Developmental Changes in the Fine Structure and Histochemical Properties of Mucous Cells in the Parotid Gland of the Infant Japanese Macaque

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Summary. Mucous cells have been known to occur in the terminal portions of the parotid gland in a few species of mammals during a limited period of their development. The aim of this study was to examine the occurrence and features of mucous cells in the parotid gland of the infant Japanese macaque.

Light microscopy revealed that mucous cells in the macaque parotid gland were present in the terminal clusters and acini at postnatal day 15, were less prevalent at day 30, and continued to decrease in number over 3 months. Mucous cells were no longer recognized in the parotid gland in 6-month-old macaques.

Electron microscopy showed that the mucous cells contained electron-lucent secretory granules and bipartite or tripartite secretory granules. By 3 months of age, there was a scarcity of mucous cells and a concomitant increase in transitional cells. These transitional cells were intermediate in structure between mucous and serous cells, and contained three types of granules: electron-lucent, bipartite or tripartite, and electron-dense. None of the cells showed apoptotic figures.

Lectin histochemistry indicated that the mucous cells in the early postnatal period had sugar residues identical in nature to those seen in the granules from mature serous cells in the glands of 3-month-old macaques.

Immunohistochemistry using an antibody against human α -amylase showed a weakly positive reactivity in the secretory granules of the mucous cells, starting from day 15. In the transitional cells, the electron-dense granules showed a stronger immunoreactivity than either the electron-lucent granules or the heterogeneously structured granules. These results suggest that the secretory granules of mucous cells have characteristics in common with those of serous cells, and that during the transitional period the mucous granules change from the initial electron-lucent to heterogeneous forms, finally becoming the electron-dense granules. The mucous cells in the parotid gland of the juvenile Japanese macaque are therefore suggested to be converted into serous cells.

In the majority of mammals, the parotid gland is classified as a serous gland, since the acini consist of serous cells (GROMET-ELHANAN and WINNICK, 1963; SCHRAMM, 1964; BALL, 1974; BENDAYAN et al., 1986; AVERY, 1994; FAWCETT, 1994). However, there have been reports that mucous cells are present in the terminal clusters and acini during both the prenatal and the early postnatal periods (ZIMMERMANN, 1927; AKIYOSHI, 1929; FAHRENHOLZ, 1937; EMI, 1939; DU PLESSIS, 1957; MUNGER, 1964; LAWSON, 1970; KOMORI et al., 1979; TAGA and SESSO, 1979; BALL et al., 1988a, b; SIVAKUMAR et al., 1998). Our previous study has demonstrated that the mucous cells in rat parotid glands appear on the first postnatal day (IKEDA and AIYAMA, 1997, 1999), and then increase to a maximal level by day 5. They start to decrease around day 8 and disappear by day 10. We also suggested that the mucous cells in problem may change into serous cells, since cells with morphological characteristics intermediate between both cells were recognized. Neither the mucous nor the transitional cells showed any morphological signs of cell death.

It thus remains to be confirmed whether the mucous cells in the parotid gland in the perinatal period might occur only in certain species. In addition, it is as yet unclear whether mucous cells from the same gland in different species share common morphological and transitional characteristics. As human juvenile glands are hardly available, it is important to investigate primates.

The objective of this study was to determine whether mucous cells are present in the parotid gland of infant Japanese macaques, and, if so, to examine their fine structure and the histochemical properties of their secretory granules.

MATERIALS AND METHODS

Animal and tissue preparation

All animal experiments followed the National Institutes of Health (NIH) Guidelines for Care and Use of Laboratory Animals. Male and female Japanese macaques — aged 15 days, 30 days, 3 months, 6 months, 1.5 years, 3 years, and 4 years — were purchased from Nippon Bio-Supp.Center. Each animal was anesthetized using an intravenous injection of pentobarbital sodium, and the parotid gland was removed. The gland was then cut into small pieces and fixed for 24 h using a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.05 M cacodylate buffer (pH 7.4). Some pieces were then rinsed in a 0.05 M cacodylate buffer and post-fixed in cacodylate-buffered 1% osmium tetroxide for 1 h. All fixed samples were dehydrated in ethanol, and embedded in Epon-Araldite.

Light and electron microscopy

Samples that had been fixed in 2.5% glutaraldehyde and 2% paraformaldehyde were cut into 2 μ m thick sections. These were stained with periodic acid-Schiff (PAS) and alcian blue, pH 3.5, for examination using light microscopy.

Ultrathin sections post-fixed in osmium tetroxide were prepared for electron microscopy by staining with uranyl acetate and lead citrate. These sections were examined using a JEOL 2000EX-II transmission electron microscope.

Silver methenamine technique

Samples that had been fixed in 2.5% glutaraldehyde and 2% paraformaldehyde were used. Ultrathin sections were oxidized by immersion in 1% periodic acid solution for 30 min. After three short rinses using distilled water, the sections were transferred to the staining solution (prepared according to RAMBOURG, 1967; KUROSUMI and INOUE, 1981), and soaked for 30 min at 60°C. Subsequently, the sections were washed with distilled water, double-stained with uranyl acetate and lead citrate, and examined using transmission electron microscopy.

Lectin histochemistry

Samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde and examined histochemically using a variety of lectins: concanavalin A (Con A), *Ulex europaeus* agglutinin-I (UEA-I), peanut agglutinin (PNA), soybean agglutinin (SBA), *Helix pomatia* agglutinin (HPA), *Dolichos biflorus* agglutinin (DBA), wheatgerm agglutinin (WGA) and *Limax flavus* ag-

glutinin (LFA) (E. Y. Laboratories Inc., San Mateo, CA).

Ultrathin sections were double-labeled using the two-side method. First, one side of the grid was dipped in a 3% H₂O₂/methanol mixture for 10 min, rinsed with distilled water, and treated with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) for 30 min to prevent nonspecific binding. The grid was then soaked overnight at room temperature in 20 μ g/ml gold-labeled lectin (E-Y Lab.), and then washed with phosphate-buffered saline (PBS). The labeling sequence was repeated on the other side using a different lectin. Finally, the grid was washed with distilled water and double-stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

Immunohistochemical staining

Samples that had been post-fixed in osmium tetroxide were used. Ultrathin sections were prepared for electron microscope examination by being immersed in a 3% H₂O₂/methanol mixture for 10 min, rinsed with distilled water, and treated with 12.5% sodium metaperiodate for 15 min to bleach osmicated tissue sections. The sections were then rinsed with distilled water and soaked in 5% normal goat serum (IBL Research Products Corp., USA) for 30 min. After being rinsed with PBS, the sections were incubated overnight at room temperature with an antibody against human α -amylase (Sigma) that had been diluted 1:500 in PBS containing 1% bovine serum albumin. The sections were subsequently rinsed with PBS, exposed to gold-labeled anti-rabbit IgG (E-Y Lab.) for 2 h and rinsed again with PBS. Finally, they were washed with distilled water and double-stained with uranyl acetate and lead citrate.

Control sections were prepared by omitting either the primary or the secondary antibody, or by incubation with immunoglobulin prepared from non-immune rabbits. These control incubations were performed under the conditions described above.

RESULTS

Light microscopy

In the youngest monkeys — the 15-day-old animals — secretory granules reacting strongly to PAS and alcian blue were observed in mucous cells of the terminal clusters and acini of the parotid gland (Fig. 1a). These mucous cells were further found in 30-day-old and 3-month-old animals. The mucous cells were most prevalent on day 15, and then decreased in number by day 30 (Fig. 1b) with a continuing decline

over 3 months. They could no longer be detected in macaques that were 6 months of age or older (Fig. 1c).

Electron microscopy

In 15-day-old macaques, the mucous cells contained secretory granules that showed either homogeneously electron-lucent, or bipartite and tripartite granules with electron-dense cores (Fig. 2a, b). Frequently images indicating exocytosis of the granules were recognized in these cells. Some cells contained only secretory granules of high electron density, and these were considered to be serous cells, although they were few in number. By day 30, the mucous granules were predominantly bipartite in structure (Fig. 2c), and serous cells with electron-dense granules had become more prevalent than at day 15 (Fig. 2f). By 3 months of age, mucous cells with electron-lucent granules had become scarce and the majority of these cells contained granules of a heterogeneous, i. e., bipartite and tripartite structure. The electron-dense cores and moderately dense portions in those granules were also increased in size (Fig. 2d). There were some cells with a mixture of granule types, i. e., electron-lucent, bipartite or tripartite, and electron-dense forms (Fig. 2e). These cells were considered to be transitional cells. None of these cells showed chromatin condensation characterizing the process of apoptosis.

By 6 months of age, all of the acini came to consist of serous cells containing electron-dense granules, and mucous cells were no longer apparent. In the samples obtained from macaques aged 1.5, 3, and 4 years, the acini were exclusively composed of serous cells without any mucous cells.

Silver methenamine technique

The electron-lucent and bipartite or tripartite mucous granules showed a positive reaction to silver methenamine. The less dense portions of the latter granules showed a reaction stronger than the dense portions. There was minimal reaction in the electron-dense granules that were found in serous cells, and the same was the case in the dense portions of the mucous granules with a heterogeneous structure (Fig. 3).

Lectin staining

Electron microscope examination of ultra-thin sections prepared from a 15-day-old monkey parotid gland showed that there were gold particles labeled with WGA, SBA and DBA on the homogeneously

Fig. 1. Light micrographs of mucous cells stained with alcian blue. **a.** Postnatal 15 days. Alcian blue-positive mucous cells (arrows). **b.** Postnatal 30 days. Alcian blue-positive mucous cells (arrows) are decreased in number compared with those seen at 15 days. **c.** Postnatal 6 months. Alcian blue-positive mucous cells have disappeared. The intercalated duct cells show weakly positive staining. $\times 270$

Fig. 2 a-f. Electron micrographs of mucous and intermediate type cells. **a.** Postnatal 15 days. Acini consist of mucous and serous cells. $\times 2,300$. **b.** Postnatal 15 days. A mucous cell containing secretory granules of homogeneously electron-lucent and a bipartite structure with a moderately dense core. $\times 20,000$. **c.** Postnatal 30 days. A mucous cell containing granules of low electron density and those of bipartite structure. The core of the bipartite granules is denser than that of the granules shown in **b.** $\times 20,000$. **d.** Postnatal 3 months. A mucous cell containing bipartite granules, which are denser than those seen in **c.** $\times 15,000$. **e.** Postnatal 3 months. A cell showing an intermediate structure between the mucous and serous cell types. Both bipartite and electron-dense granules are seen in the cell. $\times 15,000$. **f.** Postnatal 30 days. Serous cells with electron-dense granules. $\times 20,000$

Fig. 2 e and f. Legend on the opposite page.

electron-lucent and bipartite or tripartite mucous granules (Fig. 4a, b). We could thus confirm that these granules contained β -D-N-acetyl glucosamine (β -D-GlcNAc), α -D-N-acetyl galactosamine (α -D-GalNAc) and α -D-GalNAc-D-GalNAc. Labeling was intense in both the electron-lucent granules and the less dense portions of the granules with a heterogeneous structure, although the electron-dense cores in the heterogeneous granules were only weakly labeled. In addition, labeling with WGA or SBA was more intense than that with DBA. By day 30, the increasing in size of the electron-dense cores and moderately dense portions of the heterogeneous granules suppressed the frequency of the gold particles on the mucous granules compared with their appearances on day 15. By 3 months of age, the remaining mucous granules showed only a few labeled particles on their limited less dense portion. By this time, the electron-lucent granules in the transitional cells, which possessed three types of granules — electron-lucent, bipartite and electron-dense, showed a weaker reaction to the lectin staining than those in the mucous cells at 15 days or 3 months of age.

In samples from macaques aged 15 and 30 days, the serous granules were weakly labeled with WGA, SBA and DBA lectins, in concordance with the weak lectin reactivity of the electron-dense cores of the bipartite and tripartite granules. However, by 3 months of age, probably as a result of the observed

Fig. 3. Postnatal 15 days. An electron micrograph of granules stained with the silver methenamine technique. The granules of homogeneously low electron density and the less dense portions of the bipartite granules react strongly, whereas the homogeneously electron-dense granules and the dense portions of the bipartite granules show a minimal reaction. $\times 25,000$

Fig. 4. Electron micrographs of granules stained with lectins. **a.** Postnatal 15 days. The mucous granules are labeled with DBA (15-nm gold particles) (arrows) and SBA (20-nm gold particles) (arrowheads) lectins. Labeling is more intense over the electron-lucent granules and over the less dense portions of the bipartite granules compared with the electron-dense cores. $\times 36,000$. **b.** Postnatal 15 days. The mucous granules are labeled with WGA (5-nm gold particles) (arrows) and SBA (20-nm gold particles) (arrowheads) lectins. Labeling is more intense over the electron-lucent granules and over the less dense portions of the bipartite granules. $\times 34,000$. **c.** Postnatal 3 months. The serous granules are labeled with WGA (5-nm gold particles) (arrows) and SBA (20-nm gold particles) (arrowheads). $\times 29,000$

decrease in the number of the granules with bipartite and tripartite structures, most of the serous granules exhibited an intensely positive reaction to WGA, SBA and DBA lectins (Fig. 4c); this reaction remained evident beyond 3 months of age.

At all the stages of development that were investigated, both mucous and serous cells were negative to the stainings with Con A, UEA-I, PNA, HPA and LFA.

Immunohistochemical staining

In the glands from 15-day, 30-day and 3-month-old macaques, the electron-lucent granules and the bipartite or tripartite granules in the mucous cells were weakly labeled with gold particles (Fig. 5a, b). Gold particles were detected in both the less dense portions and the electron-dense cores of the heterogeneous granules. However, by 3 months of age the electron-dense granules in the intermediate cells tended to

Fig. 5. Electron micrographs of granules immunostained with an antibody to human α -amylase. **a.** Postnatal 15 days. The electron-lucent granules show a few gold particles (*arrows*). $\times 22,000$. **b.** Postnatal 30 days. The electron-lucent and bipartite granules are labeled with a few gold particles (*arrows*). $\times 20,000$. **c.** Postnatal 3 months. The granules in a transitional cell are labeled with gold particles (*arrows*). The labeling is more intense over the electron-dense granules compared with those of bipartite structures. $\times 17,000$. **d.** Postnatal 3 months. The serous granules are heavily labeled with gold particles. $\times 18,000$

immunoreact more strongly than the electron-lucent and heterogeneous granules in the same cells (Fig. 5c).

On day 15, the electron-dense granules of the serous cells were only weakly labeled with gold particles. However, the intensity of labeling in these granules was stronger in the older glands (Fig. 5d).

DISCUSSION

In the present study, mucous cells were demonstrated in the monkey parotid gland between 15 days and 3 months after birth, and these cells contained granules of homogeneously electron-lucent and bipartite or tripartite. This indicates the possibility that mucous cells may occur in the developing parotid gland of primates. Although the youngest macaque we were able to obtain was already 15 days of age, it seems likely that these cells are present from birth. The present findings strongly suggest that the mucous cells in the monkey parotid gland change their morphological characteristics to those of serous cells, the intermediate type cells representing the transitional forms. Chromatin condensation in the nucleus suggesting apoptosis was recognizable neither in the mucous cells nor in the transitional cells. Accordingly, it is believed that cell death was infrequent, if at all. Recently TAKADA et al. (2001) demonstrated evidence of increased mitotic figures of mucous cells in the parotid gland of early postnatal mice.

Glycoconjugates play an important role in tissue formation and undergo changes in their characteristics and distribution as cells differentiate and age (HIRANO, 1980; YOGESWARAN, 1983; STANLEY, 1987). The present study showed that mucous granules possessed the same sugar residues as the mature serous granules. Thus it appears that the mucous cells produce glycoconjugates until mature serous cells appear.

The present study demonstrated that the mucous cells contained amylase, and the electron-dense granules in the transitional cells showed its immunoreactivity stronger than the electron-lucent granules and the heterogeneous granules from the same cell. This suggests that the mucous cells become rich in amylase as they change morphologically into serous cells. Thus, the observed relation between the morphological characteristics and amylase labeling of secretory granules indicate that the mucous granules mature from those of low electron density to those with a heterogeneous structure, and finally to those with high electron density.

Serous cells containing only electron-dense granules showed minimal immunoreactivity for amylase on days 15 and 30, but this reaction became strong by 3 months. It is therefore suggested that both the serous and mucous granules in the monkey parotid gland contain small amounts of amylase from shortly after birth. It has been reported that the serous granules in serous cells in the rat parotid gland soon after birth have a strong affinity for gold particles labeled with neonatal submandibular gland protein B1 (BALL et al., 1988a; SIVAKUMAR et al., 1998; IKEDA and AIYAMA, 1999), although these granules are only weakly positive for amylase. Therefore, the serous granules from the monkey parotid gland several weeks after birth may have a rich supply of this still unknown protein. The serous granules started to show a strong amylase immunoreaction as well as lectin staining by 3 months, when the mucous granules had decreased markedly in number. Accordingly, maturation of the serous cells seems to occur during this period.

In sum, all these findings, i. e. transformation of mucous granules to a serous type, amylase labeling of the transforming granules, sugar residues that are common to both mucous and serous granules, and lack of apoptotic figures in the transforming cells, provide strong support for the view that mucous cells are converted into serous cells in the juvenile macaque parotid gland.

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