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Distribution profiles of keratin proteins during rat amelogenesis

M. Nakai, Y. Tatemoto, H. Mori, and M. Mori*
Department of Oral Surgery, Asahi University School of Dentistry, Hozumi-cho, Motosu-gun, Gifu, 501-02 Japan

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Summary. We examined rat cells undergoing amelogenesis for the presence of three types of keratin proteins using a polyclonal antibody to keratin (against total keratins (TK) with molecular masses ranging from 41 to 65 kilodaltons (kd) and monoclonal antibodies keratins to KL1 and PKK1 (reactive with keratins with molecular masses of 55–57 and 41–56 kd, respectively). In normal oral epithelia from young rats, the TK, KL1, and PKK1 antibodies bound to all of the epithelial strata. The epithelial cap on the top of incisors and the dental lamina of molar teeth exhibited strong TK staining, moderate staining KL1, and little or no PKK1 staining. In developing molar enamel organs, both the outer and inner enamel epithelia, the stratum intermedium, and stellate reticulum cells were all positively stained by the TK immunoreagent. In developing incisors, TK only bound strongly to stratum-intermedium cells, and no KL1 and PKK1 staining antibodies was observed in ameloblasts or the stratum intermedium.

Introduction

The occurrence of histodifferentiation during the development of tooth germs in the incisor and molar regions of rodents amply demonstrated has been (Moe 1971; Ten Cate 1962, 1980). Furthermore, recent studies have provided data concerning the lectin-binding affinities as well as the distribution of polyclonal antibodies to keratin in the developing teeth of rats and humans (Nakai et al. 1985). Tooth germs contain two different cellular elements that are of ectodermal and mesenchymal origin; the former arises from embryonic oral epithelium and therefore probably contains keratin proteins. In the present study, we examined the distribution of an immunohistochemically detectable keratin that acts as a marker of epithelial or epithelium-derived cells in rat cells undergoing amelogenesis. The distribution of keratins of different molecular weights was determined using keratin polyclonal antibodies (for 41- to 65-.kilodalton (kd) keratins) (Schlegel et al. 1980; Thesleff et al. 1984) and monoclonal antibodies, KL1 for 55- to 57-kd keratins; Viac et al. 1983) and PKK1 (for 41- to 56-kd keratins; Holthöfer et al. 1983, 1984; Kariniemi et al. 1984). The patterns obtained using the three keratin immunoreagents were then compared.

* To whom offprint requests should be sent

Materials and methods

Tooth germs of 0-, 1-, 2-, 3-, and 4-week-old Wistar rats, were studied. The lower and upper jaws were each divided into left and right parts, and were then fixed in 10% formalin for 12 h at 20° C. The materials were decalcified using an ethylenediaminetetraacetate (EDTA) solution consisting of 0.5 *M* EDTA, 4Na salt, and 5 *M* citric acid, adjusted to neutral pH (Mori et al. 1965; Takada et al. 1968). Most of the materials had became completely decalcified after 2–3 weeks, as assessed by X-ray examination. The decalcified jaw bones were washed thoroughly, dehydrated, and mounted in paraffin. Serial paraffin sections at (4 μm) were cut in order to determine the staining patterns of the three keratin antibodies; sections were also stained in order to establish the histological orientation.

Immunohistochemistry

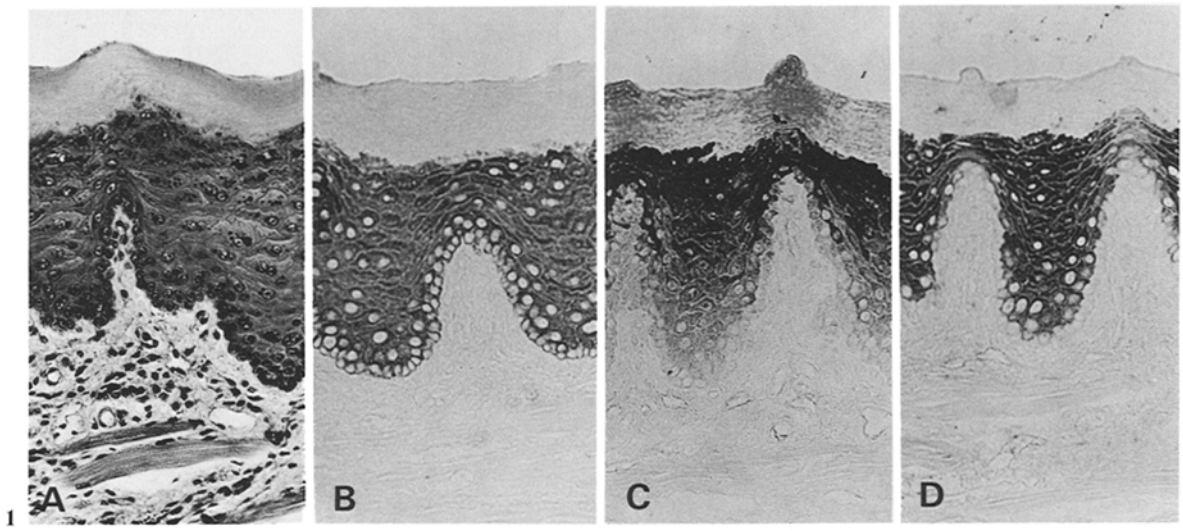
Polyclonal antibody to keratin. Anti-total keratin (TK) is the immunoglobulin fraction of rabbit antisera to the stratum corneum of the sole of the human foot, and it reacts with keratins from with molecular masses of between 41 and 65 kd.

Deparaffinized sections were treated with a methanol solution containing 0.3% H_2O_2 for 20 min in order to inactivate endogenous peroxidase. After thorough rinsing, the sections were treated with normal swine serum (1:20; Dakopatts, Copenhagen, Denmark) for 30 min. After being blotted dry with filter paper, the section were reacted with anti-TK (1:50; Dakopatts) for 1 h and rinsed three times in phosphate-buffered saline (PBS). The sections were then treated with antirabbit IgG (1:20; Dakopatts) for 30 min, rinsed three times in PBS, reacted with the peroxidaserabbit-antiperoxidase (PAP) complex (1:100, Dakopatts) for 30 min, and then rinsed well. Finally they were immersed for 5 min in a solution consisting of 0.05 *M Tris* buffer (pH 7.6) containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H_2O_2.

KL1 monoclonal antibody to keratin. KL1 was generated in a mouse in response to an immunogen isolated from human keratinized squamous epithelium; this antibody has an affinity for keratins with molecular mass ranging from 55 to 57 kd (Viac et al. 1983).

After the inactivation of endogenous peroxidase, the sections were treated with normal rabbit serum (1:20; Wheaton, USA) for 30 min and blotted dry with filter paper. They were then reacted with KL1 (1:100; Immunotech, France) for 1 h, rinsed three times in PBS, and treated with horseradish-peroxidase(HRP)-labeled rabbit antimouse immunoglobulins (1:20; Dakopatts) for 30 min. After thorough rinsing, the sections were finally immersed for 5 min in the already described DAB staining solution.

PKK1 monoclonal antibody to keratin. PKK1 was produced in a mouse following immunization with cytoskeletal proteins isolated from a pig-kidney epithelial cell line; this antibody reacts with keratins with molecular masses of 41–56 kd (Holthöfer et al. 1983).



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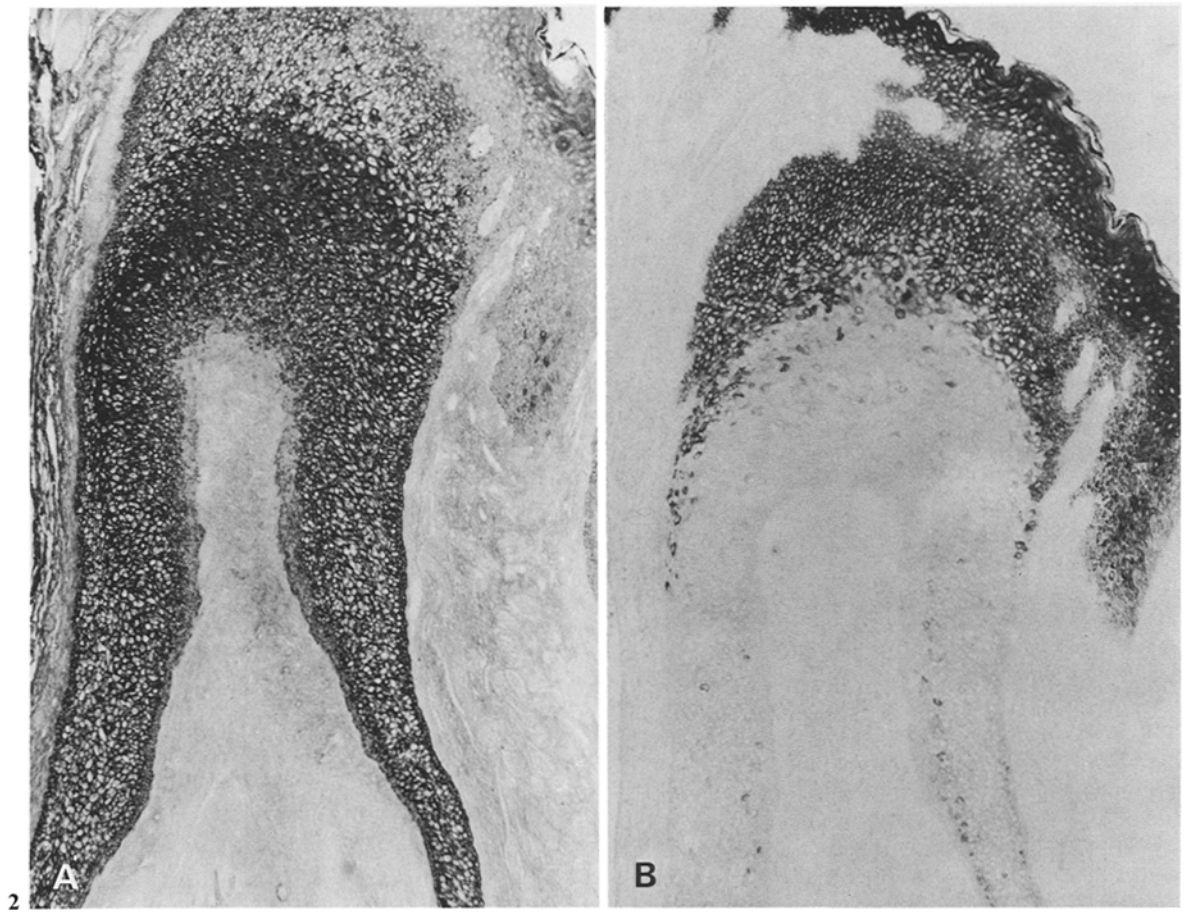

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Fig. 1 A–D. Oral mucosa in young rats. **A** H&E staining. **B** Polyclonal antibody to keratin (total keratin; molecular masses 41–65 kd); Total-keratin staining is visible in all epithelial strata. **C** Monoclonal antibody to keratin (KL1; 55–57 kd). KL1 keratin is distributed in spinous and upper-layer cells of the stratified squamous epithelia, but is absent in basal cells. **D** Monoclonal antibody to keratin (PKK1; 41–56 kd); PKK1 keratin a trace reaction is visible in basal-layer cells. ×200

Fig. 2 A, B. Epithelial caps at the top of developing incisors 5-day-old rats. **A** Total keratin is widely distributed in epithelial caps. Outer-layer cells exhibit comparatively slight TK staining, intermediate-layer cells exhibit the strongest staining, and inner-layer cells show rather weak staining. **B** KL1 keratin is present in outer-layer cells where the staining is moderate to strong, intermediate-layer cells exhibit little or no staining. ×40

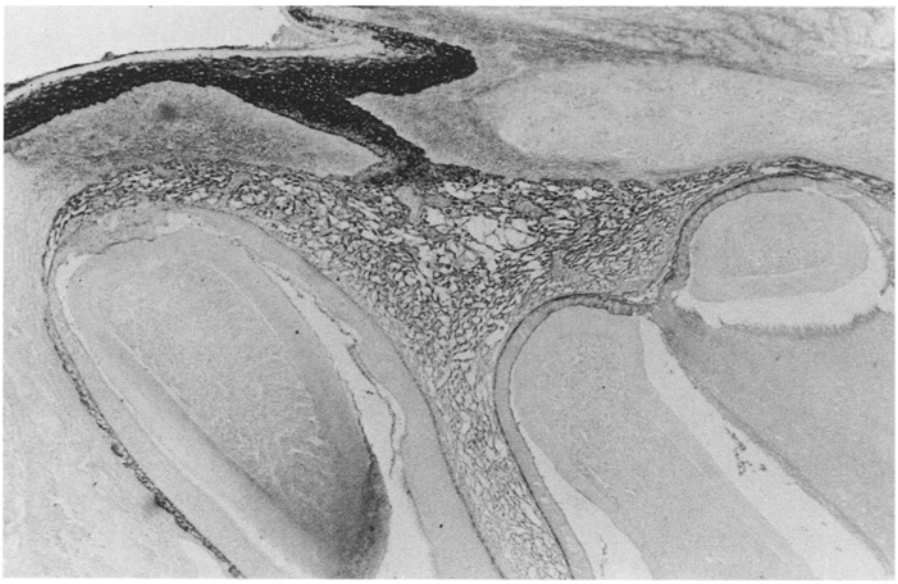

Fig. 3. Dental lamina and developing molar teeth of 5-day-old rats. Strong TK staining is confined to the oral epithelium and dental lamina of developing molars. The enamel organs exhibit positive staining for TK. ×40

Deparaffinized sections were treated for 30 min at 37° C with 100 ml PBS containing 0.1 g trypsin and 0.1 g $CaCl_2$. After the slides had been rinsed well, endogeneous peroxidase was inactivated as already described, and the sections were rinsed again thoroughly. After the sections had been treated with normal rabbit serum (1:20; Wheaton) for 30 min, they were blotted dry with filter paper and reacted with PKK1 (1:50; Labsystem, Finland) for 1 h. After being rinsed three times in PBS, the sections were reacted with HRP-labeled rabbit antimouse immunoglobulins (1:20; Dakopatts) for 30 min, rinsed well, and immersed in DAB solution to visualize the antibody binding.

Control

Normal rabbit serum and normal mouse serum were used in place of the polyclonal antibody to TK and the monoclonal antibodies to keratin (KL1 and PKK1), respectively. No reaction was seen in these sections.

Effects of decalcification on keratin staining

Neutral EDTA decalcifying solution has previously been used to detect hydrolytic and oxidative enzymes in mineralized tissue. In the present study, we used this decalcifying solution to detect keratin protein. The staining for keratin in decalcified specimens was compared to that in nondecalcified specimens. The staining intensity and distribution of both polyclonal and monoclonal (KL1 and PKK1) antibodies to keratin in the rat oral epithelia was similar in decalcified and nondecalcified paraffin sections.

Results

The oral epithelium of neonatal and young rats is composed of stratified squamous epithelial cells. The TK reagent reacted with all of the cell layers except for the superficial layers. The monoclonal antibodies KL1 and PKK1 produced a similar staining distribution, although the staining intensity for the PKK1 keratin reagent was weak (Fig. 1).

Epithelial cap of developing incisors

During postnatal development, most of the tip of the incisor is covered with an epithelial cap which is continuously connected to the oral epithelia. From its keratin staining patterns, this covering cap appeared to be divided into three

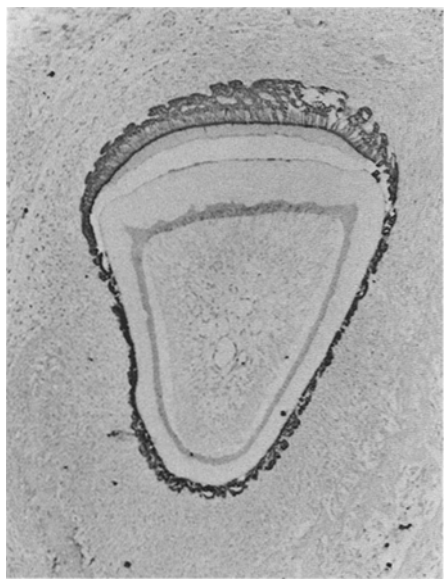

Fig. 4. Cross-section of developing incisor teeth of 3-day-old rats. Positive total-keratin staining is visible in enamel organs surrounded by the developing incisor. ×40

layers: (*1*) an outer zone adjacent to the basal cells of the oral epithelium, (*2*) an intermediate zone, and (*3*) an inner zone in contact with the outer layer of the enamel organ. The TK staining was slight to moderate in the outer layer, fairly strong in the intermediate zone, and somewhat less strong in the inner zone. On the contrary, the KL1 reaction was moderate to strong in the outer layer, in the inner layer, and absent in the intermediate zone. The monoclonal antibody to keratin, PKK1, produced slightly positive staining only in the intermediate zone (Fig. 2).

Enamel organs in the molar region

The dental lamina of developing molar germs is connected to the basal-layer cells of the oral epithelium. The staining for TK was strong in both the oral epithelium and dental lamina. In molar tooth germs, the TK staining was slight

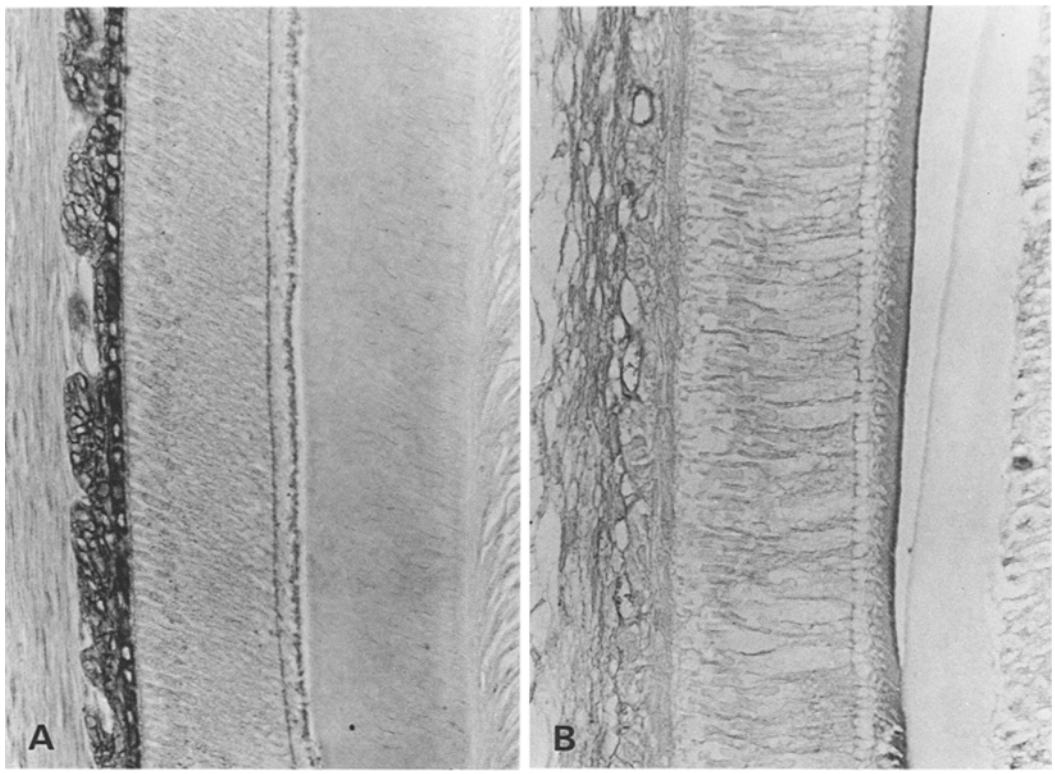

Fig. 5A, B. Higher magnification of developing incisors. **A** Total-keratin staining is confined to the stratum intermedium. All ameloblasts exhibit no reaction for keratin (5 days). **B** PKK1 staining is absent throught the whole enamel organ including ameloblasts and the stratum intermedium (10 days). × 200

to moderate in stellate reticulum cells, but was only slight in the external and internal enamel epithelia. Both monoclonal antibodies, however, failed to stain these cells (Figs. 3 and 5B).

Amelogenesis in developing incisors

The stages of differentiation that occur during amelogenesis in rat incisors may be roughly divided into four phases: young ameloblast, matrix-forming ameloblast, ameloblast with calcification, and reduced ameloblast. Young ameloblasts are located in the cervical base of the incisor and have a cuboidal or low columnar shape. Matrix- forming ameloblasts are the most active with regard to enamel protein synthesis, and these cells have a high columnar shape with nuclei situated on the basal side. At this stage, the adjacent stratum intermedium is well developed. During the calcification stage, the columnar ameloblasts became longer than those seen during the matrix-forming stage. Reduced ameloblasts are associated with terminal enamel calcification and are located at the end of the incisor. In enamel organs, the presence of keratin was only demonstrated by the TK immunoreagent, its localization being limited to stratum intermedium cells. The staining of the stratum intermedium cells adjacent to young ameloblasts

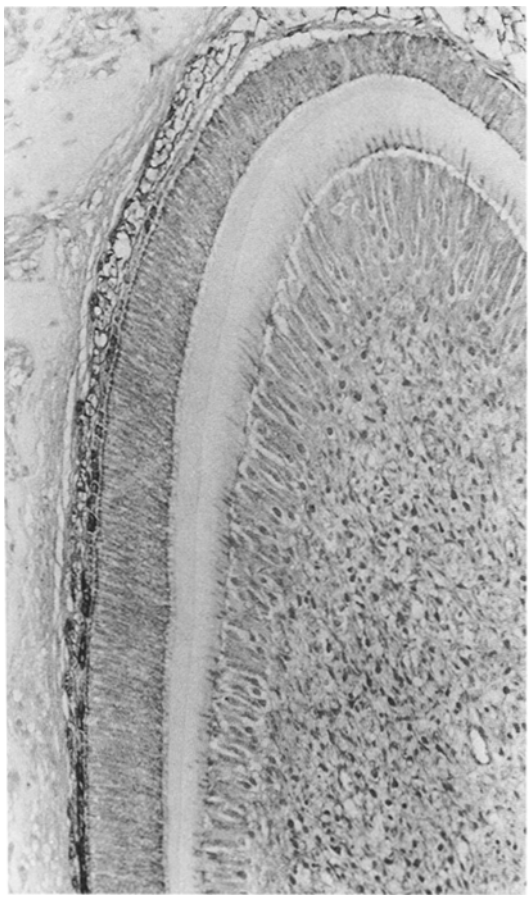

Fig. 6. Distribution of total keratin in a developing molar. In the area at the top of the molar, strong TK staining is confined to the stratum intermedium, while slight staining is visible in the stellate reticulum (5 days). × 100

Table 1. Keratin-antibody staining cells of young rats

	Total	KL1	PKK1
Oral epithelium			
Basal	+1 ~ +2	± ~ +1	0 ~ ±
Middle	+2	+2	+2
Upper	+2	+3	+2
Incisor-covered epithelium			
Outer layer adjacent to basal cells of the oral epithelium	+1–2	+2–3	0, ±
Intermediate layer	+3–4	+1–0	+1–2
Inner side	+2–3	0	0
Molar-covered epithelium			
Outer layer adjacent to basal cells of the oral epithelium	+3–4	0	0
Stellate reticulum	+1–3	0	0
External luminal layer	+1	0	0
Internal luminal layer	+1	0	0
Stratum-intermedium cells in different ameloblast stages			
1. Young	+1–2	0	0
2. Matrix formation	+3–4	0	0
3. Calcified	+2–3	0	0
4. Reduced	+1, ±	0	0

0, negative; ±, trace; +1, slight; +2, moderate; +3, strong

was weak, but a stronger reaction was seen in high-columnar ameloblasts exhibiting enamel protein secretion (Figs. 4, 5A and 6) as well as in ameloblasts at the calcified stage exhibiting reduction. All ameloblasts, irrespective of their stage of differentiation, were completely unstained by the three types of antibodies to keratin (Table 1).

Discussion

The present immunohistochemical study represents the first attempt to identify keratin in developing teeth using of monoclonal antibodies, although the distribution of TK during amelogenesis has previously been described (Lesot et al. 1982; Thesleff and Ekblom 1984). Recently, the distribution of keratins in stratified squamous epithelia of the skin and mucosa has been shown to vary according to molecular weight, i.e., low-molecular-weight keratins (as detected using to PKK1) are usually located in basal cells, whereas higher-molecular-weight keratins (revealed by KL1) are distributed in upper strata and are absent in basal cells (Sun et al. 1983, 1984; Franke et al. 1979; Schlegel et al. 1980; Banks-Schlegel et al. 1981; Löning et al. 1982; Woodcock-Mitchell et al. 1982). This differential distribution of keratins in the epithelial strata is one of characteristic features of stratified squamous epithelia.

During the stratification progress of normal human squamous epithelia, the keratin profiles from the basal cells to the upper cells showed different distribution patterns. (Fuchs et al. 1980; Banks-Schlegel et al. 1981, 1982; Woodcock-Mitchell et al. 1982; Said et al. 1983; Löning et al. 1982). However, the rat oral epithelium dose not exhibit this zonal distribution pattern of immunohistochemically detectable keratin proteins. In the present study, the TK reagent reacted with cells of the enamel organ, while the monoclonal antibodies to keratin did not react with these cells.

In human ameloblasts, the TK reaction is particularly evident in Tomes' fibers of functioning and reduced ameloblasts as well as in stratum-intermedium cells. In developing rat teeth, TK was found to be confined to the stratum intermedium and was absent from ameloblasts at all stages. These differences with regard to the localization of keratin proteins in rats and humans may reflect the absence of cross-reaction between enamel proteins and TK antibodies in rat ameloblasts.

The staining intensity of the TK antibody was strong in the dental lamina of molar teeth as well as in oral epithelial cells; however, the staining was drastically reduced in intensity in enamel organs, particularly in the outer layer of enamel epithelium adjacent to the dental lamina. This suggests that keratin expression in epithelial cells that undergo differentiation during amelogenesis becomes much lower than that seen in homologous oral epithelial cells. This reduction in the intensity of the TK staining in differentiated enamel organs was observed throughout amelogenesis.

The histochemical properties of both the epithelial cap at the top of developing incisors and the dental lamina of molar teeth have not previously been described. In the present study, we found quite strong staining by the TK polyclonal antibody, while of the monoclonal antibodies to keratin KL1 decorated only the outer and intermediate zones, whereas PKK1 produced only slight staining in the intermediate zone. We suggest that the differences in the distribution profiles of these keratin antibodies indicate dissimilarities between the oral epithelium and the epithelial cap of incisors.

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