Cytokeratin expression in palatal and marginal mucosa of cleft palate patients


a Key Laboratory of Oral Biomedical Engineering of Ministry of Education, School & Hospital of Stomatolog, Wuhan University, 65 Luoyo Road, Postal Code 430079, Wuhan, PR China
b Department of Orthodontics and Oral Biology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands
c Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands
d Department of Plastic Surgery, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands
e Department of Obstetrics and Gynecology, Erasmus University Medical Centre, PO Box 2040, 3000 CA Rotterdam, The Netherlands

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Summary

Objective: The margin of a palatal cleft is a unique anatomical site since the palatal mucosa is continuous with the nasal or nasopharyngeal mucosa. The aim of this study was to compare the expression patterns of cytokeratins and basal membrane components of the mucosa in the area of the cleft.

Design: Biopsies from the mucosa of the hard palate and from the cleft margin in the soft palate were obtained from five patients during the primary surgical closure of the cleft. The tissues were processed for haematoxylin-eosin staining and for immunohistochemistry. Antibodies against the cytokeratins (CK) 4, 7, 8, 10, 13, 16 and 18, and the basal membrane components heparan sulphate (HS) and collagen type IV (CIV) were used for immunostaining.

Results: The nasopharyngeal epithelium was thinner than the epithelium of the soft palatal mucosa, and showed less interpapillary ridges. The nasopharyngeal epithelium was stratified but expressed the keratins of a simple epithelium (CK 7, 8 and 18). The

* Corresponding author. Tel.: +31 243614005; fax: +31 243540631.
E-mail address: h.vondenhoff@dent.umcn.nl (J.W. Von den Hoff).

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expression pattern abruptly changed into that of a typical non-keratinized stratified epithelium (CK 4, 13) at the transition to the soft palatal epithelium. The epithelium of the hard palate was a fully differentiated, keratinized and stratified epithelium (CK 10, 16). The basal membrane was thinner in the nasopharyngeal epithelium, which might be related to the presence of abundant inflammatory cells. Conclusion: The area around the palatal cleft showed three different types of epithelium. There was an abrupt transition in phenotype of the epithelium from the oral side to the nasopharyngeal side.

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Introduction

A cleft palate causes severe physiological problems. The complex mechanisms of normal sucking, breathing, hearing and speech are impaired because the secondary palate has not closed. The embryonic development of the secondary palate is divided into three phases, which are characterized respectively by the formation of the palatal shelves from the maxillary processes, the elevation of the palatal shelves, and the fusion and subsequent degradation of the medial edge epithelium covering the paired palatal shelves. The complete fusion of the palatal shelves is important for the normal function of both the oral and the nasal cavities. A deviation at any stage of the formation of the secondary palate can lead to a cleft.

At the cleft margin, the anterior palatal mucosa is continuous with the nasal mucosa and the posterior mucosa with the nasopharyngeal mucosa. Normal oral mucosa can be classified into three types: (1) the oral lining mucosa, which is found on the soft palate, the ventral and lateral sides of the tongue, the floor of the mouth and the cheeks. All of these are covered by non-keratinized stratified epithelium; (2) the masticatory mucosa which occurs on the hard palate and the gingiva, and is covered by a keratinized stratified epithelium; (3) a specialized type of mucosa that covers the dorsum of the tongue and may be keratinized locally. The nasopharynx, on the other hand, is lined by a pseudostratified ciliated columnar epithelium or a stratified epithelium in the more posterior region. It has been described that, at the margin of the cleft, the mucosa of the soft palate is paler than the nasal mucosa. The demarcation line is said to be clinically visible as the so-called “white line”. However, only little is known about the histological and immunohistochemical characteristics of the marginal mucosa within the cleft.

Epithelial cells are characterized by intermediate filaments that consist of different combinations of cytokeratins. The profile of suprabasal cytokeratin expression in a particular epithelium is indicative for its degree of differentiation. Keratinized epithelium like that from skin is highly differentiated and expresses the larger cytokeratins 1 and 10. In contrast, the simple and glandular epithelia express the smaller cytokeratins 7, 8 and 18. The cytokeratin expression in skin epithelium, normal oral epithelia and respiratory epithelia has been well investigated. The basal layers of these epithelia usually express the cytokeratins 5 and 14. In the suprabasal layers of the masticatory mucosa the cytokeratins 1 and 10 are found (like in skin) but also 6 and 16. The lining mucosa of the oral cavity is characterized by suprabasal expression of the cytokeratins 4 and 13, which is also typical for the oropharyngeal mucosa. In contrast, little is known about the specific cytokeratin expression of the epithelia in the region of the palatal cleft. An epithelium can further be characterized by the structure and composition of its basement membrane (BM). The thickness and the composition of the BM can differ from normal during wound healing and under inflammatory conditions.

In the present study, the general histology and the expression of cytokeratins and BM components in the marginal epithelium from the cleft region were compared with that of the hard palate epithelium. To our knowledge, this is the first time that the transitional epithelium from the cleft margin of children with a cleft palate is described in detail.

Materials and methods

Patients

This study was carried out at the Radboud University Medical Centre Nijmegen in The Netherlands. For this study, five patients in the age of 1–2-years old with a non-syndromic cleft palate, with or without cleft lip and alveolus (CLA)P were selected. These patients were scheduled for primary surgical closure of the soft palate. All patients were routinely screened on contra-indications for surgical procedures (e.g. haemostasis problems). Normal mucosal samples from the hard palate were obtained using a 3 mm biopsy punch and samples from the cleft margin in the soft palate were excised during the closure of the cleft (Fig. 1). Samples of the cleft
margin in the hard palate were not taken as the hard palate is generally closed at a later age (if necessary). The Central Ethical Committee of The Netherlands approved these experiments. All parents signed an informed consent.

Antibodies

The cytokeratin 4, 10 and 13 antibodies (Euro-Diagnostica, Arnhem, The Netherlands) and the cytokeratin 16 antibody (Novo Casta, Newcastle upon Tyne, UK) were monoclonal mouse anti-human antibodies. The cytokeratin 7, 8, and 18 antibodies (monoclonal mouse anti-human antibodies) were kind gifts from Dr. Goos van Muijen (Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). The heparan sulphate JM 403 antibody (mouse anti-human heparan sulphate) was a kind gift from Prof. Dr. J. Berden (Department of Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands). The collagen type IV polyclonal antibody (Euro-Diagnostica, Arnhem, The Netherlands) was a rabbit anti-human antibody. The cytokeratins 7, 8 and 18 were used as markers for simple epithelia, 4 and 13 as markers for non-keratinized stratified epithelia, and 10 and 16 as markers for keratinized stratified epithelia.5,7—12

(Immuno)histochemistry

The specimens were thoroughly rinsed in saline and transferred to a tube containing cold phosphate-buffered saline (PBS) and antibiotics (penicillin, streptomycin, fungizone) immediately after removal. Samples were fixed for 4 h in 4% paraformaldehyde in 0.1 M phosphate buffer at room temperature and embedded in paraffin. Serial sections (5 μm) were cut, mounted on Superfrost-Plus slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized and rehydrated for histological analyses. Some sections were stained with haematoxylin and eosin (HE) for a general tissue survey. Immunohistochemical stainings were performed on deparaffinized subsequent sections using the avidin—biotin-peroxidase complex method. Briefly, before staining, the sections were pre-treated with 0.1% trypsin (Sigma Chemical Co., St. Louis, MO, USA) in 0.2 M Tris—HCl buffer (pH 7.2) for 10 min at 37 °C except for the CK 4, 13 and 16 staining. The sections for the latter staining were subjected to an antigen retrieval method using a microwave oven.17 Subsequently, the sections were washed in PBS and incubated in 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) in PBS for 30 min. After that, the sections were incubated with the primary antibodies at 4 °C overnight. Then the sections were incubated with either a goat or a donkey anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) except for the detection of collagen type IV, which was a goat anti-rabbit biotinylated antibody (Chemicon International Inc., Temecula, CA). Finally, the sections were stained with a standard DAB (Sigma) method and counterstained with Delafield’s haematoxylin. The general observations in the samples from the five patients are given on the basis of representative sections.

Results

Morphology

The locations where the tissue samples were taken are indicated in Fig. 1. The samples from the cleft margin showed an abrupt transition from the nasopharyngeal part to the (soft) palatal part (Fig. 2A and B). A salivary gland is present in the centre of this sample. The nasopharyngeal epithelium consisted of about 10 cell layers. The lower half of the epithelium consisted of elongated cells whilst the upper half contained more ovoid cells. Almost no interpapillary ridges were present. The connective tissue below the epithelium contained a relatively large number of lymphocytes. The epithelium from the palatal side consisted of about 15—20 cell layers and showed a clear stratification in three layers. The basal layer, adjacent to the basement membrane, was composed of cuboidal or columnar cells. Above that, the prickle cell layer consisted of larger ovoid cells. In the upper

Figure 1 Clinical picture of the cleft region. The picture shows the palatal cleft of one of the patients selected for this study (A: anterior, P: posterior). The incision lines for cleft closure are indicated with black dots. The site for taking the biopsy from the hard palate is indicated by the white circle. The marginal tissue is taken at the site indicated by the white oval.
half of the epithelium, the cells gradually became more flattened. This layer is often divided into an intermediate layer and a superficial layer. A granular layer and a cornified layer were absent from both the nasopharyngeal and the palatal mucosa.

The epithelium in the mucosal biopsies from the hard palate was a typical keratinized epithelium displaying four distinct layers (Fig. 2C and D). The thickness was about 15–20 cell layers. On the surface of the epithelium, a thin ortho-keratinized layer or cornified layer was present. Below this layer, a granular layer, prickle-cell layer and basal layer were found.

**Cytokeratins**

The distribution of the cytokeratins 7, 8, and 18 in the marginal epithelia was largely similar. All three were expressed only in the nasopharyngeal part of the marginal epithelium (Fig. 3A). Cytokeratin 7 was only expressed in the upper two-third of the epithelium whilst cytokeratins 8 and 18 were expressed down to the basal membrane. The epithelium of the central salivary gland and its duct also stained positive for these markers. All three cytokeratins were absent in the epithelium from the hard palate, although individual cells at the tips of interpapillary ridges were sometimes positive for cytokeratins 8 and 18 (not shown).

The cytokeratins 4 and 13 were mainly expressed in the epithelium at the palatal side of the cleft margin (Fig. 3B). They were found in all epithelial layers except the basal layer. The salivary gland epithelium was not stained. Some superficial expression of cytokeratin 4 occurred in the nasopharyngeal epithelium but only in the
Cytokeratin 4 was completely absent. The cytokeratins 4 and 13 were completely lacking in the hard palatal mucosa.

The cytokeratins 10 and 16 were only present in the epithelium from the hard palatal mucosa (Fig. 3C). Cytokeratin 10 was only expressed in the upper half of the epithelium but the staining was not continuous. Cytokeratin 16 was homogeneously distributed throughout all but the basal cell layers.

**Basal membrane markers**

Heparan sulphate was expressed continuously in the basal membrane of the epithelium from both the cleft margin and the hard palatal mucosa (Fig. 4A and C). Expression also occurred around the salivary glands and the blood vessels. The expression was weaker at the nasopharyngeal side of the margin then on the palatal side and appeared to decrease further to the nasal side. Collagen type IV showed more or less the same distribution in the samples as heparan sulphate but was also expressed around muscle fibers (Fig. 4B and D).

The immunohistochemical data are summarized in Table 1. The nasal and palatal side of the cleft margin have a different expression pattern of cytokeratins. At the nasal side the cytokeratins 7, 8 and 18 are highly expressed whilst some cytokeratin 4 also occurs more to the transition area. In contrast, the palatal side shows only expression of the cytokeratins 4 and 13. The cytokeratins 10 and 16 are exclusively expressed in the epithelium of the hard palatal mucosa. The two basal membrane markers are present in all epithelia.

**Discussion**

The mucosa located at the margin of the cleft in cleft palate patients represents a unique tissue. At this site, the nasal and nasopharyngeal mucosa blend into the palatal mucosa. This study was performed to analyze the marginal epithelium of the cleft. The marginal mucosa was obtained from patients at the time of primary palatal closure and was taken from the region of the soft palate during surgery. For comparison, mucosal biopsies from the hard palate were taken.

It is said that the division line between nasal mucosa and oral mucosa is clinically visible as a “white line” but this was not evident on intra-oral pictures. Surgeons often use the white line to mark out their incisions. In histological sections an abrupt transition between the two mucosa was found. The epithelium of the nasal side was thinner than that of the oral side, and showed no interpapillary ridges. However, it was up to 10 cell layers thick which may be required to resist mechanical stimulation by the

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**Figure 3** Immunohistochemistry of cytokeratins. All sections are from the same representative sample which is shown in Fig. 2. The nasal side of the cleft margin is indicated by (N) and the palatal side by (P). In panel (A), staining for the cytokeratins 7, 8 and 18 is shown which are markers for simple epithelia. The asterisks indicate a salivary gland and the arrow the salivary duct. Panel (B) shows a staining for the cytokeratins 4 and 13, which are markers for non-keratinized stratified epithelia. Panel (C) shows staining for the cytokeratins 10 and 16, which are markers for keratinized stratified epithelia. Original magnification: 2.5×.
posterior wall of the pharynx during swallowing. More anteriorly, the nasal epithelium is described to be a pseudostratified ciliated columnar epithelium. The connective tissue directly below the nasal epithelium contained large numbers of inflammatory cells. In this area, the basal membrane appeared thinner than at the oral side after staining for heparan sulphate and collagen IV. Inflammatory cells such as PMNs, are capable of producing proteolytic enzymes that degrade basal membrane components such as laminin, heparan sulphate proteoglycans and collagen type IV. In the nasal mucosa, PMNs might protect against airborne infections. In addition, their continuous presence might

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<th>Table 1 Summary of the expression patterns</th>
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The table shows a positive (+), negative (−), or intermediate (±) reaction for the indicated cytokeratins and for the basal membrane components heparan sulphate (HS) and collagen type IV (CIV) in the three types of epithelium.
cause the decrease in thickness of the basal membrane.

The cytokeratins expressed by a certain epithelium can be used as a marker for its type of differentiation. In the stratified non-keratinized epithelium at the nasal side of the cleft margin, the cytokeratins 7, 8 and 18 were expressed. They were also expressed in the salivary glands and ducts. These proteins are usually found in simple epithelia such as nasal epithelium and glandular epithelium. In this case they are also expressed by a stratified epithelium. Cytokeratin 8 and 18 were also occasionally expressed in isolated cells at the tips of interpapillary ridges in the epithelium of the hard palate mucosa. These cells probably are Merkel cells, which are often present in a so-called Merkel cell—neurile complex. These complexes seem to be involved in mechanoreception but additional functions have been suggested.

The epithelium at the oral side of the cleft margin was a stratified non-keratinized epithelium and expressed the cytokeratins 4 and 13. These proteins also occur in other non-keratinized epithelia such as the buccal mucosa. All stratified epithelia generally express the cytokeratins 5 and 14 in the basal layer which can therefore not be used as differentiation markers. In contrast, the expression of specific cytokeratins in the more superficial layers reflects the final differentiation of the tissue. The cytokeratins 10 and 16 only occurred in the stratified keratinized epithelium of the hard palate. The structure of this epithelium is similar to the epidermis of skin. Epidermis also expresses cytokeratin 10, but 16 is only expressed during wound healing or in certain hyperproliferative skin diseases. This indicates that the epithelium of the hard palate has some features in common with the epidermis in these skin conditions.

In conclusion, we have found that at least three types of epithelium occur in the area of the cleft. At the nasal side of the cleft margin a stratified non-keratinized epithelium occurs with a typical keratin expression pattern of a simple epithelium. The epithelium at the oral side is thicker and possesses interpapillary ridges. The most highly differentiated, stratified, and keratinized epithelium was found on the hard palate. The transitional epithelium from the cleft margin in the hard palate was not available since the anterior part of the cleft is usually not closed at this age.

It was shown that the type of differentiation of these epithelia is reflected by their cytokeratin expression. The most interesting finding however, was a very abrupt transition from the simple epithelium of the nasal side to the stratified epithelium at the oral side of the cleft. Next to genetic factors, the type of differentiation of a certain epithelium depends on environmental cues such as mechanical factors, the presence of inflammatory factors, and the underlying connective tissue. During the fusion of mouse embryonic palatal shelves in vitro, it was shown that the medial edge epithelium loses the expression of the simple epithelium cytokeratins 8 and 18. It is suggested that this might depend on signaling form the underlying mesenchyme through TGFRα. In cleft palate, mesenchymal signaling might be disturbed leading to an impaired differentiation and fusion of the palatal shelves.

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References


