Blood group substances as differentiation markers in human dento-gingival epithelium

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Steffensen, B., Lopatin, D. E., Caffesse, R. G., Hanks, C. T. Blood group substances as differentiation markers in human dento-gingival epithelium. Journal of Periodontal Research 1987; 22: 451–455.

The level of cellular differentiation of human oral, sulcular, and junctional epithelium was compared by immunohistochemical analysis of cell membrane-associated blood group-specific carbohydrates. Identification of the blood group A-specific carbohydrate and its two immediate precursor substances, type 2 chain H and N-acetyllactosamine, was accomplished by an indirect immunofluorescence technique. Murine monoclonal antibodies reacting specifically with the antigenic determinants of the blood group substances were used as markers. The blood group A substance, indicating the highest level of cellular differentiation, was demonstrated on the cells in the upper layers of the oral epithelium. In the sulcular epithelium, the A substance was present on a few cells only, while type 2 chain H was observed frequently. This indicates an intermediate differentiation level of sulcular epithelium. The type 2 chain H precursor, N-acetyllactosamine, the indicator of the lowest level of cell differentiation among the tested substances, was the only blood group substance detected on the junctional epithelial cells and on the basal cells of the sulcular and oral epithelium. Based upon previous studies of cell renewal and differentiation in oral epithelium, the present results indicate that the variations in distribution of the different blood group substances correspond with the regional rates of cell division and the levels of cellular differentiation. The findings also suggest that the cells in the junctional epithelium differentiate to a level similar to that of basal cells in the oral epithelium.

Accepted for publication April 23, 1987

Introduction

The epithelium of the human dento-gingival region has been divided into oral, sulcular, and junctional epithelium (1, 2). The differences in structural and functional characteristics of these epithelia, such as tissue permeability (3), variation in ultrastructure (4), rate of cellular division (5), and degree of keratinization (6), have been attributed to embryogenic development, tissue interaction and the special environment in the dento-gingival area (7, 8). The junctional epithelium retains specific qualities which are clearly distinct from the sulcular and oral gingival epithelium. However, after surgical removal, a new junctional epithelium can regenerate from the oral epithelium (2, 9–12). The nature of the processes leading to reestablishment of structure and function of the junctional epithelium is not yet fully understood (13).

Blood group-specific carbohydrate substances have been detected on epithelial cell membranes as well as on erythrocytes (14). It has been demonstrated, biochemically, that the specificity of the blood group substances is determined by variations in the structure of the terminal sugar residues on the carbohydrate chains (15, 16). Analysis of epithelial blood group substances has been shown to be useful in studies of cell differentiation of normal oral epithelium (17, 18) and in conditions of premalignancy, malignancy, and wound healing (19, 20). Current data indicate that the synthesis and elongation of the blood group-specific carbohydrates are correlated to the differentiation and maturity of epithelial cells (17, 18, 20). Thus, with increasing differentiation, the epithelial cells express more developed carbohydrates. Very few similar data are available on the level of cellular differentiation of the different epithelial components of the dento-gingival area in humans (21). Results from studies in mice indicate, however, that the junctional epithelium is less differentiated than sulcular or oral epithelium (8, 22).

The aim of the present investigation was to compare the level of cellular differentiation between human oral, sulcular, and junctional epithelium by immunohistochemical identification of three well-defined epithelial blood group substances.

Material and methods

Tissue samples

Twenty-one biopsies of marginal gingiva were obtained from 14 patients during periodontal surgery (16 specimens) or in combination with tooth extraction (5 specimens). Blood typing showed that 11 patients had blood type

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Antigen	Carbohydrate structure	Mouse monoclonal antibodies
A determinant	GalNAcα1 ↘ 3 / L-Fucα1 ↗ 2 Galβ1--4GlcNAc1--R (1--3 or)	"AH16" Type 1 or 2 chain
H determinant	L-Fucα1--2Galβ1--4GlcNAc1--R	"BE2" Type 2 chain-specific
N-acetyllactosamine	Galβ1--4GlcNAc--R	"1B2" Type 2 chain-specific

Gal = Galactose.
Gl = Glucose.
Fuc = Fucose.
Ac = Acetyl.
GlcNAc = N-acetyl-D-glucosamine.

Fig. 1. The chemical composition of the carbohydrate chains specific for the blood group substances A, type 2 chain H, and N-acetyllactosamine.

0 and 3 patients had blood type A. Biopsies from patients with these blood types were selected because previous studies have demonstrated the blood group substances type 2 chain H and N-acetyllactosamine in epithelium from individuals with blood type 0 and, in addition, the blood group A substance in persons with blood type A (20).

The patients were appraised of the study and informed consents were obtained consistent with the policies of The University of Michigan and the NIH.

Tissue processing

Sixteen specimens were fixed in a 4% neutral buffered formaldehyde solution, and 5 specimens were fixed in Bouin's fixative (23). Following fixation, the specimens, which included tooth substance, were placed in a buffered 0.5 mol/l EDTA solution (pH 7.1) for decalcification of the hard tissues. All specimens were embedded in paraffin and cut at 4–5 microns-thick sections. The sections were deparaffinized with xylene and hydrated in graded ethanol and phosphate buffered saline at pH 7.4 (PBS) before the staining procedure.

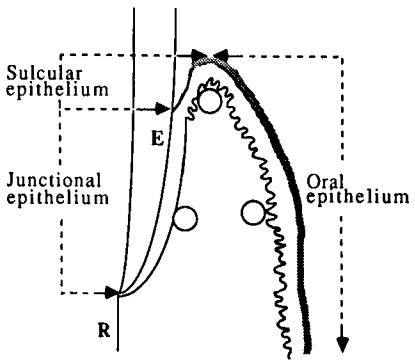

Fig. 2. Schematic drawing of a gingival tissue section illustrating the oral, sulcular, and junctional epithelium. The circles represent the areas in the subepithelial connective tissue where the inflammatory cell infiltrate was evaluated.

Similar procedures for tissue processing have previously been shown to enable clear detection of epithelial blood group substances (17, 18).

Antibodies and conjugates

The epithelial blood group substances were identified by an indirect immunofluorescent staining technique. Murine monoclonal antibodies reacting with carbohydrates specific for the complete blood group A substance (AH16) (24) and the immediate precursor substances, type 2 chain H (BE2) (25) or N-acetyllactosamine (1B2) (25) were applied to the sections as the primary antibodies. The chemical composition of the carbohydrate determinants for these blood group substances is shown in Fig. 1. (The murine monoclonal antibodies were obtained as a gift from Dr. S.I. Hakomori, Fred Hutchinson Cancer Research Center, Seattle, WA). The secondary antibodies were goat anti-mouse immunoglobulins (IgA + IgG + IgM, heavy and light chain-specific) conjugated with fluorescein isothiocyanate (FITC) for immunofluorescence visualization (Cappel Scientific Div., Malvern, PA).

Staining procedure

The tissue sections were incubated in a humidity chamber with the primary

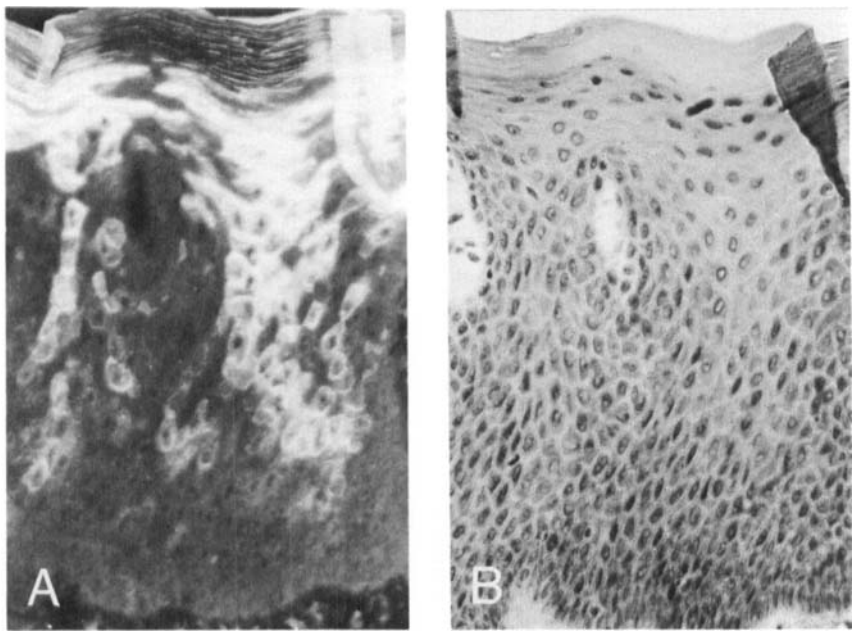

Fig. 3. Oral epithelium from a patient having blood type A (× 250). A: Immunofluorescence staining of the blood group A substance using murine monoclonal antibodies. B: Hematoxylin-eosin staining of the section shown in A.

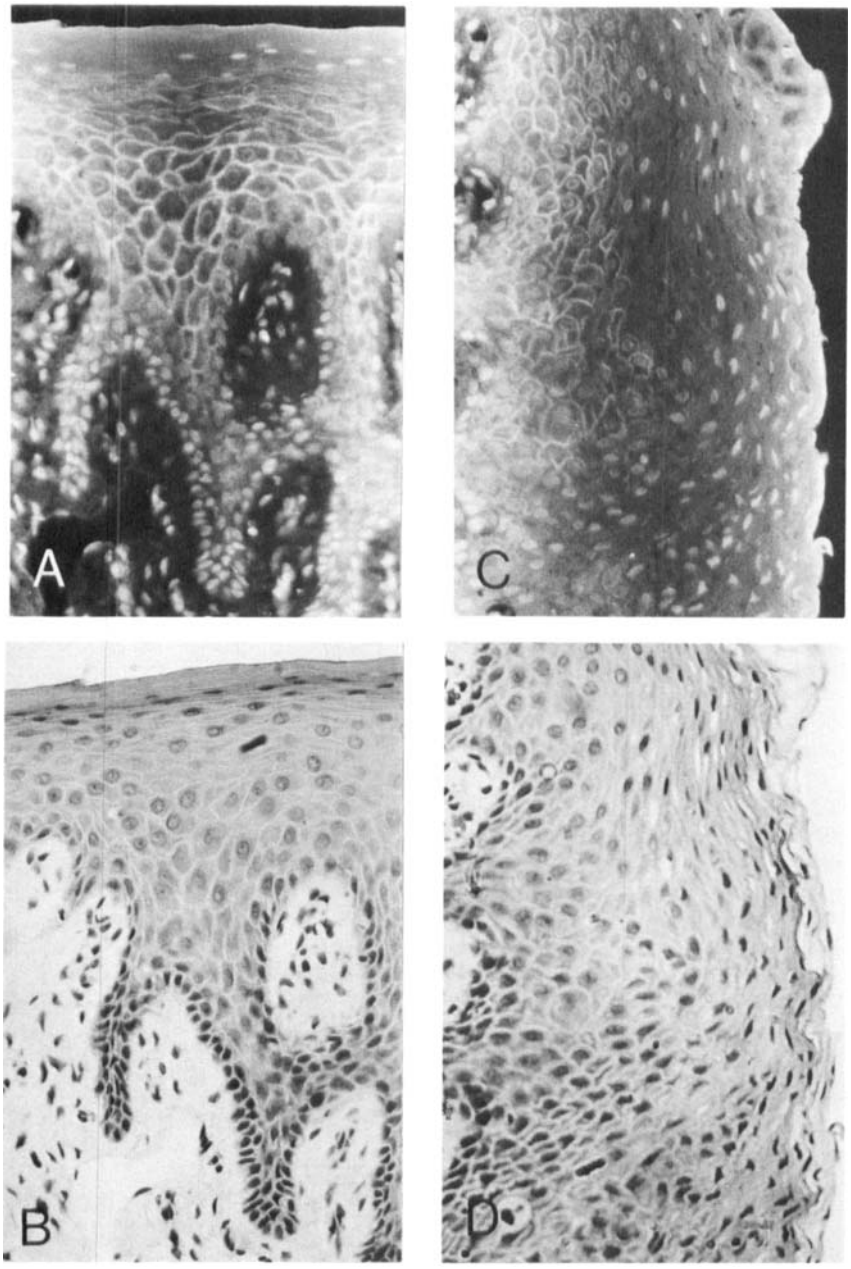

Fig. 4. Gingival epithelium from a patient having blood type O (×250). Immunofluorescent staining of the blood group substance type 2 chain H using murine monoclonal antibodies in (A) oral epithelium and (C) sulcular epithelium. B and C: Hematoxylin-eosin staining of the sections shown in A and C.

murine monoclonal antibodies at a dilution of 1:5 for 24 h at 4°C. After three rinses with PBS, each for 5 min, a 1:10 dilution of the secondary goat anti-mouse antibodies was added to the sections and they were incubated for 1 h at room temperature. Following repeated PBS rinses and slight air-drying, the sections were mounted in a glycerol/PBS-based mounting medium containing para-phenylenediamine to reduce fading (26).

Evaluation of staining reaction

Evaluation of the stained sections was performed with a Leitz epifluorescence microscope which was equipped with a filter system for FITC immuno-fluorescence (IF) and an HBO/2 200 W mercury superpressure lamp as light source. The staining reaction was evaluated qualitatively in the areas of oral, sulcular, and junctional epithelium (Fig. 2); the reaction of basal, parabasal and spinous cells was determined in each area. After examination and photography in the IF microscope, the sections were restained with hematoxylin-eosin (HE), permitting comparison of the IF staining reaction with the normal histology.

Degree of inflammation

The degree of inflammation was determined from the HE-stained sections. In each section, the total number of inflammatory cells was counted (field size = 1.3 mm² at 400×) in the connective tissue located immediately beneath a) the oral epithelium, b) the sulcular epithelium, corresponding to the region where keratinization disappeared, and c) the junctional epithelium at the midpoint between the most coronal collagen fiber attachment and the most apical part of the sulcular epithelium. These cell counts were related to the IF staining patterns (Fig. 2).

Control reactions

Control reactions included a) staining with FITC-conjugated goat anti-mouse antibodies alone, b) substitution of PBS in place of the murine monoclonal antibodies, and c) application of the hybridoma cell culture media in place of the cultured hybridoma supernatant fluid containing antibody.

Results

Blood group A substance

The *blood group A substance* was found on single cells or groups of cells in the upper spinous cell layers in the oral epithelium (Fig. 3). Only single scattered cells in the most superficial cell layers of the sulcular epithelium showed presence of the A substance. No substance A was detected in the junctional epithelium.

Type 2 chain H substance

In the ortho-keratinized oral epithelium, the *type 2 chain H substance* was demonstrated on cells in all spinous cell layers (Fig. 4A) and on parabasal cells over the connective tissue papillae. This substance was also present in the para- or non-keratinized sulcular epithelium but was, in this region, limited to the parabasal and deeper spinous cells (Fig. 4C). A clear borderline of the staining reaction for the type 2 chain H substance, corresponding to the morpho-

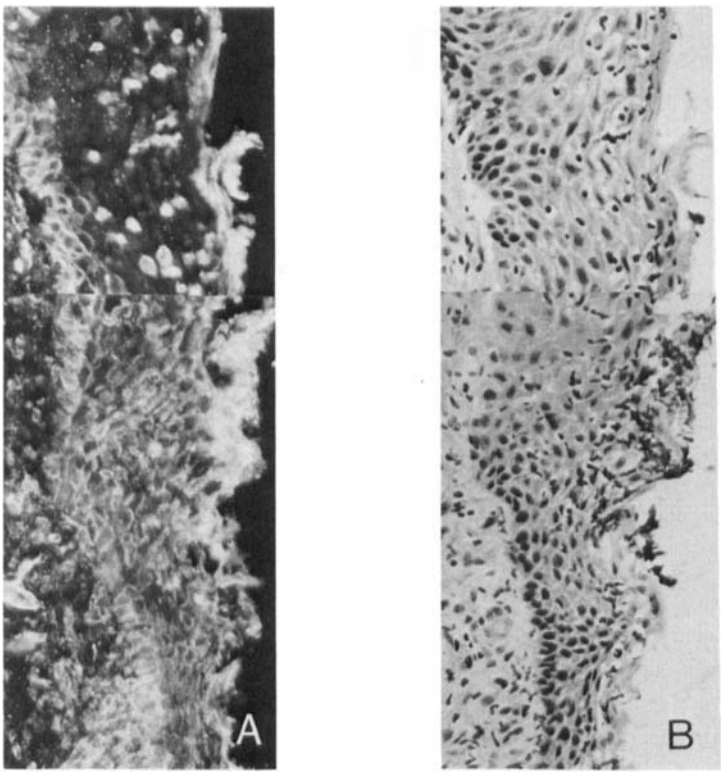

Fig. 5. Junctional epithelium from a patient having blood type O (×250). A: Immunofluorescence staining of blood group substance N-acetyllactosamine using murine monoclonal antibodies. B: Hematoxylin-eosin staining of the section shown in A.

logical border between the sulcular and junctional epithelial cells, could be observed. In the junctional epithelium, only single separate cells carried the type 2 chain H substance.

N-acetyllactosamine

N-acetyllactosamine was observed on cell in the whole width of the junctional epithelium, especially in the more apical areas (Fig. 5). Like the staining reaction for the antibodies to type 2 chain H substance, the demarcation of the staining reaction for N-acetyllactosamine corresponded to the morphological delineation between junctional and sulcular epithelium. The N-acetyllactosamine was also detected on the basal cells of the oral and sulcular epithelium, and on the parabasal cells of the oral epithelial ridges.

Degree of inflammation

The degree of inflammation in the specimens ranged from a total of 32 to 385 inflammatory cells per field (1.3 mm²). No relationship between the inflammatory infiltrate in the subepithelial connective tissue and the location of the blood group substances was observed in the examined sections.

Fixation

The staining reactions following formalin fixation were clear and distinct. Bouin's fixative caused a reaction of higher intensity but also with more background stain. The distribution of the blood group substances was similar with either of the two fixatives and the choice of fixative was judged to have no influence on the interpretation of the results.

Discussion

In the present study, it has been possible to identify three different blood group substances in the human dento-gingival epithelium, and to locate these to certain areas and epithelial layers. The method of identification was based upon an immunohistochemical reaction between cell membrane-associated carbohydrates and murine monoclonal antibodies specific for each of the carbohydrates (17, 20, 24, 25). Previously, Dabelsteen and coworkers (17, 20) have used similar methods on oral epithelium and have demonstrated that the incomplete carbohydrates are present on the least mature cells, and that the complete carbohydrates are found on more mature and more highly differentiated cells. In agreement with their findings, the results from this investigation showed that the most complete blood group substance, the A substance, was located in the upper layers of the oral epithelium and the precursor substances, type 2 chain H and N-acetyllactosamine, were present in the deeper cell layers. Thus, the location of the different carbohydrates can be interpreted as a reflection of the maturity and differentiation of the epithelial cells.

The distribution of the blood group substances in the different regions of the dento-gingival epithelium seemed to be related to the rate of cell proliferation. Thus, the least developed carbohydrate, N-acetyllactosamine, was widespread in the junctional epithelium which has a very high rate of cell division (5, 9, 27, 28). The rates of cell division in the regions where the type 2 chain H and the A antigens were detected (i.e. the sulcular and oral epithelium), are known to be lower than in the junctional epithelium (5, 9, 27, 28). In other words, the results clearly indicated a relationship between the level of cellular differentiation and the regional rates of cell division.

In studies of epithelium from other parts of the oral cavity, the location of blood group substances has been related to the type and degree of keratinization (18). This is in agreement with the findings in the present study. The type 2 chain H substance was found on cells at a higher cell layer in ortho-keratinized oral epithelium compared to the non- or para-keratinized sulcular epithelium (Fig. 4 A–D). Previous observations have demonstrated an association between the degree of keratinization and the rate of cellular division of the sulcular epithelium (6, 29). The present findings may, however, also indicate that the type of keratinization is associated not only with the rate of cellular division but also with the level of cellular differentiation as represented by blood group substances.

It has been reported that an inflammatory infiltrate in the subepithelial connective tissue influences the mitotic rate of the overlying epithelium (30).

It was, therefore, anticipated that less mature epithelial cells might be detected in areas with a high inflammatory cell count than in areas with a low degree of inflammation. However, comparisons of the distribution of blood group substances between different tissue sections in the present study did not reveal such alterations of cellular maturation due to subepithelial connective tissue inflammation. This may, in the oral epithelium, be the result of an overall low degree of inflammation. As even the least inflamed sections of the sulcular and, in particular, the junctional epithelium demonstrated blood group substances representative for cells with a low level of differentiation, a further decline may not have been detectable.

Detection and analysis of cell membrane-associated carbohydrates, i.e. blood group substances, has already for some time been used for investigations of cell behavior and differentiation in conditions of premalignancy and malignancy in the human oral epithelium (19, 20). The results from this study indicate that the method might be a valuable tool for understanding early healing and cell differentiation in the dento-gingival area.

Acknowledgements

The authors gratefully acknowledge Dr. Leif Helldén for critical review of the manuscript. This investigation was supported by USPHS grant DE-02731 from the National Institute of Dental Research, Bethesda, and the LeGro Fund from the School of Dentistry, Ann Arbor, Michigan.

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