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Wound Repair of the Oral Mucosa

Immunohistological and ^{3}H-Thymidine-Autoradiographic Observations

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Summary. The migratory and proliferative capacity of oral epithelium was studied after induction of subepithelial suction blisters in palatal rat mucosa. FITC-coupled lectins and their affinities to epithelial cells at the wound margin were analyzed by immunfluorescence microscopy.

Immunocytochemical distribution of 67 K-keratin polypeptides in different epithelial layers were studied using specific antisera against keratin proteins. By means of ^{3}H-thymidine-autoradiography the proliferation of epithelial basal cells and their migration at the wound edge was quantified by evaluation of the labelling index. Double labelling was performed, combining the autoradiographic and the keratin staining technique.

It was found that the subepithelial suction blister showed rapid repair (epithelial regeneration after 3–4 days). Peanut Agglutinin (PNA) showed a selective affinity to the basal 2–3 layers of oral rat mucosa. No modifications of lectin affinities were found in epithelial wound repair. PNA-positive keratinocytes were demonstrated in the migratory epithelial tongue.

The selective presence of 67 K-keratin (67 K) in the suprabasal epithelial cell layers was interpreted as an indication of the differentiation process in rat oral mucosa. The ^{3}H-thymidine-labelling index of basal cells increased significantly, beginning at the wound edge after 24 h. The peak of the labelling index curve was located in the vicinity of the wound after 96 h.

Double labelling technique revealed 67 K-negative and ^{3}H-thymidine-positive basal cells as well as 67 K-positive and ^{3}H-thymidine-negative cells at the migration front. These observations support findings that migrating epithelial cells in oral wound healing derive both from undifferentiated (str. basale) and from differentiated (inferior str. spinosum) cell compartments.

Key words: Wound repair – Lectin affinity – Keratinocyte differentiation – Autoradiography

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The present study was concerned with the migratory and proliferative capacity of oral epithelium. Epithelial proliferation and migration has previously been studied by means of histochemistry, autoradiography and electron microscopy (Review: Gabbiani et al. 1978; Hunter et al. 1974; Krawczyk 1971; Lindner 1967, 1982; Maibach et al. 1972; Oehlert 1969; Pollack 1979; Winter 1972). Recently immunohistological methods have been applied to changes of membraneous and cytoplasmic components during normal epithelial maturation (Dabelsteen et al. 1978) and epithelial repair (Dabelsteen and MacKenzie 1978; Gabbiani et al. 1978; Löning et al. 1980, 1982). The investigation of keratin modifications and of lectin affinity changes are of considerable interest in epithelial differentiation and migration during mucosal wound healing. For studies of keratinocyte proliferation we combined those immunohistological methods with ^{3}H-thymidine autoradiography. Using rat oral mucosa these methods were applied to elucidate the *following questions*:

1. Which lectins are useful in distinguishing epithelial compartments of different degrees of maturation and are hence of interest for the determination of migrating epithelial cells at the wound margin?
2. Are keratins of high molecular weight, normally present in differentiated keratinocytes, detectable in the epithelial outgrowth?
3. What additional information can be obtained using a double labelling technique with ^{3}H-thymidine autoradiography and keratin staining?

Material and Methods

Experimental Design. Thirty-two male Wistar-rats with an average weight of 250 g were anaesthetized by intraperitoneal application of pentobarbital (Nembutal®, 0,10 ml/100 g body weight). Subepithelial blisters (Fig. 1) were induced on the palatal mucosa using a modified suction device following a method originally described by Kiistala and Mustakallio (1967).

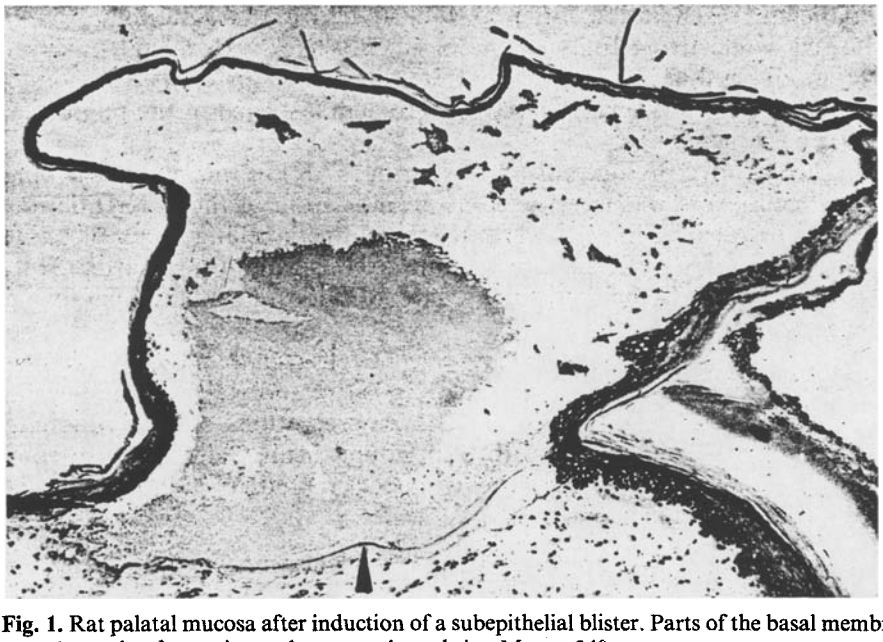

Fig. 1. Rat palatal mucosa after induction of a subepithelial blister. Parts of the basal membrane zone (*arrowhead*) remain on the mesenchymal site. Mag. × 340

Table 1. Applied lectins

Abbreviation	Lectin	Specific sugar
Con A	Jack bean	α-D-mannose α-D-glucose
DBA	Dolichos biflorus agg.	α-D-Nac-galactose
PHA	Phaseolus vulgaris agg.	D-Nac-galactose
PNA	Peanut agg.	D-galactose
RCA_1	Ricinus communis agg. I	D-galactose
RCA_2	Ricinus communis agg. II	D-galactose
SBA	Soy bean agg.	α-D-NAc-galactose D-galactose
SJA	Sophora japonica agg.	β-D-galactose β-D-NAc-galactose
HPA	Helix pomatia agg.	α-D-NAc-galactose

All lectins were used at 100 µg/ml, incubation for 60 min at room temperature.

Buffer: PBS, pH 7.2 for DBA, HPA, PNA, RCA_1, RCA_2, SBA; 0.05 M Tris, 0.15 M NaCL, pH 8.7 for SJA; 0.05 M Tris, 0.01 M $CaCL_2$, 0.01 M $MnCL_2$, 0.15 M NaCL, pH 7.0 for Con A.

Controls: Preincubation of the FITC-coupled lectins with their specific sugars (0.2 M final dilution, contact for 2 h at 4° C).

The pressure within the vacuum-chambers was maintained at 150 mmHg below the atmospheric pressure, the required suction time averaged 30 min. The maximum diameter of the blisters measured 3 mm. Immediately after removal of the cup, the blister roofs were carefully removed. The wounded areas were excised in intervals of 24 h starting immediately after injury up to 1 week. The specimens were stretched on cork for good orientation, fixed in Bouins solution and embedded in paraffin wax. A part of each sample was quickly frozen in liquid nitrogen and stored at −70° C.

Immunofluorescence

Frozen material was cut at 4 µm sections in a SLEE-cryocut. The sections were kept overnight at −20° C prior to incubation with the FITC-coupled lectins (Table 1). Sections were briefly (10 min) fixed in acetone. All lectins were used at 100 µg/ml protein and for incubation for 60 min at room temperature. Controls were done by preincubation of the FITC-coupled lectins with their specific sugars (0,2 M final dilution, contact for 2 h at 4C, Table 1).

The sections were observed under a Zeiss Ploems epifluorescence microscope equipped with a HBO 50 W mercury lamp and Zeiss filter system for FITC. Photomicrographs were taken using a Kodak Tri-X pan.

Immunocytochemistry

67K antiserum. According to a procedure previously described by Viac et al. (1978) adult (400 g) female Hartley guinea pigs were immunized with the keratin polypeptide of molecular weight of 67.000 daltons. Eight hours after the last injection the animals were bled by heart puncture. The immune serum obtained was absorbed successively with human erythrocytes and liver powder (Olson et al. 1972) and used at a dilution of 1:200 in phosphate buffered saline (PBS) (Viac et al. 1980). On the sections, immunocytochemical staining was performed using the primary antisera described above and peroxidase-conjugated link antisera (goat anti-guinea pig IgG 1:50 in PBS, NORDIC). 3′, 3′-diaminobenzidine (SIGMA) was used to reveal peroxidase activity (Graham and Karnovsky 1966). Parallel sections were stained with haematoxylin-eosin and the PAS-reaction. Control studies were performed in which the specific

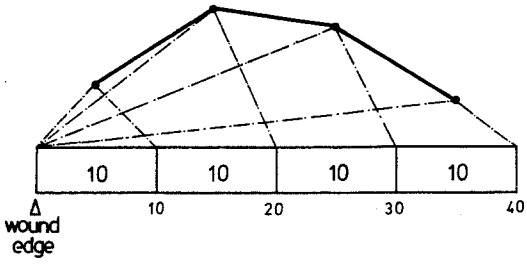

Fig. 2. Evaluation of the ^{3}H-thymidine labelling index of epithelial basal cells (further details in the text)

primary antibodies were replaced by different immune sera or peroxidase-coupled link antiserum only.

Autoradiography

For autoradiographic investigation methyl-^{3}H-thymidine (special activity 25 Ci/mmol, Amersham Buchler) was injected intraperitoneally (4 × 1 μCi/g body weight at intervals of 90 min). The animals, previously wounded as described, were killed by ether narcosis 90 min after the last isotope injection with a total incorporation time of ^{3}H-thymidine (^{3}H-t) of 6 h. The excised specimens were fixed in Bouins solution and embedded in paraffin wax following routine procedures. Using the stripping film technique, 4 μm sections were exposed for 3 weeks with Kodak fine grain autoradiographic stripping plate AR 10. Slight counterstaining was performed with haematoxylin-eosin (for further details see Albrecht 1981). At high magnification (× 1,000, oil immersion), the autoradiographs were evaluated by determination of the labelling index of the epithelial basal cells. The labelling index (l.i.) is defined as the ratio of labelled cells per total number of counted basal cells.

To investigate local changes in basal cell proliferation at the wound edge, the l.i. was computed in subsequent 10-basal-cell-intervals as a percentage of labelled cells per total number of counted basal cells at the moment (Fig. 2). Statistical analysis was done using the *U*-test (Clauß and Ebner 1979). Significant findings are indicated with a probability of error $\alpha = 0{,}05$.

Double Labelling

Keratin staining was performed prior to the autoradiographic technique.

Results

Immunofluorescence

The FITC-coupled lectins used showed different affinities to epithelial cells, summarized in Table 2: In the normal oral mucosa, the basal cell layers

Table 2. Lectin binding to the normal oral epithelium of rats

Lectin	Basal layer	Intermediate layer	Superficial layer
Con A	+	+	+
DBA	−	−	−
PHA	+	+	−
PNA	+	−	−
RCA_1	+	+	−
RCA_2	+	+	−
SBA	+	+	−
SJA	+	+	−
HPA	+	+	−

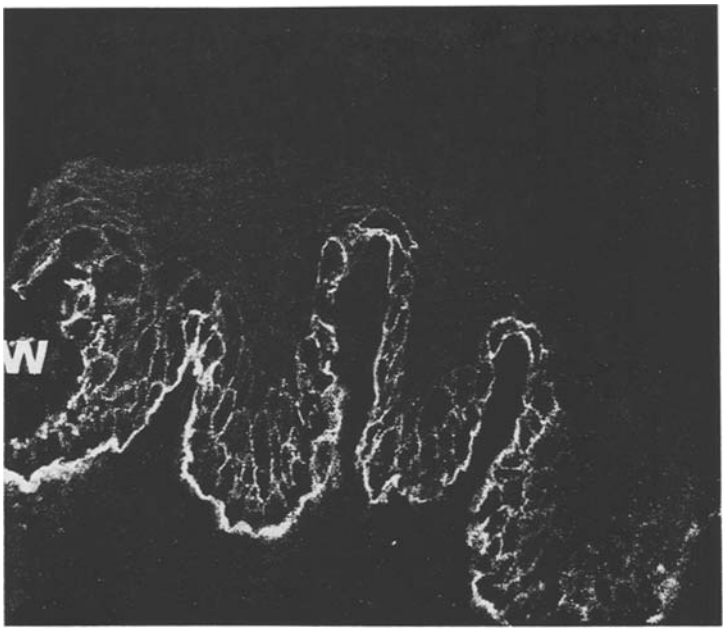

Fig. 3. Wound (left side) and wound margin (right side): Binding of PNA to basal epithelial cell layers (*W* = wound)

are labelled with all lectins except DBA. DBA was found to bind to saliva-derived substances at the surface but not to epithelial cells. The intermediate cell layers, e.g. stratum spinosum and stratum granulosum, showed distinct membrane staining with the majority of the lectins investigated. However, PNA was observed to label only the 3 basal cell layers of oral epithelium, in particular the stratum basale. The upper cell layers, e.g. stratum corneum, were only stained with ConA.

Lectin affinity studies of the wounded areas led to the following observations: There was no decreased membrane staining at the wound margins. The epithelial outgrowth was intensively labelled with PNA. This staining extended continuously to the basal layer cells distal to the wound margin (Figs. 3 and 4).

Immunocytochemistry and Autoradiography

Qualitative Findings. In normal rat oral mucosa, all epithelial cells were labelled with antibodies against large keratins (67K) except for basal layer cells, but several nuclei of these germinative cells showed an incorporation of ^{3}H-t. Focal concentrations of ^{3}H-t-labelled nuclei were observed at the tip of epithelial papillae. Epithelial cells taking up ^{3}H-t were not seen to be stained with 67K-antibodies at a total incorporation time of ^{3}H-t of 6 h (Fig. 5).

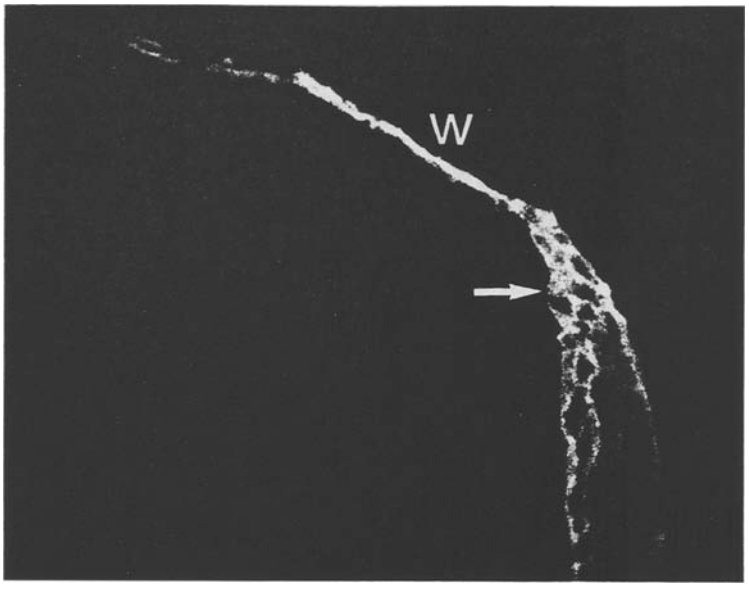

Fig. 4. Wound (*W*) and wound margin: Strong affinity of PNA to the advancing epithelial tongue (*arrow*) of the wound edge. Mag. × 550

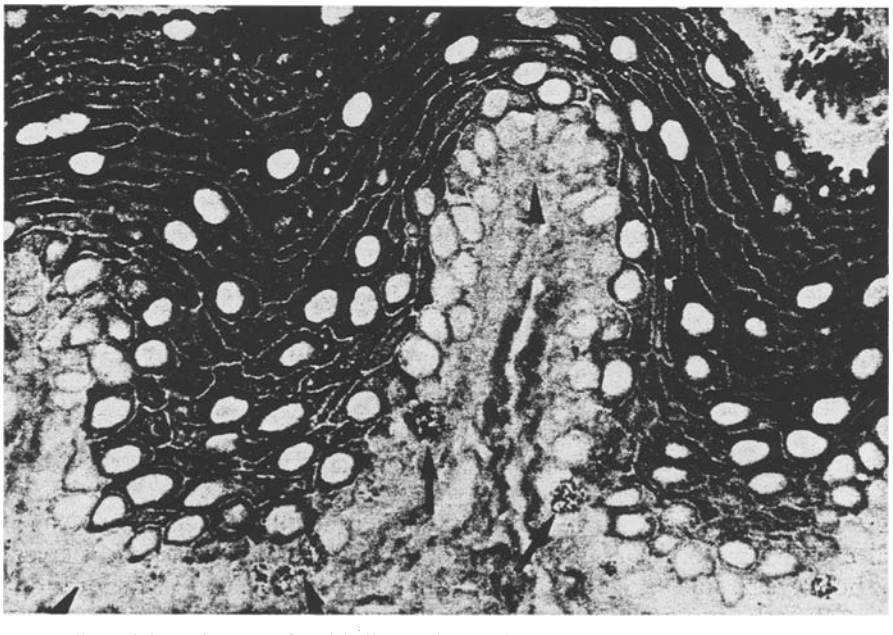

Fig. 5. Reactivity of rat oral epithelium with antibodies against 67K keratin polypeptides. Staining of suprabasal cell layers (*arrowheads* point to the basal membrane). Double labelling with ^{3}H-thymidine. Several keratin-negative basal layer cells contain nuclei with ^{3}H-thymidine incorporation (*arrows*). Mag. × 550

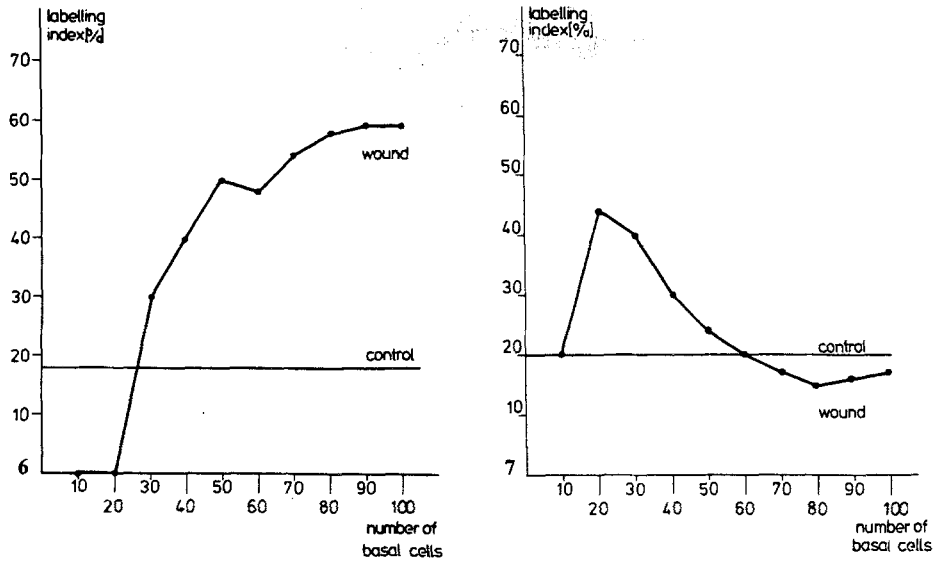

Fig. 6. Labelling index of basal cells 24 h after wounding. Maximum of labelling index was seen in a distance of 90 basal cells from the wound hiatus

Fig. 7. Labelling index of basal cells 96 h after wounding. Maximum was found in close vicinity to the wound

At the wound margin, the epithelial outgrowth consisted of 67K-positive, ^{3}H-t-negative as well as of 67K-negative and ^{3}H-t-positive keratinocytes. A large number of epithelial cells at the advancing wound edge, however, did not stain with 67K-antibodies nor were labelled with ^{3}H-t.

Quantitative Findings. A continuously increasing labelling index of epithelial basal cells was found, beginning at a distance of about 20 basal cells counted from the wound edge (Fig. 6).

Up to 96 h, the peak of the labelling index curve (Fig. 2) was located in the vicinity of the wound. The initial broad elevation 24 h after wounding (s. Fig. 6) was followed (after 96 h) by a decrease with growing distance from the wound edge initially most evident proximally (Fig. 7). About 60 cells away from the wound margin, normal values were again found.

Discussion

Superficial wounding by induction of subepithelial suction blisters is an elegant method by which to study pure epithelial wound healing (Kiistala and Mustakallio 1967). Separation of epithelium and connective tissue occurs between the basal cell membrane of keratinocytes and the basement membrane, as seen at the light microscopic level (Kiistala 1968; Ortonne et al. 1981; Pang et al. 1978). Ultrastructurally the lamina densa of the

junctional zone is well preserved and along this the epithelial cells can advance to cover the wound hiatus (Löning 1982).

One of the objects of the present study was to investigate lectin binding to normal and injured oral epithelium. Using fixed, paraffin-embedded specimens, Dabelsteen et al. (1978) demonstrated a selective loss of blood group antigens during oral wound healing as well as decreased lectin-affinity of the epithelial outgrowth (Dabelsteen and MacKenzie 1978). To these authors, the changes of cell membrane carbohydrates appeared to be related to areas of increased cell movement, a finding which may be of considerable interest with regard to the complete or partial antigen loss in some premalignant and malignant lesions of oral mucosa (Dabelsteen 1980). On the basis of frozen sections, we did not find that the investigated lectins showed less affinity to the epithelial outgrowth. This observation is interesting with respect to and in comparison with neoplastic epithelial cell growth, where decreased lectin binding has been demonstrated by us and by other research groups (Dabelsteen 1980; Löning 1982).

Another aspect of lectin affinity studies was to find out if epithelial compartments of rat oral mucosa can be distinguished by their different degree of maturation. In fact, PNA revealed a selective affinity to the stratum basale and part of the lower stratum spinosum in accordance with recent investigations on esophageal epithelium (Watanabe et al. 1981). In the wound area, all advancing epithelial cells were stained with FITC-coupled PNA. This observation may lead to the conclusion that epithelial migration originates from the 3 basal layers of oral squamous epithelium.

Arriving at this point we wanted to characterize in more detail the proliferating and migrating epithelial outgrowth. In addition to other methods like the colchicine arrested counting of mitoses (Winter 1972) ^{3}H-thymidine-autoradiography represents a valid quantitative method for studies of cell kinetics (Oehlert 1969; Schmiegelow et al. 1981). Well established autoradiographic studies on squamous epithelium cell proliferation have indicated that during epithelial wound healing the basal cell labelling index, counted in the wound area, varies in a characteristic pattern in close dependence upon regeneration time (Matoltsy and Viziam 1970; Oehlert 1969). After 24 h of wounding, our labelling curve showed consistently elevated values over a distance of about 100 basal cells counted from the wound margin. After 96 h this significant elevation was restricted to the neighbourhood of the wound edge. Using a double-labelling technique we expected further insights into the relation of basal cell proliferation and epithelial cell migration and differentiation. Epithelial cells which incorporated ^{3}H-t were not stained with high molecular keratins which are believed to be a product of differentiated suprabasal keratinocytes (Löning et al. 1980; Viac et al. 1980). At the wound edge, advancing differentiated 67K-positive epithelial cells were actually present. This finding in the oral mucosa is consistent with recently published results on human skin wounds (Ortonne et al. 1981).

We cannot exclude the possibility that within the germinative cell layer there are keratinocytes at an early stage of differentiation which are neither labelled with ^{3}H-t (l.i. of epithelial basal cells at the wound edge 40%)

nor with 67K antibodies. In accordance with previous observations of epidermal wound healing our results are in favour of migrating epithelial cells being derived from undifferentiated and differentiated cell compartments (str. basale and inferior str. spinosum).

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