane of either the nasal or medial epithelial cells in rat embryos. However, in the present study, cross-badded collagen fibrils running longitudinally and transversely in the nasal palatal mesenchyme were observed in the frontal section (Fig. 1-a and 1-c), suggesting that collagen fibrils were randomly oriented in the mouse palatal shelves. Labeling for both type Ⅰ and type Ⅲ collagen was observed on cross-banded fibrils, the mean diameters of which did not differ significantly. There has been a consensus that type Ⅰ collagen typically forms cross-banded fibrils[11–14]. However, it remains unclear whether type Ⅲ collagen forms cross-banded fibrils or thinner fibrils lacking a cross-banded pattern. Amenta et al.[13] and Martinez-Hernandez[14] reported that type Ⅲ collagen fibrils lacked cross-banded pattern, but that they had a characteristic beaded periodicity. However, we showed that, at least in the mouse palatal mesenchyme, type Ⅲ collagen formed cross-banded fibrils as well as filaments lacking a cross-banded pattern. This discrepancy may well be due to differences present in the various kinds of tissue.

The double immunostaining technique using types Ⅰ and Ⅲ collagen antibodies suggested that, in the palatal shelves, both types of collagen fibrils were co-distributed within the same fiber in addition to both types of collagen molecules being co-distributed within the same fibrils (Fig. 1-e). The co-distribution of types Ⅰ and Ⅲ collagen was reported in the human skin[34], in the rat liver[35], and in the mouse periodontal ligament[16]. In the culture of mouse embryonic palatal mesenchymal cells, almost all cells showed positive staining for type Ⅰ and type Ⅲ collagen, simultaneously, using the immunofluorescent technique[36]. These data suggest that types Ⅰ and Ⅲ collagen molecules are simultaneously secreted by the same cell and then co-assembled in the same fibers and sometimes in the same fibrils.

Fibronectin has been thought to play many roles in embryonic morphogenesis, such as migration, attachment of cells or interaction between them, or between cells and extracellular matrices. We showed the two patterns of distribution of fibronectin in the embryonic mouse palatal mesenchyme. Most of the fibronectin was localized between the mesenchymal cells and the collagen fibrils (Fig. 2-a), and within the collagen fibers (Fig. 2-b). It is well known that fibronectin has collagen-binding domains and cell-binding domains[17,18]. This suggests that fibronectin existing between the mesenchymal cells and the collagen fibrils or else among the fibrils, may participate in the adhesion of the cells and fibrils, or of the fibrils to each other and that the mesenchymal cells and collagen fibers construct a network, with fibronectin acting as at least one of the adhesion molecules. An *in vitro* study showed that human dermal fibroblasts contracted in collagen gel culture and reduced the size of the gel matrix[37]. Therefore, any contraction of the palatal mesenchymal cells, through the cell-collagen network, may participate in creating one of the intrinsic shelf forces, which elevate the shelves from the vertical to the horizontal position.

The quantitative analysis of the density of type Ⅰ and type Ⅲ collagen revealed that, both on day 14.0 and on day 16.0, the relative density of type Ⅰ to type Ⅲ collagen was higher in the nasal area than in the oral area. This means, in other words, that the relative density of type Ⅲ to type Ⅰ collagen was higher in the oral area than in the nasal area. In general, type Ⅲ collagen is dominant in distensible tissue, such as the walls of blood vessels or periodontal ligaments, suggesting that this type of collagen participates in the elasticity of the tissues[16,38]. It has been thought that hyaluronic acid and collagen form gel-fiber networks which hydrate and expand their molecular domain to produce an inrinsic force to reorient the palatal shelves[4]. If the increase of type Ⅰ collagen content raises the stiffness of collagen fiber and the increase of type Ⅲ collagen content raises the elasticity of the fiber, the relatively high content of type Ⅰ collagen in the nasal area may contribute to the hinge action of the nasal side of the shelf and the relatively high content of type Ⅲ collagen in the fibers of the oral area may contribute to the passive movement of the oral side. Therefore, this action of the network comprising collagen fibers and fibronectin may participate, at least in some part, to the intrinsic shelf force in order to elevate the shelf in the middle portion of it. Although the difference in the ratios of type Ⅰ to type Ⅲ collagen between the nasal and the oral area observed on day 14.0, still remained on day 16.0, the value in the nasal area decreased on day 16.0 to less than that on day 14.0 (Table 2). These data may relate the phenomenon of completion of hinge action in the nasal side of the shelf.

Epithelial-mesenchymal interaction is an important event for morphogenesis and exerts an effect on the basement membrane structure and turnover for epithelial cell differentiation in various tissues[26]. After the fusion of the palatal shelves, the nasal side of the epithelium differentiates ciliated cells, while the oral side differentiates stratified squamous cells. Medial edge epithelium ceases DNA synthesis and dies, or else transforms into mesenchymal cells on day 16 following elevation[23–25]. In the present study, the distribution of fibronectin within the basement membrane of the palatal epithelium prior to shelf elevation showed no differences between the nasal, medial, and oral areas (Fig. 3), suggesting that fibronectin in the basement membrane does not directly play an important role in the differentiation of palatal epithelial cells. We also found that fibronectin was localized both in the lamina lucida and in the lamina densa (Fig. 3). Considering that an indirect labeling procedure was used in the present study, we compared the histogram of actual gold particles with that of the model which is described in the legend of Figure 3. Results suggest that fibronectin was distributed both in the lamina lucida and in the lamina densa in all three of the areas observed. Although various kinds of cells synthesize fibronectin *in vitro*, only a few cells, including hepatocytes[39] and dental mesenchymal

cells[40], have been reported to synthesize it *in vivo*. We showed the presence of labeling for fibronectin on the rough endoplasmic reticulum of palatal mesenchymal cells, suggesting that these cells synthesize fibronectin *in vivo* as well as *in vitro*[36].

In this study, we revealed the supramolecular organization of type Ⅰ and type Ⅲ collagen, and fibronectin, and their interrelationship in the developing mouse palate. These observations provide some basic information towards understanding the role of types Ⅰ and Ⅲ collagen and fibronectin in the elevation of the palatal shelf.

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抄録：マウスの口蓋形成過程においてⅠ型およびⅢ型コラーゲンとフィブロネクチンの分布を免疫組織化学的手法を用いて検索した。Ⅰ型およびⅢ型コラーゲンは横紋を有するコラーゲン細線維に共存していた。口蓋突起内でのコラーゲンの分布は場所により異なっていて，Ⅰ型コラーゲンは口腔側よりも鼻腔側に多く分布していたが，Ⅲ型コラーゲンにはそのような差が認められなかった。Ⅲ型に対するⅠ型の割合は場所や胎齢により異なっていて，口腔側よりも鼻腔側に多く，さらに鼻腔側では胎齢16日よりも14日に多かったが，口腔側においては胎齢による差が認められなかった。フィブロネクチンは不定形物質として間葉細胞の表面やコラーゲン線維の間に存在し，細胞間質においては線維状構造物として認められた。また口腔上皮の基底膜にも存在していた。以上のことからⅠ型およびⅢ型コラーゲンとフィブロネクチンは密接に関係しながら口蓋形成に関与しているものと考えられた。

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