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Elastin in oral connective tissue modulates the keratinization of overlying epithelium

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Abstract

Aim: One of the most discernable differences between keratinized and non-keratinized oral mucosas is the quantity of elastin they contain in the connective tissues. Whether elastin modulates the keratin expression of oral epithelial cells is unknown.

Methods: Four specimens containing both keratinized and non-keratinized mucosas were processed for immunohistochemical (IHC) stainings for elastin and four keratins. Six keratinized and non-keratinized portions of oral mucosas were dissected and cultured on an organ culture system. Purified elastin and elastase were added separately to the media. After 14 days, the mucosas were examined for four keratin expressions. Cell cultures of keratinized and non-keratinized gingival fibroblasts were established and tested for elastin expression. Oral mucosa equivalents were then engineered and tested for keratin expression.

Results: Keratinized epithelium exclusively expressed keratin-1 and -10 (K1/10), while non-keratinized epithelium expressed keratin-4 and -13 (K4/13). Only non-keratinized fibroblasts expressed elastin in cell culture. Both the native and the engineered keratinized gingiva changed phenotypes and expressed K4/13 when treated with exogenous elastin. On the contrary, the native non-keratinized mucosa started to express K1/10 when elastase eradicated inherent elastin.

Conclusions: Our study demonstrated that the elastin in the oral connective tissue is important for the non-keratinized phenotypes of overlaying epithelium.

Key words: elastase; Elastin; gingival; keratin; keratinization

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The oral mucosa can be divided into three categories: lining mucosa, masticatory mucosa, and specialized mucosa (Gartner 1994). Gingiva is the masticatory mucosa, which is rigid, tough, and tightly bound to underlying bone by dense connective tissue. It is covered with (para)keratinized epithelium, and immediately surrounds a tooth. Alveolar mucosa is the non-keratinized mucosal lining that covers the basal part of the alveolar process and continues without demarcation into the vestibular fornix and the floor of the mouth (Weinberg & Eskow 2003). Under a light microscope, epithelial layering in keratinized gingiva and non-keratinized alveolar mucosa reveals minor differences. At the molecular level, various keratin (K) family members are expressed in these two sites. Keratin is a family of about 21 polypeptides that are divided into acidic and basic subfamilies. Keratin molecules are usually expressed in pairs. The K4/K13 pair of keratins is typically expressed by lining mucosa associated with the properties of flexibility and elasticity, whereas the K1/K10 pair expressed by masticatory mucosa, hard palate, and skin is associated with rigidity and toughness (Sawaf et al. 1991). It has long been recognized that oral connective tissue can determine overlying epithelial differentiation (Kollar & Baird 1970, Karring et al. 1975). For example, 1 month after free grafts of connective tissue from either the keratinized gingiva or the non-keratinized alveolar mucosa had been transplanted into areas of the alveolar mucosa in

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seven monkeys, the gingival connective tissue grafts became covered with keratinized epithelium, while the alveolar mucosa transplants were covered with non-keratinized epithelium (Karring et al. 1975). Because the connective tissues determine the fate of epithelial differentiation and expression of different keratins, it is important to identify what components in the connective tissues of gingiva and alveolar mucosa make the difference. One known significant difference is the number of elastic fibres in the two connective tissues. A mature elastic fibre consists of a predominant elastin core and a surrounding microfibrillar sleeve (Kielty 2006). The lamina propria and submucosa of the alveolar mucosa contain numerous elastic fibres, while the elastic fibres are present only in association with blood vessels in the connective tissue of gingiva (Lindhe et al. 2003). Therefore, in the present study, we used several assays to test our hypothesis that (a) elastin in connective tissue promotes a non-keratinized phenotype (K4/13 positive), while the absence of elastin is associated with a keratinized phenotype (K1/K10 positive).

Materials and Methods

Collecting oral mucosa samples and isolating oral fibroblasts and keratinocytes

Ten human oral mucosa samples – distal wedges for crown lengthening on lower second molars – consisting of both keratinized gingiva and non-keratinized alveolar mucosa were obtained from our periodontal surgery clinic. A signed informed consent form previously approved by the Institutional Review Board of our university was obtained from each patient. Four of the samples were fixed in 4% paraformaldehyde (PFA) and then embedded in paraffin for future immunohistochemistry (IHC). The other six samples were used for tissue culture experiments. A small piece (5×5 mm) of each sample was excised to establish cell cultures. The methods used to isolate gingival fibroblasts, alveolar mucosal fibroblasts, and oral keratinocytes are described elsewhere (Igarashi et al. 2003). The fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml). The oral keratinocytes were maintained in a serum-free medium (Keratinocyte-SFM; Gibco, Carlsbad, CA, USA) supplemented with the same antibiotics and antimycotic. All the cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells used in this study were within passages 3–5.

IHC

Paraffin-embedded samples were cut into 4 μm sections and placed on silane-coated slides, which were then deparaffinized and rehydrated with serial xylene and ethyl alcohol. The slides were then incubated for 10 min. in 3% H_2O_2 in methanol, to quench endogenous peroxidase activity, and rinsed three times in phosphate-buffered saline (PBS) for 5 min. each. For K1, 4, 10, and 13, the antigens were retrieved using heat treatment (10 mM citrate buffer, pH 6.0). For elastin, proteinase K (20 μg/ml, 37°C, 10 min.) was used to unmask the antigen. After the slides had been washed with PBS three times for 5 min. each and blocked for 20 min., they were incubated with primary antibodies for elastin and K1, 4, 10, and 13 in a concentration of 2 μg/ml overnight at 4°C. The primary antibodies for K4, 10, and 13 were purchased from Neomarkers (Labvision, Fremont, CA, USA), and for K1 and elastin, from Abcam (Cambridge, MA, USA). The following day, the slides were incubated with biotin-conjugated secondary antibodies and streptavidin-horseradish peroxidase according to the manufacturer's instructions (Dako, Carpinteria, CA, USA). Finally, peroxidase activity was detected using a chromogen kit (Zymed, San Francisco, CA, USA) and then counterstained with Mayer's haematoxylin.

Immunocytochemistry (ICC) and Western blotting

The gingiva fibroblasts, alveolar mucosa fibroblasts, and oral (gingival) keratinocytes were cultured separately on cover glasses for 1 week. The cells were then fixed in methanol at -20°C for 5 min. and left to air dry. After they had been washed three times in PBS, 3% H_2O_2 in PBS was added to quench endogenous peroxidase. The following procedures without antigen retrieval were the same as for the IHC. For Western immunoblot, the cell lysates were collected from 10 cm culture plates after addition of 1 ml lysis buffer for each plate. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on gels of 12% acrylamide. For each lane, 40 μg of protein from different cell lysates and 10 ml of molecular weight standards were applied to the gel. After electrophoresis, transferring, and blocking, the primary antibody against elastin was incubated with the membrane for 1 h at 37°C at a concentration of 0.1 μg/ml. The polyclonal rabbit anti-mouse β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the internal control for equal loading. After the membrane had been washed, secondary antibody with horseradish peroxidase was incubated with the membrane for 1 h at 37°C at a concentration of 0.05 μg/ml. After the membrane had been given a final wash with PBST, it was developed using an enhanced chemiluminescence kit (Amersham Bioscience, Piscataway, NJ, USA) and exposed on X-ray films.

Transwell tissue culture

Transwell membrane culture systems (0.4 μm pore size) yield results close to those of the conventional Trowell-type system in an organ culture (Ramsdell et al. 2006, Giuliani et al. 2008). We used surgical scalpels to separate keratinized and non-keratinized portions of oral mucosa into six pieces of gingival and six alveolar mucosal tissue. Three samples of each were used as native controls. To investigate whether exogenous elastin protein influences the keratin phenotypes of keratinized gingiva, purified protein of elastin from human lungs (Calbiochem, Merck KGaA, Darmstadt, Germany), at a concentration of 200 μg/ml, was added to the culture media (a 1:1 mixture of DMEM and K-SFM) of the lower wells for the three gingival samples. The other three gingival samples, the control group, were cultured without elastin. The tissues on membrane were cultured with a medium–gas interface and maintained for 2 weeks. The medium was changed every 2 days. At the same time, three alveolar mucosal samples were treated with 15 U/ml of human neutrophil elastase (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 6 h to remove endogenous elastin. The treated and control alveolar mucosa samples were then transferred to the transwell tissue culture systems and maintained for 2 weeks. The medium (a 1:1 mixture of

DMEM and K-SFM) in the lower well was changed every 2 days. At the end of 2 weeks, the samples were removed from the membrane, fixed in 4% PFA, processed for paraffin embedment, and prepared for IHC.

Tissue-equivalent culture

Fibrin gels containing 5×10^5 gingival or alveolar mucosal fibroblasts were prepared using a modified procedure described elsewhere (Okazaki et al. 2003): we used fibrin gel rather than collagen gel to imitate lamina propria. After mixing 1 ml of fibrinogen (50 NIH U/ml in 0.15 M NaCl) (Calbiochem) and 100 μL of bovine thrombin (3 mg/ml) (Sigma-Aldrich), the mixture was added with 5×10^5 fibroblasts, gently vibrated for a few seconds, poured into the transwell inserts, and allowed to gel for 1 h at 37°C. DMEM was then added to the lower well; each well was covered for overnight incubation at 37°C. The medium was removed 24 h later and the gels were plated with 1×10^6 gingival keratinocytes (at passage 3 or 4) in SFM medium, and the keratinocytes were allowed to adhere for 24 h. From the second day, medium (a 1:1 mixture of DMEM and K-SFM) was added only to the lower well until it just touched the underside of the transwell membrane. Cultures were then maintained for 3 weeks at the air/liquid interface; the medium was changed every 2–3 days. At the end of the experiment, the gel was fixed, embedded, and processed for IHC assays.

Results

IHC of K1/10, K4/13, and elastin in native oral mucosa

The entire gingival epithelium appeared to be parakeratinized, alveolar mucosa was not keratinized. The expression patterns of examined keratins and elastin in four patients were highly similar. K1 and 10 were expressed in the suprabasal layer of keratinized gingiva, while K4 and 13 were expressed in the suprabasal layer of non-keratinized alveolar mucosa. K1 and 10 appeared in pairs, as did K4 and 13. However, when the IHC micrographs from serial sections of the same patient were superimposed, the expression areas of paired keratins were not completely identical (Fig. 1a–d). Elastin was expressed homogeneously and abundantly in the connective tissue of non-keratinized alveolar mucosa.

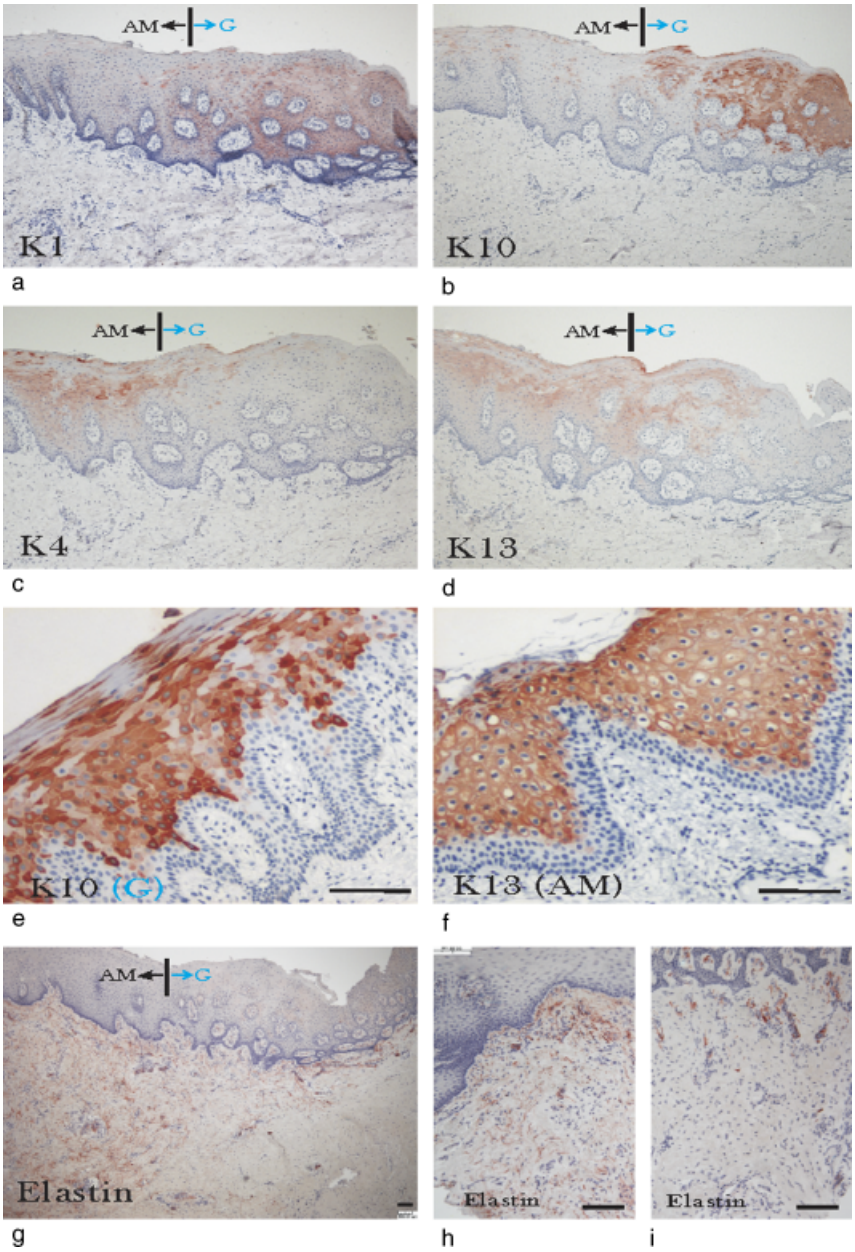

Fig. 1. Immunohistochemistry for keratin 1 (a), keratin 10 (b and e), keratin 4 (c), keratin 13 (d and f), and elastin (g–i) in the oral mucosa consisting of both keratinized and non-keratinized parts. AM, non-keratinized alveolar mucosa; G, keratinized gingiva. Microphotograph (e) shows a close-up view of keratinized-gingiva immunostained for keratin 10. Microphotograph (f) shows a close-up view of alveolar mucosa immunostained for keratin 13. Microphotographs (h) and (i) are show close-up views of alveolar mucosa and gingiva from figure (g). Scale bar = 100 μm.

On the contrary, it was detectable only in association with blood vessels in the connective tissue of keratinized gingiva (Fig. 1e–g).

K1/10 and K4/13 expression in tissue cultures when elastin concentrations were changed

When the tissue fragments of oral mucosa were cultured on the transwell membranes for 2 weeks, the superficial layer of the epithelial cells gradually detached and the thickness of the whole epithelium decreased. The rete ridge was less prominent than native oral mucosa (Figs 2 and 3). Because the expression patterns of paired keratins were so similar, we show only the results of K1 and 4. After 2 weeks of culturing, the keratinized gingiva still expressed K1/10 and not K4/13 (Fig. 2a and c). When the

gingiva was treated with 200 μg/ml of purified elastin protein, the epithelial cells started to express K4/13. The expression of K1/10 was less intense than that in untreated controls (Fig. 2b and d). After 2 weeks of culturing, the non-keratinized mucosa still expressed K4/13, not K1/10. When purified neutrophil elastase was added to remove inherent elastin in the connective tissue of alveolar mucosa, there seemed to be fewer cells in the connective tissue (Fig. 3b and d). In some areas, the basal layer of epithelium detached from the underlying connective tissue. K1/10 expression occurred and K4/13 expression significantly decreased (Fig. 3b and d). The immunostaining of elastin in the cultured gingiva fragments was consistently negative after the defined culture period. The expression of elastin in the cultured alveolar mucosa was still positive, but the intensity was decreased compared with the fresh alveolar mucosa (data not shown).

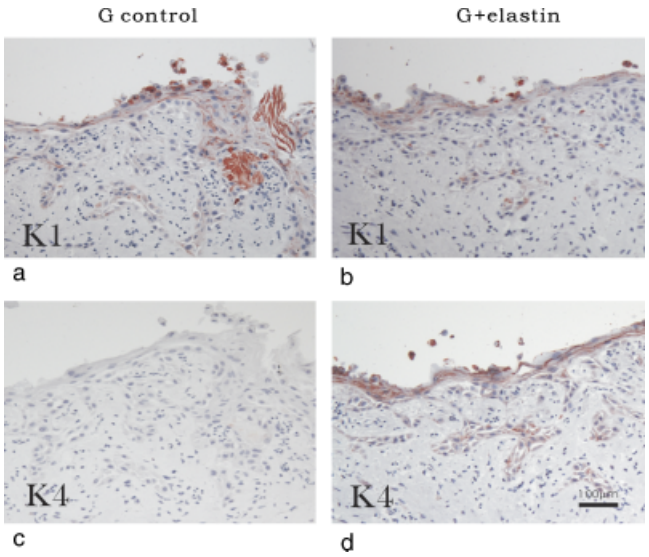

Fig. 2. Immunohistochemistry for keratin 1 (a and b) and keratin 4 (c and d) in keratinized gingival culture treated with or without exogenous elastin protein. The photographs shown are representative of the sections from six specimens. G, keratinized gingiva. Scale bar = 100 μm.

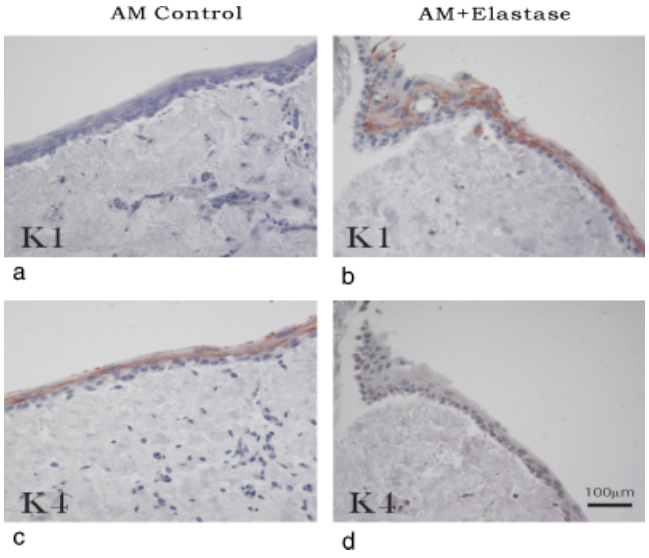

Fig. 3. Immunohistochemistry for keratin 1 (a and b) and keratin 4 (c and d) in non-keratinized alveolar mucosa treated with or without elastase. Purified elastase was added to degrade inherent elastin in the mucosa specimen. The photographs shown are representative of the sections from six specimens. AM, non-keratinized alveolar mucosa. Scale bar = 100 μm.

ICC and Western blotting for oral mucosa fibroblasts and keratinocyte

Because there are many different cell types and extracellular components in the connective tissue of oral mucosa, it is difficult to clarify what cells or components are critical for oral keratinization. We tried to narrow the target to fibroblasts, which are the predominant cells in connective tissue. The morphologies of the cell cultures of gingival fibroblasts and alveolar mucosal fibroblasts were different (Fig. 4a). The gingival fibroblasts were more bipolar and spindle shaped. When they reached 100% confluence, they gathered to form parallel arrays and whorls. Alveolar mucosal fibroblasts from the same passage as the gingival fibroblasts were more flattened and multipolar, and their nuclei were larger and rounder. When they reached confluence, the cells gathered in random directions, not in parallel as the gingival fibroblasts did (Fig. 4a). The ICC showed that only the alveolar mucosal fibroblasts were immunoreactive for elastin. Positive staining occurred in the extracellular matrix (Fig. 4b). Only the alveolar mucosal fibroblasts showed a positive band for immunoblotting. The molecular weight of elastin that we detected was about 70 kDa (Fig. 4c). The results of both ICC and Western blotting proved that only fibroblasts from the connective tissue of non-keratinized alveolar mucosa expressed elastin (Fig. 4).

Characterizing a tissue-equivalent culture that imitates oral mucosa

To determine whether elastin influences the expression of keratins in vitro, we engineered oral mucosal equivalents using gingival or alveolar mucosal fibroblasts in fibrin gels and oral keratinocytes on the surfaces of gels. After 3 weeks of culture in an air–liquid interface, the epithelial components became multilayered. However, they were not as thick as in genuine oral mucosal tissue cultures. We found no obvious stratum corneum or rete ridge. We then added 200 ng/ml of purified human elastin to the medium for the gingival equivalent test group. The alveolar mucosal equivalent was used as a positive control (Fig. 5a and d). The IHC of non-keratinized keratins (K4/13) showed that the

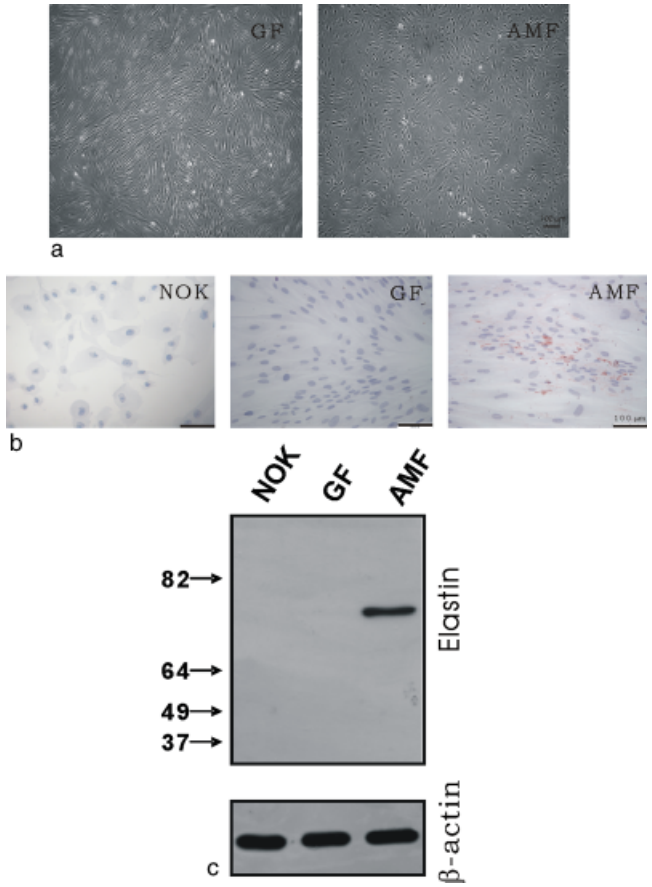

Fig. 4. The in vitro characterization of gingival and alveolar mucosal fibroblasts expressing elastin. The appearances of the two fibroblast cultures were different under the phase-contrast microscope (a). The immunocytochemistry of three different types of oral cells showed that only AMF had a positive reaction (b). The immunoblot for elastin revealed that only AMF had a visible band about 70 kDa long. β-actin was the internal control. The results shown are representative of the experiments from six specimens. GF, gingival fibroblasts; AMF, alveolar mucosa fibroblasts; NOK, normal oral keratinocytes from gingiva. Scale bar = 100 μm.

gingival equivalent without elastin treatment did not express K4/13 (Fig. 5b and e). However, after elastin treatment, the gingival equivalent, like the alveolar mucosal equivalent, expressed K4/13 (Fig. 5c and f).

Discussion

Clinically, gingival augmentation surgery is intended to increase the width of keratinized tissue and improve periodontal health in certain situations, such as progressive gingival recession, placement of a restoration with an intracrevicular margin, orthodontic complications, and mucositis of dental implants (Oates et al. 2003, Chung et al. 2006). Most of the gingival augmentation procedures are surgical and need two surgical sites: a donor and a recipient. These procedures are usually quite uncomfortable for patients (Oates et al. 2003). If we more completely understood the molecular mechanisms of the keratinization of oral mucosa, we would be better able to develop less invasive procedures to augment keratinized gingiva.

It has long been known that the underlying mesenchyme has an instructive influence over the overlaying epithelium (Kollar & Baird 1970, Karring et al. 1975, Mackenzie & Hill 1984). Many factors, including Vitamin D_3, Ca^{2+}, interferon γ, transforming growth factors, integrins, and retinoids in the supporting connective tissue, are known to influence the gene expressions that are responsible for the heterogeneity of epithelial tissue. In the present study, we found that elastin, a fibrous protein rich in the connective tissue of non-keratinized oral mucosa, is important for maintaining the non-keratinized phenotype of oral mucosa. A decrease in the amount of elastin caused a phenotypic conversion to keratinized oral mucosa. Interestingly, several oral and dermal pathologies with hyperkeratosis show elastolysis or elastic fibre degeneration. These pathologies include actinic cheilosis, lichen planus, Costello syndrome, and Acrokeratoelastoidosis of Costa (Boisnic et al. 1995, Fiallo et al. 1998, Bloor et al. 2000, Hatamochi et al. 2000, Huber & Terezhalmy 2006). Actinic cheilosis is a diffuse degenerative change of the vermilion border of the lips as a result of sun damage. It is characterized by hyperkeratosis and solar elastosis (degeneration of collagen and elastic fibres caused by ultraviolet light). The lichen planus is characterized by hyperkeratinization and severe inflammatory changes. It has been reported that elastic fibres were scarce or absent in the inflammatory zone in all the examined lesions (Dahlbäck & Sakai 1991). The reduced elastin due to degradation by elastase or ultraviolet light might be partially responsible for the conversion of the non-keratinized phenotype to the keratinized one. The data from our study showed that elastin is indirectly important for the non-keratinized phenotype of oral mucosa. However, what molecules are upstream and downstream of elastin in oral mucosa remains unclear. Retinoic acid, the active form of vitamin A in epithelia, inhibits morphological structures and the expression of several markers of keratinized epithelia, such as K1 and profilaggrin. In contrast, retinoic acid up-regulates keratin 13 and 19, which are normally found in non-keratinized epithelia (Kopan et al. 1987, Asselineau et al. 1989). There is more recent evidence (Kautsky et al. 1995) that retinoic acid regulates oral epithelial differentiation is that partially mediated by an indirect, fibroblast-mediated effect. Moreover, it has been found that there is a distinct difference between gingiva and buccal mucosa in the expression of cellular retinoic acid-binding protein I (CRABP-I). Fibroblasts in the gingiva stained positively for CRABP-I while those in the buccal mucosa exhibited no staining for CRABP-I (Berkovitz & Maden 1993). The physiological role of CRABP is to regulate the intracellular level of free retinoic acid in the cell. Therefore, the CRABP-I in the gingival fibroblast may retain more retinoic acid

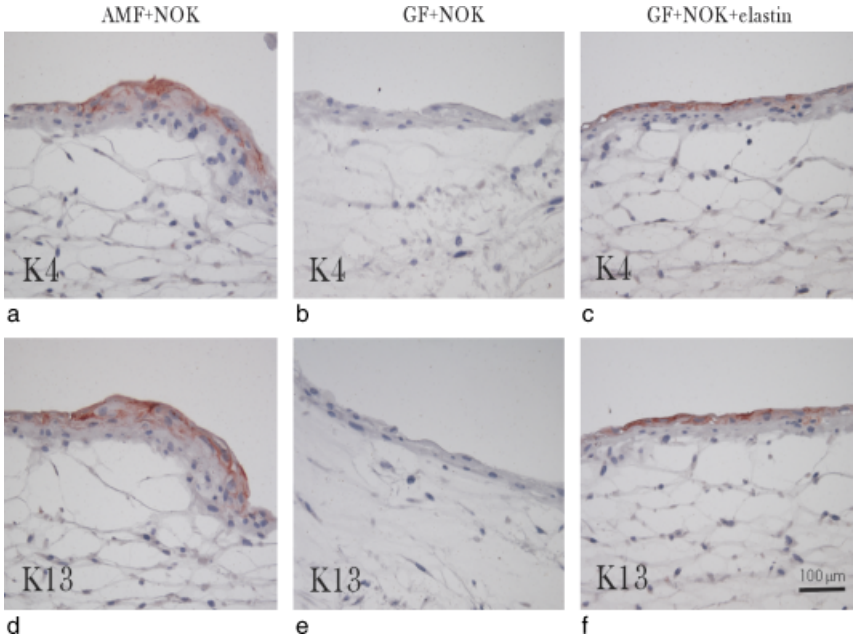

Fig. 5. Immunohistochemistry for non-keratinized keratins (K4 and 13) in the tissue equivalents of oral fibroblasts and keratinocytes. The tissue equivalent of alveolar mucosal fibroblasts in the fibrin gel and overlaid with gingival keratinocytes was used as a positive control (a and d). The tissue equivalent of keratinized gingival fibroblasts and keratinocytes with (c and f) or without (b and e) added exogenous elastin were cultured for 3 weeks. Then the equivalents were processed for the immunohistochemistry of K4 and 13, which are usually expressed in non-keratinized epithelia. GF, gingival fibroblasts; AMF, alveolar mucosal fibroblasts; NOK, normal oral keratinocytes from gingiva. Scale bar = 100 μm.

in the cell and release less retinoic acid into the extracellular matrix. Interestingly, exogenous retinoic acid can up-regulate elastin expression in different kinds of cell cultures: lung fibroblasts, vascular smooth muscle cells, and embryonic skin fibroblasts (Liu et al. 1993, Hayashi et al. 1995, Tajima et al. 1997). In summary, it is plausible that the connective tissue of non-keratinized alveolar mucosa has more retinoic acid in a free form. More retinoic acid promotes the expression of elastin in alveolar mucosal fibroblasts and indirectly upregulates the expression of specific non-keratinized keratins (K4/K13). As far as we can search in the literature, no study provides direct evidence that an alteration in the pattern of differentiation-specific keratins invariably leads to the modulation of cornified layer production. However, studies of some oral hyperkeratotic pathologies, such as lichen planus, non-dysplastic keratoses, submucosa fibrosis, and leukoplakia, consistently showed that K1/K10 aberrantly appeared in the lesions on buccal mucosa, colocalized with hyperkeratotic areas, and therefore provide indirect evidence (Boisnic et al. 1995, Bloor et al. 2000, Fillies et al. 2007, Lalli et al. 2008, Jacques et al. 2009).

Fibronectin may be one of the candidate molecules downstream of elastin in the non-keratinized phenotype of oral mucosa. Elastin increases both total protein and fibronectin biosynthesis in the cell culture of human skin fibroblasts (Fodil-Bourahla et al. 1999). Fibronectin added to the methylcellulose culture of skin keratinocytes largely inhibits the terminal differentiation of human keratinocytes (keratinization). The effect of fibronectin is concentration- and time-dependent and is mediated by a receptor of the integrin family (Adams & Watt 1989). In our unshown data, direct addition of elastin in the oral keratinocyte culture did not change the keratinized phenotypes of the cells. Therefore, elastin may up-regulate fibronectin in oral mucosa fibroblasts and inhibit the keratinization of mucosal epithelia indirectly.

In conclusion, our study confirmed that the epithelium of non-keratinized alveolar mucosa specifically expressed K4/K13, while that of keratinized gingiva expressed K1/K10. There was a huge amount of elastin in the connective tissue of non-keratinized alveolar mucosa, but only a limited amount in the blood vessels of the connective tissue of keratinized gingiva. When exogenous elastin was added to the tissue culture of keratinized gingiva, the epithelium started to express K4/K13. On the contrary, when elastase was added to degrade the inherent elastin in non-keratinized mucosa, the overlaying epithelium switched to the keratinized phenotype. The results of our study provided evidence that elastin is important for the non-keratinized phenotype of oral mucosa. If a specific blocking agent of elastin is discovered, it will be possible to develop a non-surgical treatment to augment keratinized gingiva.

Acknowledgements

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Clinical Relevance

Scientific rationale for study: The underlying connective tissue can determine the phenotypes of oral epithelia. A discernable difference between keratinized and non-keratinized oral connective tissues is the quantity of elastin they contain: it is abundant in the latter but scarce in the former.

Principal findings: Elastin is important in determining the non-keratinized phenotypes of overlaying epithelium. Eradication of elastin in non-keratinized oral mucosa favoured their expression of keratinized keratins.

Practical implications: With more understanding of the molecular mechanisms in the keratinization of gingival epithelia, non-surgical or less invasive techniques may be developed and clinically applied to replace conventional mucogingival surgery.