原　　著

IMMUNOCYTOCHEMICAL LOCALIZATION OF PAROTIN SUBUNIT IN SUBMANDIBULAR GLANDS OF THE JAPANESE MONKEY, *MACACA FUSCATA*

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Abstract : To determine the intracellular localization of parotin subunit in monkey submandibular glands, parotin subunit antiserum labeling and immunogold labeling were performed at both light and electron microscopic levels. Parotin subunit was found to be distributed along tonofibrils of striated duct cells, and to be localized intensely in middle electron-dense areas and less intensely in the dense core of secretory granules of serous acinar cells.

Therefore, parotin subunit is secreted by serous cells and speculated to play a role in the formation of tonofibrils in duct apical region.

Key words : parotin, monkey, submandibular gland, localization, acinar secretory granule

INTRODUCTION

Parotin, isolated from the bovine parotid gland[1], was assumed to be synthesized in the parotid gland acini and absorbed by the striated ducts[2]. The hypothesis has not been clarified. Parotin exhibits hypocalcemic action[1], as well as hypoglycemic[3] and bone healing[4] effects in diabetic rats. A parotin subunit, isolated from parotin[5], is a homotrimer (45 kDa)[6, 7] possessing strong hypocalcemic action[5].

Parotin has been shown to be localized in bovine parotid gland[8]. Parotin subunit has been detected in ducts of bovine[8, 9], human[9] and rat parotid glands[10] and submandibular glands[5], and also in various organs of the guinea pig[9]. When the enzyme-labeled antibody method and the immunofluorescence-labeled antibody method were used to examine the localization of parotin subunit in the salivary glands, kidney, and pancreas of the Japanese monkey, reactivity was found to be highest in striated duct cells of submandibular glands among salivary glands[7]. Striated ducts of the monkey submandibular glands were longer and more numerous than those of parotid glands.

However, whether parotin subunit is synthesized or absorbed by the duct cells of these monkey organs can not be verified at light microscopic level. Further study of the electron-microscopic immunocytochemical localization of parotin subunit in submandibular glands was undertaken using immunogold method.

MATERIALS AND METHODS

Tissue preparation

Submandibular glands from five adult male Japanese monkeys, *M. fuscata,* were examined. Normal salivary glands were obtained from two of these animals during oral pathology and oral surgery studies. The submandibular glands were removed under 25 mg/kg sodium pentobarbital anesthesia before lethal over-dosing with the anesthetic. The experiment was carried out with the approval of our institutional animal care committee[16].

Bovine parotin subunit and rabbit anti-bovine parotin subunit antiserum (polyclonal) were obtained from Asuka Pharma. Co., Tokyo, Japan. The cross-reactivity of the rabbit anti-bovine parotin subunit antiserum with bovine parotin subunit and with extract of not only parotid glands but also the submandibular glands was examined before[7].

Light-microscopic immunocytochemistry

The submandibular glands were cut into pieces (approximately $1cm^2 \times 0.5cm$ blocks) and fixed in Bouin's fixative for 3-5 h. After fixation, 4μm thick paraffin sections were prepared, deparaffinized and stained by the protein A-gold technique[13,14] as follows. The sections were placed in phosphate buffered saline (PBS) for 10 min, blocked with 1% bovine serum albumin (BSA) in PBS for 30 min, and then incubated overnight with 0.01 mg/ml rabbit anti-bovine parotin subunit antiserum in PBS supplemented with 0.5% BSA at 4°C[11]. After washing in PBS three times for 5 min each, the sections were blocked with 1% BSA in PBS for 30 min and incubated with undiluted protein A-gold (15 nm, Jansen Life Sciences Products, Beers, Belgium) overnight. They were then rinsed in PBS three times for 5 min each and mounted in glycerin jelly.

As controls, some sections were incubated with normal rabbit serum, PBS or antiserum preabsorbed with bovine parotin subunit (working antiserum 1 ml: antigen 200μg) overnight, instead of primary anti-rabbit parotin subunit antiserum.

Electron-microscopic immunocytochemistry

The submandibular glands were minced, immediately immersed in 4% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 1-2 hr at 4°C, and embedded in LR White resin (London Resin, England). Thin sections on nickel grids were stained by the protein A-gold technique14). The thin sections were hydrated on a drop of PBS for 5 min and blocked with 1 %BSA in PBS for 30 min. The grids were incubated with rabbit anti-bovine parotin subunit antiserum (1:100 diluted with 0.5% BSA in PBS) for 1 hr, then transferred to washing buffer (0.5 M NaCl, 0.02 m Tris-HCl, 0.1% Tween 20) for 5 min three times each and blocked with 1% BSA in PBS for 30 min. The grids were then incubated with protein A-gold (1:10 diluted with PBS) for 30 min, washed with PBS, then with distilled water, and dried. They were then stained with 2% uranyl acetate in 0.15 M oxalic acid (pH7) for 10 min, followed by lead citrate (in accordance with the prescription for the Auroprobe EM, Janssen) for 1 min before viewing in a JEOL 1200 EX electron microscope at 80 kV. Controls involved either replacing the primary antiserum with normal rabbit serum or preabsorbing the primary antiserum with an excess of the antigen overnight at 4°C.

RESULTS

Light-microscopic localization of parotin subunit

Immunostaining with the anti-parotin subunit antiserum was observed in apical region of the duct cells: the striated duct cells were intensely stained (Fig. 1). Weak to strong

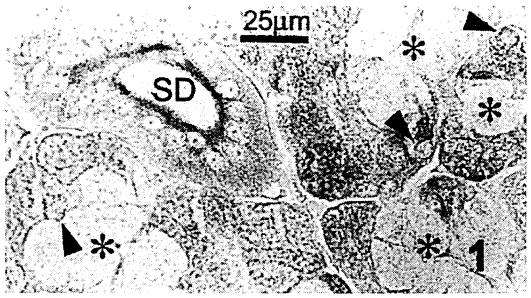

Fig. 1. Immuno-gold staining for parotin subunit at the light microscopic level. Apical region of monkey submandibular striated duct cells (SD) shows strong staining. In acini, weakly to strongly stained cells with unstained round nuclei (arrow heads) surround unstained cells (*). ×330.

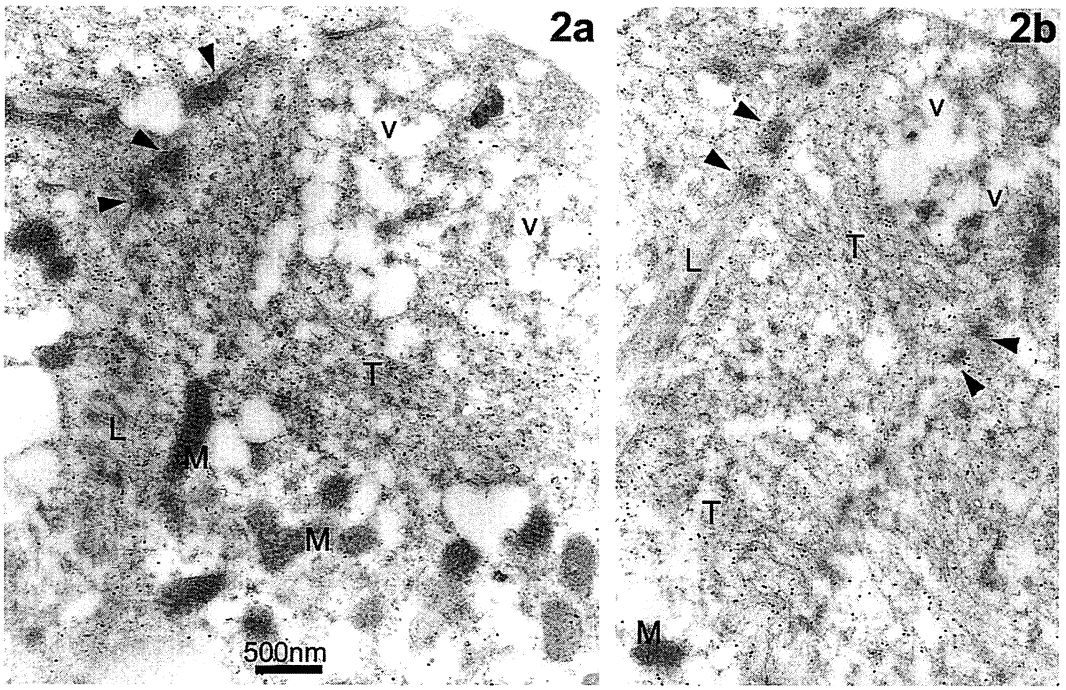

Fig. 2. Immuno-gold labeling for parotin subunit at the electron-microscopic level. ×17.500. Apical region of striated duct cells shows many labeling particles along tonofibrils (T) that run to connect desmosomes (arrow heads), and few labeling particles on vacuoles (v) and cytoplasm beneath lumen. a. Mitochondrion (M) is scarcely labeled. b. A cell showing labeling along double-layered tonofibrils (T) and along lateral membrane (L).

immunostaining was exhibited in acinar cells with unstained round nuclei.
Both controls using preabsorbed antiserum and normal rabbit serum instead of primary antiserum showed no specific immunoreactivity (data not shown).

Electron-microscopic immunocytochemical localization of parotin subunit

Immunolabeling with gold particles for anti-parotin subunit was often observed along tonofibrils which run among both sides of desmosomes of supra-lateral membranes (Fig. 2a, b) and sometimes comprise a double layer at apical region in striated duct cells (Fig. 2b). The labeling particles were few on vacuoles, and cytoplasm including secretory granules (or vesicles), Golgi complex, rough endoplasmic reticulum and mitochondria.

In the mature secretory granules of serous acinar cells, the middle electron-dense area was labeled (Fig. 3a). The core of the secretory granules was labeled less intensely than the middle electron-dense area (Fig. 3a). Middle electron-dense area of immature secretory granules was labeled weakly (Fig. 3b). A few labeling particles were found on Golgi

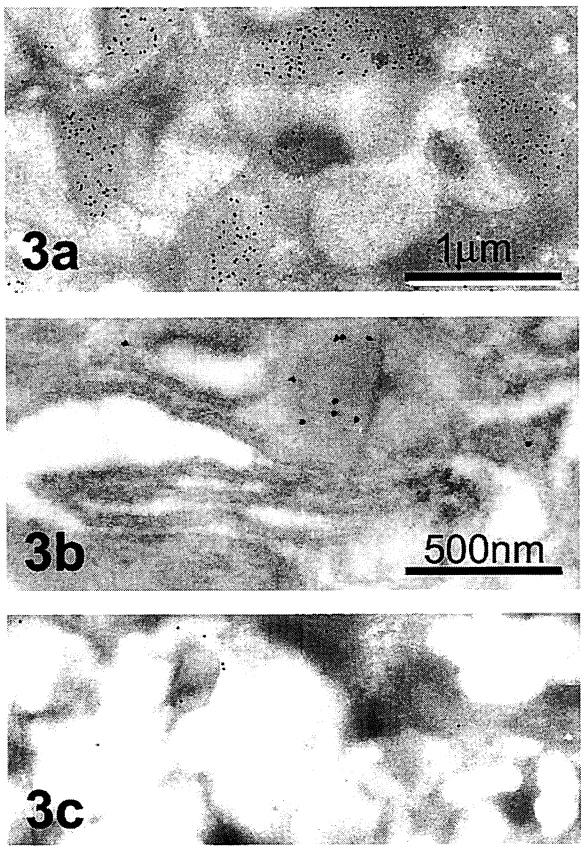

Fig. 3. Immuno-gold labeling for parotin subunit on acinar cells at the electron-microscopic level. a. Serous cell secretory granules showing intense labeling in middle electron-dense area and weak labeling in dense core. ×10.000. b. Golgi complex in serous cell. Weak labeling is observed on middle electron-dense area of immature secretory granule. A few labeling particles are observed on Golgi stack. ×20.000. c. Mucous cell secretory granules are unlabeled. ×10.000.

complex without immature secretory granules. The secretory granules of mucous cells were unlabeled (Fig. 3c).

The controls were essentially free of gold particles (data not shown).

DISCUSSION

Immunolabeling with anti-parotin subunit in apical region of striated duct cells was observed along tonofibrils and near desmosomes of supra-half lateral plasma membranes, differing from the case of kallikrein which was localized in the small granules[15] and not in acini[16]. The tonofibrils running transversely from desmosomes are considered to be cytokeratin intermediate filaments in epithelia. The parotin has been detected in the ducts forming and keratinized portions of preomorphic adenoma in human salivary glands[7, 9, 11]. The hypocalcemic activity of parotin subunit[5] is suggested to affect keratinization and preomorphic differentiation. Thus, the parotin subunit is suspected to be functionally related to cytokeratin. The parotin subunit-related proteins in the *M. fuscata* salivary glands might have affinity for the tonofibrils but cannot be identified as the 45 kDa cytokeratin of tonofibrils as discussed before[7].

On the other hand, parotin subunit was found in neither secretory granules (vesicles) nor cytoplasm including vacuoles beneath lumen. Therefore, the parotin subunit was neither produced nor endocytosed in the striated duct cells. Whether electrolyte-like transport of parotin subunit was conducted by the supra-half of lateral plasma membrane cannot be determined by the present method.

In the acini, middle electron-dense part and electron-dense core of the serous cell secretory granules were labeled with the protein A-gold of this study, but had shown no reaction with either indirect immunofluorescence or indirect immunoperoxidase methods of previous study[7]. These differences in reactivity of the serous cells are considered to be attributable to differences in the sensitivity and concentration of the conjugated materials with the secondary antibody; i.e. the concentration of peroxidase- or fluorescence-conjugated secondary antibody was not sufficient to allow detection of parotin subunit in serous cell secretory granules. Alternatively, parotin subunit in the secretory granules exists at such a low level or is masked in some way that it is difficult to detect depending on the technique being used. Weak reactivity was also reported in acinar cells of the bovine[9] and the rat[10] parotid gland with anti-parotin subunit at high concentration. The distribution of parotin subunit in the present monkey submandibular serous cell secretory granules is similar to that of amylase in human submandibular serous cell secretory granules. However, amylase is not localized in striated ducts[18, 19], differing from parotin.

In conclusion, these data support the possibility that parotin subunit is originally secreted by serous cells. Parotin subunit antiserum may cross-react with S parotin isolated from bovine, porcine, and equine submandibular glands[1]. Study about electron-microscopic immunocytochemical localization of parotin subunit in bovine submandibular glands and also in monkey parotid glands remains in the future. It is hypothesized that parotin subunit participates in duct cell formation with cytokeratin in the apical region. After parotin subunit is secreted by salivary glands, suspected to be absorbed at epithelium of small

intestine (Dr. Ishizaka suggestion), reached via blood vessels to duct cells of some organs, and plays a role for maintenance of structure: parotin subunit may be related with tonofibrils (cytokeratin intermediate filaments) which construct salivary gland luminal walls to resist expansion on secretion.

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