原　　著

LOCALIZATION OF PAROTIN SUBUNIT IN THE SALIVARY GLANDS, KIDNEY AND PANCREAS OF THE JAPANESE MONKEY, *MACACA FUSCATA*

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Abstract : Parotin subunit was localized immunohistochemically in the apical region of the duct cells in monkey salivary glands using anti-parotin subunit polyclonal antiserum by both indirect immunofluorescence and indirect immunoperoxidase techniques. Most intense reactivity was observed in the striated duct cells among the duct system cells. In addition, specific immunoreactivity was also detected in the collecting tubules of the kidney and the cells of the pancreatic duct system.

It is supposed that parotin subunit plays a role in duct formation.

Key words : parotin, monkey, salivary gland, kidney, pancreas

INTRODUCTION

Parotin was biologically isolated from the bovine parotid gland. Parotin-like substances, such as S-parotin from bovine, porcine and equine submandibular glands, saliva parotin from human saliva, uro-parotin from human urine[1] and serum-parotin from bovine serum[2] have also been purified. These parotin and parotin-like substances generally act on the mesenchymal tissue, especially the hard and connective tissues, to promote their development and growth, and have hypocalcemic activity[1]. Furthermore, a parotin subunit was isolated from parotin[1] using sodium dodecyl sulfate (SDS). The amino acid composition of the subunit was similar to that of parotin. The subunit possessed strong hypocalcemic action. However, parotin is not identical to calcitonin since its hypocalcemic mechanism is apparently different[3] and it shows no cross reactivity with either calcitonin or NGF antisera[4]. Nor can it have identity with kallikrein since it does not affect blood pressure[1].

Parotin has been found in the duct system cells of bovine[5], human[4] and rodent parotid glands and in the rat submandibular gland[3], also in various organs in the guinea pig[4], but not in the monkey. The usefulness of parotin has been demonstrated in human clinical treatment of various diseases as chondrodystrophia and spongylitis deformans[6]. Parotin subunit immunoreactivity has been detected in tumor cells of human salivary glands[7] and thus primate studies are important for ascertaining the properties of parotin. Therefore, the localization of parotin subunit in the salivary glands and additionally in the kidney and pancreas of the Japanese monkey, *macaca fuscata* was immunohistochemically studied.

MATERIALS AND METHODS

Tissue preparations

Parotid, submandibular and sublingual glands, kidneys and pancreas 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 organs were removed under 25 mg/kg sodium pentobarbital anesthesia before lethal over-dosing with the anesthetic, cut into pieces (approximately 1cm2 x 0.5cm blocks) and fixed in Bouin's fixative for 3 to 5 hr. The experiment was carried out with the approval of our institutional animal care committee as described previously[8]. After fixation, 4-m thick paraffin sections were prepared, deparaffinized and stained by indirect immunofluorescence, indirect immunoperoxidase techniques.

Bovine parotin subunit and rabbit anti-subunit antisera (polyclonal) were obtained from Teikoku Hormon Mfg. Co., Ltd., Tokyo, Japan. The molecular weight of the parotin subunit monomer was 45 kDa and that of the dimmer was 90 kDa on sodium SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The amino acid composition of the subunit is similar to that of bovine parotin; the main constituent amino acids were glutamic acid, aspartic acid and leucine, the N-terminal amino acid was glycine as determined by the dimethyl amino naphthalene sulfonyl chloride method, and the C-terminal amino acid was Leu. The carboxyl terminal sequence was -Val-Ser-Ala-Thr-Leu-OH as determined by digestion of carboxypeptidase A and the isoelectric point was pH 4.53).

Gel electrophoresis and immunoblots

Tissue extracts were obtained from submandibular and parotid glands homogenized in SDS-sample buffer and collected with 50% acetone. Proteins were electrophoretically transferred from gels onto nitrocellulose membranes[9, 10]. Each cross reactivity of the anti-parotin subunit antisera with parotin subunit and the extracts was examined by immunoblotting.

Immunohistochemical staining

Indirect immunofluorescence technique: Sections were immersed in phosphate buffered saline (PBS) for 10 min and then reacted overnight at 4℃ with the primary anti-serum followed by 0.01 mg/ml rabbit anti-parotin subunit serum in PBS supplemented with 0.5% bovine serum albumin (BSA)[7]. Tissues were rinsed in PBS 3 times for 5 min each and then reacted for 2 hr with the secondary antibody followed by fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin (Cappel, USA, 1:20 dilution in PBS). The tissues were rinsed again in PBS 3 times for 5 min each and mounted in glycerin jelly.

Indirect immunoperoxidase technique: Sections were immersed in PBS for 10 min, then in 0.5% periodic acid for 10 min and reacted with the primary antiserum as described above. Tissues were transferred to the secondary antibody followed by horseradish peroxidase-labeled anti-rabbit IgG (Cappel, USA, 1:50 in PBS) for 15 min. They were rinsed again in PBS 3 times for 5 min each and reacted with substrate medium composed of 20 mg 3, 3'-diaminobezidine 4 HCl in 75 ml 0.05 M tris HCl buffer (pH 7.6) containing 0.01% H2O2. The sections were washed in tap water, routinely dehydrated, cleared, and mounted in glycerin jelly.

Controls: The tissues were incubated for immunostaining with each of normal rabbit serum, PBS and antiserum preabsorbed with an excess of parotin subunit (working antiserum 11 ml: antigen 200-g) overnight at 4℃, instead of the primary antiserum.

RESULTS

Gel electrophoresis and immunoblotting Rabbit anti-bovine parotin subunit rabbit antiserum reacted with not only bovine parotin subunit but also extracts of M. fuscata submandibular and parotid glands (Fig. 1). The molecular weights of the proteins recognized in the extracts were greater than that of the bovine parotid subunit main band.

Parotid gland Immunostaining with the anti-parotin subunit antibody was observed in the duct cells: intense immunoreactivity was observed in the striated duct cells by both indirect immunofluorescence (Figs. 2a and b) and indirect immunoperoxidase techniques (Fig. 2c). In the duct cells, the apical region was most intensely stained. However, by the immunoperoxidase technique, none was observed in the intercalated duct cells (Fig. 2d).

Submandibular gland The staining observed in the ducts (Figs. 3a-g) was similar to that in the parotid gland. In part of the striated duct, the swollen apocrine-like projection into the lumen exhibited specific immunofluorescence (Fig. 3c). A few of the duct cells were unstained. These cells may have been in a refractive stage of cell cycle or be different from

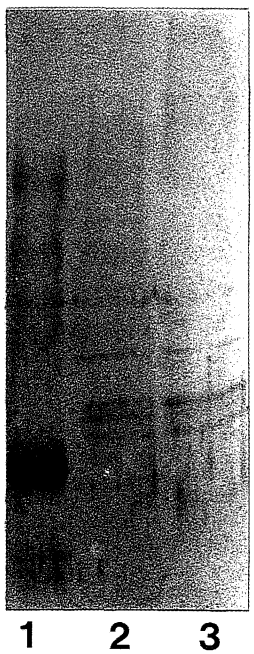
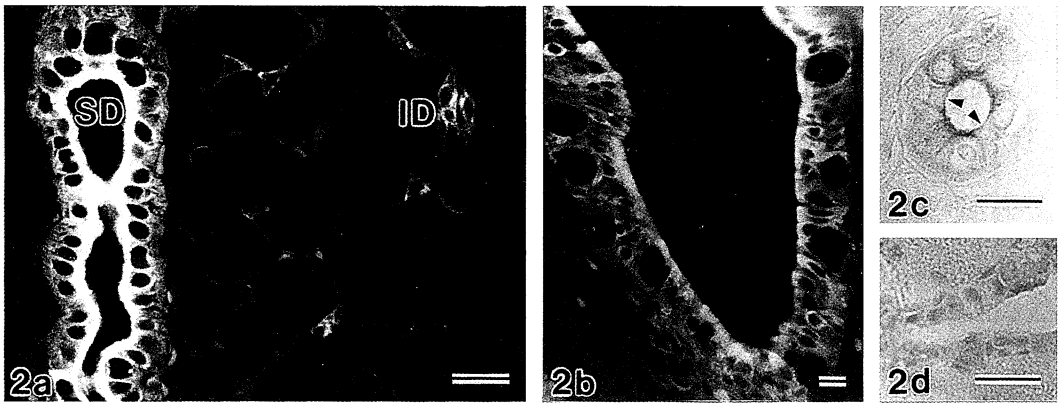
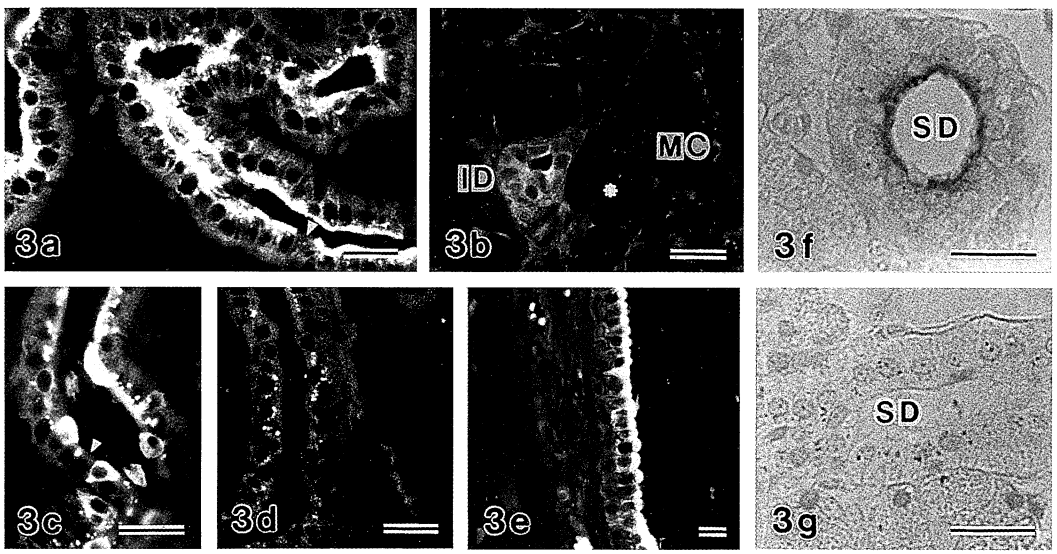


Fig. 1. Immunoblots with rabbit anti-bovine parothin subunit antiserum. The main reactive band of bovine parotin subunit (1) has a molecular weight of 45 kDa. Molecular weights of immunoreactive proteins in monkey submandibular (2) and parotid gland (3) extracts are comparatively greaterr than that of the bovine parotin subunit (1).



Figs. 2a-d. Sections of parotid gland stained by indirect immunofluorescence (a, b) and indirect immunoperoxidase (c, d) techniques. The apical region of the duct cells is intensely immunostained. a Immunoreactivity in intercalated duct (ID) is weak compared with that in striated duct (SD). b Staining intensity is reduced in excretory duct. c Specific staining is observed in the apical region of striated duct. A few duct cells are unstained (arrowheads). d Intercalated duct is unstained. *Bars*=25μm.



Figs. 3a-g. Submandibular gland stained by immunofluorescence (a-e) and immunoperoxidase (f, g) techniques. Intense staining in the apical region of duct cells was observed. A few duct cells show no reactivity (arrow-heads). a Striated ducts are long and numerous. b Intercalated duct (ID) is weakly stained. c Apoclin-like projections in the striated, swelling into luminal cavity, shows reactivity. d Control staining with anti-parotin subunit preabsorbed with parotin subunit. Non-specific fluorescense of coarse granules is observed in the supra-nucleus regions of striated duct. e Exeretory duct. f Striated duct. g Control staining using PBS instead of parotin subunit antiserum shows a lack of immunoreactivity. *Bars*=25μm.

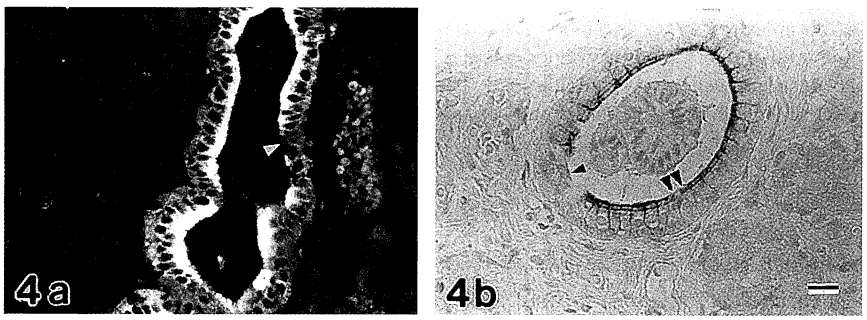
the stained cells in type.

Both controls using preabsorbed antiserum and normal rabbit serum instead of primary antiserum showed non-specific immunoreactivity in coarse granules of the supra-nucleus region in the striated duct cells[8], and did not exhibit fluorescence (Fig. 3d). Controls for specificity of the immunoperoxidase labeling in which normal rabbit serum or PBS were used, showed no specific immunoreactivity (Fig. 3g).

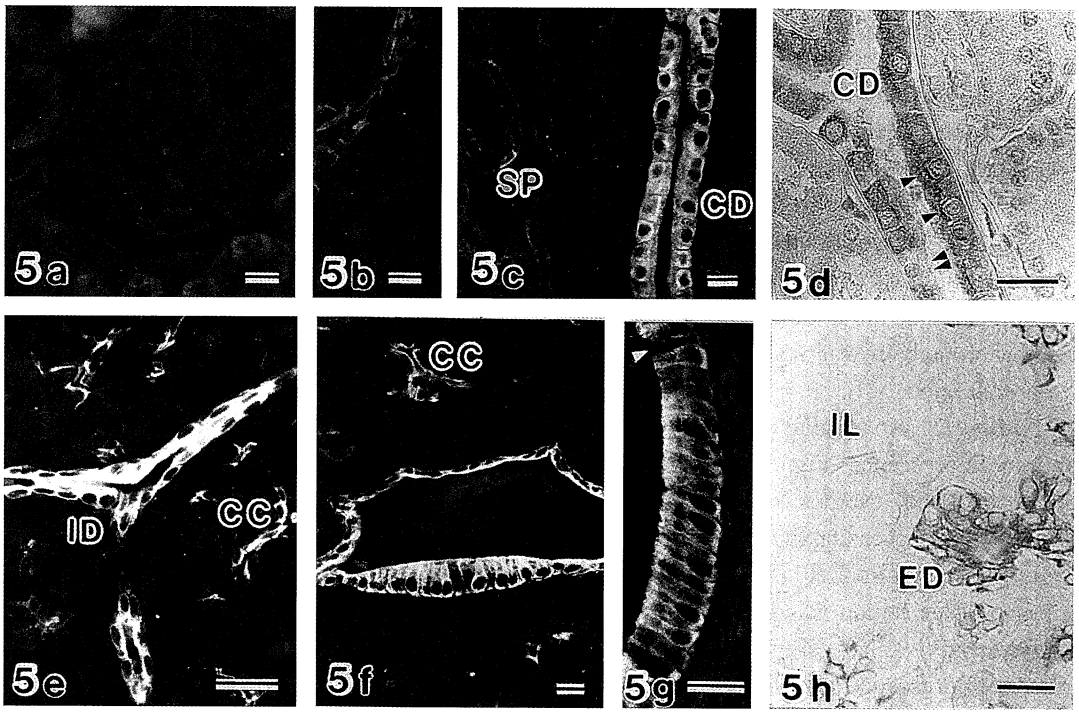
Sublingual gland The apical regions of the duct system cells were also intensely stained (Figs. 4a and b). In part of the interlobular duct, apical cytoplasm exhibited specific immunostaining in a double ring pattern, and also the supra-half of the lateral plasma membrane portion was stained using the immunoperoxidase technique (Fig. 4b). A few cells were unstained.

Kidney A similar staining pattern was observed in the kidney by both immunofluorescence (Figs. 5a-c) and immunoperoxidase (Fig. 5d) techniques. Glomeruli and of the proximal tubules were unstained (Fig. 5a). The apical region of distal tubule cells (Fig. 5b), and occasionally the plasma membranes of straight portions of proximal tubules were weakly stained (Figs. 5b and c). Collecting tubules were stained strongly, and their supra-nuclear region was often stained most intensely (Figs. 5c and d).

Pancreas No significant differences were observed between the results of immunofluorescence and immunoperoxidase techniques. Cells in the duct system such as centroacinar cells, intercalated (Fig. 5e), interlobular (Fig. 5f) and excretory (Figs. 5g and h) duct cells showed specific staining, with the apical regions of these cells showing the most intense staining. In the cuboidal or columnar cells of the interlobular and excretory duct cells, the basal portions were stained also (Figs. 5f-h). No staining reaction was observed in the acinar cells of the islets of Langerhans (Fig. 5h).



Figs. 4a, b. Sublingual gland stained by immunofluorescence (a) and immunoperoxidase (b) techniques. a Striated duct. b Immunostaining in double ring-like structure and supra-half lateral plasma membranes of apical region in an interlobular duct. Some unreastive cells are senn (arrowheads). *Bar*=25μm..



Figs. 5a-d. Kidney stained by immunofluorescence (a-c) and immunoperoxidase (b) techniques. a The glomerulus shows no reactivity. Proximal convoluted tubules are seen with no fluorescence. b Distal convoluted tubules (DC) is stained weakly in apical and very weakly in basal regions. c The supra-nuclear region of the collecting tubles (CD) show the brightest staining. Plasma membranes of straight proximal tubules (SP) show weak fluorescence. d The collecting tubule is reactive, with the supra-nuclear region most (arrowheads). An unstained cell is indicated (double aroowheads). *Bars*=25μm.

Figs. 5e-h. Pancreas stained by immunofluorescence (e-g) and immunoperoxidase (h) techniques. e The apical region of centroacinar cells (CC) and intercalated duct cells (ID) exhibit specific immunofluorescence. f The apical region of flattened cells and the whole cytoplasm of cuboidal or columnar cells in the interlobular duct exchibited fluorescence. g The whole cytoplasm of excretory duct cells are reactive. An unstained cell (arrowhead) is seen. h The islet of Langerhans (IL) is unstained, while the excretory duct (ED) near the islet is short and positively stained. *Bars*=25μm.

DISCUSSION

Immunohistochemical localization of parotin subunit has been demonstrated in duct system cells of salivary glands, kidney and pancreas of various mammalian species: parotin subunit has been shown to be localized in primates such as the human[11] and the Japanese monkey, *M fuscata*, in the present study, not only the parotid, but also submandibular and sublingual glands, and in other species such as guinea pig parotid, submandibular and sublingual glands, kidney and pancreas[4], rat parotid gland[11] and bovine parotid gland[4]; parotin has been shown to be localized in bovine parotid gland[5].

No significant differences were observed between the three major salivary glands of M. fuscata. The cause of the lack of staining reaction in intercalated ducts using immunoperoxidase in the present study of *M. fuscata* salivary glands may be attributable to

the concentration of peroxidase-conjugated secondary antibody used being low enough to discern differences in labeling intensity, and that the intensity of immunoreactivity in the intercalated ducts was low compared with that in the other ducts in this species.

The staining appeared strongest at light microscopic level in the apical region of striated duct cells in the *M. fuscata* salivary glands using all three immunohistochemical staining methods, likely in rat parotid gland[11] and similar to the case of kallikrein[8,12]. In the present case of parotin subunit, immunoreactivity was often revealed in apical double ring structure and in the supra-half lateral membrane of the sublingual gland. Parotin has been detected in the ducts forming and keratinized portions of pleomorphic adenoma in human salivary glands[4, 7,13], in which cytokeratin and intercellular matrix components such as proteoglycan are abundant[14]. Calcium induces production of proteoglycan[15] and keratinization[16,17], and Ca^{++}-ATPase has been shown to be localized on the lateral plasma membrane of these ducts[14]. Therefore, the hypocalcemic activity of parotin subunit[3] is suggested to affect keratinization and pleomorphic differentiation. On the other hand, the molecular weight of one acidic cytokeratin (type 1) in human simple epithelia is 45 kDa[15], the same as that of bovine parotin subunit. However, the isoelectric point of the 45kDa keratin is pH 5.7[19], different from that of parotin subunit (pH 4.5) which is more acidic. In addition, immunoblotting assay in the present study showed that the anti-parotin subunit antiserum reacted with proteins in the extracts of monkey salivary glands of greater molecular weights than the reacted main band of bovine parotin subunit. The proteins recognized in the monkey salivary glands may be bovine parotin subunit derivatives which are modified bovine parotin subunit with some radicals. Therefore, it is suspected that the parotin subunit-related proteins in the *M. fuscata* salivary glands cannot be identified as the 45 kDa cytokeratin.

Furthermore, strong immunoreactivity for parotin subunit was observed in the collecting tubules and weak immunoreactivity in the distal tubules and the straight portions of proximal tubules of the *M. fuscata* kidney, and has been found in distal tubules of the guinea pig[4]. Parotin subunit immunoreactivity in these duct system cells is suspected to be related to uro-parotin isolated from human urine[1]. In the *M. fuscata* pancreas, strong immunoreactivity was present in the duct system cells but not in the islets of Langerhans in the rat[11] and guinea pig[4]. Therefore, parotin subunit is supposed to participate in duct formation not only in monkey salivary glands but also in the kidney and pancreas. However, parotin subunit may participate in a variety of function[5] in various species. To clarify whether parotin subunit is synthesized or absorbed by the duct cells of these monkey organs, further study of electron-microscopic immunocytochemical localization of parotin subunit in organella on exocytosis or endocytosis in the duct cells of these monkey organs is required.

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