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Postnatal Growth and Differentiation of the Salivary Gland

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Summary. **In order to determine the postnatal proliferation potential of the salivary gland cells, we labeled the rat submandibular gland with Brdu (5-bromo-2-deoxyuridine). We detected cells in the proliferation cycle and examined populational changes of cell types during a period of 98 days after birth. BrdU labeling was observed in all segments of the gland: ductal, terminal tubule, myoepithelial and acinar cells. Acinar cells, ductal cells, and myoepithelial cells, characterized by S-100 protein positive cytoplasm, increased in number, and their final population reached approximately 60 and 30.9%, respectively. In contrast, terminal tubule cells, which could clearly be identified by their Masson's trichrome positive granules, occupied 43% of the whole gland at birth, decreased in ratio and disappeared at day 41. The results suggest that the salivary constituent cells with specific functions duplicate in their own locations through the postnatal growth of salivary glands, and that in the salivary gland there is no proliferating zone in which some reserve cells serve as multiple cell types. Based on these results, we have added discussion on oncogenetic pathways of salivary tumors.**

INTRODUCTION

The salivary gland is a representative compound tubulo-alveolar gland; its structure is the most complicated among exocrine glands of the digestive system. In contrast, gastrointestinal glands are generally composed of simple tubules and are known to contain within a proliferating zone such as the mucous neck cells in the gastric gland.[1,2] However, we do not know whether a similar proliferating zone exists in the salivary gland or every constituent cell duplicates as its own location in the salivary unit.

It has been widely accepted that the highly variable histology of salivary gland tumors depends on the complicated structure of the gland.[3,4] This idea has led many pathologists to the reserve cell theory on the tumorigenesis of salivary glands, which has also been widely discussed.[5-8] This theory is based on a hypothesis that some pluripotential cells serve as reserve cells for the origin of salivary neoplasms as well as germinal centers for each constituent of adult salivary tissues. However, such a pluripotential reserve cell has never been demonstrated morphologically in the salivary gland.[7-9]

Elucidating the proliferation system of salivary epithelial cells should prove important understanding not only their normal differentiation processes but also the oncogenetic pathways in the salivary glands. Based on our previous histological and ultrastructural studies on the development of the rat submandibular, we have suggested that no pluripotential cell focus serving different cell types is recognizable after a certain stage of embryogenesis.[9]

In order to obtain further evidence for this idea, this study aimed to determine the proliferation potential in epithelial cells during the postnatal development of the salivary gland. We studied the distribution pattern of S-phase cells in the rat submandibular gland by the BrdU (5-bromo-2'-deoxyuridine) labeling method and identified which types of salivary cells might possess proliferation potential. We further examined the cell types composing the salivary gland at various stages of the postnatal development. Our special interest was whether differentiated myoepithelial cells might keep their proliferation potential in the postnatal development, because they seem to play an important role in salivary tumorigenesis.[9-11]

MATERIALS AND METHODS

A total of 156 female Sprague-Dawley strain rats, during their first 98 days after birth, were given an intraperitoneal injection of BrdU (100 mg/kg body weight, Sigma, St Louis, MO, USA).[12-14] Five rats were killed every two days until day 29 and days 37, 41, 56, 98. Bilateral submandibular gland tissues were removed 30 min after the injection, and immediately fixed with 70% ethanol for 24 h at room temperature. The tissues were embedded in paraffin. Serial 4 μm paraffin sections were cut, one each stained with hematoxylin and eosin and Masson's trichrome for identification of terminal tubular cells, the others being used for immunohistochemistry.

BrdU immunohistochemistry

After deparaffinization with xylene, sections were rinsed in 0.01 M phosphate-buffered saline (PBS), and were digested with 0.05% actinase E (Kaken Pharmaceutical Products, Tokyo), followed by hydrolysis with 2 N HCl 1 h at room temperature. The sections were then blocked with 5% normal goat serum in PBS and subsequently immunostained by the biotin-streptavidin peroxidase method, using a Stravigen (B-SA) kit (Biogenex Laboratories, San Ramon, CA, USA). The primary antibody against BrdU (mouse monoclonal, Becton Dickinson Monoclonal Center, Mountain View, CA, USA) was diluted at 1:50. The reaction products were developed by 3,3'-diaminobenzidine (DAB). The sections were counterstained with hematoxylin.

Evaluation of BrdU labeling index

The proliferation potential of salivary cells was expressed by the labeling index, which was defined as the percentage of labeled nuclei in a given cell population 30 min after the administration of BrdU. Five squares (250 μm$\times$250 μm) on each sections at magnification $\times$400 were randomly selected. The ratios of labeled nuclei to the total within the squares were averaged.

BrdU immunoelectron microscopy[14]

The BrdU labeled submandibular gland tissues were minced and fixed with periodate-lysine-paraformaldehyde fixative for 15 h at 4℃. They were then washed in PBS containing graded sucrose and finally in 20% sucrose- 5% glycerol, and embedded and frozen in OCT compound (Miles Laboratories Inc., Elkhart, IN, USA). Frozen 6 μm sections were cut and mounted on poly-L-lysine coated glass slides. The sections were first treated with 0.05% actinase E, followed by hydrolysis with 2 N HCl for 1 h at room temperature and incubated overnight with the antibodies as described above. After incubation with peroxidase-streptavidin, the sections were fixed with 1% glutaraldehyde for 10 min at room temperature, followed by incubation with DAB for 30 min, then with H_2O_2-containing DAB. After postfixation with 2% osmium tetroxide, the sections were embedded in Epon 812. Ultrathin sections were cut and observed with a Hitachi H-300 electron microscopy.

S-100 protein immunohistochemistry

We utilized S-100 protein as an immunohistochemical marker for myoepithelial cells of the rat submandibular gland.[15,16] Prior to immunostaining, sections were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. The sections were immunostained in the same manner as mentioned above, using rabbit anti S-100 protein antibodies (prediluted, BioGenex Laboratories) and biotinylated goat anti-rabbit IgG (prediluted, BioGenex Laboratories).

Double staining for BrdU and S-100 protein[17]

S-100 protein immunohistochemistry was carried out basically by the method mentioned above. Sections previously immunostained for S-100 protein were thoroughly washed with PBS and digested with 0.05% actirase E, followed by hydrolysis with 2 N HCl for 1 h at room temperature. After blocking with normal goat serum, the sections were stained for BrdU with an immunogold technique, using gold conjugated goat anti-mouse IgG (BioCell Research Laboratories, Cardiff, UK) and the silver enhancement reaction (BioCell silver enhancing kit, BioCell Research Laboratories). BrdU uptake was indicated by black-colored silver grains, whereas the occurrence of S-100 protein was demonstrated in brown.

Double staining with BrdU and Masson's trichrome

The BrdU labeling is better evaluated against the background of Masson's trichrome staining, as this enables us to identify terminal tubules by specifically visualizing the cytoplasmic granules in these particular tubules. However, this double staining was practically impossible, because aniline blue-stained granules were diminished after the BrdU immunostaining procedure. In this study, therefore, after Masson's trichrome staining, representative areas including terminal tubules were photographed. Successively, the sections were washed in 0.1 N HCl in ethanol to decolor

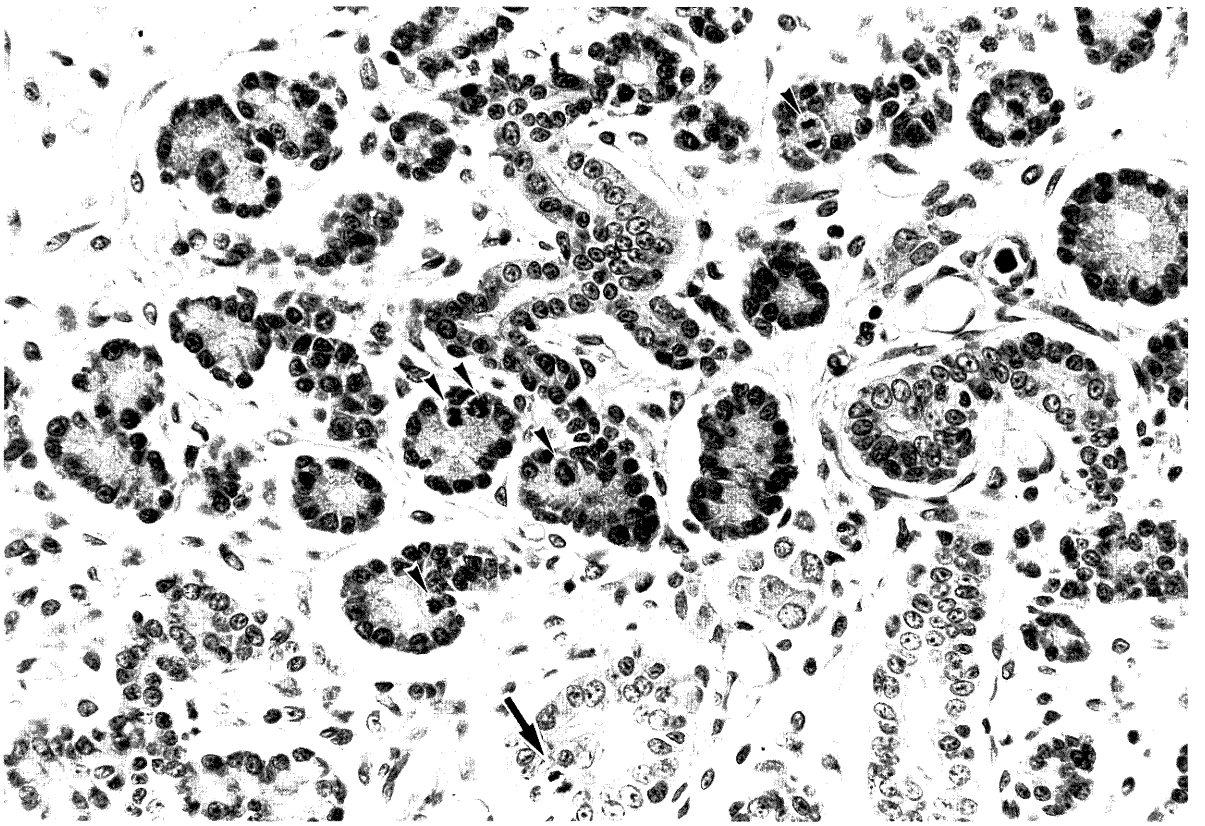

Fig. 1. Rat submandibular gland at birth. Intralobular ducts and terminal tubules are embedded in mucoid stroma. Mitoses are seen most frequently in and around terminal tubules (arrowheads) and in duct cells (arrow). hematoxylin and eosin stain. ×400

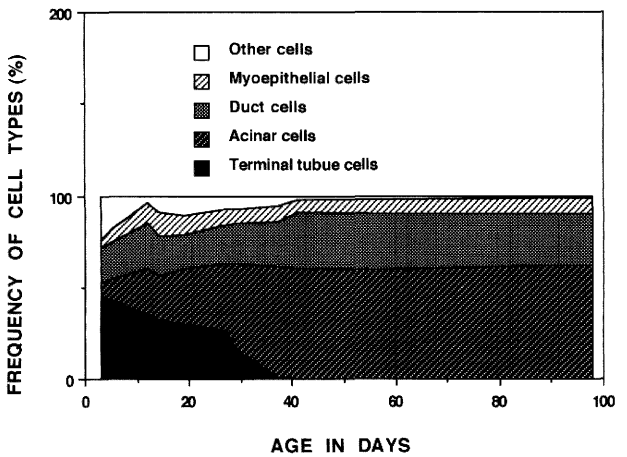

Fig. 2. Percentages of cell type population of the rat submandibular gland for 98 days after birth.

the nuclear staining, and then processed for the same BrdU immunostain method as described above. Photomicrographs were taken again of the same areas on BrdU labeled sections, to be compared with those from Masson's trichrome-stained sections. BrdU-labeled nuclei were distinct from non-labeled nuclei, although the latter were still brown colored after destaining.

Counting of salivary component cells

Scheduling, fixation and embeddeding were same as described in the evaluation of BrdU labeling index. Each salivary constituent cell was identified by using the above-mentioned staining method. Three random squares (250 μm×250 μm) were selected at a magnification of ×400 for the count of nuclei of the acinar, terminal tubule, duct and myoepithelial cells. The frequency of each cell type to the total number of cell nuclei was expressed in percentages. We calculated the mean of the values from the three squares of each section.

RESULTS

Population of salivary cell types

At the first day of age, the submandibular gland was still immature histologically. The intralobular stroma was loose and contained mucoid materials. Since ductal differentiation was poor, it was difficult to distinguish each segment of the duct system (Fig. 1). A characteristic component to the rat subman dibular gland at this stage was the terminal tubules. Terminal tubules were located proximal to the duct

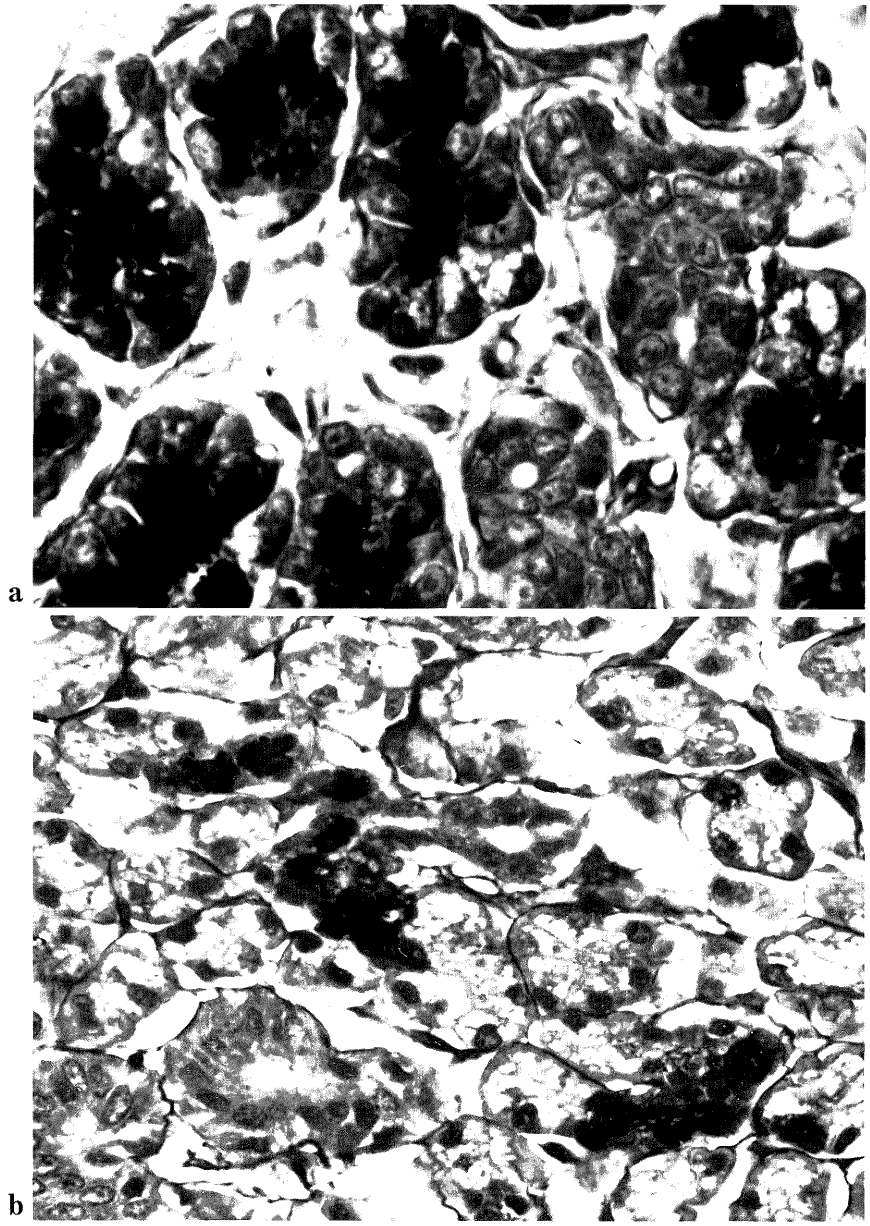

Fig. 3. Structural changes of terminal tubules during postnatal development of the rat submandibular gland. Masson's trichrome stain, ×640. **a.** Eight days of age. Most terminal tubule cells are filled with definite granules. Acinar cells have scanty cytoplasms. **b.** Thirty-seven days of age. Terminal tubule cells, adjacent to intercalated ducts, are flattened and still contain weakly stained granules.

system, and their proximal ends were continuous to acinar buds. Fig. 2 shows the relative population change among four types of salivary constituent cells: acinar, terminal tubular, ductal (including intercalated, granular convoluted, striated and excretory ducts), and myoepithelial cells. Terminal tubule cells were predominant at the early stages but decreased in number by day 37, while the population of acinar cells showed a reverse transition, elevating to about 60% of all salivary cells by the same point in time. Numbers of ductal and myoepithelial cells gradually increased after birth, and reaching a plateau around day 40. The following describes the details of the populational changes of each component of the submandibular gland in the course of development.

Terminal tubule cells

We identified terminal tubule cells with Masson's trichrome stain. At day 1, the cytoplasm of terminal tubule cells stained diffusely blue. By day 8, the

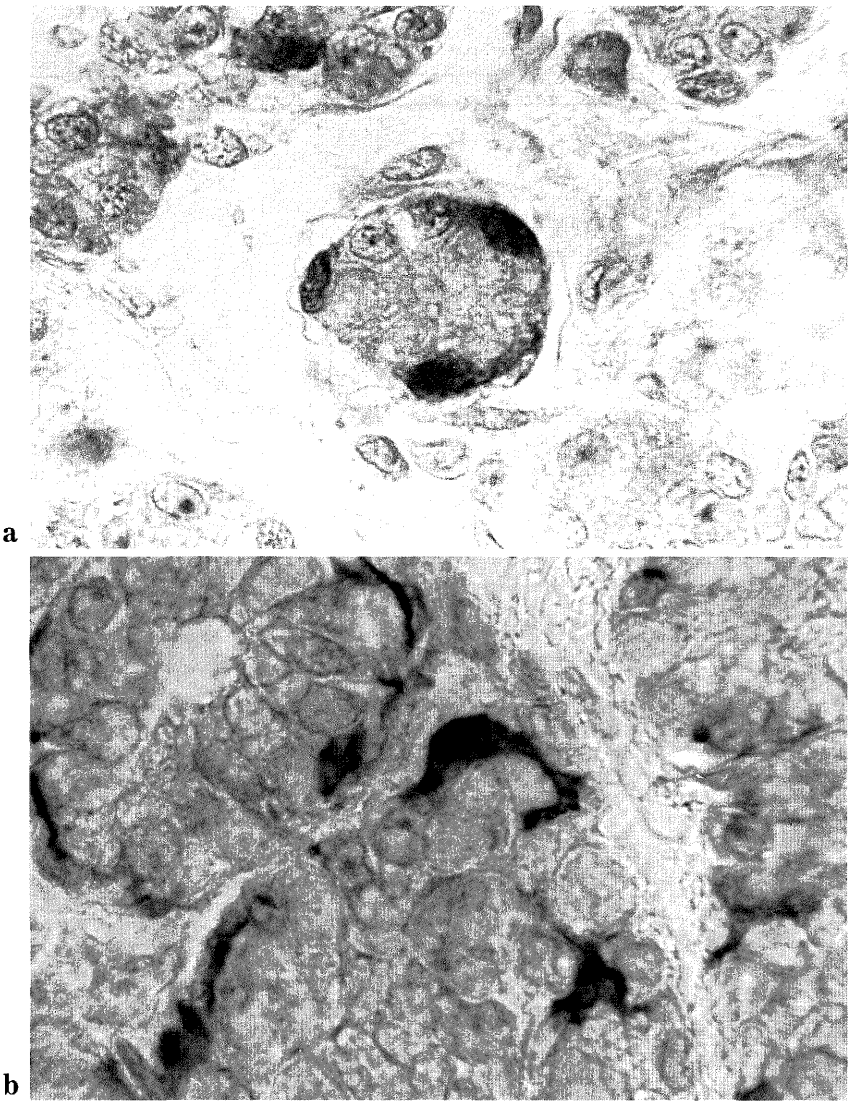

Fig. 4. Myoepithelial cells in early stages of rat submandibular gland development. Immunoperoxidase stain for S-100 protein. Counter stain with hematoxylin. **a.** Three days of age. Positively stained cells are seen around terminal tubules. ×1000. **b.** Seven days of age. Positive cells show the characteristic pattern of myoepithelial cell distribution around tubulo-acinar structures. ×1,000

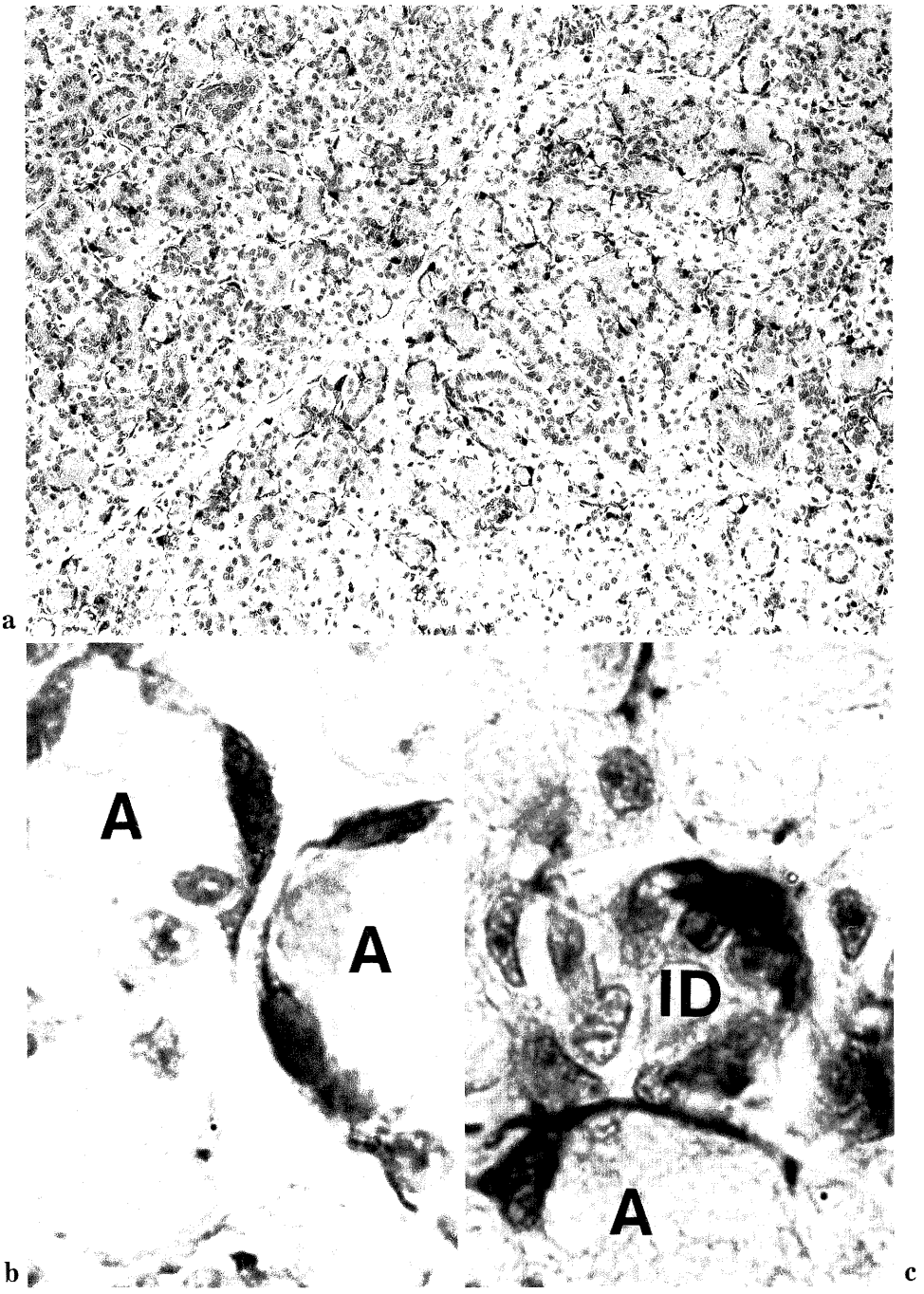

Fig. 5. Myoepithelial cells of the rat submandibular gland at 27 days of age. Immunoperoxidase stain for S-100 protein. Counter stain with hematoxylin. **a.** Acini are surrounded by myoepithelial cells. ×200. **b.** Myoepithelial cells encircling acini (A). ×1,000. **c.** Myoepithelial cells surrounding an intercalated duct (ID) and acini (A). ×1,000

cytoplasmic staining became definitely granular (Fig. 3a). Terminal tubule cells occupied 43% of the whole gland at day 1; their population decreased gradually until day 27 (25%), and then rapidly sank down to 0.5% at day 37. Parallel to the decrease in their population, the cytoplasmic granules were reduced in number and size, becoming concentrated at the apical end of the cells. After day 37, the terminal tubule cells were rarely recognizable, although flattened terminal tubule cells containing a smaller amount of granules were occasionally located at the most proximal branching of intercalated ducts (Fig. 3b).

Acini

In contrast to the terminal tubule cells, the population of acinar cells continued to increase through the developmental course observed. Acinar cells were distinct with Masson's trichrome negative cytoplasm from terminal tubule cells, in addition to their characteristic shape. Mitotic figures were quite frequent among them. Their population at day 3 was 6%, and increased to 47.5% at day 27, and up to 60% at day 41, when acinus formation was almost completed. Terminal tubule cells were occasionally present between acini and intercalated ducts. After day 41, the population was maintained at around 60%, and acinar volume continued to increase thereafter (Fig. 2).

Duct system

The duct system was composed of interlobular excretory ducts and intralobular ducts. The latter consisted of intercalated ducts and striated ducts. Granular convoluted ducts started to develop after day 35. Intralobular ducts represented the duct system in this study, because squares for cell counts on histological sections were placed within lobules. The population of the total duct system was 18.9% at day 3, and gradually elevated to 32.1% at day 41. After then, it was maintained at around 30% (Fig. 2).

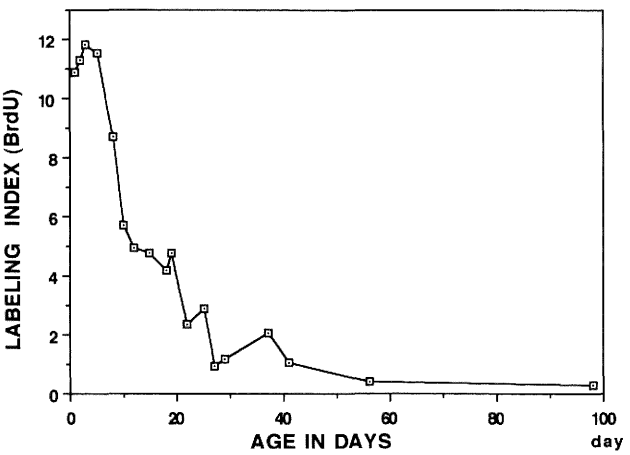

Fig. 6. BrdU-labeling index in the rat submandibular gland during the period of 98 days after birth.

Myoepithelium

Myoepithelial cells were identified by their location (basal side of acini and ducts) and their immunoreactivity for S-100 protein. S-100 protein positive cells were not recognized at birth in the submandibular gland. However, scattered immunostaining for S-100 protein appeared by day 3 (Fig. 4a), and by day 7, S-100 protein-positive cells were distributed around the tubulo-acinar structures (Fig. 4b). Their population was 3.8% at day 3, and 8.8% at day 7. This was once elevated up to 13% on day 15, to reach a plateau around 9% after day 20 (Fig. 2). The characteristic distribution of S-100 protein-positive cells (Fig. 5a) at the periphery of acinar cells (Fig. 5b), especially of intercalated ducts (Fig. 5c), came to be recognized by day 27.

BrdU labeling index

The overall BrdU labeling index of the submandibular gland was 11.0 during the first week of age, and declined to 4.8 at day 15, to 2.4 at day 22, and to 1.1 at day 41 (Fig. 6). After 56 days of age, it continued in the range of 0.1-0.2 until the last day of the experiment. Mitotic figures and BrdU labeling were recognized in all segments of the duct system, terminal tubules, and acini.

We could not count BrdU labeling in each cell type through the developmental course, due to technical difficulties. However, the labeling was observed in 3.72% of the total nuclei of the terminal tubule cells at day 8, but decreased to as low as 0.35% at day 19 (Fig. 7a, b), whereas 6.8% of the labeling was obtained in acinar cells at the same time. In the duct system, BrdU labeling was detected in intralobular duct cells as well as both basal and luminal cells of interlobular excretory ducts (Fig. 8a-c). BrdU labeling was also shown in myoepithelial cells with simultaneous S-100 protein positiveness (Fig. 9). At day 19, 1.7% of the myoepithelial cells were stained positively for BrdU. BrdU incorporation in myoepithelial cells was confirmed at the electron microscopic level. BrdU was labeled in the cells, which were located between acinar or ductal cells and the basement membrane facing the extracellular matrix (not shown).

DISCUSSION

The rapid renewal of epithelial cells of gastrointestinal glands is well known. In the gastric mucosa, undifferentiated cells in the proliferating zone of the isthmus,[1,2] which is located between the foveolae and fundic glands, migrate in both upper and lower directions and differentiate to the surface mucous cells and parietal or mucous neck cells, respectively. Mucous neck cells further differentiate into chief cells. Similar germinal cells have been shown in the intestinal glands.[1,18] In contrast to such simple tubular glands, information on cell renewal is quite limited in exocrine glands with more complicated glandular structures, such as the salivary glands and pancreas. As for the rodent pancreas, whose exocrine portion is composed of a compound tubulo-alveolar gland, there have been some ^{3}H-thymidine autoradiographic investigations[19-22] showing that every cell type retains its proliferation potential after birth although the ^{3}H-thymidine labeling rates varied with cell types. These data[19-22] suggest that there is no particular proliferating zone in the pancreas.

In this study we have shown the low renewal rate of epithelial cells of the adult rat submandibular gland under physiologic circumstances. The BrdU labeling study indicated that cell renewal was not limited to any special cell types, but occurs in all types. Acinar cells frequently incorporated BrdU in neonates from birth. The acinar cell population could be increased by the proliferation of existent acinar cells,[23-25] although acinar cells are fully differentiated and functioning cells. In the previous studies utilizing ^{3}H-thymidine labeling, the salivary acinar cells were reported to be labeled and the labeling was more prominent when rats were stimulated with isoproterenol.[26]

Since the nature and ultimate fates of the terminal tubule cells of the rat submandibular gland have not been fully clarified, two possible courses of fate have been postulated for them. One is the transformation

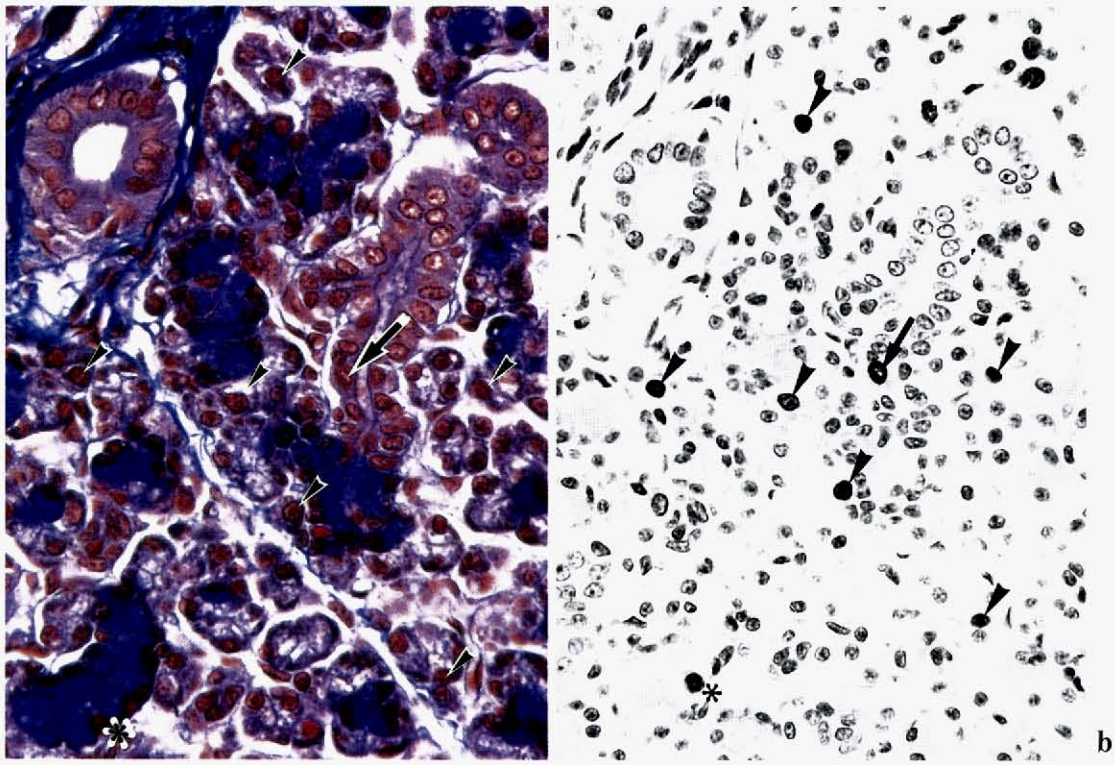

Fig. 7. Comparison between Masson's trichrome and BrdU labeling in the rat submandibular gland at 19 days of age. Proliferating cells are identified as acinar cells. Masson's trichrome and immunoperoxidase stain for BrdU on the same section. ×400. **a.** Masson's trichrome stain. Acinar cells later labeled for BrdU are shown by arrowheads, intercalated duct cell by the arrow, and terminal tubule cells by the asterisk. **b.** BrdU immunoperoxidase stain after decolorization for Masson's trichrome stain. BrdU labeled cells are indicated as above.

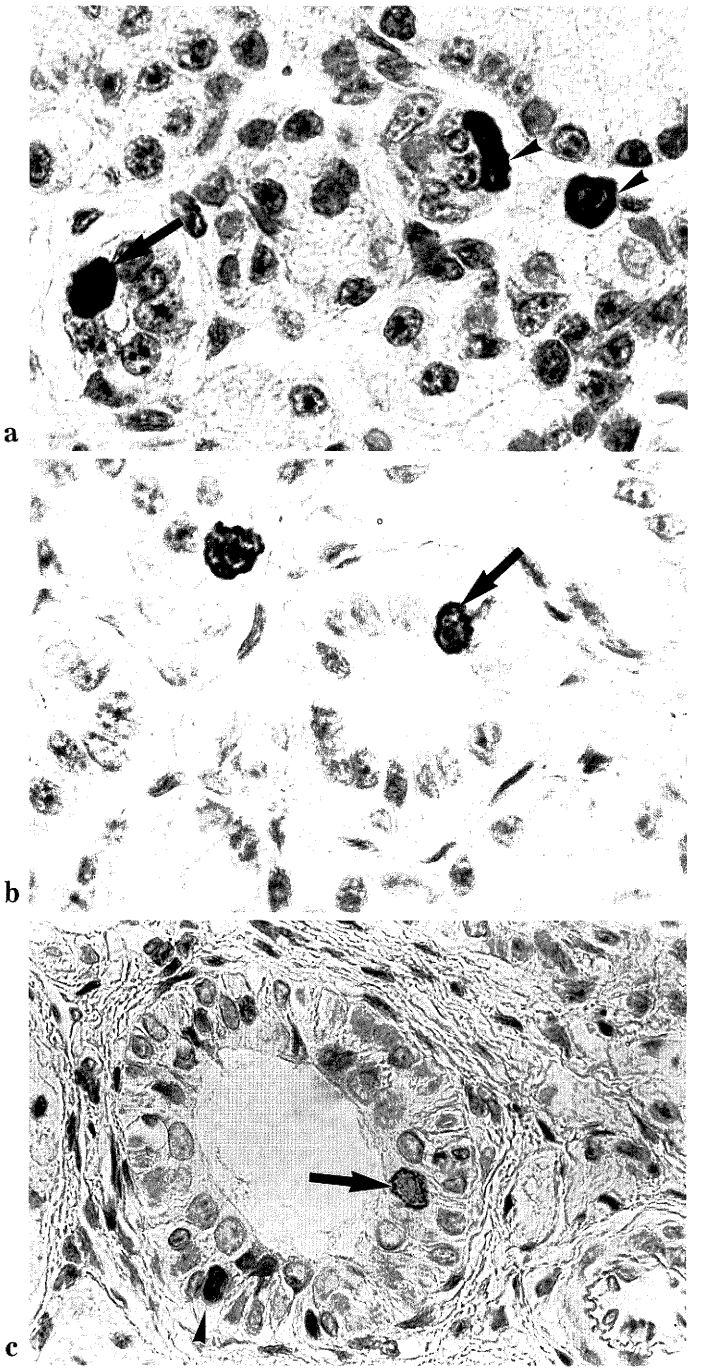

Fig. 8. Immunoperoxidase stain for BrdU in the submandibular gland at 19 days of age. Counterstain with hematoxylin. ×1000 **a.** Nuclei of intercalated ducts (arrow) and basally situated cells of acinar-intercalated duct complex (arrowheads) are labeled by BrdU. b. An intralobular striated duct cell incorporates BrdU in its nucleus (arrow). **c.** Both basal cell (arrowhead) and luminal cell (arrow) of the interlobular duct are also labeled.

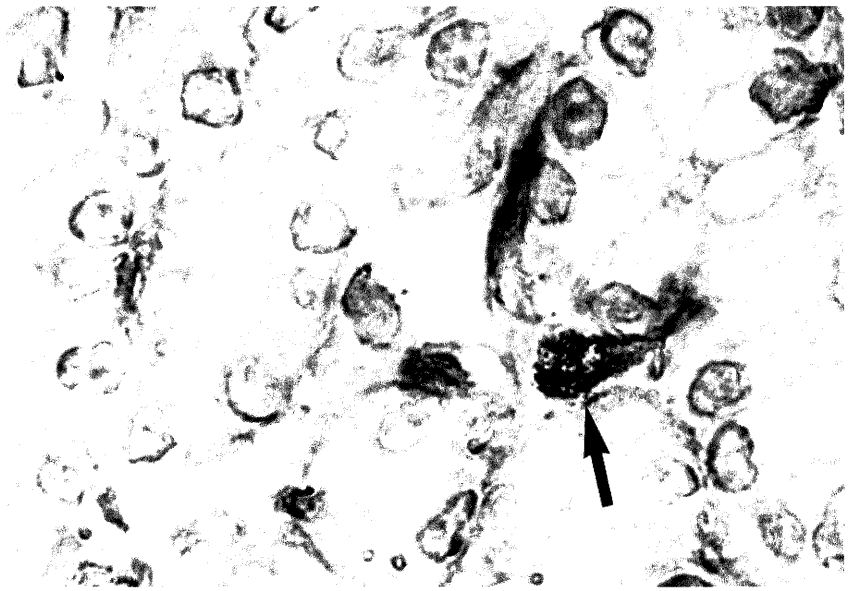

Fig. 9. Double immunopeoxidase stain for S-100 and BrdU in the rat submandibular gland at 19 days of age. An acinar myoepithelial cell (arrow) with BrdU-labeled nuclei is simultaneously positive for S-100 protein. ×1,000

into the cells of the intercalated duct,[9,27-29] and the other is that into the proacinar and acinar cells.[24,26,28,30] However, there has been no morphological evidence supporting the idea that the terminal tubule cells give rise to the acinar cells.[23,24] Yamashina and Barka[29] reported different endogenous peroxidase activities between submandibular acinar cells and terminal tubule cells in 17-18 day old rat fetuses, and suggested that there was no transitional form between them. They concluded that these two cell types acquire their own cellular characteristics in the fetal stage, taking into consideration also the finding that isoproterenol affects the secretion of proacinar cells but not that of terminal tubule cells. However, Strum[30] observed that terminal tubule cells of the rat submandibular gland seemed to contain peroxidase-positive granules temporarily in order to fulfill the early need for the substitution of acinar cells until the acinar cells increased in number. In the present study, terminal tubule cells decreased in number during the postnatal development of the rat submandibular gland. Their signal for duplication became lower after 19 days of age and thereafter their population decreased to the point of disappearance. Similar results were obtained by Jacoby et al.[27] and Chang[24] in the postnatal submandibular gland of the rat. The results suggest that the terminal tubule cells are not able to differentiate into other cell types in the postnatal growth of the submandibular gland.

Both luminal and basal cells of the ducts in various locations were shown to have the ability for self-renewal in this study. Dardick et al.[31] reported a similar result in the rat submandibular gland, using ^{3}H-thymidine labeling during the first 15 days after birth. Basal cells should not be regarded as stem cells of luminal epithelial cells of interlobular excretory ducts, excluding the mucosal orifice of the salivary ducts.

Another interesting result in the present study is that myoepithelial cells were also shown to be able to duplicate. Previous histological observations in the rodent submandibular gland have suggested that only developing myoepithelial cells have a mitotic activity,[32,33] but that fully developed myoepithelial cells loose the ability to divide. Myoepithelial cells have been suggested to be derived from terminal tubules at the fetal stage.[9,32-34] However, once differentiated into myoepithelial cells, they seem to proliferate independently from other cell types after birth.[9,33,34] Sapino[17] showed, using *in vivo* BrdU labeling, that mature mammary myoepithelial cells of 30 day old mice were able to proliferate under estrogen and progesterone stimulation. Dardick et al.[11,31] reported the existence of modified myoepithelial cells, whose proliferation potential was ascertained at the base of striated and excretory ducts. These observations, including ours, imply that the myoepithelial proliferation of the salivary gland does not necessarily require its progenitor cells for self-renewal after birth.

Since every cell type of salivary gland has been

revealed to have a proliferation potential, it is concluded that no proliferating zone exists in the salivary glands. This coincides with the results hitherto obtained from investigations in the exocrine pancreas. This also suggests that salivary gland tumors originate from differentiated and functioning constituent cells of the salivary gland. The reserve cell theory on the oncogenesis of the salivary gland has spread in the textbook teaching of the salivary tumor pathology.[3-8] This theory, which is based merely on the postulation of certain reserve cells being responsible for neoplastic transformation, and not at all on any experimental data, is now refuted by the results of the present study.

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