## Divalent cation-mediated epithelial union with connective tissue

L. J. SCALETTA[1] and D. K. MacCALLUM[2], [1]*School of Dentistry, Case Western Reserve University, Cleveland, Ohio 44 106, and* [2]*Departments of Oral Biology and Anatomy, University of Michigan, Ann Arbor, Mich. 48 104, USA*

Union between stratified squamous epithelium and the underlying connective tissue is believed to be effected by basal epithelial cell hemidesmosomes attaching to the subjacent basal lamina [1–6]. Although the precise physical architecture and chemical nature of this relationship remain to be determined, it appears that in skin the attachment is relatively strong, since it resists separation in vesiculation [7, 8] and inflammation [9, 10] and under all but the most extreme mechanical stress [11]. Studies on corneal epithelium have also demonstrated the strength of the hemidesmosome attachment to the basal lamina [12, 13]. The present study investigates the role of divalent cations in maintaining this union in human oral mucosa.

### *Materials and Methods*

Freshly excised surgical specimens of human oral mucosa were washed twice in calcium- and magnesium-free phosphate-buffered saline (PBS) and then trimmed to approx. $5 \times 2 \times 2$ mm. Tissue slices were then incubated at 37°C in an isotonic solution of phosphate-buffered saline containing 20 mM tetrasodium ethylenediamine tetraacetate dihydrate (EDTA) (6.83 g NaCl; 0.2 g KCl; 1.15 g $Na_2HPO_4$; 0.2 g $KH_2PO4$; 0.12 ml 1% phenol red; 7.6 g $Na_4$-EDTA; made to 1 l with distilled water and gassed with 5% $CO_2$ to pH 7.3–7.4). After a 50 to 60 min incubation in PBS–EDTA at 37°C the epithelium could be easily separated from the connective tissue with fine forceps, using a dissecting microscope. Control specimens of oral mucosa were incubated for 4 h in PBS–EDTA containing 20 mM $Ca^{2+}$ ion (as $CaCl_2$), or for 4 h in $Ca^{2+}$-and $Mg^{2+}$-free PBS, after which time separation of epithelium from connective tissue was attempted. Other tissue slices were incubated in PBS–EDTA for 1 h and then either transferred directly to, or partially separated and then transferred to, a divalent cation supplemented medium, Earle balanced saline (EBS). After a 1 h incubation at room temperature, separation was again attempted. Several specimens from each experiment were fixed directly (untreated) and examined ultrastructurally as normal controls of our fixation and embedding procedures.

Tissues from all groups were fixed at room temperature for 2 h in 2% paraformaldehyde and 2.5% glutaraldehyde in half-strength Millonig buffer [14]. Specimens were postfixed in Millonig 2% $OsO_4$, stained en bloc in 0.5% uranyl acetate [15], dehydrated in graded concentrations of ethyl alcohol and propylene oxide prior to embedding in a Maraglas–D.E.R. 732 mixture [16]. Sections were stained with uranyl acetate and lead citrate and viewed with either a Phillips EM 300 or Siemens Elmiskop Ia electron microscope.

### *Results and Discussion*

The relationship of hemidesmosomes to the basal lamina as seen in untreated preparations was as described by others [3, 5, 6]. In these preparations fine filaments were observed to traverse the space between the cell membrane and the basal lamina. The hemidesmosomes possessed peripheral densities which were associated with these filaments (fig. 1*a*). In specimens which were separated, the basal lamina remained with the connective tissue portion (fig. 1*b*). Sites of hemidesmosome attachment to the basal lamina were no longer apparent following tissue separation. Examination of the epithelial portion of the separated preparation revealed extracellular tufts of generally filamentous material emanating from the hemidesmosomes, and an absence of hemidesmosomal peripheral densities. Other portions of the basal cell membrane were devoid of extracellular material (fig. 1*c*). Desmosomes connecting epithelial cells appeared unaltered by EDTA treatment.

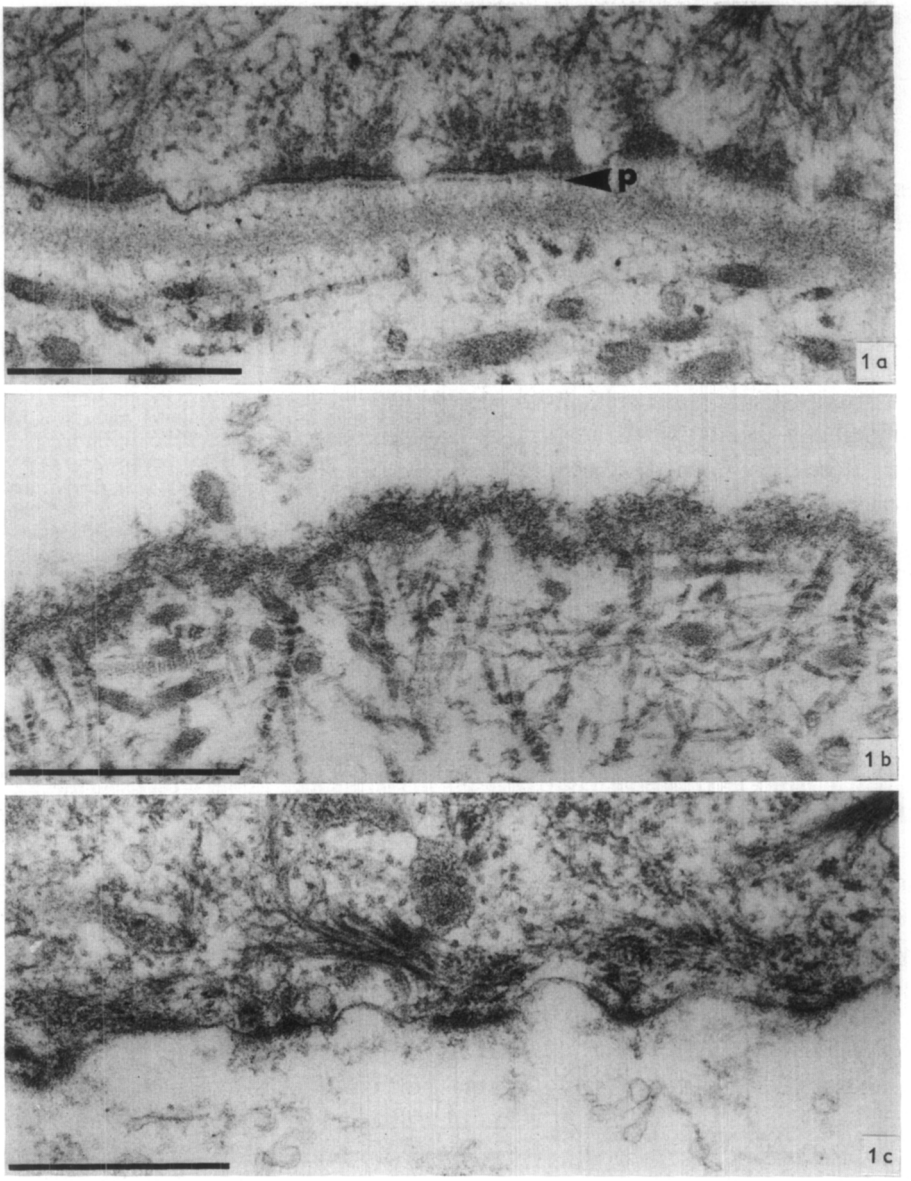


*Fig. 1.* (*a*) Contact region between epithelial basal cell and the basal lamina. Several hemidesmosomes are evident, the peripheral density of one is marked by arrow *P* ( × 64 200); (*a*) Connective tissue portion of a separated preparation carries with it an intact basal lamina ( × 60 000); (*c*) hemidesmosomes of basal cells in a separated preparation. A fine filamentous material emanates from the hemidesmosomes but peripheral densities are absent × 50 000. The line represents 0.5 $\mu$m.

Tissues incubated in $Ca^{2+}$ supplemented PBS–EDTA would not separate nor would tissues incubated in $Ca^{2+}$-, $Mg^{2+}$- free PBS. In each instance, there appeared to be some minor tissue shrinkage. However, the morphology of the hemidesmosomes and their relationship to the basal lamina remained the same as in untreated preparations.

Samples, partially separated following a 50 min incubation in PBS–EDTA, when placed in a divalent cation containing solution (EBS) for 1 h could no longer be separated. In fact, opposing surfaces of the separated tissues became noticeably more adherent to one another within a few minutes following return to EBS. Following 1 h exposure to divalent cations, the fine structure of partially separated preparations resembled control preparations in the unseparated regions and resembled completely separated preparations in those regions where the epithelium had been lifted from the connective tissue. EDTA treated but unseparated tissues which were post-incubated in EBS for 1 h, not only resembled untreated controls ultrastructurally but could not be separated into their epithelial and connective tissue components. Tissues similarly treated but post-incubated in $Ca^{2+}$- and $Mg^{2+}$-free EBS could be separated even after several hours in the divalent cation-free saline.

Our conclusion from these experiments is that divalent cations, probably calcium and/or magnesium, are necessary for the union of stratified squamous epithelium to connective tissue. A similar observation was made with respect to the union of ectoderm with mesoderm in developing chick limbs [17]. The following observations support the hypothesis that the nature of the union between epithelium and connective tissue is (at least in part) a divalent cation mediated association of hemidesmosomal filaments with the basal lamina: (1) Following tissue separation hemidesmosomal filaments, while being structurally altered, always remain associated with the epithelial cells. The basal lamina, on the other hand, remained firmly attached to the connective tissue portion of the treated tissues. (2) An increased adhesiveness develops between the opposing surfaces of EDTA separated epithelium and connective tissues when they are returned to divalent cation supplemented medium (EBS). Further, a firm union which defies separation by routine methods is reestablished between epithelium and connective tissues of EDTA treated but unseparated oral mucosa when the specimen is postincubated in EBS. This union is not reestablished in divalent cation-free saline; epithelium and connective tissue may be separated even after several hours of incubation in this medium.

It is also worth noting that there is a disappearance of the peripheral densities from hemidesmosomes following separation. It is possible that divalent cations may be in part responsible for the stability of these morphological regions whose biological significance remains to be determined. However, such speculations must await definitive studies on the physical and chemical structure of the hemidesmosome. In any event, divalent cation participation in this type of union appears to be localized (hemidesmosome) and in that respect differs from the well documented but poorly understood roles which divalent cations play in the phenomena of cell adhesiveness and cell membrane hardening [18].

This research was supported by USPHS grants DE-2697, DE-00180, DE-32,968, DE-2731 and a University of Michigan Cancer Research Grant.

## *References*

1. Odland, G F, J biochem biophys cytol 4 (1958) 529.
2. Farquhar, M G & Palade, G E, J cell biol 26 (1965) 263.
3. Stern, I B, Periodontics 3 (1965) 224.
4. Kelly, D E, J cell biol 28 (1966) 51.
5. Schroder, H E & Theilade, J, J periodont res 1 (1966) 95.
6. Frithiof, L, Acta pathol microbiol Scand, suppl. 200 (1969) 1.
7. Pearson, R W, Ultrastructure of normal and abnormal skin (ed A S Zelickson) p. 320. Lea & Febiger, Philadelphia, Pa (1967).
8. Wilgram, G F, Ultrastructure of normal and abnormal skin (ed A S Zelickson) p. 335. Lea & Febiger, Philadelphia, Pa (1967).
9. Thilander, H, J periodont res 3 (1968) 303.

10. Gavin, J B, J periodont res 5 (1970) 19.
11. Van Scott, E J, J invest derm 18 (1952) 377.
12. Jakus, M A, Invest ophthalmol, 1 (1962) 202.
13. Kenyon, K R, Invest ophthalmol 8 (1969) 156.
14. Millonig, G, J appl phys 32 (1961) 1637.
15. Karnovsky, M J, J cell biol 35 (1967) 213.
16. Erlandson, R A, J cell biol 22 (1964) 704.
17. Goel, S C & Jurand, A, J cell sci 3 (1968) 373.
18. Manery, J F, Mineral metabolism (ed L L Comar & F Bronner) vol. 3, p. 406. Academic Press, New York (1969).

Received April 28, 1971

Revised version received July 26, 1971

**Protoplasts isolated from callus cells of maize endosperm.** Formation of multinucleate protoplasts and nuclear division

F. MOTOYOSHI, *Institute for Plant Virus Research, 959, Aoba-cho, Chiba, Japan*

*Summary*

About 50% of the total protoplasts enzymatically isolated from callus cells of maize endosperm were multinucleate, suggesting that fusion of single protoplasts occurred during the protoplast isolation process. Some of the protoplasts cultured in vitro showed enlargement, budding, and nuclear division during 6 days of culture. Nuclear division in the multinucleate protoplasts was usually synchronous. No evidence of protoplast proliferation has yet been obtained.

It has been described how protoplasts from different plant species can fuse [1], and some of the fused protoplasts of tobacco leaf cells regenerate the cell wall and repeatedly divide when cultured in vitro [2]. In this study, the author obtained evidence indicating fusion and nuclear division in protoplasts isolated from callus cells of maize endosperm.

*Material and Methods*

Callus cells (TM34) used in this work were obtained from a hybrid endosperm of maize [3] and subcultured every 30 days for about 7 years on White's agar medium with added 0.5% dried extract of yeast (Daigo Chemical Co., Osaka, Japan). To digest the cell wall, 500 mg of the cells, which were obtained around 10 days after transfer of the callus on the agar medium, were suspended in 25 ml of an enzyme mixture consisting of 5% cellulase (Cellulase Onozuka CLA696, Kinki Yakult Manuf. Co., Nishinomiya, Japan), 2% Macerozyme (ME43, Kinki Yakult), 0.01% potassium dextran sulfate (Meito Sangyo Co., Nagoya, Japan), and 0.6 M mannitol, and incubated at 37°C in a 100 ml flasks on a reciprocal shaker (60 rpm) in the dark. The enzyme mixture had been previously adjusted to pH 5.4 with 1N HCl and sterilized by filtration through a Millipore filter (PH, Millipore Co., Bedford, Mass.) After incubation for 3.5 h, the suspension was filtered through a stainless steel mesh (150 mesh), and the protoplasts were collected by low speed centrifugation (ca. 70 g, 5 min), followed by washing three times with 15 ml of 0.6 M mannitol by centrifugation (ca 70 g, 2 min).

In other work the author attempted to isolate protoplasts from tobacco calluses, but the cell wall was not completely digested, and after enzyme treatment 10 to 20% of the protoplasts were observed to be surrounded by a cell wall. Dead cells, cell fragments with a cell wall, and living protoplasts surrounded by a cell wall could not be removed from the protoplast preparation by the washing procedure, since they sedimented together with protoplasts by centrifugation (unpublished). The cell wall of the maize callus was, however, more efficiently digested, although some tightly associated large clumps

Table 1. *Frequency of protoplasts and cellulase-untreated cells with different numbers of nuclei*

| Protoplasts | | | Cellulase-untreated cells | | |
|---|---|---|---|---|---|
| No. of nuclei per protoplast | No. of protoplast | Percent of protoplasts | No. of nuclei per cell | No. of cells | Percent of cells |
| 1 | 264 | 52.8 | 1 | 355 | 85.1 |
| 2 | 97 | 19.4 | 2 | 54 | 12.9 |
| 3 | 44 | 8.8 | 3 | 5 | 1.2 |
| 4 | 29 | 5.8 | 4 | 2 | 0.5 |
| 5 | 24 | 4.8 | 5 | 0 | 0.0 |
| 6 | 12 | 2.4 | 6 | 0 | 0.0 |
| 7 | 10 | 2.0 | 7 | 1 | 0.3 |
| 8 | 7 | 1.4 | | | |
| 9 | 4 | 0.8 | | | |
| 10 | 4 | 0.8 | | | |
| 11 | 2 | 0.4 | | | |
| 14 | 1 | 0.2 | | | |
| 15 | 1 | 0.2 | | | |
| 28 | 1 | 0.2 | | | |
| Total | 500 | 100.0 | Total | 417 | 100.0 |