Histochemical Studies of Oral Epithelium

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Introduction

Histochemical studies of normal and pathological oral mucosa have been reported by many investigators, such studies as dealing chiefly with the localization of glycogen, nucleic acid and phosphatase. In a previous paper, the histochemical distribution of various hydrolytic enzymes and dehydrogenases in the human gingiva and oral epithelium of rodents has been reported. In the present studies it is of interest and significance to report on the distribution and localization of hydrolytic enzymes, dehydrogenases and other various substances, and further on the correlation among them.

Materials and Methods

Human oral epithelium was obtained from patients who had undergone extractions of pathological teeth and surgical operations for tumor treatment of the jaw. As the specimens of human material, buccal mucosa and gingiva under clinically normal and pathological conditions were used. Other specimens, such as oral and tongue epithelium of both sexes of mice, rats, guinea pigs, rabbits and dogs were examined. The fresh tissues were cut into thin sections (10–20 μ) in a cryostat (−20°C) according to Coon’s modification of the Linderstrom Lang method, and were dried at room temperature (20°C). For the demonstration of dehydrogenases and diaphorase, unfixed fresh preparations were used, but for the hydrolytic enzyme the specimens were fixed in 10% neutral formalin for 10 minutes and rinsed briefly in distilled water. Tissue from the same specimens was also fixed in a 10% formalin solution or 80% alcohol, paraffin sections were made hematoxylin eosin staining, the periodic acid Schiff reaction for polysaccharides and toluidine blue stain for metachromasia performed.

Demonstration of Enzymes

Twenty serial sections each were prepared from the freshly frozen material in a cryostat (−20°C) and two sections each were incubated according to the following methods.
Alkaline phosphatase\(^{1,2}\): $\alpha$-Naphthyl acid phosphate (10mg) was dissolved in 0.1M Clark and Lub's buffer (20 ml) at pH 9.2 and Diazob Blue B (20 mg) added. The fixed section was incubated in the fresh solution at 20°C for 20 to 30 minutes, and a positive reaction was determined by the formation of a black color with a yellow or brown "background".

Acid phosphatase\(^{1,2}\): The incubation solution was prepared employing 0.1M acetate buffer at pH 5.8 instead of the Clark and Lub's buffer for alkaline phosphatase. The fixed section was allowed to react in the freshly prepared incubation solution at 20°C for one hour. The sites of enzyme activity were determined by the appearance of dark brown to black azo dye with light brown "background".

Esterase: $\beta$-Naphthyl acetate (10mg) was dissolved in 1 ml acetone and added to 0.1 M Michaelis buffer (20 ml) at pH 7.2, then Diazob Blue B added, the mixture shaken, filtered, and incubated at 20°C for 30 minutes.

$\beta$-Glucuronidase, $\beta$-Galactosidase and $\beta$-Glucosidase: In demonstration of these three $\beta$-glycosidases, the following methods were used; the post-incubation azo coupling method described by Seligman et al (1954)\(^4\) using 6-bromo-2-naphthyl $\beta$-D-glucuronide was employed for $\beta$-glucuronidase, the colorimetric estimation for $\beta$-galactosidase and $\beta$-glucosidase reported by Cohen et al (1952)\(^5\) using 6-bromo-2-naphthol $\beta$-D-galactopyranoside and 6-bromo-2-naphthol $\beta$-D-glucopyranoside, and histochemical demonstration of $\beta$-galactosidase published by Rutenberg et al (1958)\(^6\) using the same substrate. Incubation in each substrate for the three $\beta$-glycosidases was for six hours. After incubation, the sections were rinsed in tap water and transferred to a cold (4°C) freshly prepared 0.02M phosphate buffer solution (pH7.5) containing Diazob Blue B (1mg/1ml).

Aminopeptidase: Both the histochemical method of Burstone and Folk (1955) and Nachlas et al (1957) for demonstration of aminopeptidase activity were used. L-leucyl-$\beta$-naphthylamide hydrochloride and DL-alanyl-$\beta$-naphthylamide hydrochloride were used as the substrates in both methods. Burstone utilized diazotized o-aminoazotoluene (Garnet GBC) and diazotized p-nitro-anisidine or p-nitro-p-amino-2,5-dimethoxy-diphenylene (Black salt K). In our present study, Diazob Blue B was employed instead of Garnet GBC, and phosphate buffer at pH 6.8 used for the substrate solution.

One ml of 1% stock substrate solution (8mg/1ml), distilled water (40ml), 0.2M phosphate buffer (10ml) and 30mg of Diazob Blue B was mixed and shaken. Incubation was for one hour at room temperature by the Burstone method, but at 37°C for one hour according to the Nachlas method. However, the results reported in this paper were obtained by the following incubation solution; substrate solution (8mg/1cc) 1ml, acetate buffer (pH 6.5, 0.1M) 10ml, 0.85% sodium chloride 8ml, 2 $\cdot$ 10$^{-3}$ M potassium cyanide 1ml, 10mg of Diazob Blue B, was mixed the sections incubated for one hour at 37°C.

By the method of Nachlas et al, the sections were rinsed in distilled water for a short time, and after chelating in 0.1M copper sulfate, mounted in balsam.
Succinic dehydrogenase, Nachlas et al method (1957) was used. Equal volumes of 0.2M phosphate buffer at pH 7.6 and 0.2M sodium succinate were combined, and the same volume of Nitro-BT aqueous solution (1mg/1ml) added. Unfixed fresh frozen sections were incubate for 15 minutes at 37°C, then fixed in 10% formalin, washed in water, dehydrated, and mounted in balsam.

TPN and DPN diaphorase; The method of Nachlas et al (1958) was employed. For demonstration of TPN diaphorase the following mixture was used: 0.6ml of sodium DL-isocitrate 2.5M, 0.5ml of sodium L-malate (2.5M), 0.3ml manganese chloride (0.005M), 0.2ml of TPN aqueous solution (5mg/1ml), 1.1 ml of veronal acetate buffer (0.0M) at pH 7.4 and 0.3ml of Nitro BT solution (5mg/1ml).

Results

The reaction of alkaline phosphatase employing the azo coupling method in the oral and gingival epithelium of both the human and experimental animals was almost negative. Alkaline phosphatase activity was demonstrated in the capillaries and fibers in subepithelial connective tissues and high enzyme activity was observed in the cells and ground substances of the regenerative connective tissue.

Acid phosphatase was contained in the epithelium, especially highly stainable in the superficial layer of human oral mucosa, and also in the hornified layer and granular cell layer in the buccal and tongue epithelium of rodents. The tongue epithelium of the rat, guinea pig and dog showed a moderate acid phosphatase reaction in the basal cell layer. Esterase activities were found in all layers of oral epithelium, except for the hornified layer or superficial layer. The connective tissue was almost negative or very slightly positive while strong activity was seen in the muscle. The three glycosidases activities (β-glucuronidase, β-galactosidase and β-glucosidase) were shown to be of the same distribution in oral epithelium. Enzymatic reactions were seen in the cells from the basal cell layer to granular cell layer. The connective tissue cell was found to contain some activity, while in the muscle a strong enzyme reaction was observed. Aminopeptidase reaction appeared in the basal cell layer, a marked enzymatic activity seen in the basal cells of the human, dog, guinea pig and rat, while in mouse they showed almost no reaction. The succinic dehydrogenase activity was determined to be microcrystal in form in the cytoplasm of the basal cells and prickle cells in the oral epithelium employing Nitro-BT and neotetrazolium chloride. High activity was observed in basal cells and adjacent spinal cells which gradually decreased toward the superficial layers, and completely disappeared in the hornified layers. Sometimes, a significantly visible reaction was seen in the st. granulosum, this phenomena being artefacts caused by sulfhydryl groups contained in these cells. Triphosphopyridine nucleotide and diphosphopyridine nucleotide diaphorase were contained in the cell plasma of the whole epithelium except for the hornified layer,
the activities also being the same by the use of Nitro BT method. The localization of hydrolytic and dehydrogenative enzymes in the oral and tongue epithelium can be summarized as in the following table.

The Localization of Various Enzymes in Human Oral Epithelium

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>St. basale</th>
<th>St. spinosum</th>
<th>St. granulosum</th>
<th>S. corneum</th>
<th>Subepithelial Connective Tissue</th>
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<tbody>
<tr>
<td>Alkaline Phosphatase</td>
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<td>+ ~ +</td>
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<tr>
<td>Acid Phosphatase</td>
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<td>+ ~ +</td>
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<tr>
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<td>+</td>
<td>+</td>
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<tr>
<td>Aminopeptidase</td>
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<tr>
<td>Glycosidase</td>
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<tr>
<td>β-Glucuronidase</td>
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<td>β-Glucosidase</td>
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<td>TPN and DPN diaphorase</td>
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Discussion

In the histochemical studies of oral epithelium up to the present, the distribution of glycogen and phosphatase activity has chiefly been studied.

Engel et al. (1950)\(^1\) have reported on the glycogen of the pathogenesis of desquamative gingivitis, and Turesky et al. (1951)\(^2\) described by histochemical methods of the glycogen deposit in normal and inflammatory human gingiva. Dewar (1955)\(^3\) observed the localization of keratin, carbohydrate, alkaline phosphatase and nucleic acid in 56 patients, Trott (1957)\(^4\) reported glycogen detection in the human gingiva of 140 cases, and our previous experiments showed the histochemical distribution of polysaccharides and nucleic acid of the gingiva in 324 cases with various types of periodontal diseases. These reports show that the existence of a distinguishable relationship between the degree of inflammation and content of glycogen in gingival epithelium.

In the previous report, PAS positive polysaccharides of the human gingiva was observed diffusely in the st. spinosum and st. granulosum. DNA content as a rule was constant, but RNA was dominant in the basal cell layer and a tendency of the RNA to be decreased was seen in the epithelium. The histochemical observations of the sulfhydryl group (SH) and disulfide group (SS) in gingival epithelium have been reported by Tresky et al. (1957)\(^5\), Cabrini and Carranza (1951)\(^6\), Ring et al. (1950)\(^7\) and Staple (1957)\(^8\) reported on alkaline phosphatase distribution in the gingiva and Cabrini and Carranza (1958)\(^9\) described the acid phosphatase in the human gingiva. Histochemical studies of succinic dehydrogenase have been reported by Montagna (1955)\(^10\), Braun-Falco (1955)\(^11\), Argyris (1956)\(^12,13\) in the skin, and Kawakatsu et al. (1960)\(^14,15\) in the oral epithelium. In general, succinic dehydrogenase activity in the epithelial tissue was localized strongly in the basal cell layer and gradually decreased toward the
superficial layers.

In the enzymatic histochemical determination in the oral epithelium of the human and rodents, it was observed that no alkaline phosphatase, but on the other hand, acid phosphatase was contained in the epithelium and the strong activity was shown in the cornified layer. Esterase reaction was seen diffusely in the cytoplasm of epithelium cells except for the hornified layer. The cellular localization of $\beta$-glucuronidase using the Fishman and Baker method in the gingiva was demonstrated in the basal cell layer according to Tanaka. In this study, $\beta$-glucuronidase activity employing the post-incubation azo coupling method was demonstrated in the basal cell layer to granular cell layer, differing from observation of the localization of enzymatic activity by the Fishman and Baker method and azo dye method.

Development of the epithelial cell suggested that it was formed by nucleoprotein, hydrolytic enzymes and dehydrogenases in the basal cell, and the prickle cell in the st. spinosum showed polysaccharide synthesis, the metabolic energy being obtained from glycolysis in the adjacent metaspinocyte during the production of keratohyalin. Polysaccharide occurrence is high in the spinocyte, but in the granular cell layer no reaction is seen by amylase and trichloroacetic acid treatment, and keratohyalin of the granular cell layer contained a high amount of sulphydryl group. These findings show a significant correlation among the polysaccharide, sulphydryl group and keratohyalin of granular cell. These phenomena on the unknown constitutions of oral epithelium are closely related to the distribution of enzymatic activities and various substances. Such studies of the localization of various enzymes in the oral epithelium are indispensable in order to define the fundamental metabolism of the epithelium.

**Summary**

1. No alkaline phosphatase activity was observed in the oral epithelium of human and experimental animals under normal conditions, but the enzymatic activity of the capillary and inflammatory connective tissue was shown moderately or strongly.
2. Acid phosphatase was localized in the epithelium, especially, in the superficial layer, and in strong activity was observed in st. granulosum and st. corneum of oral epithelium.
3. Esterase reaction was observed throughout the whole layer of the oral epithelium except for the hornified layer.
4. The localization of $\beta$-glucuronidase, $\beta$-galactosidase and $\beta$-glucosidase was the same, the enzyme reaction being located in st. germinativum and st. granulosum by the post coupling method technique.
5. Aminopeptidase activity was presented in the st. basale and adjacent spinocellular layer.
6. Succinic dehydrogenase distributed strongly in the cytoplasm of the basal cell and gradually decreased toward the superficial layer. On the other hand, TPN and DPN diaphorase activities were dominant throughout all layers.
of the oral epithelium except for the hornified layer.

References

1) Mori, M., T. Mizushima, K. Fujita and T. Tani, Igakunoayumi. 32, 661, 1660 (in Jap.)

Discussion

Dr. Mizutani: The tetrazolium reactions of dehydrogenases must be exactly differentiated from the nonenzymatic reduction by SH-groups especially in the hair-follicles and papilla of the tongue. How do you think about the discrepancy of the findings by two kinds of ß-glucuronidase procedures?

Dr. Mori: (1) It is reasonable to decide the strong tetrazolium reduction in the epidermis as SH groups rather than dehydrogenases. (2) It needs further studies to compare the two methods for ß-glucuronidase.