Over-expression of MMP-3 in the fissured tissue of cleft lip and palate

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Abstract

Objective: In order to elucidate the retrogressive degeneration of orofacial cleft, the fissured tissues of prenatal and postnatal cleft lip and palate were examined by histological and immunohistochemical methods.

Design: Totally 42 cases of prenatal (n=17) and postnatal (n=25) cleft lip and/or palate were examined in comparison with 10 cases of normal lip and oral mucosa using immunohistochemical stainings of MMP-3, MMP-9, MMP-10, cathepsin G, PCNA, E-cadherin, TGase 2, HSP-70, vWF, and VEGF.

Main Outcome Measures: In the fissured tissue the sebaceous glands were strongly positive for PCNA and grew into the underlying fibromuscular tissue (24/42). Some hyperplastic sebaceous glands of prenatal cleft lip produced infundibular follicular cyst (9/17). The skin and mucosal epithelia from the postnatal cleft lip and palate (10/25) showed severe basal hyperplasia (11/25) and melanocyte infiltration (7/25).

Results: The immunostaining of MMP-3 and HSP-70 were strongly positive in the hyperplastic sebaceous glands and nearby atrophying muscle bundles of the fissured tissue, while MMP-9, MMP-10, and cathepsin G were almost negative. The immunoreactions of the other antibodies used in this study were similar between in the fissured tissues and in the normal controls.

Conclusions: These data suggest that the over-expression of MMP-3 is closely related to the sebaceous gland hyperplasia, epithelial dysplasia, and the muscle degeneration, and that the over-expression of MMP-3 in the fissured tissue may continuously aggravate the cleft condition in the later life.

Key words
Human, Fetal, Adult, Cleft lip and palate, Sebaceous gland, MMP-3

INTRODUCTION

Cleft lip and palate are most frequent congenital malformation in oral and maxillofacial region[1]. In general, the palatogenesis can be divided by the exogenous factors as well as the intrinsic factors. The exogenous factors are usually related to the growth of tongue, nasal cartilage and primary palate, Meckel's cartilage and cranial base. The intrinsic factors are usually related to cell migration, epithelial-mesenchymal transformation, synthesis and accumulation of extracellular matrix, and cell division[2]. Palatal mesenchymes originated from neural crest cells are gradually located in the inner side of left and right maxillary process, and rapidly grow transversely to fuse each other to form palatal processes and shelves. However, the merging processes of palate and lip should undergo rapid tissue degradation and regeneration in a short time. Thus, cleft formation is not directly resulted from fusion failure in the merging of palatal process, mesial and lateral nasal process which begins in the 6th week of gestation, but also the cleft can be resulted from the fusion defects in the tri-dimensional organization of palatal shelves and lip in the conjunction with the growth of adjacent structure including the secondary

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palate and nasal capsule which begins in the 7th week of gestation[3-4]. However, the fusion defect of cleft lip and palate produced abnormal orofacial structure and caused the continuous tissue degradation. In this study, in order to elucidate the retrogressive degeneration of cleft lip and palate tissues, we had screened different kinds of antibodies against the extracellular matrix proteins in the fissured tissues of prenatal and postnatal human cleft lip and palate by immunohistochemistry, and found that MMP-3 was characteristically over-expressed in the hyperplastic sebaceous glands and nearby muscle bundles.

MATERIALS AND METHODS

Cleft tissue specimens

17 cases of fetal cleft lip and palate tissue were obtained from fetal autopsy materials in the Department of Pathology, Seoul National University Hospital, and 25 cases of fresh fissured tissues of postnatal cleft lip and palate were collected postoperatively in the Department of Oral and Maxillofacial Surgery, Kangnung National University Dental Hospital (KNUDH). And for the negative control 10 cases of normal lip and oral mucosa tissues were also obtained from the biopsy materials in the Department of Oral Pathology, KNUDH. The specimens were fixed in 10% buffered formalin and embedded with paraffin, and sectioned and examined by hematoxylin and eosin staining. Among 25 postnatal human cleft lip and palate tissues 11 cases were obtained from palatorrhapy, 8 cases from cheilorrhapy, and 6 cases from scar revision (Table 1).

Immunohistochemistry

Antibodies including PCNA (proliferation cell nuclear antigen) and MMP-3 (matrix metalloproteinase-3), MMP-9, MMP-10 (DAKO, Denmark), cathepsin G, TGase-2 (transglutaminase-2), E-cadherin (Uvomorulin), and HSP-70 (heat shock protein-70) (Santa Cruz Biotechnology, USA) were used for immunohistochemical staining. Biotinylated secondary antibody and streptavidin-peroxidase were followed by colorization with DAB (3,3' -diaminobenzidine, Sigma, USA) (Table 2).

Briefly, the paraffin section was treated with each antibody by indirect immunohistochemical method using labeled streptavidin-biotin staining technique (DAKO, LSAB K0681, Copenhagen, Denmark). DAB was precipitated by peroxidase reaction in dark brown color, and the result was observed with weak hematoxylin counter staining. For the negative control the microsections were applied with pre-immune rabbit serum and simultaneously processed with the above immunostainings. The immunohistochemical methods and histological details are basically same with the previous study[5]. Microscopic features were analyzed by digital Image analyzer (Image pro-4.0, Media Cybernetics, Silver Spring, MD, USA) statistically.

RESULTS

Histological observation

In the fissured tissues of prenatal (14/17) and postnatal (13/25) cleft lip and palate the sebaceous glands were deeply proliferative into the underlying fibroadipose and muscle tissues. The hyperplastic sebaceous glands in the fissured tissues of prenatal cleft lip and palate produced multiple infundibular follicular cysts (9/17) with the retention of sebum in the sebaceous ducts. The fissured tissues of the postnatal cleft lip and palate showed severe basal hyperplasia of epithelium (11/25), melanocyte infiltration (7/25), submucosal fibrosis (4/25), and muscle degeneration (9/25), but there appeared only weak inflammatory reaction (Fig. 1).

Immunohistochemical examination

The hyperplastic sebaceous glands in the fissured tissue of cleft lip were strongly positive for PCNA and continuously grew into the underlying muscle tissue. Then, MMP-3 was strongly positive in the sebaceous gland and nearby degenerating muscle bundles of the postnatal (9/25) cleft tissues, while MMP-9, MMP-10, and cathepsin G were weak or almost negative in the sebaceous gland and muscle tissues but they were consistently positive in the macrophages infiltrated into the fibromuscular tissues. In the cross section of the muscle bundles the positive reaction of MMP-3 was usually observed in the degenerating foci in the periphery of muscle bundles. HSP-70 was also diffusely positive in the fissured tissues of cleft lip and palate, especially localized at the degenerating muscle bundle similar to the immunoreaction of MMP-3. On the other hands, the immunoreactions of E-cadherin, TGase-2, vWF and VEGF were relatively weak in the fissured tissues of cleft compared to the normal lip and oral mucosa(Fig. 2).

Table 1. Cleft lip and palate tissues used in this study

No.	File No.	Age	Clefts / Surgery / Histology
	Prenatal cleft lip and palate		
		weeks* / sex	
1	RCM331	33 / F	MCLP / auptosy / sebaceous gland hyperplasia with infundibular follicular cysts
2	RCM367	27 / F	BCLP / autopsy / sebaceous gland hyperplasia, fatty infiltration into loose connective tissue
3	RCM373	36 / F	BCLP / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
4	RCM516	32 / M	BCL and BAC / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
5	A80-006	29 / M	UCLP / autopsy / sebaceous gland hyperplasia
6	A80-024	22 / M	UCLP / autopsy / submucosal fibrosis
7	A81-031	30 / M	BCL and BICP / autopsy / sebaceous gland hyperplasia with infundibular follicular cyst, submucosal fatty infiltration
8	A81-054A	35 / M	UCLP (conjoined twin) / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
9	A81-054B	35 / M	UCLP (conjoined twin) / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
10	A82-039	33 / F	BCLP / autopsy / sebaceous gland hyperpasia with fatty infiltration
11	A86-013	32 / F	BCLP / autopsy / sebaceous gland hyperplasia with fibroadipose tissue
12	A87-036	41 / F	MCLP / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
13	A87-042	25 / F	PICP / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
14	A87-052	32 / M	BCLP / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
15	A87-066	40 / M	BCLP / autopsy / sebaceous gland hyperplasia with infundibular follicular cysts
16	A87-106	37 / F	UCLP / autopsy / submucosal fibrosis
17	A88-034	38 / M	BCLP / autopsy / sebaceous gland hyperplasia
	Postnatal cleft lip and palate		
		years / sex	
1	KNU-01	21 / F	BCLP / palate repair / basal hyperplasia and melanocyte infiltration
2	KNU-02	14 / M	UCLP / palate repair / sebaceous gland hyperplasia, muscle degeneration with fibrous-fatty tissue
3	KNU-03	4 / M	CP / palate repair / submucosal fibrosis
4	KNU-04	25 / F	UCL / scar revision / basal hyperplasia and melanocyte infiltration, sebaceous gland hyperplasia
5	KNU-05	4 / F	BCLP / palate repair / submucosal fibrosis
6	KNU-06	6 / F	BCLP / palate repair / basal hyperplasia, melanocyte infiltration
7	KNU-07	13 / F	UCL / scar revision / basal hyperplasia and granulation tissue, sebaceous gland hyperplasia
8	KNU-08	21 / F	UCLP / scar revision / sebaceous gland hyperplasia and muscle degeneration
9	KNU-09	3 / F	UCL / cheiloplasty / sebaceous gland hyperplasia, muscle degeneration, and fibrofatty tissue
10	KNU-10	26 / F	BCLP / palate repair / basal hyperplasia and submucosal fibrosis
11	KNU-11	24 / M	UCL / scar revision / sebaceous gland hyperplasia and muscle degeneration, infundibular follicular cysts
12	KNU-12	30 / M	BCLP / scar revision / basal hyperplasia, granulation tissue, and fibrofatty tissue, sebaceous gland hyperplasia
13	KNU-13	21 / F	UCL / cheiloplasty / basal hyperplasia, granulation tissue, and fibrofatty tissue, sebaceous gland hyperplasia, melanocyte infiltration
14	KNU-14	11 / M	CP / palate repair / sebaceous gland hyperplasia and fibrofatty tissue
15	KNU-15	8 / F	UCLP / palate repair / sebaceous gland hyperplasia, muscle degeneration, and fibrofatty tissue
16	KNU-16	1 / F	UCL / cheiloplasty / sebaceous gland hyperplasia and muscle degeneration
17	KNU-17	26 / F	UCLP / palate repair / basal hyperplasia with severe inflammation
18	KNU-18	62 / F	UCL / cheiloplasty / basal hyperplasia and granulation tissue, melanocyte infiltration
19	KNU-19	21 / M	UCL / cheiloplasty / basal hyperplasia and granulation tissue, melanocyte infiltration
20	KNU-20	35 / F	UCLP / scar revision / sebaceous gland hyperplasia and chronic inflammation
21	KNU-21	63 / F	BCLP / palate repair / muscle degeneration and chronic inflammation
22	KNU-22	41 / M	UCL / cheiloplasty / submucosal fibrosis and granulation
23	KNU-23	63 / M	UCLP / palate repair / fibrofatty tissue and chronic inflammation
24	KNU-24	72 / F	BCL / cheiloplasty / basal hyperplasia and muscle degeneration, melanocyte infiltration
25	KNU-25	1 / F	UCLP / cheiloplasty / sebaceous gland hyperplasia and muscle degeneration, infundibular follicular cysts

*: gestational age

Abbreviation: BCLP: bilateral cleft lip and palate, UCLP: unilateral cleft lip and palate, MCLP: median cleft lip and palate, CP: cleft palate, UCL: unilateral cleft lip, BCL: bilateral cleft lip, BICP: bilateral incomplete cleft palate, PICP: posterior incomplete cleft palate, BAC: bilateral alveolar cleft

Table 2. Immunohistochemical detection using different antibodies in the fissured tissues of prenatal and postnatal cleft lip and palate

tissues / antibody	PCNA	cathepsin-G	MMP-3	MMP-9	MMP-10	TGase-2	E-cadherin	HSP-70	vWF*	VEGF*
Cleft fissured tissue										
hyperplastic epithelium	++	-	+	±	-	+	±	+	-	-
hyperplastic sebaceous gland	+	-	+++	+	-	+	±	++	-	-
underlying connective tissue	±	±	++	±	-	±	-	+	±	±
infiltrated fatty tissue	-	+	++	+	-	-	-	-	±	±
adjacent muscle tissue	-	+	+++	+	-	±	-	++	-	-
Normal lip and oral mucosa										
epithelium	+	-	±	-	-	+	+	±	-	-
connective tissue	±	-	±	-	-	+	-	±	+	+

Degree of expression: -; negative, ±; rare, +; slight, ++; moderate, +++; severe, *: expression in endothelial cells

DISCUSSION

Cleft lip and palate are formed in the early fetal stage by inhibiting the morphogenesis of orofacial structure[3] and the failure of complete organization of lip and / or palate also produces the cleft lip and / or palate in the later fetal stage[6-8]. However, the cleft may give striking impact on the following growth of entire orofacial structure. Abnormal development of primary palate causing cleft lip secondarily inhibits the fusion of the secondary palate. Cleft lip is formed when the fusion fails among median nasal prominence, lateral nasal prominence, and maxillary prominence. One of the possible causes of such a deficiency is contact failure between each ends of opposite ventral prominence. These problems may be caused by the growth defect of one or both prominences. Moreover, complete cleft lip occurs by the delay of the mesenchymal cell proliferation in the median nasal prominence and maxillary prominence. And incomplete cleft lip occurs when the proliferation of mesenchymal cell delays a little in the mesenchymal center[9].

During the embryonal and early fetal period the cleft areas were most active growth sites for the swelling, elongation, and adhesion between each orofacial components, and then a lot of vasculature and cytological expressions of different growth factors were concentrated in the distal ends of the embryonal processes. Because the unwanted cleft produces functional losses and abnormal frameworks of orofacial structure, the adaptational growth of the cleft tissue should be followed to support the basic orofacial functions. However, some useless cleft tissues will undergo cellular atropy, degeneration, and / or apoptosis rapidly. These cytological phenomena may occur shortly after the failure of embryonal process closure, but the abnormal orofacial structure needs a long time for the remodeling and adaptation, which may extended until the postnatal pubertal age[10-14].

As the fissured tissue obtained from the prenatal and postnatal cleft lip and palate may still has some sequela of abortive cleft formation, we collected the fissured tissue from the prenatal and postnatal cleft lip and palate and made serial sections of them for microscopic observation. The fissured tissues obtained from prenatal and postnatal cleft lip and palate showed the dysplastic epithelium infiltrated with a lot of melanocytes, that is identical with other author's reports[15-16]. Contrast to the UV light stimulation, trauma, and chemicals such as nicotine, the fissured tissues of the cleft lip and palate were closely associated with the anatomical defects in fibro-epithelial organization and maturation[17]. Therefore, it is presumed that the continuous dysplastic changes of the fissured epithelia are relevant to the abnormal ectomesenchymal interaction which is a major causative factor of cleft during the embryonal period[18-21].

The palatogenesis is a highly complex process controlled by mutual interaction between palatal epithelial cells and mesenchymal cells, which makes designation of the correct location, extracellular matrix remodeling, and fusion of palatal shelves. Collagen is an essential component of the palatal development, and it plays an important role as an extracellular matrix in the palate forming stage. Disappearance of medial edge epithelium which needs at the stage of making mesenchymal continuity crossing over the secondary palate is the final important stage of palate formation. Remodeling of extracellular matrix such as collagen is important in the aspect of growth and reformation of the connective tissue. The turnover and rearrangement of type 1 collagen, which is major component of palatal matrix, is regarded as important stage in palate formation. Expansion of amino acids cross-linked with collagen can affect palatal shelf elevation. This is because cross-linking increases tissue' s own hardness. It is also known that the palatal mesenchymal cells secrete extracellular matrix simultaneously carrying MMPs in order to remodel the extracellular matrix. Thus, the proteolytic degradation of extracellular matrix by MMPs is very important for the palatal fusion[22-23]. Both type 1 collagen and denatured collagen can be degraded during palate forming stage[24]. Among MMPs the MMP-3 is known as an active protease activating the proteolytic process to degrade the denatured matrix and also known as a differentiating factor for sebaceous gland growth and an unscheduled apoptosis factor[25], while the MMP-9 and MMP-10 are usually related to the cellular interaction for persistent inflammatory reaction. In this study we found the MMP-3 was predominantly expressed in the degenerating muscle bundles in the absence of inflammatory cell infiltration, and that the proliferative sebaceous glands grown into the underlying fibromuscular tissue showed the strong positive reaction of MMP-3. Especially the muscular degeneration was frequently occurred in the vicinity of the sebaceous glands. Therefore, we presume that by some reason not identified in this study the sebaceous gland becomes proliferative with the over-expression of MMP-3 and also secreted some amount of MMP-3 into the adjacent fibromuscular tissue, and resulted in fibromuscular degeneration in the fissured tissue of cleft lip.

Tanino et al published the results of comparing two palatoplasty methods in two stage palatorrhapy. They found that the deep tissues of cleft lip and palate remained through simple excision of oral mucosa can cause histological degeneration continuously and also cause relapse such as oronasal fistula, and can maintain lasting clefting environment. To prevent more progressive degeneration of these cleft lip and palate tissue, early surgical treatment like normal tissue graft on cleft lip and palate area must be preceded to inhibit lasting muscle atrophy or degeneration[26-27]. This clinical application of normal tissue graft may be implicated not only to recover the orofacial functions but also to prevent the over-expression of MMP-3 in the fissured tissue of cleft lip and palate.

In further immunohistochemical screening using different antibodies relevant to cellular proliferation, differentiation, angiogenesis, degeneration, and apoptosis, TGase-2 (a cross-linking enzyme), E-cadherin (a cell adhesion protein), VEGF and vWF (angiogenesis relating factors) were relatively weak in the fissured tissues of prenatal and postnatal cleft lip and palate compared to the normal mucosa and lip. On the other hands, heat shock protein-70 (HSP-70) known to a kind of shaferon protein which protects itself from different stresses likely thermal shock was strongly positive in the degenerating muscle bundles at which the MMP-3 was also strongly co-localized. This finding may indicate that the degenerating muscle bundles are under stressed condition, become atrophied and finally degraded by MMP-3.

Although the present study mainly examined the histological features of the prenatal and postnatal cleft tissues and performed the immunohistochemical stainings, the fissured tissues of orofacial clefts characteristically disclosed the hyperplastic sebaceous glands, diffuse fatty infiltration, and the basal hyperplasia and melanosis of the fissured epithelium. Among the antibodies against different intracellular and extracellular proteins MMP-3 was overexpressed in the sebaceous glands and localized at the degenerating muscle bundles in the fissured tissue, while those of MMP-9, MMP-10, cathepsin G were entirely sparse. These data suggest that the over-expression of MMP-3 is closely relevant to the sebaceous gland hyperplasia and the muscular degeneration, and that the continuous expression of MMP-3 in the fissured tissue during the postnatal period may further degenerate the involved muscle bundles and fibro-adipose tissue. Therefore, we presumed that although there may exist the innate defense mechanism against the retrogressive changes of oro-facial tissues, the phenomenon of MMP-3 over-expression itself may indicate the continuous aggravation of the cleft condition in the later life.

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FIGURES ①

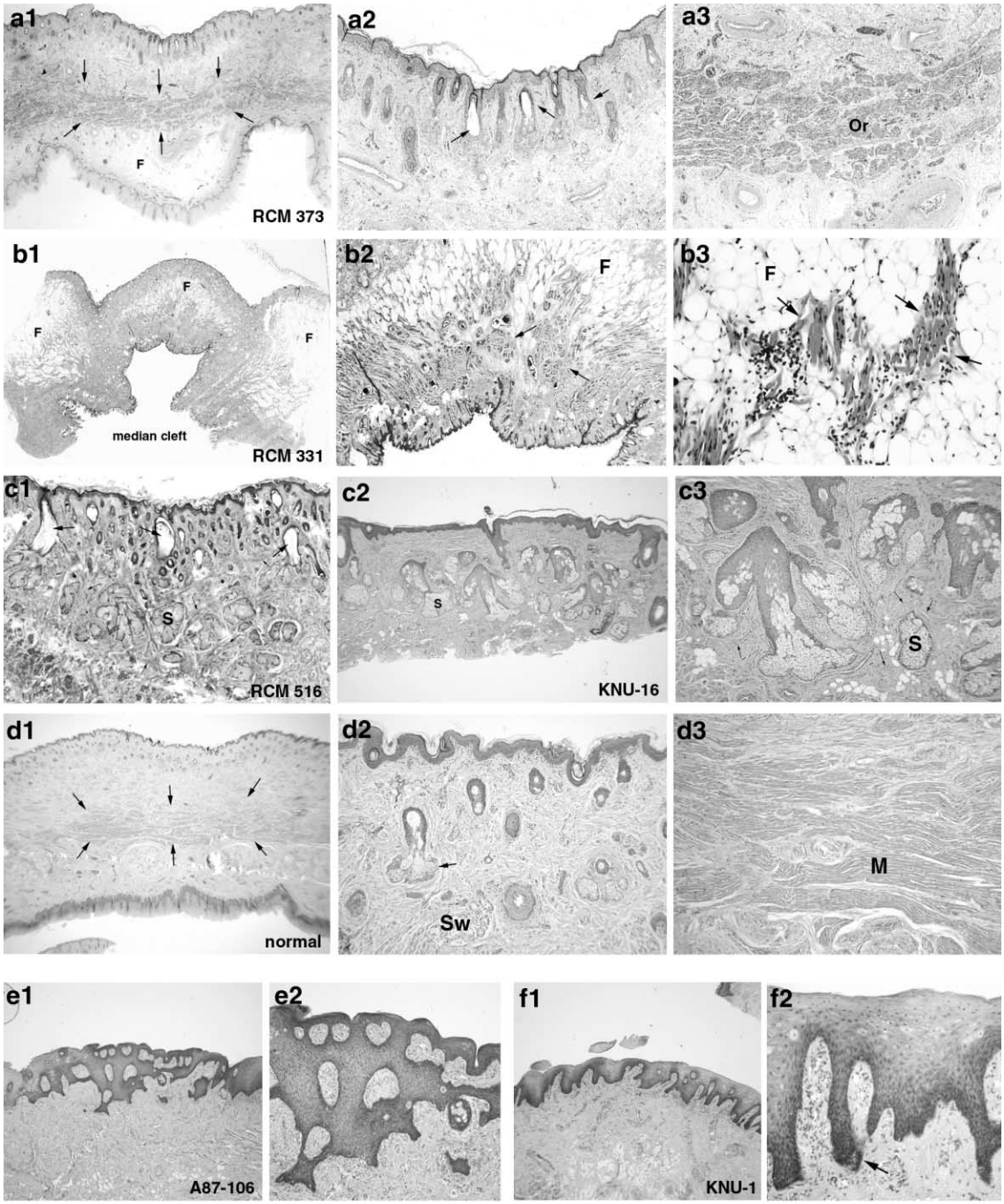

Fig. 1. a: longitudinal microsections from philtrum area of bilateral cleft lip (RCM 373), a1: noted the thin orbicularis oris muscle (arrows) and diffuse fatty infiltration (F). a2: noted infundibular follicular cyst formation (arrows), a3: high magnification of a1, the orbicularis oris muscle (Or) bundles were thinned and partly separated. b: longitudinal microsections from philtrum area of median cleft lip (RCM 331), b1: noted fatty infiltration (F) instead of orbicularis oris muscle, b2: noted the sebaceous hyperplasia (arrows) and fatty infiltration (F), b3: noted atrophying muscle bundles surrounded by fatty tissues. c: cross microsections of lip (RCM 516), c1: noted the sebaceous hyperplasia (S) with multiple infundibular cyst formation (arrows), c2: microsection of fissured tissue of cleft lip (KNU-16), severe sebaceous gland (S) hyperplasia, c3: high magnification of c2, d: longitudinal section of normal lip (32 weeks old fetus), d1: noted the bundles of orbicularis oris muscle (arrows), d2: normal development of hair follicle and sebaceous gland (arrow), sweat gland is also noted, d3: noted the thick muscle bundles (M), e1: fissured tissue from prenatal cleft palate (A87-106), noted the severe basal hyperplasia and submucosal fibrosis, e2: high magnification of e1, f1: fissured tissue from postnatal cleft palate (KNU-1), noted the dysplastic epithelium and submucosal fibrosis, f2: high magnification of f1, noted the melanocyte infiltration (arrow).

FIGURES ②

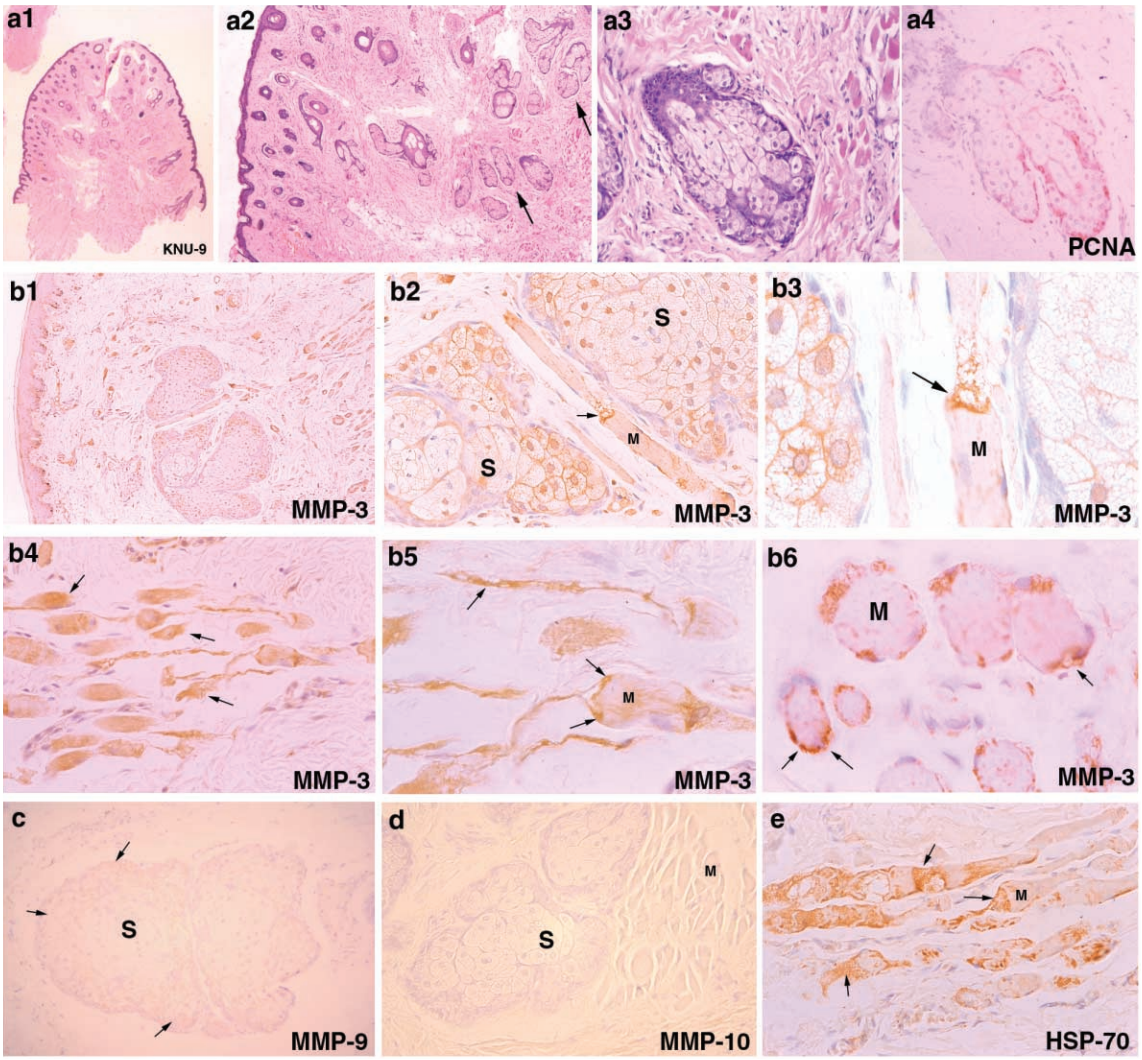

Fig. 2. a: cross section of the fissured tissue of cleft lip, a1: noted the sebaceous hyperplasia and the absence of orbicularis oris muscle, a2: noted the infiltrative growth of sebaceous gland (arrows), a3, a4: PCNA reaction in the hyperplastic sebaceous gland. b: microsections from cleft lip area (KNU-16), immunostaining of MMP-3, b1: diffuse positive reaction in the fissured tissue, b2: strong positive in the sebaceous gland (S) and nearby muscle bundle (M), b3: high magnification of d2, showing strong MMP-3 reaction in the degenerating site (arrow) of muscle bundle (M), b4: MMP-3 was condensely localized at the degenerating muscle bundles (arrows), b5: high magnification of d4, MMP-3 was localized at the degenerating site (arrows) of muscle bundle (M), b6: cross section of degenerating muscle bundles (M), positive reaction in the periphery (arrows), c: immunostaining of MMP-9, sparse reaction (arrows) in sebaceous gland (S), d: immunostaining of MMP-10, almost negative in the sebaceous gland (S), e: immunostaining of HSP-70, strongly positive in the degenerating muscle bundles (arrows).