Immunohistochemical Detection of Filaggrin in Preneoplastic and Neoplastic Lesions of the Human Oral Mucosa

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The distribution pattern of filaggrin in lesions of human oral mucosa was studied with the use of an anti-filaggrin serum raised in rabbits. A peroxidase–antiperoxidase method for the detection of filaggrin was applied to specimens from 9 cases of leukoplakia, 5 cases of verrucous carcinomas, 2 cases of carcinoma *in situ*, and 5 cases of invasive carcinoma. Areas of normal mucosa with different stages of keratinization were available in the same biopsy specimens. The granular layer of normal orthokeratinized epithelium was positive, whereas the horny layer was negative. Parakeratinized and nonkeratinized epithelia stained less than orthokeratinized epithelium. In leukoplakia and verrucous carcinoma, the reaction was irregular both in the granular and the cornified layers. Carcinoma *in situ* had a virtually negative reaction, and invasive carcinoma exhibited a slight positive reaction in the more differentiated areas. The immunohistochemical demonstration of altered filaggrin patterns in oral lesions correlates well with the degree of epithelial dysplasia and could be a helpful tool in grading white lesions and neoplasms of the oral mucosa. (Am J Pathol 1985, 119:456–461)

FILAGGRIN is a protein present in stratified squamous epithelia. It was first isolated and characterized in 1977 and named stratum corneum basic protein[1] or histidine-rich basic protein.[2,3] The existence of a phosphorylated precursor of filaggrin in keratohyalin granules and its interaction with keratin intermediate filaments have been demonstrated.[4–8] These data led to the formulation of the hypothesis that after secretion from the keratohyalin granules, filaggrin would form the supporting matrix of keratin filaments needed to complete the differentiation of cornified epithelial cells. Filaggrin has been localized in tissue sections with the use of immunohistochemical methods. It has been detected in the granular and horny layers of normal epidermis and oral mucous membranes, and the intensity of the reaction in rat epithelia is greater in the skin than in normal oral mucosa.[9] In the latter, the amount and localization of the reaction product seemed to be related to the anatomic site and thickness of the horny layer.

In skin tumors experimentally induced in mice, the immunohistochemical detection of filaggrin showed an irregular reaction in papillomas and no reaction in most carcinomas.[10] A similar reaction pattern was found in human skin tumors.[11] In this report, we have analyzed the patterns of distribution of filaggrin in preneoplastic and neoplastic lesions of the human oral mucosa and in areas of normal human mucosa with different types of differentiation.

Materials and Methods

Anti-filaggrin serum was raised in rabbits by injection of purified filaggrin from newborn mouse epidermis. The complete procedure of isolation and characterization of the antigen and the production and reactivity controls of the antiserum was done by the methods of Mamrack et al.[10] In brief, filaggrin was

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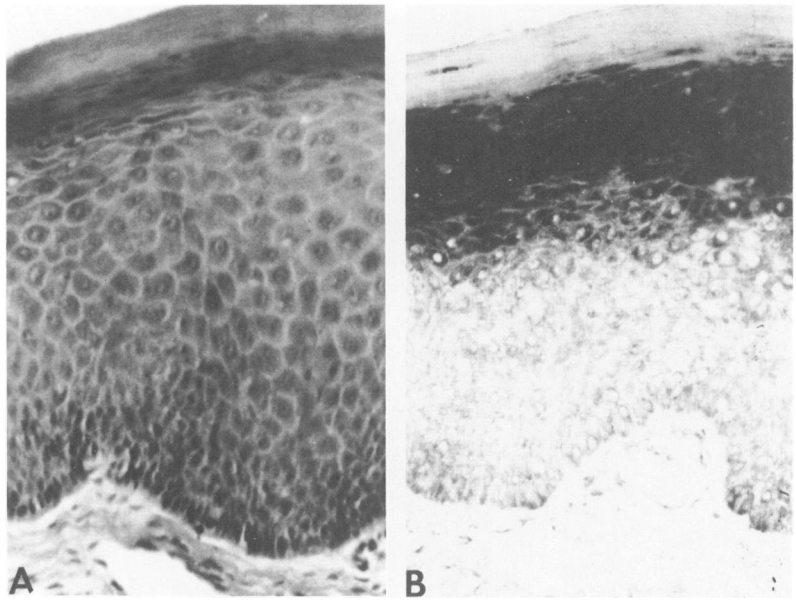

Figure 1—Normal orthokeratinized mucosa. **A**—H&E staining. **B**—Intense filaggrin reaction in granular area and absence of staining in horny layer. (×130)

purified by the method of Steinert et al.[12] An 8 M urea skin extract was treated with diethylaminoethyl (DEAE) cellulose, and the supernatant was subjected to gel electrophoresis. Filaggrin (0.5 mg/0.5 ml phosphate-buffered saline [PBS]) was mixed with 2.5 ml Freund's complete adjuvant and injected subcutaneously on the back of a New Zealand white rabbit. One week after the third immunization, the rabbit's serum was obtained and precipitated with ammonium sulfate. The 0–45% cut was then dialyzed against 10 mM K_2HPO_4, pH 8.0 and loaded onto a DEAE-cellulose column equilibrated in the same buffer. The excluded peak containing IgG was concentrated to the original protein concentration by ultrafiltration. The preimmune sera was similarly treated.

The peroxidase-antiperoxidase (PAP) technique[13] was performed on sections from 24 biopsies, which were fixed in neutral formalin and embedded in paraffin. Cross-sections of newborn rat heads were fixed in Zenker solution, processed as described by Dale and Ling,[5] and run as positive controls. The following lesions were studied: homogeneous leukoplakia (9 cases),

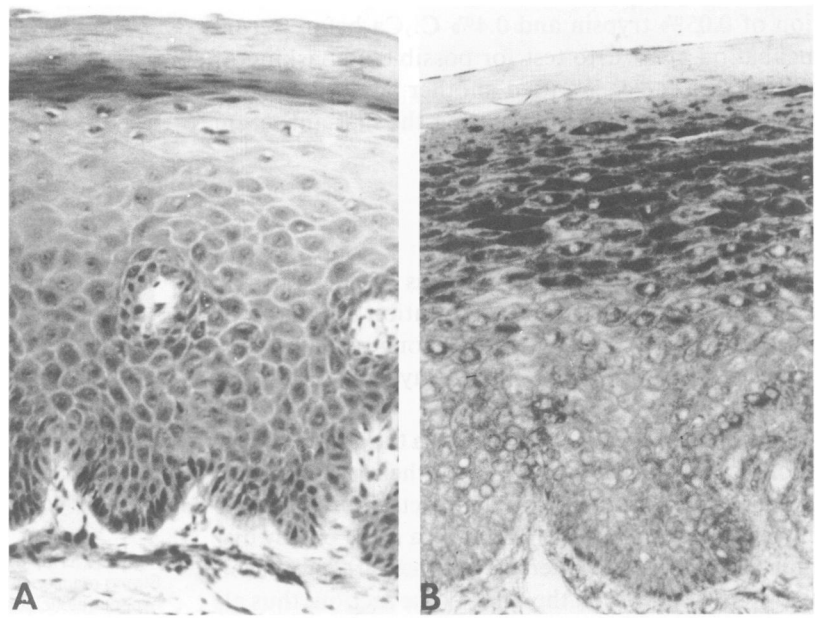

Figure 2—Normal parakeratinized area in the same specimen as in Figure 1. **A**—H&E staining. **B**—Patchy reaction in granular layer. (×130)

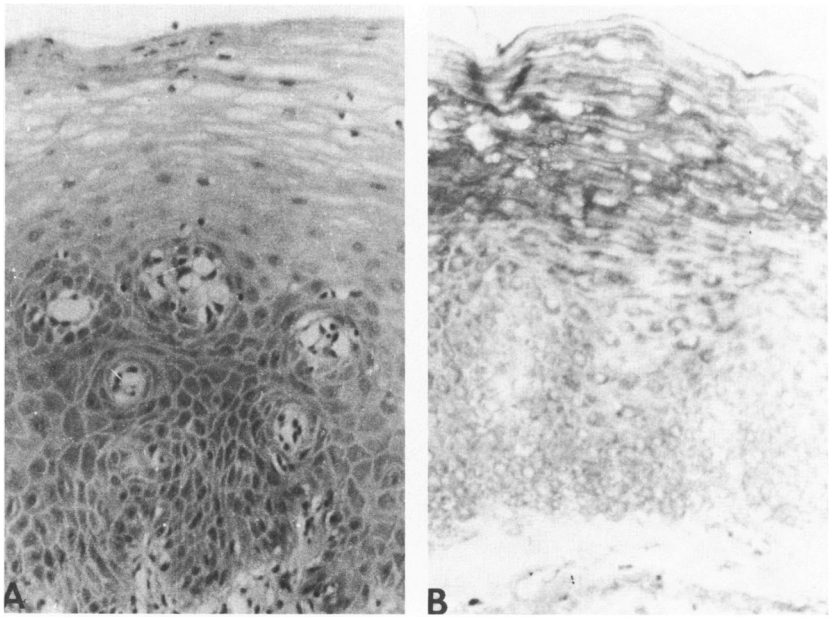

Figure 3—Normal nonkeratinized mucosa. **A**—H&E staining. **B**—Faint reaction in the surface layers. (×130)

carcinoma *in situ* (3 cases), verrucous carcinoma (5 cases), and invasive carcinoma (7 cases). Normal nonkeratinized, parakeratinized, and nonkeratinized epithelia (3 cases of each type) were also available from the biopsy blocks. We treated sections with 1.5% hydrogen peroxide to eliminate endogenous peroxidase and successively incubated them in the anti-filaggrin serum (1:20), goat anti-rabbit immunoglobulin serum from Dako-Chemetron Laboratory (Buenos Aires, Argentina) (1:500), and anti-goat rabbit PAP complex from Cappel Laboratories, (West Chester, Pa) (1:1000). Sites of peroxidase binding were revealed with the diaminobenzidine reaction. An adjacent section from each block was pretreated for 8 minutes with a solution of 0.05% trypsin and 0.4% Cl_2Ca before serum incubation at 37 C to test for possible unmasking of antigenic sites.[14,15] We used another adjacent section as a negative control by replacing the first antiserum with preimmune serum.

Results

The reaction in the tissue sections of newborn rats was similar in intensity and distribution to that originally reported by Dale et al.[5] A strong positivity was observed in cornified and granular layers of the epithelia of the tongue and palate.

A very intense reaction appeared in the granular layer of orthokeratinized human oral epithelium (Figure 1). In parakeratinized epithelia the reaction product had the same localization but exhibited a more patchy distribution (Figure 2). When ortho- and parakeratinized areas were available in the same tissue section, thus allowing a comparison of reaction intensity, the parakeratinized mucosa showed a weaker staining. Stratum corneum was negative in all cases of normal mucosa. Nonkeratinized mucosa showed a faint reaction in the spinous layer, increasing slightly in the superficial layers (Figure 3). Trypsin incubation prior to the immunochemical reaction did not modify the immunostain.

A discontinuous but strong reaction was observed in granular and horny layers of leukoplakic epithelia (Figure 4). This reaction was more intense in areas of greater hyperkeratosis. The superficial spinous cells exhibited a weaker and patchy staining in 3 of the 9 cases under study. All three cases of carcinoma *in situ* were

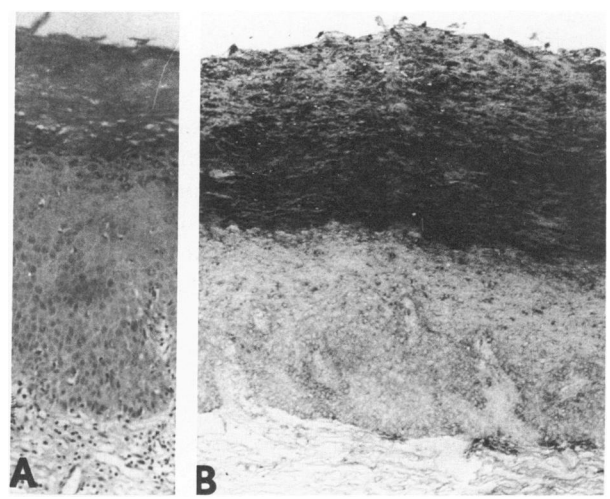

Figure 4—Homogeneous leukoplakia. **A**—H&E staining. **B**—Strong reaction in granular layers and patchy and intense reaction in the hyperkeratotic stratum corneum. (×45)

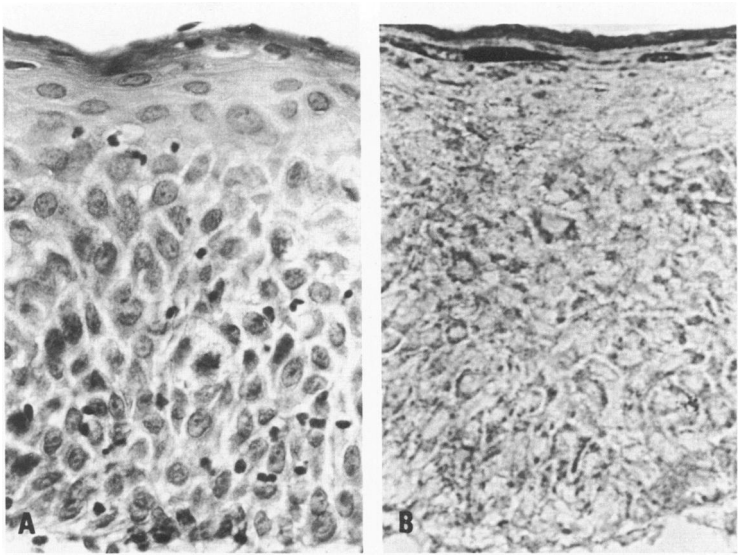

Figure 5—Carcinoma *in situ*. **A**—H&E staining. **B**—A faint reaction was observed in isolated spinous cells and superficial areas. (×80)

virtually negative, with the exception of a faint reaction in isolated superficial areas (Figure 5). Verrucous carcinomas showed similar but more pronounced pattern changes than those observed in leukoplakia, except for the basal layer. Patchy staining was seen in all other layers of the verrucous areas (Figure 6). On the other hand, a very weak or negative reaction was observed in invasive carcinoma (Figure 7). The only sites of positive reaction were found in some horny pearls of well-differentiated areas.

Discussion

This study demonstrated that, as previously shown in the rat oral mucosa,[9] filaggrin is a normal component of surface layers of human oral epithelia. A reduction or absence in the amount of filaggrin in the differentiated layers of lesions of the oral mucosa could also be detected. These changes correlated well with the degree of atypia of white lesions or with the aggressive behavior of tumors. This reached a maximum in the cases of invasive squamous carcinomas that were characterized by a total absence of filaggrin in the moderately and poorly differentiated tumor areas. These observations coincide with similar filaggrin distributions found in experimental murine[10] and human skin neoplasms.[11] Although the more immature cells of both normal preneoplastic and neoplastic lesions were always filaggrin-negative, the horny and granular layers of the different epithelia did not exhibit a uniform filaggrin immunostain pattern. In our series, the stratum corneum of normal orthokeratinized epithelia was always negative. In leukoplakias and verrucous carcinoma, the hyperkeratotic horny layer showed an intense and patchy reaction to filaggrin antiserum. The granular layer of these lesions had a less uniform but more intense reaction than the normal epithelia. This pattern of reaction, which is similar to that found in experimental papillomas[10] and human keratoacantomas,[11] is in accordance with the finding of kerotohyalin granules of irregular shape and distribution in human leukoplakia.[16,17] The homogeneous reaction of the granular layer and the positivity of the horny layer would indicate that the differentiation process in hyperkerotic lesions produces a less mature horny substance chemically or structurally different from the normal horny cell material.

A heterogeneous filaggrin distribution was also found

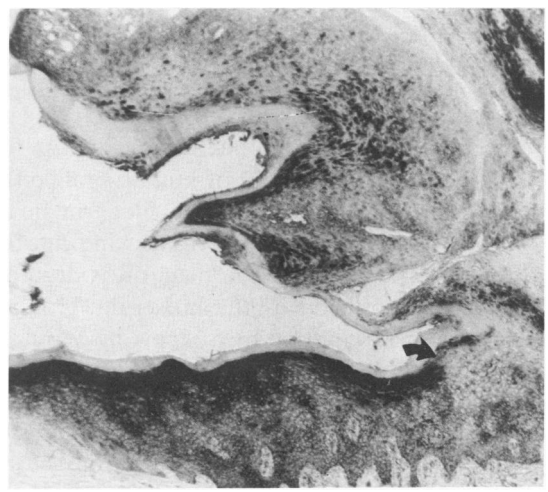

Figure 6—Verrucous carcinoma. Homogeneous pattern of reaction in normal mucosa which changes to an irregular patchy staining near the verrucous area (*arrow*). (×15)

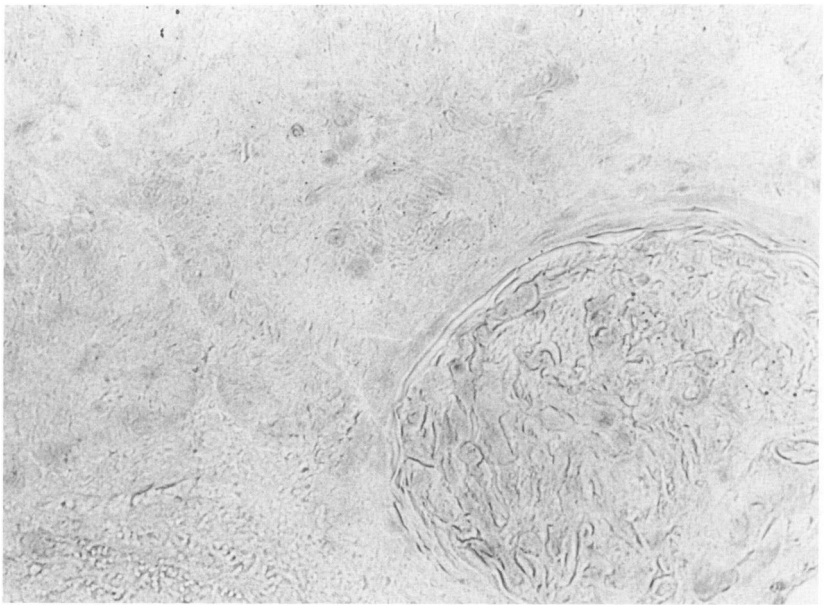

Figure 7—Invasive carcinoma. Note the negative staining of the invading carcinoma cells near a nerve cross-section (*lower left corner*). (× 130)

by Dale et al in the rodent oral mucosa.[9] These authors found a positive filaggrin reaction in the stratum corneum of the oral epithelium of newborn rats but failed to detect this protein in the hard palate epithelium of adult animals. Filaggrin was first isolated from the stratum corneum of rodent epidermis[1] and was believed to be a keratin matrix protein.[8] The failure to detect it in the surface layers of all stratified squamous epithelia has prompted several hypotheses. Dale et al[8] have postulated that either the protein is lost at the time of terminal maturation or is chemically masked, or the compact nature of the stratum corneum prevents the access of antibodies during the immunohistochemical procedure. The pretreatment with trypsin unmasked keratin filaments in the dense stratum corneum of human oral tissues.[15] Pretreatment used in the present study did not improve filaggrin detection in the negative areas. However, pretreatment with trypsin, which has been very efficient in unmasking and producing immunoreaction in previously undetectable keratin filaments in the stratum corneum of human oral epithelia,[15] was not effective in detecting filaggrin in the negative areas of the horny layer. This may indicate that in normal human oral epithelia filaggrin is degraded and cannot be identified with the antiserum. This possibility is further supported by a report by Scott and Harding[18] which shows that a complete proteolysis of filaggrin takes place under normal circumstances in the epithelium. This complete degradation could be related to the process of complete orthokeratinization, the lack of which would permit the detection of filaggrin in the surface layer of the incompletely keratinized or nonkeratinized epithelia, such as those of the normal buccal mucosa and of some white lesions of the oral mucosa.

In conclusion, the presence of filaggrin in the stratum corneum and also in the stratum granulosum is probably related to the degree of keratinization or maturation of the epithelial cells. Because preneoplastic and neoplastic lesions of the oral epithelia usually exhibit simultaneous alterations of keratinization and cell maturity,[19,20] the decrease or absence of filaggrin in these lesions suggests that the distribution pattern of this protein could be used as a marker of differentiation, of atypia in white lesions, and of tumors of the oral epithelia.

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