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Original article

Brush biopsy of human oral mucosal epithelial cells as a quality control of the cell source for fabrication of transplantable epithelial cell sheets for regenerative medicine

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ABSTRACT

Autologous oral mucosal epithelial cell sheets have been used for treating epithelial defects such as cornea and esophagus. The cell source of patients' oral mucosal epithelial cell sheet should be examined in normality because it has individual difference. In this study, oral mucosal epithelial cells were less invasively collected by brush biopsy from the buccal, gingival, labial, and palate mucosa of four healthy volunteer donors without anesthesia, and analyzed the keratin expressions by western blotting and the obtained results were compared with those by immunohistochemistry of each of the native tissues. All of the oral mucosal epithelial cells expressed keratin 4 (K4) and K13, which were mucosal stratified squamous epithelial cell markers. K1 and K10, keratinized epithelial cell markers, were also detected in keratinized tissues such as gingival and palate mucosa. The markers of epithelial basal cells such as p63 and K15 were not detected by brush biopsy-western blotting. Although this method does not include basal layers of oral mucosa, protein expressions of upper layer of lesion area are different from normal. Therefore, brush biopsy-western blotting was extremely less invasive and would contribute to quality control of the fabrication of autologous oral mucosal epithelial cell sheets.
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1. Introduction

We have successfully performed clinical applications of cell sheet based regenerative medicine in cornea [1] and esophagus [2] utilizing temperature-responsive cell culture surfaces [3,4]. Autologous oral mucosal epithelial cell sheets are used to promote wound healing and prevent stricture formation after the resection of superficial esophageal neoplasms by endoscopic submucosal dissection. This treatment is more effective than conventional treatments from the point of view of rapid wound healing and prevention of esophageal stenosis. According to the regulation for translational research by Japan's Pharmaceutical and Medical Devices Agency, transplantable cell sheets for the clinical use are fabricated to comply with the good-manufacturing-practice (GMP)-grade quality control (QC) for patient's safety [5]. The establishment of GMP-grade QC procedure requires assessing the management of manufacturing site, source, protocol, and products [6]. Especially, cell source of patient oral mucosal condition is often unstable due to individual difference. Therefore, the acquisition of the oral epithelial properties prior to fabricate cell sheets has an advantage in quality control [6,7]. Esophagus cancer occasionally coincides with oral cancer because the possible risk factors of both cancers are known to be smoking and alcohol intake [8,9], so when preparing autologous oral mucosal epithelial cell sheets, the oral cavity of patient should be carefully examined its property. For obtaining the source cells, the patient's oral biopsies were often performed after visual diagnosis. However, visual diagnosis is

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difficult to distinguish normal oral mucosa and premalignant lesion such as leukoplakia even by experienced doctor. Thus, sometimes, oral mucosal tissue has to be analyzed by immunohistology for definitive diagnosis. The protein expressions of premalignant lesion such as leukoplakia and oral lichen planus decreases K4 and K13 expressions and increases K1, K8, K10, K14, and K15 expressions in prickle cells of middle layer [10–16]. In addition, markers showing the abnormality of oral cavity are not only keratin expression but also epidermal growth factor receptor 4 (EGFR4) express in leukoplakia and alpha-defensin in candidiasis [17,18]. However, oral mucosa is resected as a cell source for cell sheets by spindle-shaped biopsy [19], and it is also resected as a specimen in order to examine oral mucosal epithelial cell properties. That is, implying that patient would be burdened by the twice biopsies. For reducing the patient's burden due to twice biopsies, therefore, we attempted to establish a simple test of apical oral mucosal cells which could analyze protein expressions. We focus on brush biopsy which could collect oral epithelial tissue with less invasiveness [20,21]. In this article, we examined combination of the brush biopsy and protein expression analysis of western blotting. Furthermore, we examined keratin expressions which representative epithelial marker by western blot analysis for investigative usefulness.

2. Methods

2.1. Preparation of samples for western blotting

This study was approved by the Institutional Review Board of Tokyo Women's Medical University, Tokyo, Japan. Oral mucosal epithelial cells and written informed consent was obtained from 4 healthy volunteers. Specimens were obtained from the buccal, gingival, labial, and palate mucosa of four healthy volunteers without smoking habit by dental brushes, which scraped the tissue 10 to 20 times with a brushed area of approximately 20 × 20 mm (400 mm^2) [Fig. 1]. Cells adhered to the bristles of brush were collected by agitating in 5.0 mL of Dulbecco's phosphate buffered saline (PBS) in a 50-mL centrifuge tube. The cells were transferred into a 15-mL centrifuge tube and centrifuged at 270 g for 5 min at 4 °C and the supernatant was removed from the tube. In addition, whole human oral epithelial tissue was collected at the time of a tooth extraction as a positive control lysate. The collected oral epithelial tissue was separated from connective tissue using dispase. These collected cells or tissue were solubilized with 237.5 μL Laemmli's sample buffer containing 12.5 μL 2-mercaptoethanol, and the solution was transferred into a 1.5-mL polypropylene micro tube [22]. For shearing DNA in the cells, the solution was sucked and pushed out 15 times through a 21-gauge needle attached to a 2.5-mL syringe, and sonicated for 3 min. The samples in the solution were denatured by heating for 5 min at 95 °C. The samples were centrifuged at 12000 g for 5 min at 4 °C and the supernatant was collected from the tube. The supernatant was stored at −80 °C until further use for western blotting analysis.

2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

SDS-PAGE was performed by standard methods. The sample solution (10 μL) was loaded onto a polyacrylamide gradient gel (4–12% Bis-Tris Gel, Life technologies, Japan) and 2-(*N*-morpholino) ethanesulfonic acid buffer (running buffer; Life technologies). Electrophoresis was performed at 200 V and 120 mA for 40 min at room temperature with the running buffer. Separated protein bands were transferred to nitrocellulose membrane (Life technologies) for 6 min by a gel transfer device (Life technologies). After transfer, the membrane was blocked with 2% skim milk (membrane blocking agent, GE Healthcare, Japan) in tris buffered saline with tween 20 (TBS-T) for 1 h at 4 °C. For analyzing the transferred proteins, antibodies, which are shown in Table 1, were diluted in blocking buffer, and the membrane with antibody solution were incubated for overnight at 4 °C. The membrane was washed with TBS-T 6 times by the following time schedule 2, 2, 15, 5, 5, and 5 min, then incubated with peroxidase-labelled anti-mouse antibody or peroxidase-labelled anti-rabbit antibody (GE Healthcare) for 1 h at room temperature. After being washed with TBS-T 6

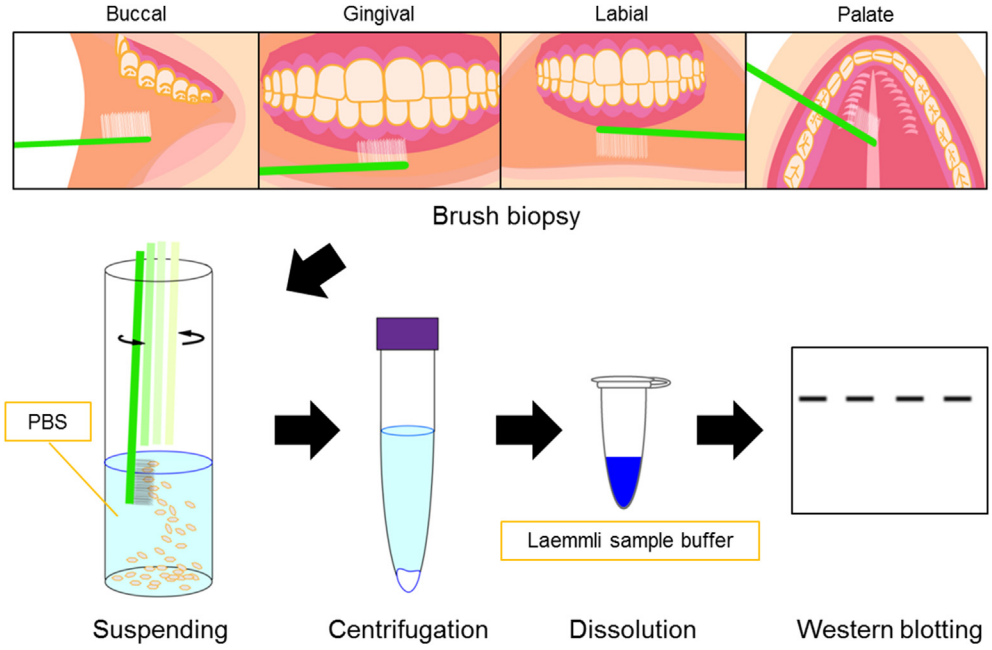

Fig. 1. Schematic diagram of the sampling method of brush biopsy. Samples were acquired from buccal mucosa, gingival mucosa, labial mucosa, and palate mucosa of oral cavity by using dental brushes. The dental brush was rotated for 10 to 20 revolutions. Collected cells were immediately agitated in PBS buffer. Cell suspension was centrifuged and supernatant was removed. Cells dissolved in Laemmli sample buffer.

Table 1
Antibodies used for detecting keratin expressions in oral mucosal cells.

Name	Clone	WB dilution	*IHC dilution	Cat No./Vendor
keratin 1	34βB4	1/1000	1/100	NCL-CK1, Leica
keratin 2	Ks 2.342.7.4	1/1000	1/100	65191, Progen
keratin 4	6B10	1/1000	1/20	ab9004, Abcam
keratin 5	XM26	1/1000	1/100	NCL-CK5, Novocastra
keratin 6	Ks6.KA12	1/1000	1/100	sc-58735, Santa cruz
keratin 7	LDS-68	1/1000	1/50	C6417, Sigma
keratin 8	M20	1/1000	1/50	sc-52324, Santa cruz
keratin 9	Ks9.70/Ks9.216	1/1000	1/20	651104, Progen
keratin 10	LHP1	1/1000	1/50	NCL-CK10, Novocastra
keratin 12	N-16	1/1000	1/50	sc-17098, Santa cruz
keratin 13	1C7	1/1000	1/50	ab22685, Abcam
keratin 14	RCK107	1/500	1/50	sc-23878, Santa cruz
keratin 15	LHK15	1/100	1/100	sc-47697, Santa cruz
keratin 16	LL025	1/1000	1/20	ab8741, Abcam
keratin 17	EP1623	1/1000	1/100	ab109725, Abcam
keratin 18	CK-18	1/1000	1/50	ab82254, Abcam
keratin 19	RCK108	1/1000	1/100	M0888, Dako
keratin 20	EPR1622Y	1/1000	1/100	ab76126, Abcam
p63	4A4	1/1000	1/100	sc-8431, Santa cruz

*IHC: Immunohistological chemistry analysis of oral mucosal tissue samples.

times, the membrane received 1000 μL luminol, chemiluminescence reagent for detecting of horseradish peroxidase (ECL Prime, GE Healthcare) on membrane for 5 min. The membrane was photographed by a chemiluminescence imager (LAS 4000; GE Healthcare).

2.3. Immunohistochemical analysis of oral mucosal tissue

Normal human oral mucosal tissues embedded in optimal cutting temperature (OCT) blocks or paraffin blocks were obtained from Analytical Biological Services (ABS, Wilmington, DE, USA). The tissues were cut into 5-μm thick sections, and dried for 1 h at room temperature and washed PBS for 10 min. For blocking an endogenous peroxidase activity, the sections were incubated with Peroxidase blocking regent (Dako), washed with PBS for 10 min, and blocked by blocking solution containing 5% donkey serum at room temperature over 1 h. The sections were then treated with one of the primary antibodies (Table 1) for overnight at 4 °C. Sections were washed 3 times for at least 5 min with PBS, and then incubated for 1 h with peroxidase-labelled anti-mouse antibody or anti-rabbit antibody at room temperature. After being washed 3 times, the sections were visualized by 3,3′-diaminobenzidine (DAB) for up to 10 min, and nuclei were co-stained with hematoxylin.

3. Results

3.1. Oral mucosal epithelial cells collection using brush biopsy

Oral mucosal epithelial cells were collected by scraping with a dental brush without bleedings under unanesthetized. The collected cells were squamous epithelial cells and were shown to be dead by trypan blue assay [Fig. 2]. Then, protein was extracted from the harvested cells and subjected to keratin expression analysis by western blotting. Brush biopsy-western blotting was able to reproducibly detect 18 different epithelial keratins expressed in the cells collected from independent 4 volunteer donors.

3.2. The expressions of oral mucosal stratified epithelial squamous cell markers

Immunohistological staining revealed the keratin expression pattern of buccal, gingival, labial, and palate, and these patterns were found to be similar to that of obtained by the present brush biopsy-western blotting method (Table 2). By brush biopsy-western blotting analysis, the expressions of keratin 4 (K4), 5 (K5), 6 (K6), and 13 (K13), which are known to be mucosal stratified squamous differentiated epithelial cell markers [23], were detected in oral mucosal cells regardless of the collected sites [Fig. 3]. These keratin-expression patterns were reproducibly obtained in the cells harvested from 4 volunteer donors by brush biopsy. Immunohistochemistry revealed that native human oral tissues, K4, K6 and K13 were expressed in the suprabasal to upper cell layers except the basal cell layer [Fig. 3]. On the other hand, K5 expression was observed from the basal cell layer to upper layer, including basal layer, in all oral tissue samples.

3.3. The expressions of keratinized markers

Keratin 1 (K1) and keratin 10 (K10), a keratinized epithelial cell markers [23], and keratin 16 (K16), which is known to be expressed in squamous epithelia [23], were detected in keratinized tissue

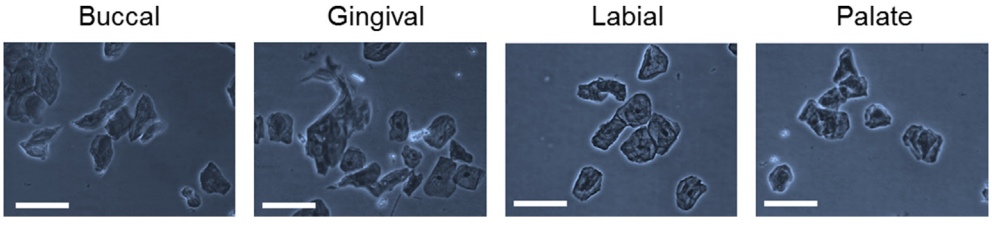

Fig. 2. Morphological analysis of oral mucosal cells collected by brush biopsy. Oral mucosal cells collected by dental brush were stained with trypan blue. Oral mucosal cells from four regions by brush biopsy were dead cells. Scale bars indicate 100 μm.

Table 2
Keratin pattern formed in oral mucosal cells.

	Buccal				Gingval				Labial				Palate			
	WB	IHC			WB	IHC			WB	IHC			WB	IHC		
		Basal	Middle	Upper		Basal	Middle	Upper		Basal	Middle	Upper		Basal	Middle	Upper
Keratin 1	(+)	−	(+)	(+)	+	−	+	+	−	−	−	−	+	−	+	+
Keratin 2	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+
Keratin 4	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+
Keratin 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Keratin 6	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+
Keratin 7	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Keratin 8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Keratin 9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Keratin 10	−	−	−	−	+	−	+	+	−	−	−	−	+	−	+	+
Keratin 12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Keratin 13	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+
Keratin 14	−	+	−	−	−	+	−	−	−	+	−	−	+	+	−	−
Keratin 15	−	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−
Keratin 16	−	−	−	−	+	−	+	+	−	−	−	−	+	−	+	+
Keratin 17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Keratin 18	(+)	+	(+)	(+)	(+)	+	(+)	(+)	(+)	+	(+)	(+)	(+)	+	(+)	(+)
Keratin 19	(+)	+	(+)	(+)	(+)	+	(+)	(+)	(+)	+	(+)	(+)	(+)	(+)	(+)	(+)
Keratin 20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 2 shows qualitative cytokeratin pattern. +: Always observed in substantial amounts. (+): Observed in minor or variable amounts. −: Not detected. WB: western blotting analysis in oral mucosal cells collected by brush biopsy. IHC: Immunohistological analysis in oral mucosal tissue samples.

such as gingival and palate mucosa [24] by brush biopsy-western blotting [Fig. 4]. Furthermore, K1 expression was weakly detected in the labial mucosa of three of four donors (donor No.1, 3 and 4), K1 and K16 expressions were only detected in the buccal mucosa of donor No.4. In immunochemical staining, K10 and K16 were expressed in the upper to middle cell layers of gingival and hard palate, whereas not expressed in other oral tissues. K1 expression was detected in the gingival and hard palate mucosa, and faintly observed in buccal and labial mucosa. Keratin 2 (K2) expression was detected in the palate mucosa of all 4 donors by brush biopsy-western blotting, while the expression was observed in the gingival and hard palate of upper to middle layers of immunohistochemical specimens.

3.4. The expressions of basal cell markers

The expressions of keratin 15 (K15), and p63 known as a basal cell marker were not detected in brush biopsy-western blotting,

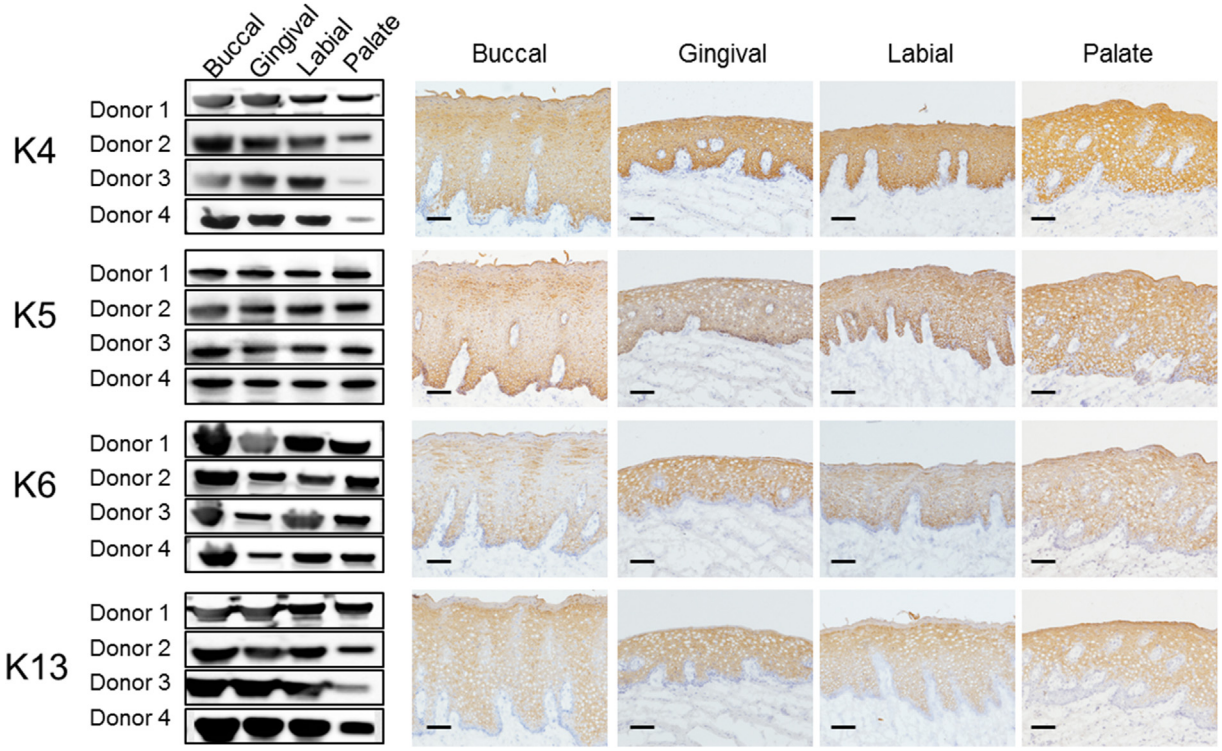

Fig. 3. Brush biopsy-western blotting analysis and immunohistological staining of mucosal stratified squamous epithelial cells markers. Brush biopsied specimens of buccal mucosa, gingival mucosa, labial mucosa and palate mucosa were analyzed by western blotting, and normal tissues were analyzed by immnohistological staining. The markers are keratin 4 (K4), keratin 5 (K5), keratin 6 (K6) and keratin 13 (K13). Scale bars indicate 100 μm.

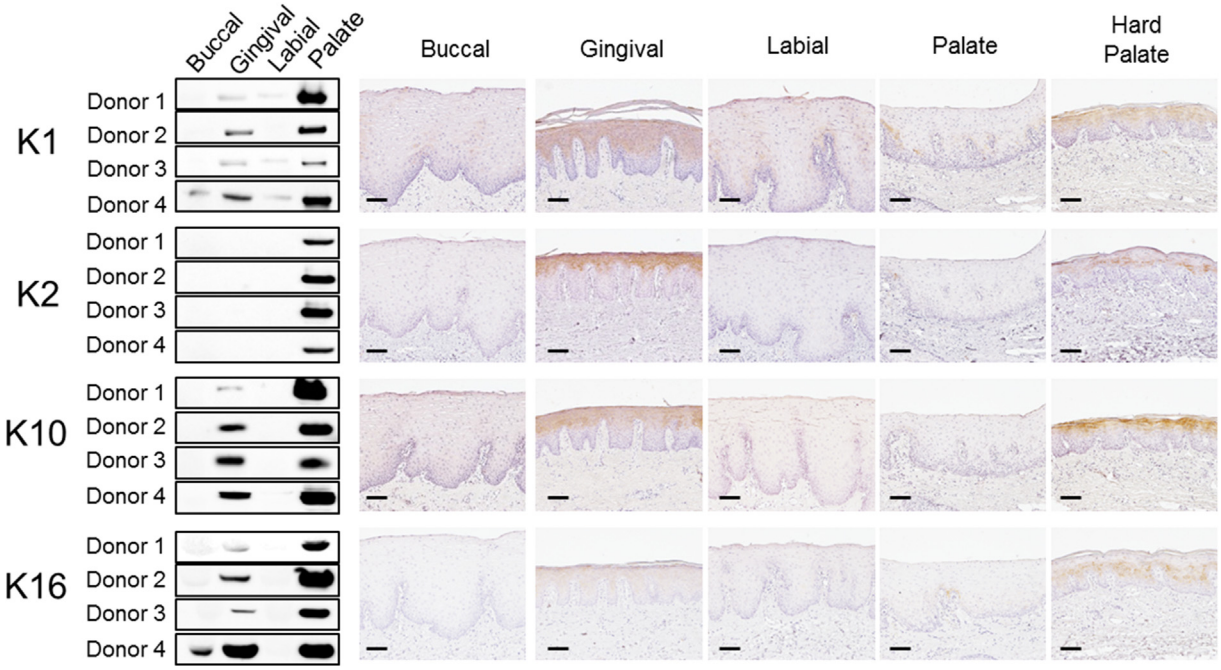

Fig. 4. Brush biopsy-western blotting analysis and immunohistological staining of keratinized epithelial cells markers. Brush biopsied specimens of buccal mucosa, gingival mucosa, labial mucosa and palate mucosa were analyzed by western blotting, and normal tissues were analyzed by immnohistological staining. The markers are keratin 1 (K1), keratin 2 (K2) and keratin 10 (K10) and keratin 16 (K16). Scale bars indicate 100 μm.

although positive control which whole oral epithelial tissue was detected [Fig. 5]. Keratin 14 (K14), also known as a basal cell and suprabasal marker was detected only at palate in brush biopsy-western blotting [Fig. 5]. In immunohistochemical analysis, K14, K15 and p63 expressions were observed in the basal to suprabasal cell layers of all type of oral mucosal tissues.

3.5. The expressions of other keratins

Keratin 7 (K7) was detected in buccal mucosa, while faintly detected in the gingival and labial mucosa, particularly in the donor No.3 and No.4 in brush biopsy-western blotting. In palate mucosa, K7 expression was not detected in all 4 specimens by brush biopsy-western blotting (Fig. 6).

In immunohistochemical staining, K7 expression was observed over the epithelial layers of 4 types of oral mucosa. Keratin 18 (K18) and keratin 19 (K19) expressions were reproducibly detected in buccal, gingival, and labial mucosa in brush biopsy-western blotting. In palate mucosa, K18 and K19 expression was hardly or faintly detected in brush biopsy-western blotting. In immunohistochemical analysis, K18 was broadly expressed over the epithelial layers, and K19 was expressed in apical and basal layers. However, palate mucosa had low K18 and K19 expressions (Fig. 6). No other keratins such as K8 expressed in simple epithelial, K9, a palmoplantar epidermal differentiation keratin, K12, keratin found in the corneal epithelium, K17, keratin in basal/myoepithelial cells and inducible in “activated” keratinocytes, K20, urothelium and Merkel cell, were detected in brush biopsy-western blotting and immunohistochemical staining (data not shown).

4. Discussion

In this study, a brush biopsy-western blotting method was established to analyze the keratins which are representative as the human epithelial marker. Brush biopsy method is used for several adjunct diagnostic tests such as histological analysis including toluidine staining, Papanicolaou staining, and qRT-PCR analysis [20,21]. However, toluidine and Papanicolaou staining methods have low specificity [25,26]. In addition, the isolation of total RNA from brush biopsy is difficult, because a high RNase activity originated from bacteria found in the oral cavity [27,28]. Cells collected by brush biopsy were dead cells [Fig. 2], which means that it could be existing on the surface and be about to peel off [29]. This result suggested that protein assay such as western blotting is thought to be better than mRNA assay such as qRT-PCR.

The results in this study indicated that brush biopsy collect the sufficient amount of protein to analyze the keratin expressions by western blotting. This study investigated keratin expression patterns in epithelial cells collected from oral cavity including buccal, gingival, labial, and palate mucosa. Keratins are typical intermediate filament proteins in epithelia and expressed in highly specific patterns, which are related to the epithelial type and stage of cellular differentiation [23,24,30]. For investigating whether brush biopsy-western blotting was able to detect a tissue-specificity such as keratinized or non-keratinized epithelium, so keratin expression patterns of buccal, gingival, labial and palate were compared. The expressions of mucosal stratified squamous epithelial cell markers such as K4, K5, K6, and K13 were detected in all specimens collected from 4 volunteers [Fig. 3], and the expressions of keratinizing epithelial cell markers such as K1 and K10 were found in gingival and palate mucosa, which are known to be cornified epithelial tissues [Fig. 4]. These results suggested that brush biopsy-western blotting could detect tissue-specific keratin expressions. K1 expression was detected in labial mucosa, which is known as non-keratinizing epithelium, suggesting that the specimen was contaminated with adjoining keratinized labial-skin cells, which is continuously found from labial mucosa [31]. Moreover, K1 and K16 expressions were only detected in the buccal mucosa of donor No.4 by brush biopsy-western blotting. In this study, immunohistochemical staining with K1 and K16 antibodies were not observed in buccal mucosa. In previous study, immunohistological staining and in situ hybridization experiment showed buccal mucosa sparsely

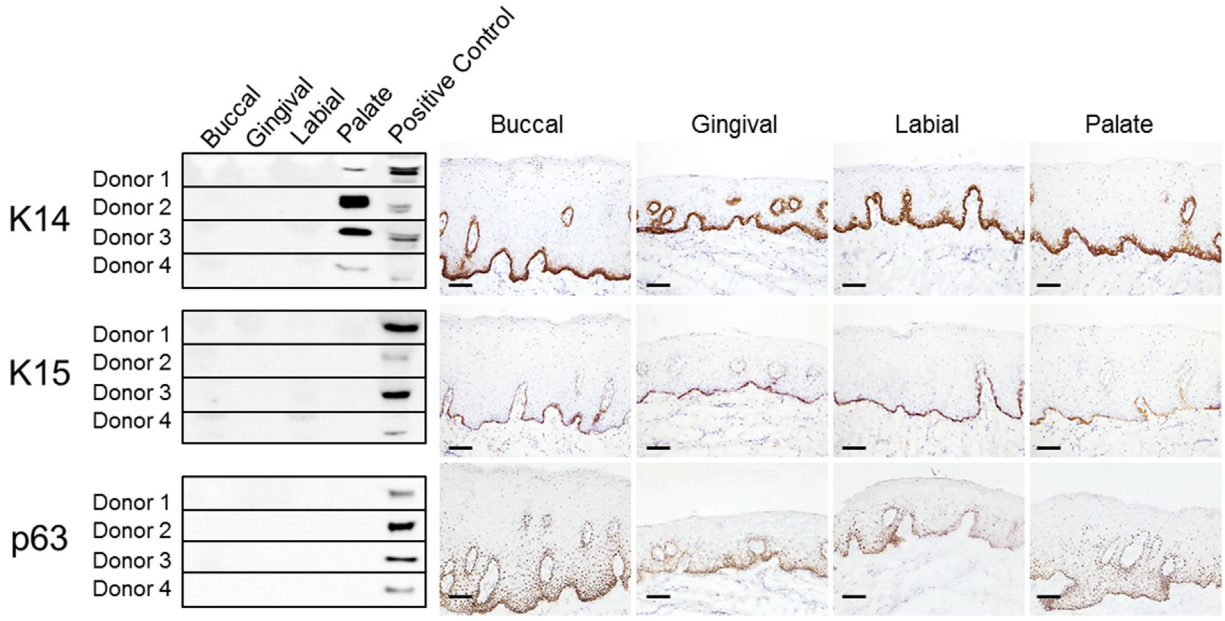

Fig. 5. Brush biopsy-western blotting analysis and immunohistological staining of basal/suprabasal cells markers. Brush biopsied specimens of buccal mucosa, gingival mucosa, labial mucosa and palate mucosa were analyzed by western blotting with positive control which human oral epithelial tissue. In addition, normal tissues were analyzed by immnohistological staining. The markers are keratin 14 (K14), keratin 15 (K15) and p63. Scale bars indicate 100 μm.

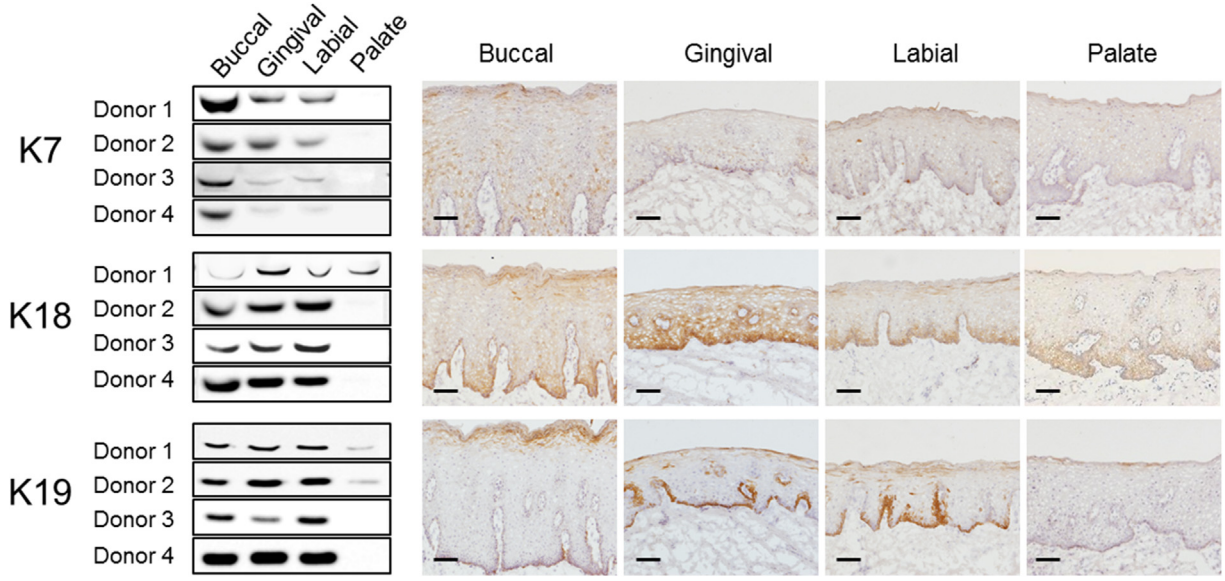

Fig. 6. Brush biopsy-western blotting analysis and immunohistological staining of simple epithelial cells markers. Brush biopsied specimens of buccal mucosa, gingival mucosa, labial mucosa and palate mucosa were analyzed by western blotting, and normal tissues were analyzed by immnohistological staining. The markers are Keratin 7 (K7), keratin 18 (K18) and keratin 19 (K19). Scale bars indicate 100 μm.

expressed K1 and its mRNA [10]. Furthermore, K16 was also shown to be expressed sparsely at normal buccal tissue in previous study [15]. Therefore, K1 and K16 expressions in donor No.4 were speculated to be adequate.

Cell markers distinguishing the keratinization of epithelial cell were detected in brush biopsy-western blotting, while basal cell markers such as K14, K15 and p63 were not detected in buccal, gingival and labial. These results indicated that brush biopsy successfully collected cells from upper layer of the oral mucosa without defect including basal cell layers. Therefore, the brush biopsy was considered to be a noninvasive procedure for patients. Interestingly, K14 was detected in brush biopsied palate epithelia. If palate epithelial cells of suprabasal and basal layer were collected by brush biopsy, p63 and K15 were also detected by western blotting. There is a possibility to detect other keratin by nonspecific reaction. However, molecular weight of the detected bands reacted with K14 antibody indicated almost same molecular weight of K14 (50 kD). Therefore, further investigations are necessary to reveal the reason of this result.

In addition, K7, K18, and K19 expressions are found in various tissues and associated with differentiation states [23]. In this study, K7, K18, and K19 sparsely expressed in epithelial cell layers, then,

these keratins were also detected by brush biopsy western blotting. The cells collected by brush biopsy were suitable for qualitative analysis of protein expressions by western blotting.

Oral mucosal epithelial cell sheet was made from undifferentiated cells existing in basal cell layer, which were not collected by brush biopsy. However, apical cell-analysis is expected to gain convincing evidence of basal cell quality of oral mucosal epithelium by brush biopsy western blotting. Stratified squamous epithelium is maintained and provided from basal cells by proliferation and differentiation. Epithelial carcinoma is derived from basal cells of DNA damage due to endogenous or exogenous factors [32], and basal cell abnormalities are inherited to upper layer cells. Thus, we suggest that brush biopsy-western blotting is useful for performing a simple test of upper layer of buccal epithelial cells to analyze some protein expressions such as K1, K4, K10, K13, K14, K15, EGFR 4 and alpha-defensin.

For these reasons, the brush biopsy-western blotting shown in this study might contribute to the preparation of autologous oral mucosal epithelial cell sheets under the highly quality control condition for clinical applications.

Disclosure statement

Teruo Okano is a founder and director of the board of CellSeed Inc., licensing technologies and patents from Tokyo Women's Medical University. Teruo Okano and Masayuki Yamato are stakeholders of CellSeed Inc. Tokyo Women's Medical University is receiving research fund from CellSeed Inc.

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