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Author(s) Alternative	Muramatsu, T; Uekusa, T; Masaoka, T; Saitoh, M; Hashimoto, S; Abiko, Y; Jung, HS; Shimono, M
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Differential expression and localization of connexins 26 and 43 in the rat gingival epithelium*

Takashi Muramatsu^{1,2}, Tomoko Uekusa², Takayasu Masaoka^{1,2,3}, Masato Saitoh⁴, Sadamitsu Hashimoto^{1,2}, Yoshihiro Abiko⁴, Han-Sung Jung⁵, and Masaki Shimono^{1,2}

¹Oral Health Science Center HRC7, ²Department of Pathology, ³Department of Periodontology, Tokyo Dental College, Chiba, and ⁴Department of Dental Science, Institute of Personalized Medical Science, Health Sciences University of Hokkaido, Sapporo, Japan; and ⁵Department of Oral Biology, Research Center for Orofacial Hard Tissue Regeneration, College of Dentistry, Brain Korea 21 Project for Medical Science, Yonsei Center of Biotechnology, Yonsei University, Seoul, South Korea

Summary. We investigated the expression and localization of connexins (CX) 26 and 43 in the rat gingival epithelium. RT-PCR analysis revealed CX26 gene expression in both the upper and lower layers of the gingival epithelium and in the total epithelial layer, whereas CX43 gene expression was limited to the lower layer and the total epithelial layer. Immunoreactivity for CX43 was observed in the membranes of adjacent cells from the basal layer to the middle of the prickle cell layer, while immunoreactivity for CX26 was observed in the granular cell layer and lower part of the squamous cell layer. Merged images revealed the co-localization of CX26 and CX43 in the middle of the prickle cell layer. By immuno-electron microscopy, gap junctions appeared curved, hemi-circular, or annular within the cytoplasm, and gold particles indicating the

presence of CX43 were localized at the outer edges of these cytoplasmic formations. These results suggest that CX43 is associated with the regulation of cell proliferation and that increased CX26 expression is associated with differentiation of keratinocytes. Thus, degradation of CX43 is considered to play an essential role in differentiation of the rat gingival epithelium.

Introduction

The oral mucosal epithelium protects the organism against external insult from mechanical, chemical, or thermal stress, and serves as a barrier against water. Histologically classified as stratified squamous epithelium, it is mainly composed of keratinocytes and is characterized by well-developed intercellular junctions such as desmosomes, adherens junctions, tight junctions, and gap junctions (Shimono and Clementi, 1976; White *et al.*, 1984). In general, the basal layer of the stratified squamous epithelium contains undifferentiated proliferating keratinocytes. These cells migrate to the prickle cell layer, with differentiation occurring as proliferation declines. During this process, structural and adhesive proteins such as E-cadherin along with tight and gap junction proteins are believed to be important markers of tissue integrity, proliferation, differentiation, and communication (Fuchs and Byrne, 1994; Kretz *et al.*, 2003).

Gap junctions are transmembrane channels that directly link neighboring cells and mediate the reciprocal exchange of metabolites and ions of less than 1 kDa in molecular weight, including second messengers such as cAMP and

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Address for correspondence: Takashi Muramatsu, DDS, Ph.D., Department of Pathology, Tokyo Dental College, 1-2-2, Masago, Mihama-ku, Chiba, 261-8502, Japan
Tel: +81-43-270-3782, Fax: +81-43-270-3784
E-mail: tmuramat@tdc.ac.jp

Ca²⁺. Gap junction-mediated intercellular communication is believed to play an essential role in the maintenance of homeostasis, coordination of secretion, morphogenesis, cell differentiation, and growth control in multicellular organs (Flagg-Newton and Loewenstein, 1979; Beyer *et al.*, 1987). Ultrastructurally, it has been shown that gap junctions occur between oral stratified squamous epithelial cells, which suggests that these gap junctions coordinate proliferation and differentiation (Shimono and Clementi, 1976; White *et al.*, 1984).

Connexins (CXs) comprise a family of gap junction proteins that form hexameric assemblies in the plasma membranes of adjacent cells, creating intercellular channels. They are distinguished according to their molecular weights as predicted from their corresponding cDNAs (Beyer *et al.*, 1987). Earlier investigations reported differential localizations of CX26 and CX43 in the rat and human epidermis and oral epithelium, and proposed that changes in CX expression indicated

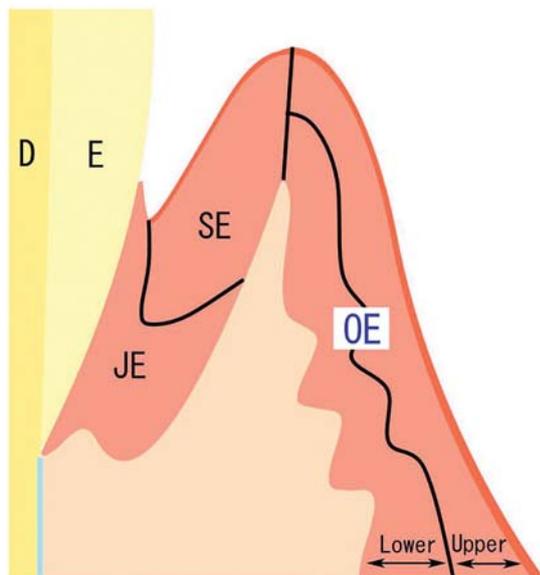


Fig 1. Schema of upper and lower layers of the oral mucosal epithelium. The oral epithelium was cut by laser microdissection. Upper and lower layers of the epithelium were obtained by laser microdissection. The upper layer was composed of cells from the middle of the prickle cell layer to the granular cell layer, and the lower layer of the oral epithelium contained cells from the basal cell layer to the middle of the prickle cell layer. Furthermore, total epithelium was also cut and used for RNA extraction and RT-PCR analysis. D: dentin, E: enamel, JE: junctional epithelium, SE: sulcus epithelium, OE: oral epithelium

differentiation of the epidermis and oral epithelium (Kamibayashi *et al.*, 1993; Saitoh *et al.*, 1997). However, mRNA expression of CXs during differentiation of oral epithelial cells has yet to be fully investigated. Moreover, ultrastructural localization of CXs during differentiation of oral epithelial cells remains to be clarified.

The purpose of this study was to investigate the expression and localization of CX26 and CX43 in the rat oral gingival epithelium using RT-PCR, immunofluorescence, and immuno-electron microscopy, and discuss changes in CX expression during proliferation and differentiation.

Materials and Methods

Animals and tissue preparation

All experiments were carried out in accordance with the Guidelines for the Treatment of Animals established by Tokyo Dental College. Four-week-old male Sprague-Dawley rats (150–180 g each) were used in the study. The rats were anesthetized with an intraperitoneal injection of 2.5% sodium thiopental (100 mg/kg) before the experiments. The palatal gingiva was excised between M1 and M2 (n=25), embedded in OCT compound, and quickly frozen in isopentane that had been refrigerated in liquid nitrogen. Frozen sections 6 μ m in thickness were prepared for immunofluorescence. Frozen sections 8 μ m in thickness were also prepared and mounted on glass slides with films for laser microdissection and RT-PCR analysis. For immuno-electron microscopy, additional rats (n=15) were sacrificed, and perfusion-fixation with 1% paraformaldehyde in a 0.1 M phosphate buffer (PB) was performed transcardially as previously reported (Muramatsu *et al.*, 1996).

Laser microdissection

Frozen sections for laser microdissection analysis were fixed in 100% methanol for 3 min, washed with 0.01% diethyl pyrocarbonate (DEPC)-treated distilled water, and stained with 0.1% toluidine blue. The lower and upper layers of the epithelium and total epithelial layer were microdissected using the AS LMD Laser Microdissection System (Leica Microsystems, Tokyo) as shown in the schema (Fig. 1). In preliminary experiments, we confirmed that the lower layer of the oral epithelium contained cells from the basal cell layer to the middle of the prickle cell layer, and that the upper layer was composed of the cells from the middle of the prickle cell layer to the granular cell layer.

RT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) and was amplified with the NanoAmp RT-IVT Labeling kit (Applied Biosystems, Foster City, CA, USA), after which amplified RNA (aRNA) was synthesized. The aRNA was reverse-transcribed into complementary DNA (cDNA), and PCR reactions were carried out using the Takara RNA PCR Kit (Takara, Tokyo). Primer sequences used to detect rat CX26, CX43, and β -actin (as an internal control) are shown in Table 1.

Primary antibodies

Anti-CX26 and anti-CX43 antibodies were purchased from Chemicon International (Temecula, CA, USA).

Immunofluorescence

Indirect immunofluorescence was carried out on the unfixed cryosections according to our previously reported method (Muramatsu *et al.*, 1996). The sections were blocked with 10% normal goat serum for 30 min at room temperature (RT) and then incubated with anti-CX26 rabbit polyclonal antibody (dilution 1:250) or anti-CX43 mouse monoclonal antibody (dilution 1:250) at 4°C overnight. After incubation with the primary antibody, the sections were incubated for 2 h at RT with Alexa fluor568-conjugated anti-rabbit IgG (dilution 1:200; Molecular Probes, Eugene, OR, USA) or Alexa fluor488-conjugated anti-mouse IgG (dilution 1:200; Molecular Probes). Specimens were observed using a fluorescence microscope (Axiophot 2, Carl Zeiss, Oberkochen, Germany). As a negative control, immunofluorescence was also carried out without the primary antibody and an absorption test was also performed.

Immuno-electron microscopy

Specimens were cut into small pieces and post-fixed in the same fixative for 5 h. For pre-embedding immuno-electron microscopic analysis, small pieces were cut to 8 μ m in thickness using a cryostat. The sections were incubated in CX43 antibody (1:250) at 4°C overnight. Sections were then washed in PBS for 6 h at RT and incubated with 5-nm colloidal gold-conjugated anti-mouse IgG (BBInternational, Llanishen, Cardiff, UK; dilution 1:20) at 4°C overnight. The samples were dehydrated and embedded in EPON 812 (TAAB, Reading, UK). Selected areas were trimmed using a light microscope, and ultra-thin sections (80 nm) were mounted on mesh grids. The sections were then examined under a transmission electron microscope (Hitachi H-7100, Tokyo) after staining with uranyl acetate and lead citrate.

Results

Laser microdissection and RT-PCR

The RT-PCR analyses of the homogenates from the rat microdissected gingival epithelium and controls are shown in Figure 2. CX26 gene expression was detected in the upper and lower layers and in the total layer of the gingival epithelium. CX26 gene expression in the liver, which was a positive control, was distinct, whereas no gene expression was detected in the heart, which was a negative control. CX43 gene expression was detected in the lower layer and in the total layer of the gingival epithelium, but was not observed in the upper layer. Expression of CX43 was detected in the heart as a positive control, while no expression was found in the liver, which was the negative control. Furthermore, β -actin expression used as an internal control, was positive in all samples, including the liver and heart.

Table 1. Primer sequences for the detection of specific connexin26, connexin43, and beta-actin

	Primer sequences	Product size
Connexin26	F: 5'-GGTGTGGGGAGATGAGCAAG -3' R: 5'-GACTTCCCTGAGCAATACCT -3'	540 bp
Connexin43	F: 5'-AAAGGCGTTAAGGATCGCGTG -3' R: 5'-GTCATCAGGCCGAGGCCT -3'	438 bp
β -actin	F: 5'- TGTATGCCTCTGGTCGTACCAC -3' R: 5'- CTCCTGAGCGCAAGTACTCTGT -3'	804 bp

Immunofluorescence

Immunoreactivity for CX26 (green) was observed in the plasma membranes of the upper layer, while no reaction was seen in the lower layer (Fig. 3a). On the other hand, immunoreactivity for CX43 (red) was present in the cell-to-cell contact membranes between adjacent cells in the lower layer. However, no reaction was seen in the upper layer (Fig. 3b). Merged images revealed different patterns of immunoreactivity in the upper and lower layers of the total epithelial layer, and yellow staining was seen in the middle area of the total layer, indicating the co-localization of CX26 and CX43 (Fig. 3c). Interestingly, immunoreactivity for CX43 was also observed in the cytoplasm in the middle of the prickle cell layer (Fig. 3d). No immunoreactivity was seen in the orthokeratinized layer (superficial layer), nor when normal goat serum was used instead of the primary antibody (data not shown).

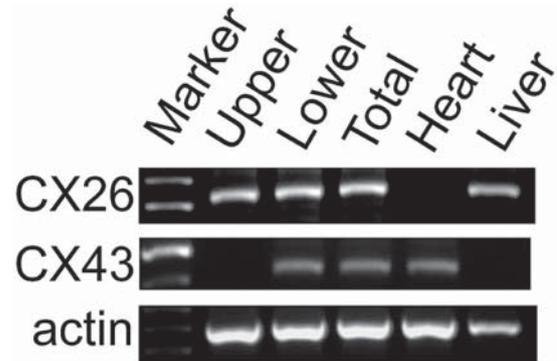


Fig. 2. RT-PCR analysis of the oral mucosal epithelium. CX26-gene expression was detected in the upper and lower layers and in the total layer of the gingival epithelium. CX43-gene expression was detected in the lower layer and in the total layer of the gingival epithelium, but was not observed in the upper layer. Note that CX26 expression was detected in both the lower and upper layers of the epithelium.

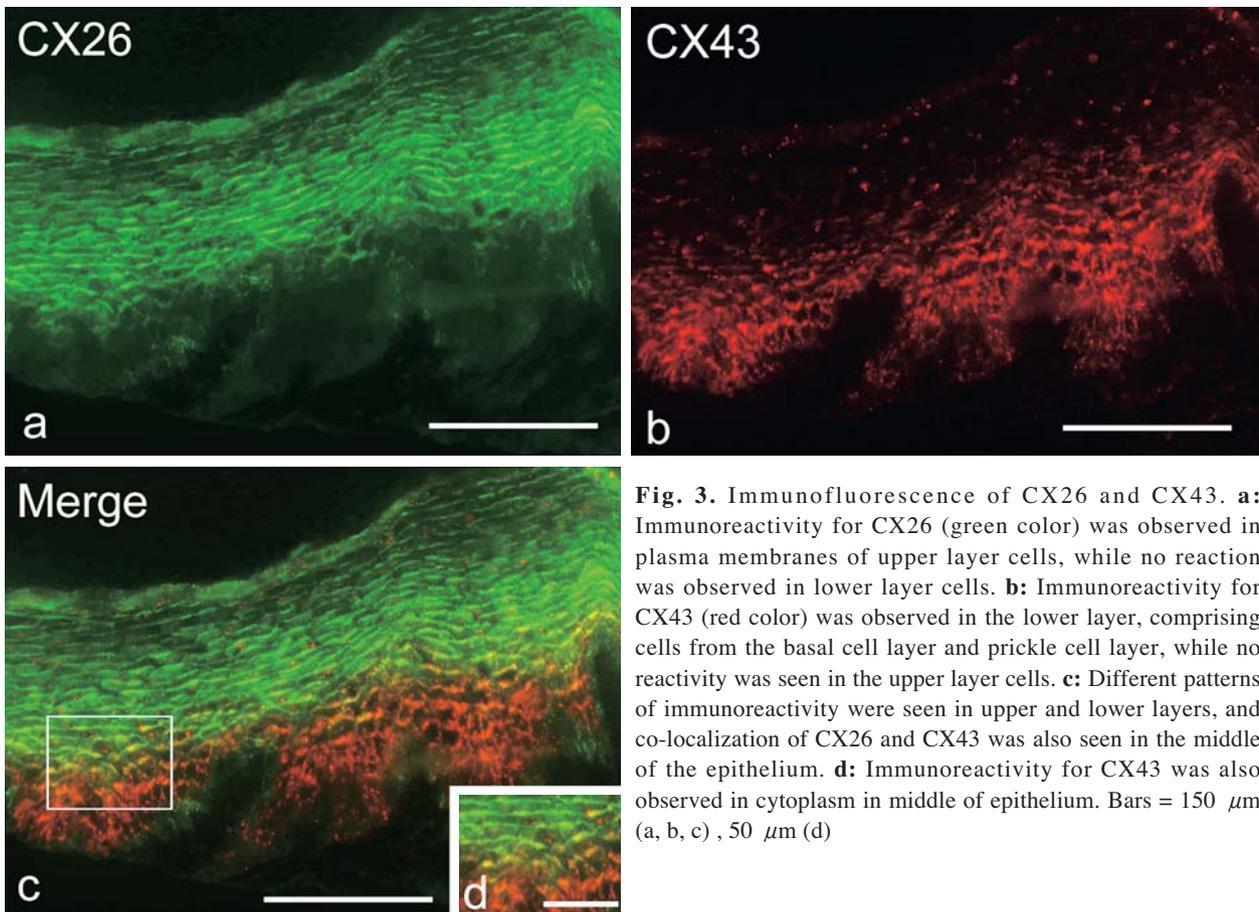


Fig. 3. Immunofluorescence of CX26 and CX43. **a:** Immunoreactivity for CX26 (green color) was observed in plasma membranes of upper layer cells, while no reaction was observed in lower layer cells. **b:** Immunoreactivity for CX43 (red color) was observed in the lower layer, comprising cells from the basal cell layer and prickle cell layer, while no reactivity was seen in the upper layer cells. **c:** Different patterns of immunoreactivity were seen in upper and lower layers, and co-localization of CX26 and CX43 was also seen in the middle of the epithelium. **d:** Immunoreactivity for CX43 was also observed in cytoplasm in middle of epithelium. Bars = 150 μ m (a, b, c), 50 μ m (d)

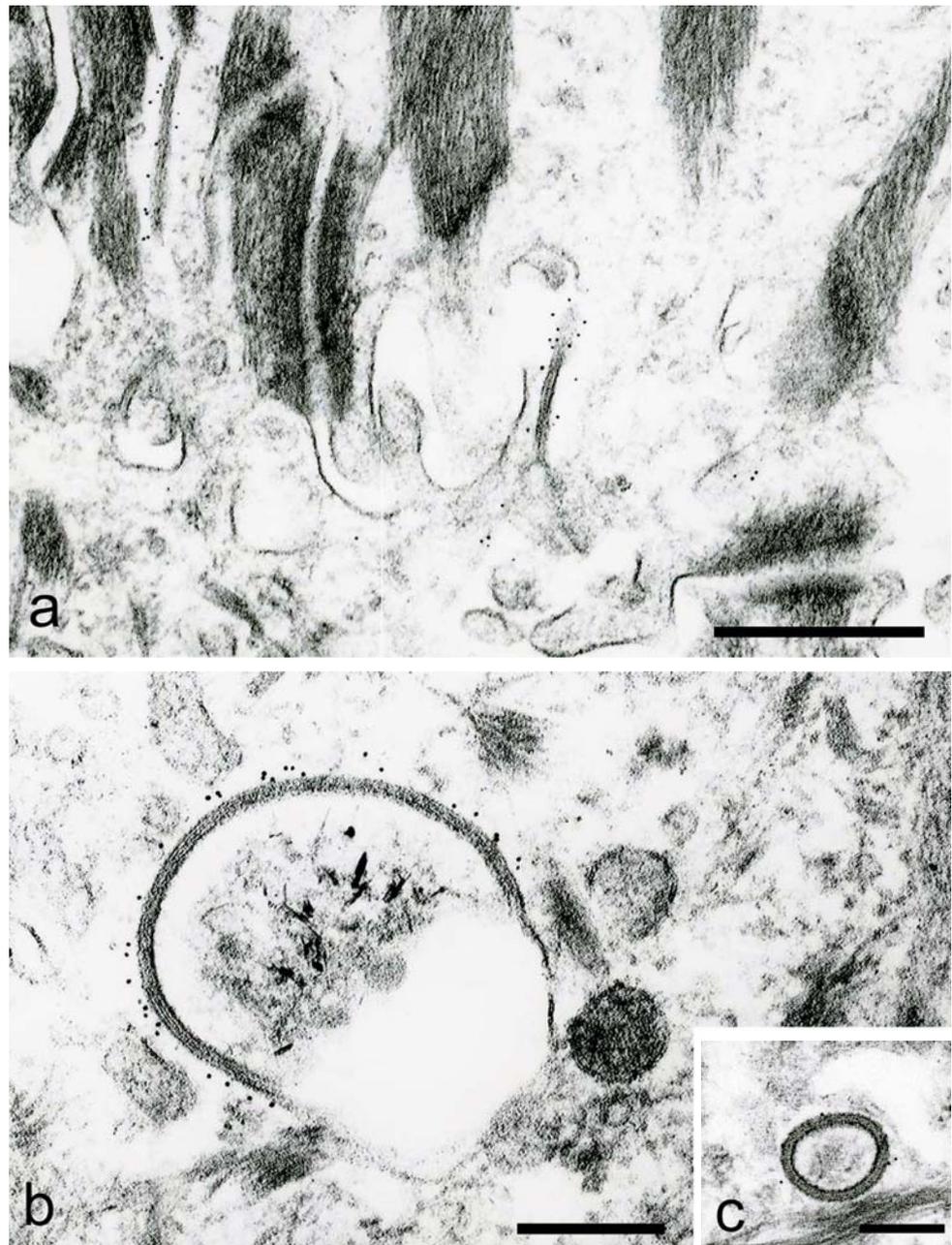


Fig. 4. Immuno-electron microscopy. **a:** Gold particles indicating CX43 were detected at closely apposed plasma membranes of the epithelium (middle area). Gold particle size = 5 nm. **b, c:** Gap junctions were hemi-circular or annular, and were localized in the cytoplasm. A subjacent cytoplasmic halo was prominent on only one side. Gold particles indicating CX43 were localized at the outer side of curved, hemi-circular and annular gap junctions, but not at the inner side. Gold particle size = 5 nm. Bars = 500 nm (a), 200 nm (b), 100 nm (c)

Immuno-electron microscopy

Immuno-labeling for CX43 was seen at gap junctions between adjacent prickle cells in the basal area of the gingival epithelium. Gold particles indicating CX43 were detected at closely apposed plasma membranes. Immuno-gold labeling was evident on both cytoplasmic sides of the linear gap junctions (Fig. 4a). In the middle of the epithelium, the gap junctions were curved, hemi-circular (Fig. 4b), or annular (Fig. 4c), and were localized in the cytoplasm. Cytoplasmic filaments were observed close to the convex surface of the annular gap junctions, and a subjacent cytoplasmic halo was prominent on only one side. Gold particles indicating CX43 were localized at the outer side of the curved, hemi-circular, and annular gap junctions, but not at the inner side (Fig. 4b).

A summary of our RT-PCR and immunohistochemical analysis is shown in Table 2.

Table 2. Summary of CX26 and CX43 expression in the rat gingival epithelium

	mRNA		Protein	
	lower	upper	lower	upper
CX26	+	+	-	+
CX43	+	-	+	-

Discussion

Intercellular communication via gap junctions plays a major role in the maintenance of normal growth, proliferation, and differentiation (Yamasaki and Naus, 1996). Keratinocytes in the oral epithelium are connected by gap junctions that can be detected by electron microscopy (Shimono and Clementi, 1976; White *et al.*, 1984), and the oral epithelium can be used as a good model for cell proliferation and differentiation. Expression of CXs in the oral epithelium has been reported. However, the precise expression and localization of CXs in the oral epithelium remains to be clarified.

Our results showed that CX43 was localized in the lower epithelial layer, which is composed of basal cells and prickle cells, where proliferative cells dominate. An earlier report also showed that transfection of the CX43 gene slowed proliferation in BSC-OF cells derived from oral basaloid squamous cell carcinoma (Shima *et al.*, 2006). Zhang *et al.*, (2003), indicating that CX43 binds S-phase kinase-associated protein-2 to inhibit the

degradation of cell cycle regulator p27. This suggests that the role of CX43 in the lower layer of the oral epithelium is as a negative regulator of the cell cycle, inhibiting excessive proliferation. Furthermore, our results also showed that reactivity for CX26 occurred in the upper area of the prickle cell layer in epidermal and corneal keratinocytes, which is consistent with the findings of earlier studies (Kamibayashi *et al.*, 1993; Goliger and Paul, 1994; Choudhry *et al.*, 1997; Wiszniewski *et al.*, 2000). These results suggest that CX43 is associated with the regulation of cell proliferation, and that increased CX26 expression is associated with the differentiation of keratinocytes.

The present study revealed that the expression of CX26 mRNA commenced from the lower layer, while the CX26 protein was expressed in the upper layer, implying that CX26 was regulated. Interestingly, Rouan *et al.*, (2001) reported that a mutant CX26 significantly inhibited not only the intercellular conductance of co-expressed wild-type CX26, but also that of wild-type CX43, i.e., there was trans-dominant inhibition of CX43 by mutant CX26. Furthermore, a recent study using CX43 RNAi demonstrated a reduction in CX26 and an impaired differentiation of the epidermis (Langlois *et al.*, 2007). Our results, taken together with those of these earlier reports, indicate that expression of CX26 may be associated not only with regular differentiation, but also with regulation of the CX43 expression in oral keratinocytes, and that CX26 and CX43 may collectively co-regulate epithelial differentiation. However, further studies, including those using RNAi knockdown and/or an impaired function status of CX26, will be necessary to clarify this relationship.

In several electron microscopic studies, double-membrane intracellular structures termed annular gap junctions were identified in the cytoplasm (Shimono and Clementi, 1976; White *et al.*, 1984; Jordan *et al.*, 2001; Leithe *et al.*, 2006; Piehl *et al.*, 2007). These annular gap junctions, believed to be involved in degradation, are important factors in epithelial differentiation and the stratification of keratinocytes (White *et al.*, 1984). Recent electron microscopic analysis revealed that annular gap junctions occurred during degradation and were associated with clathrin-dependent endocytosis (Leithe *et al.*, 2006; Piehl *et al.*, 2007) and lysosomal (Naus *et al.*, 1993; Musil *et al.*, 2000) and proteosomal (Laing and Beyer 1995; Musil *et al.*, 2000) pathways. Our immunofluorescence results showed a spotty cytoplasmic localization of CX43 in the middle of the prickle cell layer. In order to investigate this ultrastructural finding further, we performed immuno-electron microscopy using an anti-CX43 antibody. Our results revealed that the hemi-

circular and annular gap junctions were immunoreactive for CX43. Interestingly, gold particles identifying CX43 were not localized at the halo side of these curved, hemi-circular, or annular gap junctions. This suggests that the degradation of CX43-composed gap junctions occurs at the halo side earlier, and that degradation, as seen in annular gap junctions, is an important factor in the differentiation of the gingival epithelium.

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