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Title: Isolation of oral epithelial progenitors using collagen IV

[Running title: Isolation of oral epithelial progenitors]

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Abstract

OBJECTIVE: Although oral mucosal epithelial stem cells are thought to reside in the basal layer, such cells have not yet been isolated. We isolated a population of rabbit oral epithelial progenitor cells containing putative stem cells.

MATERIALS AND METHODS: Epithelial cells harvested from rabbit buccal mucosa were allowed to adhere to dishes coated with collagen IV for periods ranging from 10 min to 16 h. The properties of individual cell populations were evaluated using BrdU, Ki-67, integrin β1, integrin α6 and keratin 13 using colony forming efficiency.

RESULTS: Cells that adhered to collagen IV coated dishes within 10 min were enriched about 6-fold in terms of BrdU incorporation, Ki-67, Integrin α6, and Integrin β1 were strongly expressed. Interestingly, Keratin 13 was faintly expressed. The CFE of rapidly adherent cells among oral epithelial cells was significant compared with other cell populations at 20, 60, 120 min and 16 h.

CONCLUSIONS: These results suggested that rabbit oral epithelial cells could be isolated by depending on adhesiveness to collagen IV, especially when segregated according to progenitor cell properties. Putative progenitor cells with stem cell properties were most effectively harvested within 10 min. Our separation procedure should be a useful tool with which to isolate epithelial stem cells for regenerative medicine.

Introduction

Adult stem cells have various distinct morphological and phenotypic features, as well as growth potential. These include slow cycling or long cell cycle time, small size with poor differentiation and primitive cytoplasm, high proliferative potential after wounding or placement in culture and ability for self-renewal and functional tissue regeneration (Blau *et al*, 2001).

Cultured oral mucosal epithelial cells have recently been transplanted to treat various epithelial defects (Nishida *et al*, 2004; Feinberg *et al*, 2005). The source of successful transplantation has been attributed to the healing potential of progenitor cell populations among oral epithelial cells that contain stem cells. Such stem cells comprise only a small subpopulation of basal cells, hairy epidermal stem cells residing in the hair follicle bulge (Janes *et al*, 2002; Alonso and Fuchs, 2003) and cornea in the limbus (Lavker and Sun, 2000). However, the localization of stem cells in the oral epithelium is not understood. Furthermore, a pure population of oral epithelial stem cells must be isolated and modified for application to the regeneration of epithelial tissues.

Jones and Watt (Jones and Watt, 1993; Jones *et al*, 1995) suggested that epidermal stem cells might adhere to basement membrane proteins more than other basal cells and that such adhesion might be mediated through the differential expression of specific integrins. They also suggested that about 40% of the basal cell population expressed considerable integrin β1, and they postulated that this population contained stem cells in the human epidermis. Bick-

enbach and Chism (Bickenbach and Chism, 1998) reported the partial enrichment of epithelial stem cells using dishes coated with several types of matrix. Epidermal stem cells from murine epidermal keratinocytes adhered to several integrin ligands, collagens or other extra cellular matrix within 10 min, and these accounted for about 10% of total basal cells and 100% of BrdU label retaining cells (LRCs) (Bickenbach, 1981; Cotsarelis *et al*, 1989; Cotsarelis *et al*, 1990).

In human oral mucosal epithelium, the stem cells have been thought to be in the basal layer, in which localization of the cells expressing stem/progenitor cells-related markers, PCNA, Ki-67 (Tomakidi *et al*, 1998), cytokeratins (K5/14, K19) (Presland and Dale, 2000), integrins (α2, α3, α6, β1 and β4) (Jones *et al*, 1993) but not differentiation markers, K1/10, K4/13 have been reported. We adopted these notions together with a few modifications to separate progenitor cells among rabbit oral mucosal epithelial cells.

Here, we isolated epithelial progenitor cells to determine whether a progenitor population containing putative rabbit oral epithelial stem cells could be partially separated over time according to adhesiveness to collagen type IV. In conclusion, progenitor populations containing putative stem cells can be isolated within 10 min using collagen IV and then oral mucosal epithelial stem cells and be generated.

Materials and methods

Animals and tissue preparations

Adult Japanese white rabbits (2.0 ~ 2.5 kg) were purchased from Shiraishi Laboratory Animals (Tokyo, Japan). Buccal tissues isolated from rabbits sacrificed under anesthesia induced by an intravascular injection of Pentobarbital sodium (50 mg/ mL) were cut through the horizontal meridian, frozen and sectioned for immunohistochemistry. The protocols involving animals proceeded according to the guidelines for the treatment of experimental animals at Tokyo Dental College.

Adhesion of oral epithelial cells to collagen IV

Oral epithelial cells were isolated as described (Nakamura *et al*, 2003) with some modification. In brief, submucosal connective tissues were removed with scissors, incubated with 2.4 IU dispase II (Roche, Indianapolis, IN, USA) at 4°C for 16 h and then dispersed with 0.05% trypsin-0.53 mM EDTA (Sigma, St. Louis, MO, USA) at 37°C for 10 min to isolate single cells. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. To evaluate adhesion properties, single cell suspensions of rabbit oral epithelial cells in medium were allowed to attach to 100 mm dishes (Becton Dickinson, Lincoln Park, NJ, USA) coated with collagen IV in an incubator for 10, 20, 60 and 120 min and 16 h. Putative stem cells were enriched as follows. Cells that attached to collagen IV coated dishes within 10 min were referred to as rapidly adherent cells (RAC). The cells that remained unattached within the first

10 min were then transferred to other collagen IV coated dishes for an additional 16 h. Cells that adhered within this period were referred to as slowly adherent cells (SAC). Remaining unattached cells were collected as non-adherent cells after 16 h (NAC). Unfractionated cells that were not separated according to adhesive properties served as controls.

Colony forming efficiency (CFE)

The proliferative potential of the cell populations selected by adhesion to collagen IV was evaluated on a feeder layer of mitomycin C (MMC; Sigma)-treated 3T3 fibroblasts (ATCC CCL92; ATCC, Rockville, MD, USA) as described (Rheinwald and Green, 1975; Tseng *et al*, 1996). Each selected cell population was seeded at least in triplicate, at a density of $1x10^3$ cells/cm^2 into 100 mm dishes containing a 3T3 fibroblast feeder layer. The CFE was calculated as a ratio (%) of the number of colonies at day 12 generated by the number of epithelial cells. Growth capacity was evaluated on day 12 when cultured cells were stained with 1% Rhodamine B.

Immunohistochemistry

Immunohistochemistry proceeded as described (Yoshida *et al*, 2005). Cytospin preparations (Auto Smear CF-120; Sakura, Tokyo, Japan) on glass slides were incubated with primary monoclonal antibodies against integrin β1 (Chemicon International Inc, Temecula, CA, USA), integrin α6 (FITC conjugated; Abcam, Cambridge, UK), Ki-67 (DAKO Cytomation,

Glostrup, Denmark) and Keratin 13 (American Research Products, Belmont, MA, USA) were for 1 h. The slides were then incubated with Cy3 conjugated secondary antibodies (Chemicon) for 30 min and counterstained with 4',6-diamidino-2- phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan) for 3 min. Stained cells were assessed by point counting under a light microscope at ×200. More than 500 epithelial cells were counted in 6 - 8 representative fields. This number (500 counted cells) was considered the minimum required to obtain representative data (Goodson *et al*, 1998). Positive cell rates are expressed as the number of positively labeled cells/ the total number of cells ×100%.

Detection of BrdU LRCs

Pulse-chase experiments with BrdU proceeded as described (Taylor *et al*, 2000; Togo *et al*, 2006). Four week-old rabbits (Shiraishi Laboratory Animals) were injected subcutaneously with 50 mg/kg/day BrdU (Sigma) for 5 days. Four weeks later, the animals were sacrificed and buccal tissue was removed for the cell separation using collagen IV coated dishes. LRC in the separated cells were detected by immunohistochemistry with anti BrdU antibody (Abcam) and Rhodamine-conjugated secondary antibodies (Chemicon). The ratios of BrdU positive cells was determined by point counting as described above.

Statistical analysis

Data from RAC were statistically compared with those from SAC, NAC and Controls using

Student’s t-test and $P < 0.05$ was considered significant.

Results

Adhesion properties of oral epithelial cells

The adhesion properties of oral epithelial cells were evaluated by incubation on collagen IV coated dishes for 10, 20, 60, 120 min and 16 h (Figure 1). About 13% of epithelial cells adhered to collagen IV within 10 min. The adherent cell population increased about 20%, 42% and 50%, over 20, 60 and 120 min respectively. Finally, the adherent cell population increased to about 62% after 16 h of incubation. These results indicated that the populations of cells with adhesion properties became larger the longer oral epithelial cells were incubated on collagen IV. To evaluate each specific property, oral epithelial cells were separated into three populations based on time taken to adhere to collagen IV. We found that RAC and SAC accounted for 13% and 50%, respectively, of the whole population of harvested oral epithelial cells.

RAC possess higher proliferative potential

To evaluate growth capacity, the cells of each population selected by adhesion to collagen IV were seeded in triplicate at a density of $1x10^3$ cells/cm^2 into 100 mm dishes containing 3T3 fibroblast feeder layers for 12 days. Compared with Controls and SAC, the numbers of RAC colonies significantly increased (Figure 2A) whereas those of NAC developed fewer cell colonies. Furthermore, SAC generated slightly more cell colonies than Controls. The CFE values from four adhesion experiments with oral epithelial cells are summarized in Figure

2B. The CFE was highest among RAC (8.8%) and significantly higher than that of the Control (5.1%). The SAC (6.7%) values were also higher than the Control. The CFE was lowest among NAC (2.4%). The CFE in the RAC was about double that of the Control, and about 4-fold higher than NAC. The RAC further reached confluence within 10 to 14 days, whereas Control and SAC proliferated more slowly and reached confluence within 12 to 18 days. The colonies generated by NAC did not grow further and eventually died.

Progenitor cell properties of RAC expressing molecular markers

To compare the phenotype of individual cell populations with unfractionated whole populations, we used Ki-67 as a marker of nuclear protein and thus of proliferation, integrin α6, integrin β1, BrdU to indicate stem cells and Keratin 13 as a marker of differentiation. Normal oral mucosal tissues served as histological controls (Figure 3A, F, K, P, U)**.** Ki-67 positive cells were located in the basal and suprabasal layers of the oral mucosa (Figure 3A). More positive cells were found among RAC than in any other cell population (Figure 3B-E). Ki-67 positive cells were the most prevalent (57%) among RAC (Figure 4A). The numbers of positive cells were significantly larger than in the Control (38%), followed by SAC (35%) and NAC (6%). Integrin α6 positive cells located in the basement membrane of the basal layer of oral mucosa (Figure 3F), and more of these cells were found in RAC than in any other cell populations (Figure 3G-J). The RAC also possessed the highest ratio of integrin α6 positive cells (69%), compared with Controls (51%), SAC (51%) and NAC (24%) (Figure 4B). In-

tegrin β1 positive cells were essentially restricted to the basal layer and weakly stained in the prickle cell layer (Figure 3K). The ratio of intensely integrin β1 positive cells was highest (42%) among RAC than in the Control (18%), SAC (31%) and NAC (9%) (Figure 3L – O, Figure 4C). The numbers of cells that were normally integrin β1 positive were equal in the four populations. In contrast, cells were K13 positive in the prickle cell layer of the oral mucosa (Figure 3P) and more of these cells were positive among NAC than in the Control, RAC and SAC (Figure 3Q-T). The ratio of K13 positive cells was the lowest among RAC (20%), being significantly lower than in SAC (31%), Control (45%) and NAC (63%) (Figure 4D). BrdU positive cells were rarely found in the basal layer of the oral mucosa (Figure 3U), and more cells were BrdU positive among RAC than in Control, SAC and NAC (Figure 3V-Y). The Control contained 1.6% BrdU positive cells (Figure 4E) and RAC contained the highest proportion of BrdU positive cells (8.6%) compared with the Control (1.6%). Interestingly, SAC had significantly more (4.8%) BrdU positive cells than the Control, whereas NAC had very few (0.4%).

Discussion

We found that RAC adhered to collagen IV coated dishes within 10 min and accounted for 13% of the total population of rabbit oral epithelial cells. These cells also had greater proliferative potential than any other populations in culture. In addition, RAC contained many Ki-67 positive cells than other populations from buccal tissues. These results indicated that RAC have significantly higher proliferative potential *in vivo* and *in vitro*. On the contrary, NAC contained few Ki-67 positive cells, and included cells that did not adhere to collagen IV after 16 h. These results indicated that the timing of stem cell attachment to collagen IV is of fundamental importance as an isolation procedure *in vitro*. Other clues regarding stem cells have been generated by immunochemical studies.

Cells that were normally integrin β1-positive comprised about 80% of the oral basal cell population equally among all four sub-populations. However, more cells were intensely integrin β1-positive among RAC than in any other sub-population. Considering a previous study of the epidermis, our results indicated that these cells might include stem cells in the oral epithelia, but the population would not be pure. Another study of stem cells in human epidermis has suggested that integrin α6 positivity represents one necessary feature of epidermal stem cells (Li *et al*, 1998). The RAC contained significantly more integrin α6-positive cells than any other sub-population in the present study. In contrast, RAC seemed to contain fewer K13-positive cells. We and others have demonstrated K13 expression in the non-keratinized differentiated epithelium. Therefore, RAC might contain a few

differentiated cells. The NAC contained many differentiated cells, and then lost those populations with adhesion properties. In addition, RAC contained remarkably more BrdU-positive cells compared than any other sub-population. These findings indicated that RAC contained several slow cycling cells such as stem cells. Overall, our results including CFE and the expression profiles of several putative markers indicated that potent progenitor populations are included among putative stem cells found in RAC.

Bickenbach and Chism (1998) reported that RAC form large colonies and a structurally complete epidermis in organotypic culture. We have also attempted to produce possible culture sheets with enriched populations of progenitors, but we could not identify the timing required to producing such sheets. This might be due to the absence of ECM molecules, growth factors and the optimal media for stem cell growth.

Stem cells usually remain quiescent in a specific niche from which they are selected and transformed into proliferating cells (Watt and Hogan, 2000; Moore and Lemischka, 2006). Our results showed that the RAC population comprised 13% within 10 min, which is considerably higher than estimated number of oral mucosal epithelial stem cells. The RAC population contained only 8.6% of slow-cycling BrdU-positive cells and their proliferative potential was about double that of unfractionated cells *in vivo* and *in vitro*. The expression of putative stem cell surface markers (integrin α6 and β1 bright cells) was significantly higher among RAC than other sub-populations. These indicate that the RAC population partially conformed to the criteria for adult stem cells (1) (3) described above and had the suggested

properties of stem cells. Therefore, RAC might have progenitor population properties but they do not completely consist of stem cells. Consequently, our study suggested that collagen IV enrichment is a powerful tool with which to isolate progenitor populations containing putative stem cells for oral mucosal epithelial cells. Pure oral mucosal epithelial stem cells cannot be isolated because no specific marker has yet been identified. Further selection with other cell surface markers is essential to obtain a more enriched population.

In conclusion, our findings demonstrated that progenitor populations containing putative rabbit oral mucosal epithelial stem cells could be partially enriched by adhesion to collagen IV in 10 min. The RAC population enriched with specific putative stem cell properties might become useful for transplantation to treat diseases and damaged epithelium with a basal stem cell deficiency. This population could be used to explore new markers and improve stem cell identification, and further refine isolation methods to obtain pure stem cells in the future.

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Figure legends

Figure 1 Rabbit oral mucosal epithelial cells adhering to collagen type IV for 10, 20, 60, 120 min and 16 h.

Ratios of adherent cells at 10 min (about 13%), increased at 20 min (20%), at 60 min (42%), at 120 min (50%) and reached 62% after 16 h of incubation. Data represent means ± S.D. of 7 individual experiments.

Figure 2 Colony forming efficiency (CFE) on 3T3 fibroblast feeder layers at day 12 generated by four populations of rabbit oral mucosal epithelial cells after adhesion to collagen IV.

(**A**) Staining with 1% Rhodamine B shows growth capacity of four isolated cell populations. More colonies were generated by in RAC than Control, SAC and NAC. (**B**) CFE on day 12 was highest among RAC (8.8%), which was significantly higher than that of Control (5.1%), SAC (6.7%) and NAC (2.4%). Data represent means ± S.D. of 4 individual experiments.

*, $P < 0.05$; **, $P < 0.01$ compared with RAC.

Figure 3 Immunohistochemical staining of normal oral mucosa and four separated cell populations.

(**A**) Ki-67 expression in basal (arrowhead) and suprabasal (arrow) layers of normal rabbit oral mucosa. (**B-E**) More cells (arrowheads) are Ki-67 positive in RAC than in any other population. Almost all cells were positive among NAC. (**F**) Integrin α6 expressed in base-

ment membrane of the basal layer of oral mucosa (arrowheads). (**G-J**) Highest expression pattern and more integrin α6-positive cells (arrowheads) in RAC than in Controls, SAC and NAC. (**K**) Integrin β1 is expressed in basal layer (arrow) and less so in prickle cell layer (arrowhead) of oral mucosa. (**L-O**) Equal numbers of cells are normally integrin β1 positive (arrowheads) among all cell populations examined. Otherwise, cells typically expressed considerably more integrin β1 (arrows) in RAC and SAC. (**P**) Keratin 13 is expressed in prickle cell layer (arrowheads) and upper side of oral mucosa. (**Q-T**) More cells were K13 positive (arrowheads) among NAC than Control and SAC. Almost all cells are positive in RAC. (**U**) BrdU-positive cells (arrowheads) that were chased for 28 days were rare in basal layer of oral mucosa. (**V-Y**) More cells were BrdU positive (arrowheads) in RAC than in Controls and SAC. Almost all cells were positive among NAC. Counterstaining with DAPI. Bars 25 μm.

Figure 4 Ratio of cells positive for Ki-67, integrin α6, integrin β1, Keratin 13, and BrdU among 4 populations isolated from rabbit oral mucosal epithelial cells by adhesion to collagen IV.

(**A**) Ratios of Ki-67-positive cells were highest in RAC (57%) and significantly higher than in Control (38%), SAC (35%) and NAC (6%). (**B**) Ratios of integrin α6-positive cells were highest in RAC (69%) and significantly higher than in Control (51%), SAC (51%) and NAC (24%). (**C**) Ratios of intensely integrin β1 positive cells is highest in RAC (42%) and sig-

nificantly higher than in SAC (31%), Controls (18%) and NAC (9%). (**D**) Ratios of Keratin 13-positive cells were lowest in RAC (20%) and significantly lower than in SAC (31%), Controls (45%) and NAC (63%). (**E**) Ratios of BrdU-positive cells were highest among RAC (8.6%) and significantly higher than in SAC (4.8%), Controls (1.6%) and NAC (0.4%). Data represent means ± S.D. of 3 individual experiments. *, $P < 0.05$, **, $P < 0.01$ compared with RAC.

Figure 1

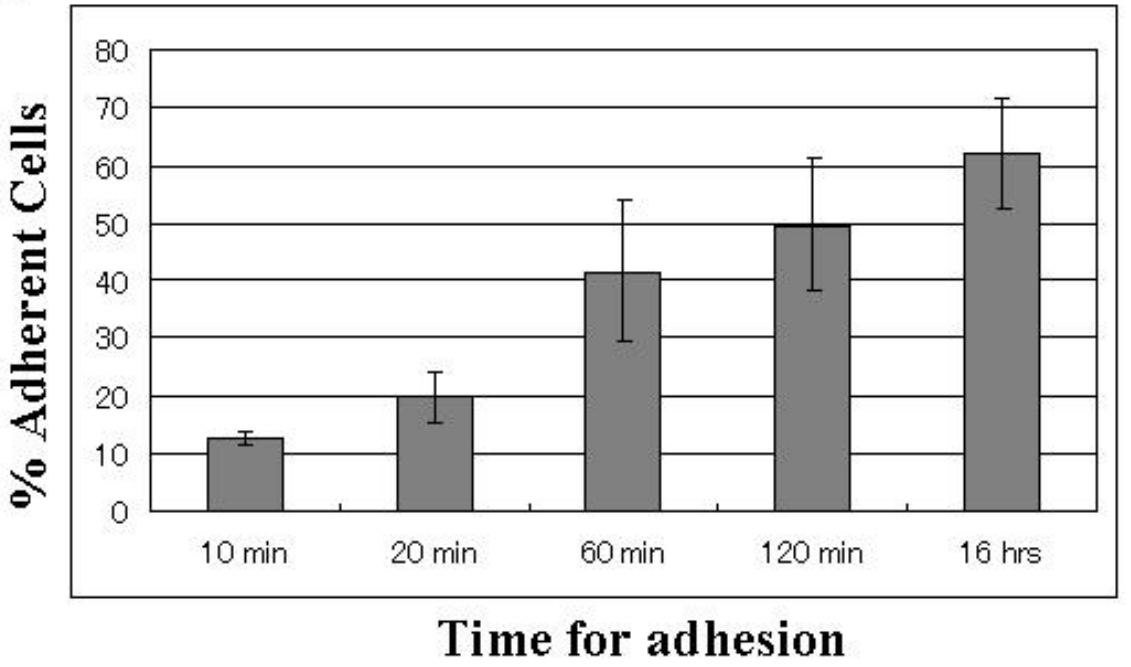
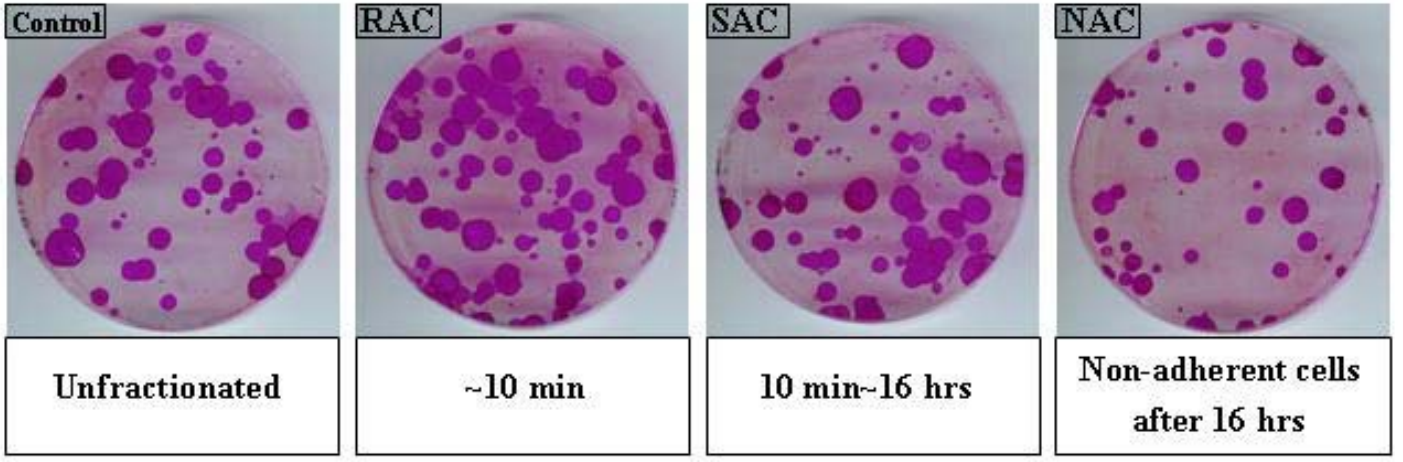

% Adherent Cells
80
70
60
50
40
30
20
10
0
10 min
20 min
60 min
120 min
16 hrs
Time for adhesion

Figure 2

A


Control
RAC
SAC
NAC
Unfractionated
~10 min
10 min~16 hrs
Non-adherent cells after 16 hrs

B

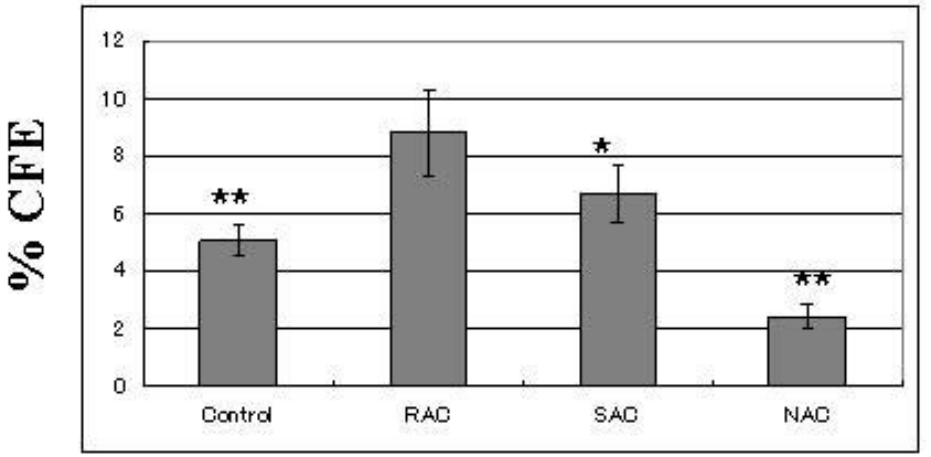
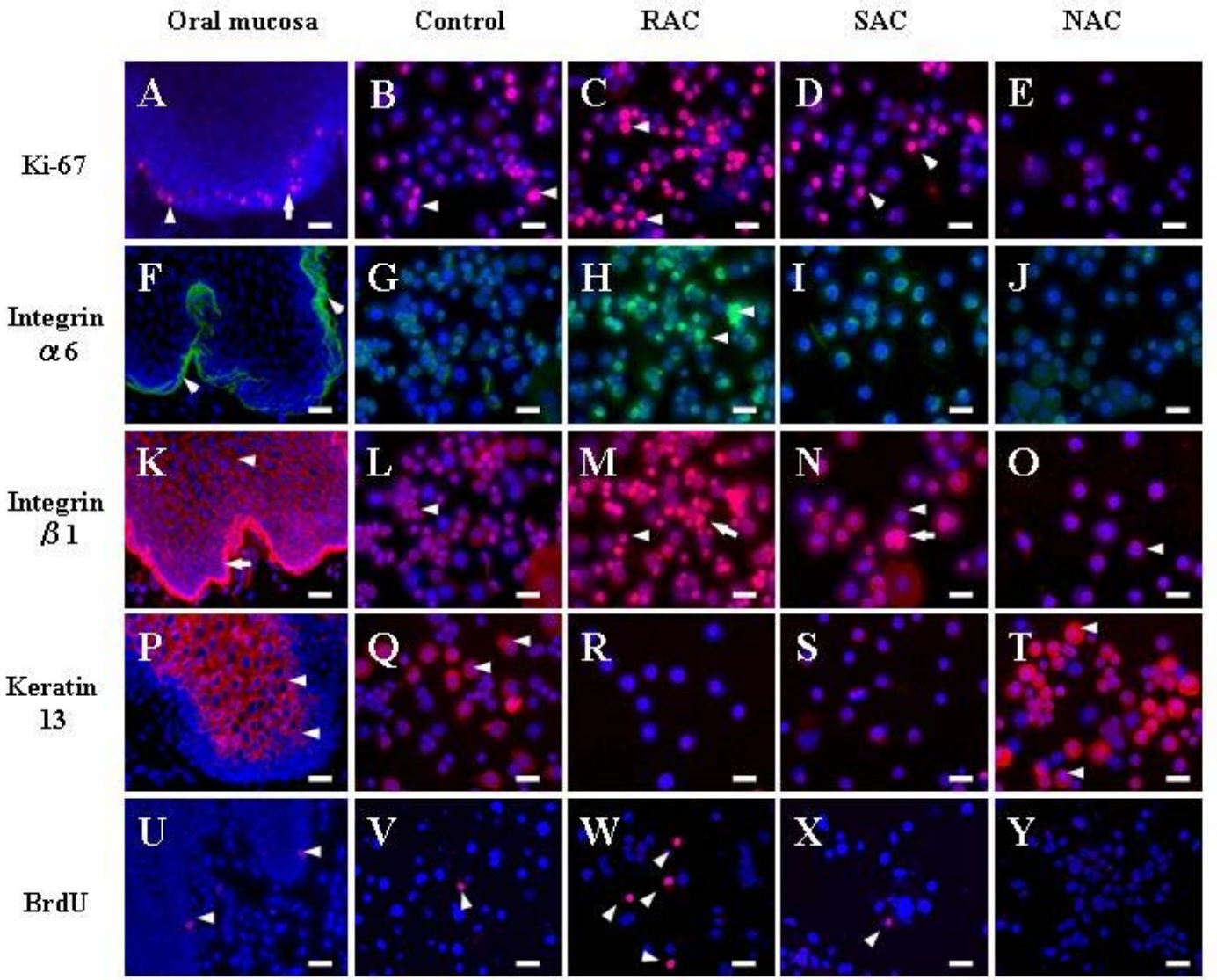

% CFE
12
10
8
6
4
2
0
**
*
**
Control
RAC
SAC
NAC

Figure 3


Oral mucosa
Control
RAC
SAC
NAC
Ki-67
Integrin α6
Integrin β1
Keratin 13
BrdU
A
B
C
D
E
F
G
H
I
J
K
L
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N
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Q
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S
T
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W
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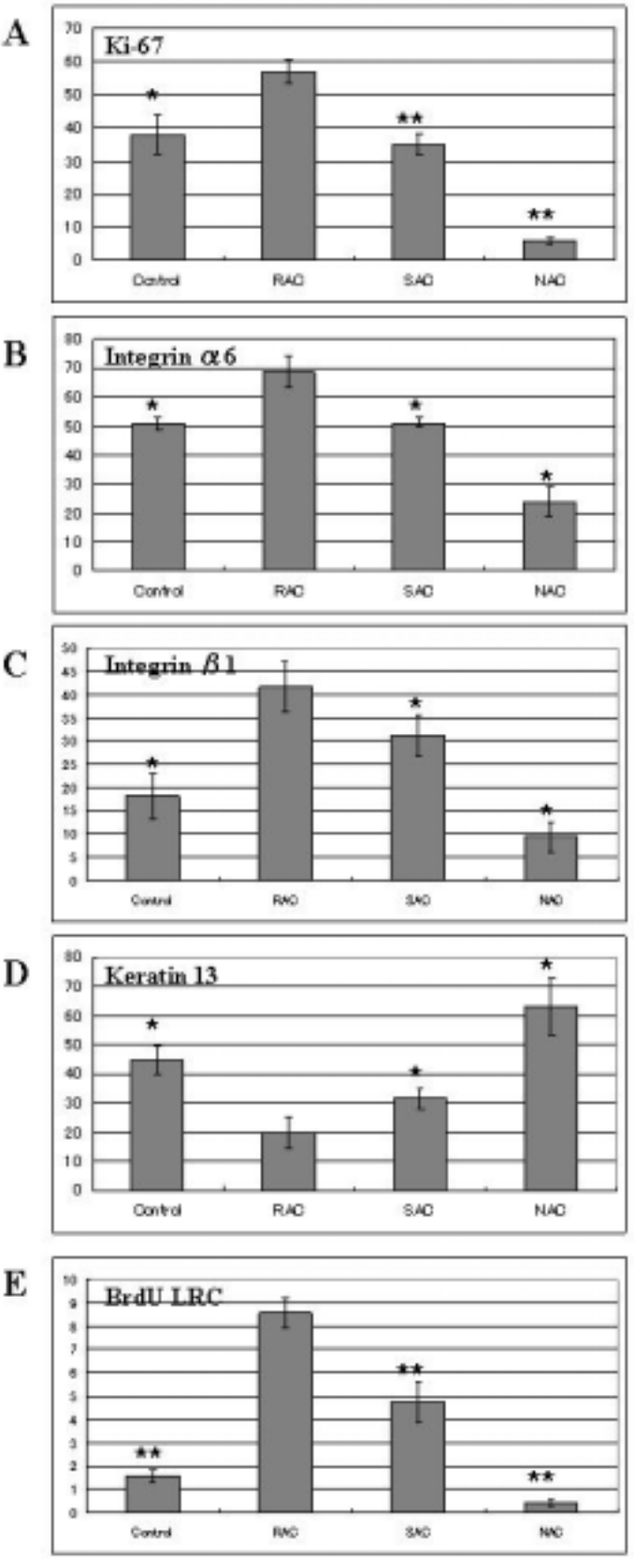
Y

Figure 4


A
Ki-67
Control
RAC
SAC
NAC
B
Integrin α6
Control
RAC
SAC
NAC
C
Integrin β1
Control
RAC
SAC
NAC
D
Keratin 13
Control
RAC
SAC
NAC
E
BrdU-LRC
Control
RAC
SAC
NAC