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Immunohistochemical distribution of S-100 protein and glial fibrillary acidic protein in normal and neoplastic salivary glands

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Summary. Immunohistochemical localization of S-100 protein, its α and β subunits, and glial fibrillary acidic protein (GFAP) in normal and neoplastic salivary glands was studied by the peroxidase-antiperoxidase method and immunoblot analysis. Positive immunostaining for S-100 protein was observed in pleomorphic adenoma, adenolymphoma, tubular adenoma, adenoid cystic carcinoma, acinic cell tumour, adenocarcinoma and carcinoma in pleomorphic adenoma. S-100 protein was localized in myoepithelial cells, epithelial cells of intercalated ducts and serous acinar cells of normal salivary gland. Both α and β subunits of S-100 protein showed almost identical distribution in normal and neoplastic salivary glands, but skeletal muscle cells were α-positive/β-negative whereas Schwann cells and fat cells were α-negative/β-positive in the stroma and neighbouring tissue. GFAP was only found in pleomorphic adenoma and its malignant counterpart. Immunoblot analysis showed that the GFAP-related antigen consisted of several polypeptide bands with a molecular weight ranging between 35,000 to 50,000 daltons.

Key words: Salivary gland – Tumours – S-100 protein – Glial fibrillary acidic protein – Immunohistochemistry

Introduction

The immunohistochemical identification of antigens with highly specific antibodies has provided important information during studies in many fields, including the diagnostic assessment of human malignancies. A variety of antigens demonstrated in salivary gland tumours have proven to be useful for diagnostic studies (McDicken and Scott 1981; Gusterson et al. 1982). S-100 protein and glial fibrillary acidic protein (GFAP) were recently added to this group of antigens (Nakazato et al. 1982b).

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S-100 protein, first isolated from the bovine brain by Moore (1965), is a low molecular weight acidic protein with calcium-binding capacity. It is a dimer of two subunit molecules, α and β, and at least three species of dimers (i.e. S-100ao ($\alpha\alpha$), S-100a ($\alpha\beta$) and S-100b ($\beta\beta$) were isolated from mammalian brain (Isobe et al. 1983). S-100 protein is localized in a wide variety of tissues and cells including normal and neoplastic salivary glands (Nakajima et al. 1982; Nakazato et al. 1983; Hara et al. 1983; Takahashi et al. 1984).

GFAP is known to be the major constituent polypeptide of glial filament, an intermediate filament exclusively found in glial cells. It is mainly found in astrocytes and ependymal cells of the brain (Yamaguchi 1980) as well as in glial cells of rat myenteric plexus (Jessen and Mirsky 1980). A few reports have described its extraneural distribution (Bock et al. 1977).

The authors have been described the distribution of S-100 protein and GFAP-related antigen in pleomorphic adenoma of the salivary glands (Nakazato et al. 1982b). In this report we demonstrate the localization of these antigens in other types of salivary gland tumours. In addition, we demonstrate the differences of distribution between the two subunits of S-100 protein, and the identity of GFAP-related antigen of the pleomorphic adenoma with GFAP of the brain.

Materials and methods

Materials. Biopsy specimens were obtained from 48 cases of salivary gland tumours which included 29 pleomorphic adenomas, 6 monomorphic adenomas (4 adenolymphomas and 2 tubular adenomas) and 13 malignant epithelial tumours (4 adenoid cystic carcinomas, 4 acinic cell tumours, 2 carcinomas in pleomorphic adenomas, 1 mucoepidermoid tumour and 1 adenocarcinoma). In addition, 9 specimens of normal salivary glands (5 submandibular glands, 2 parotid glands and 2 minor salivary glands of the palate) were collected at autopsy and used as controls. All tissues were fixed with phosphate-buffered formalin and embedded in paraffin. Sections 3 μm were used for histological and immunohistochemical staining.

Antibodies. The preparations and characterizations of antisera against S-100 protein and GFAP were previously described (Yamaguchi 1980; Nakajima et al. 1980; Nakazato et al. 1982b). A dot-immunobinding assay demonstrated that the S-100 protein antiserum reacted strongly with the S-100b and S-100a proteins and weakly with S-100ao protein (unpublished observation). Monospecific antibodies against α and β subunits of S-100 protein were prepared by affinity chromatography (Takahashi et al. 1984). Swine antiserum against rabbit IgG, and soluble complex of horseradish peroxidase/rabbit anti-horseradish peroxidase were purchased from DAKO Immunoglobulins Ltd. (Copenhagen, Denmark).

Immunohistochemistry. Immunohistochemical localization of S-100 protein, its α and β subunits, and GFAP was studied by Sternberger's peroxidase antiperoxidase (PAP) method (Nakazato et al. 1982a, b). The dilutions of antiserum and each incubation condition were as follows: anti-S-100 protein and anti-GFAP sera, 1:1,000, overnight at 4° C; anti-rabbit IgG and soluble PAP complex, 1:20, 1 h at room temperature. Monospecific antibodies were used at a final concentration of 50 μg/ml.

No specific staining was observed in control sections incubated with normal rabbit serum (1:1,000), normal rabbit IgG (50 μg/ml) and antisera absorbed with each specific antigen (1:1,000).

Immunoblot analysis. Soluble extracts of unfixed, frozen tissue of tumours were prepared from two cases of pleomorphic adenoma as described previously (extract A, Nakazato et al. 1982b). Extracts of human brain and GFAP purified from human spinal cord were also used. Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 7.5% separation and 5% stacking gel (Laemmli 1970). After the electrophoresis, the polypeptides were transferred to a nitrocellulose paper (Towbin et al. 1979) and immunostained with the PAP method using anti-GFAP antiserum.

Results

Pleomorphic adenoma

The S-100 protein has been demonstrated in the tumour cells of pleomorphic adenoma by the PAP method using conventional antiserum (Nakazato et al. 1982b). Sections incubated with monospecific antibody against the β subunit of S-100 protein showed identical immunostaining patterns as those stained with conventional antiserum (Fig. 1b). The reaction products were found in both the nuclei and cytoplasm of tumour cells. Monospecific α antibody also stained the tumour cells, but the reaction was less intense than that with the β subunit antibody or conventional antiserum (Fig. 1a).

GFAP was also found in all cases of pleomorphic adenoma (Fig. 1c). The identity of the GFAP-related antigen of pleomorphic adenoma and the GFAP of the brain was confirmed by the immunoblot analysis. The GFAP purified from human spinal cord was separated into several bands with molecular weight between 38,000 to 51,000 daltons (Fig. 1d). In 2 cases of pleomorphic adenoma, 6 to 7 polypeptide bands were positively stained with GFAP antiserum. Their molecular weights ranged between 35,000 and 51,000 daltons.

Monomorphic adenomas

In the adenolymphoma S-100 protein was found in some of lymphoid tissue cells but not in the epithelial elements. When sections were incubated with either conventional antiserum or β subunit antibody, the immunoreactivity was observed in dendritic reticulum cells outside the germinal center. However, α subunit-positive cells were located mainly in the germinal center. Neither epithelial nor lymphoid tissues were positive for GFAP.

Tumour cells of tubular adenoma were immunostained for S-100 protein. The staining intensity varied from cell to cell; some had intense immunoreactivity, whereas others were only weakly positive or completely negative. Monospecific antibody against β subunit stained duct lining cells as well as outer-layered cells (Fig. 2b). In contrast, granular reaction deposits produced with α subunit antibody were confined to the cytoplasm of outer-layered cells (Fig. 2a). Tumour cells and stroma were negative for GFAP.

Malignant epithelial tumours

In adenoid cystic carcinomas, a layer of cuboidal cells was positively stained with conventional S-100 antiserum as well as β subunit antibody (Fig. 2d). Tumour cells lining large cystic spaces or cells arranged at the periphery

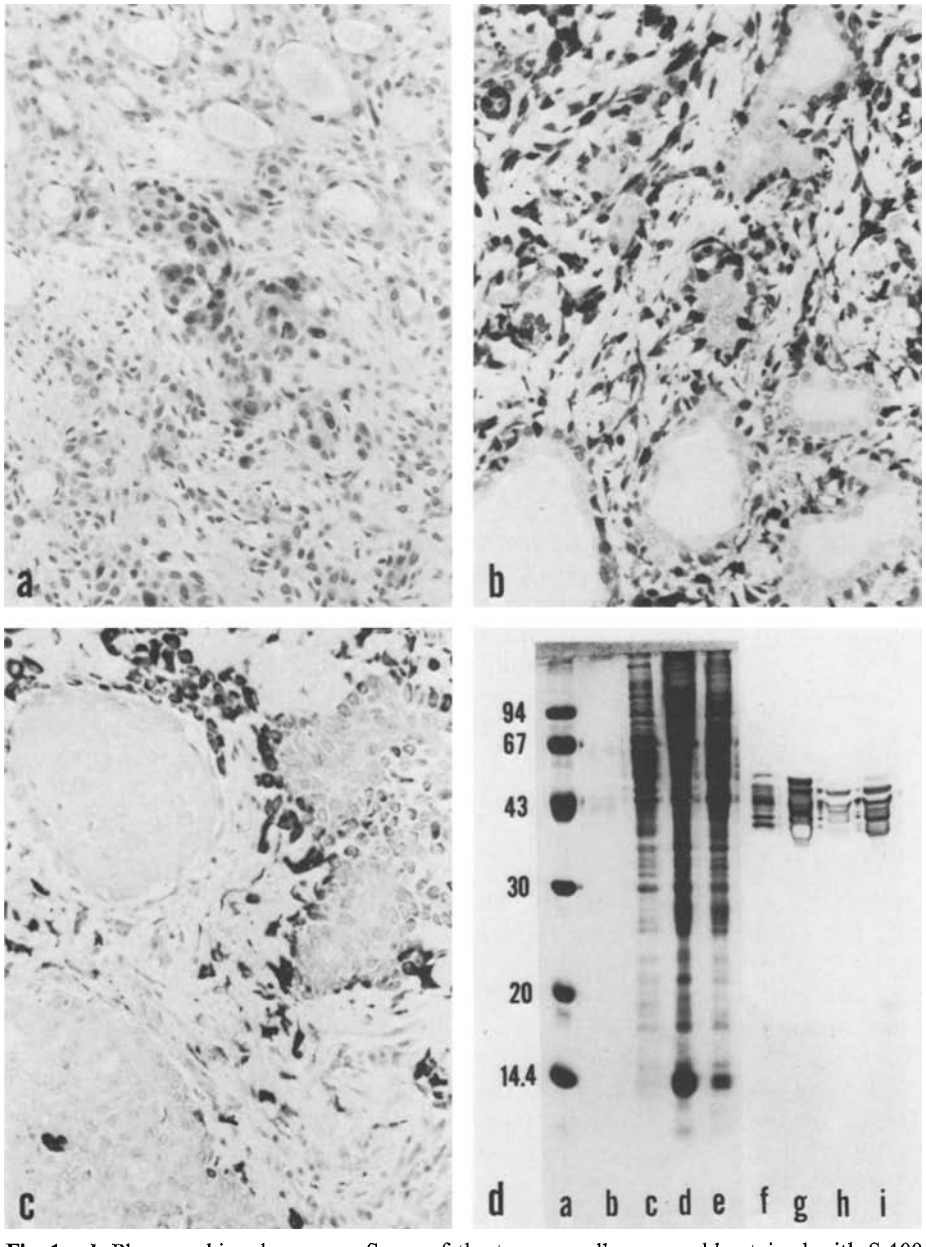

Fig. 1a–d. Pleomorphic adenoma. **a** Some of the tumour cells are weakly stained with S-100 protein α subunit antibody. **b** Intense immunoreactivity for S-100 protein β subunit is found in tumour cells, but duct lining cells are negative. **a** and **b** Immunoperoxidase and haematoxylin, $\times 210$. **c** Some of the tumour cells are positive for GFAP. GFAP-immunoperoxidase and haematoxylin, $\times 210$. **d** Immunoblot analysis. *lane a:* molecular weight standard, *lane b* and *f*: purified GFAP, *lane c* and *g:* extract of human brain, *lane d, e, h* and *i:* extracts of pleomorphic adenoma. *lane a–e:* SDS-PAGE gel stained with Coomassie, *lane f–i:* immunoblot stained with anti-GFAP serum.

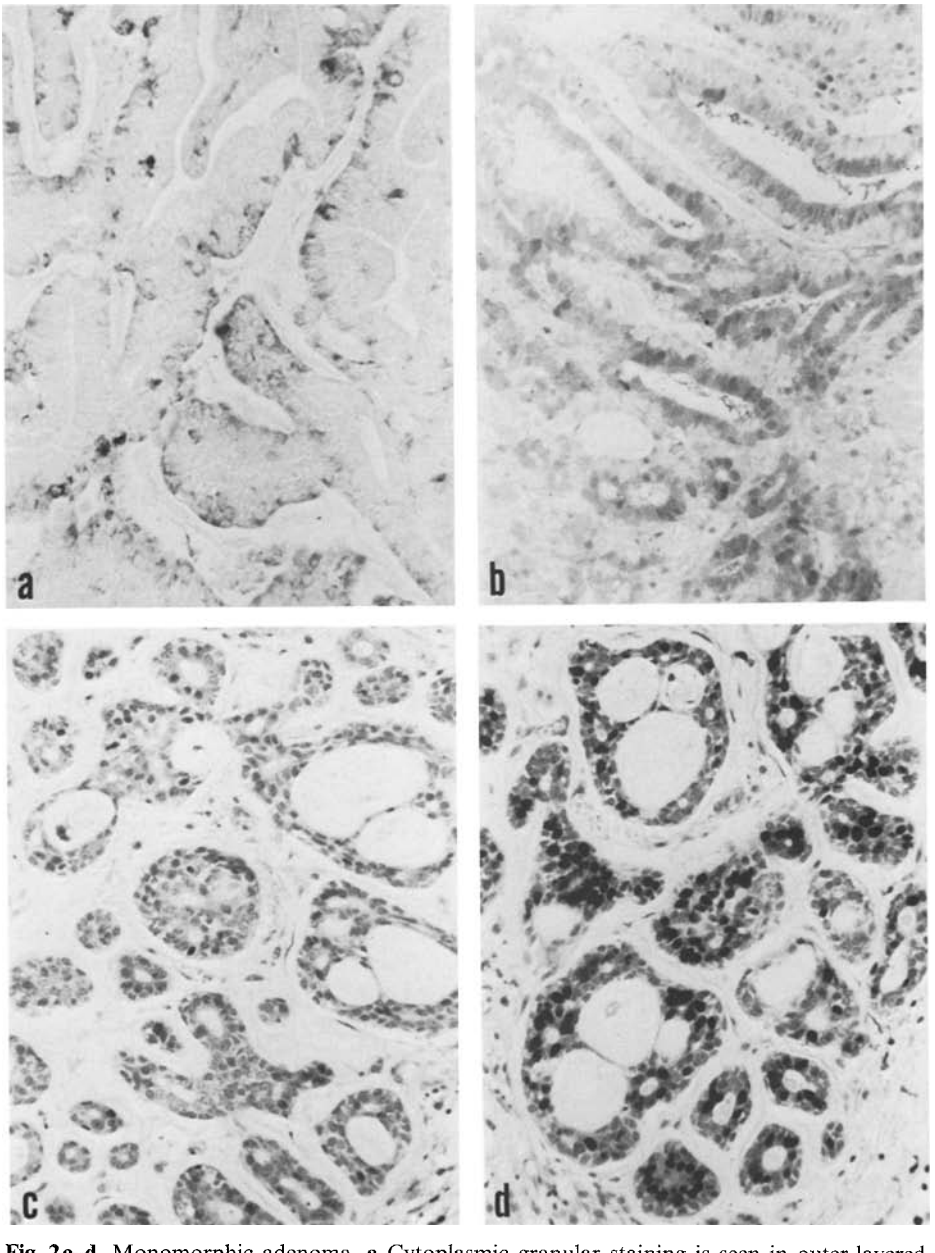

Fig. 2a–d. Monomorphic adenoma. **a** Cytoplasmic granular staining is seen in outer-layered cells. S-100 α-immunoperoxidase, × 220. **b** Duct lining cells are weakly immunostained with S-100β subunit antibody. Immunoperoxidase, × 220. **c** and **d** Adenoid cystic carcinoma stained with S-100 α subunit (**c**) or β subunit (**d**) antibodies. Almost identical immunostaining is seen, but the intensity with α subunit antibody is less than that with β subunit antibody. **c** and **d** Immunoperoxidase and haematoxylin, × 200

of tumour cell nests however, showed no immunoreactivity for S-100 protein. Identical immunostaining was obtained with monospecific α antibody, but the reaction was less intense (Fig. 2c). GFAP did not stain tumour cells.

Four cases of acinic cell tumour were examined. The tumour cells of 2 cases had positive immunostaining for S-100 protein (Fig. 3a). Both the nuclei and cytoplasm of the cells were stained with S-100 protein antiserum and β subunit antibody. α subunit antibody stained the cytoplasm of the tumour cells weakly. Cells in the other 2 cases remained negative for S-100 protein. GFAP immunoreactivity was not detected.

Neither the mucin-producing cells nor cells with squamous differentiation in the mucoepidermoid tumour had any immunoreactivity for S-100 protein or GFAP (Fig. 3b).

Tumour cells of the adenocarcinoma were positively stained with S-100 protein antiserum as well as α and β subunit antibodies (Fig. 3c, d). GFAP was not found in tumour cells.

The two cases of parotid gland tumour were characterized by the coexistence of atypical cell nests and benign myxomatous or chondroid areas. Atypical cells were arranged in irregular tubules or solid sheets and showed definite pleomorphism (Fig. 4a). There were many mitotic figures in these areas. On the basis of histological features, a diagnosis of carcinoma in pleomorphic adenoma was made. Atypical epithelial cells did not show any immunoreactivity for S-100 protein and GFAP. Spindle-shaped or stellate cells in the myxomatous area, however, demonstrated intense immunoreactivity for both S-100 protein and GFAP (Fig. 4b and c).

Normal salivary glands

In the major and minor salivary glands, myoepithelial cells were intensely immunostained with S-100 β subunit antibody. A weak staining was also observed in epithelial cells of intercalated ducts and in serous acinar cells (Fig. 5b). The epithelial cells of striated ducts and mucinous acinar cells were consistently negative. In the stroma of salivary glands and neighbouring mesenchymal tissues, Schwann cells of the peripheral nerve and fat cells were intensely stained with β subunit antibody (Fig. 5d). α subunit immunoreactivity was found in the epithelial cells of intercalated ducts and occasionally in serous acinar cells (Fig. 5a). This staining was less intense than of the β subunit antibody. An intense immunostaining of the α subunit was observed in striated muscle cells of the surrounding mesenchymal tissues, however, Schwann cells and fat cells were negative (Fig. 5a and c).

Immunostaining for GFAP was not detected in normal salivary glands or in the surrounding mesenchymal tissues.

Discussion

The results of this investigation indicate that several types of salivary gland tumour contain S-100 protein and that GFAP is restricted to the pleomorphic adenoma and its malignant counterpart.

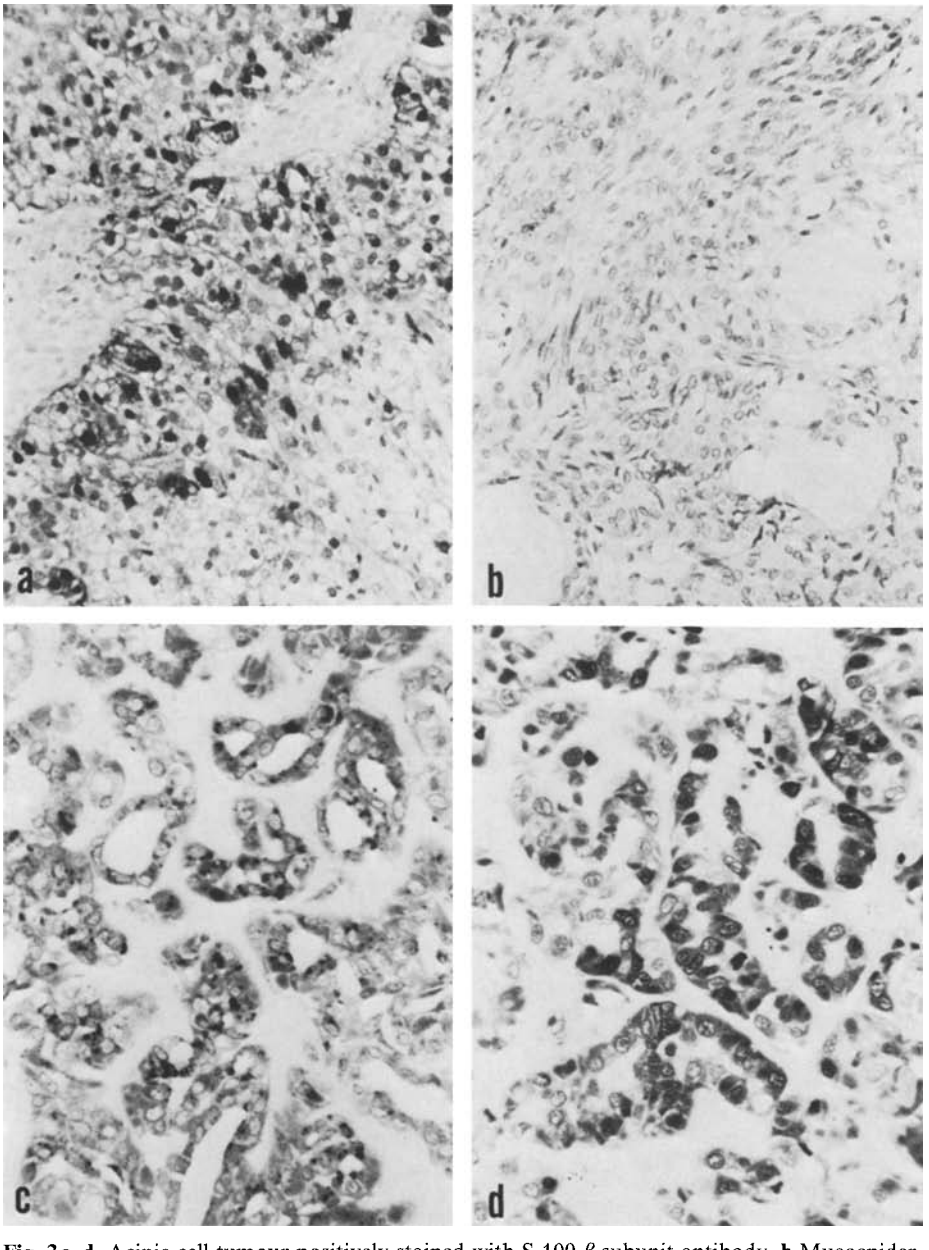

Fig. 3a–d. Acinic cell tumour positively stained with S-100 β subunit antibody. **b** Mucoepidermoid tumour is negative for S-100 protein. **a** and **b** Immunoperoxidase and haematoxylin, $\times 200$. **c** and **d** adenocarcinoma stained with S-100 α subunit (**c**) or β subunit (**d**) antibodies. Cytoplasmic granular staining is observed in **c** and diffuse cellular staining in **d**, **c** and **d** Immunoperoxidase and haematoxylin, $\times 250$

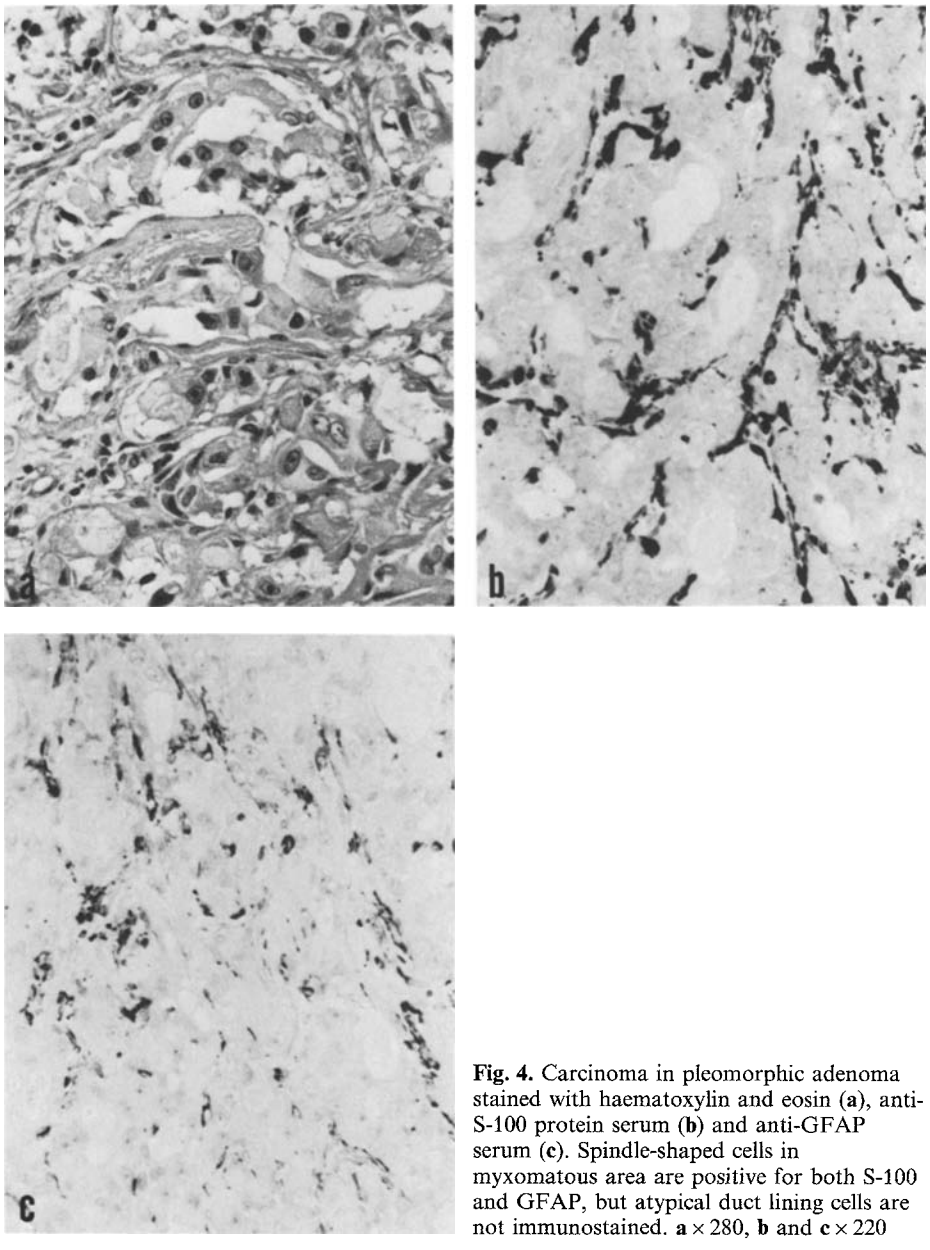

Fig. 4. Carcinoma in pleomorphic adenoma stained with haematoxylin and eosin (**a**), anti-S-100 protein serum (**b**) and anti-GFAP serum (**c**). Spindle-shaped cells in myxomatous area are positive for both S-100 and GFAP, but atypical duct lining cells are not immunostained. **a** × 280, **b** and **c** × 220

In normal salivary glands, myoepithelial cells showed strong immunoreactivity for the S-100 β subunit antibody and were weakly stained with the α subunit antibody. In addition, a weak immunoreactivity for both α and β subunits was detected in epithelial cells of intercalated ducts and in occasional serous acinar cells.

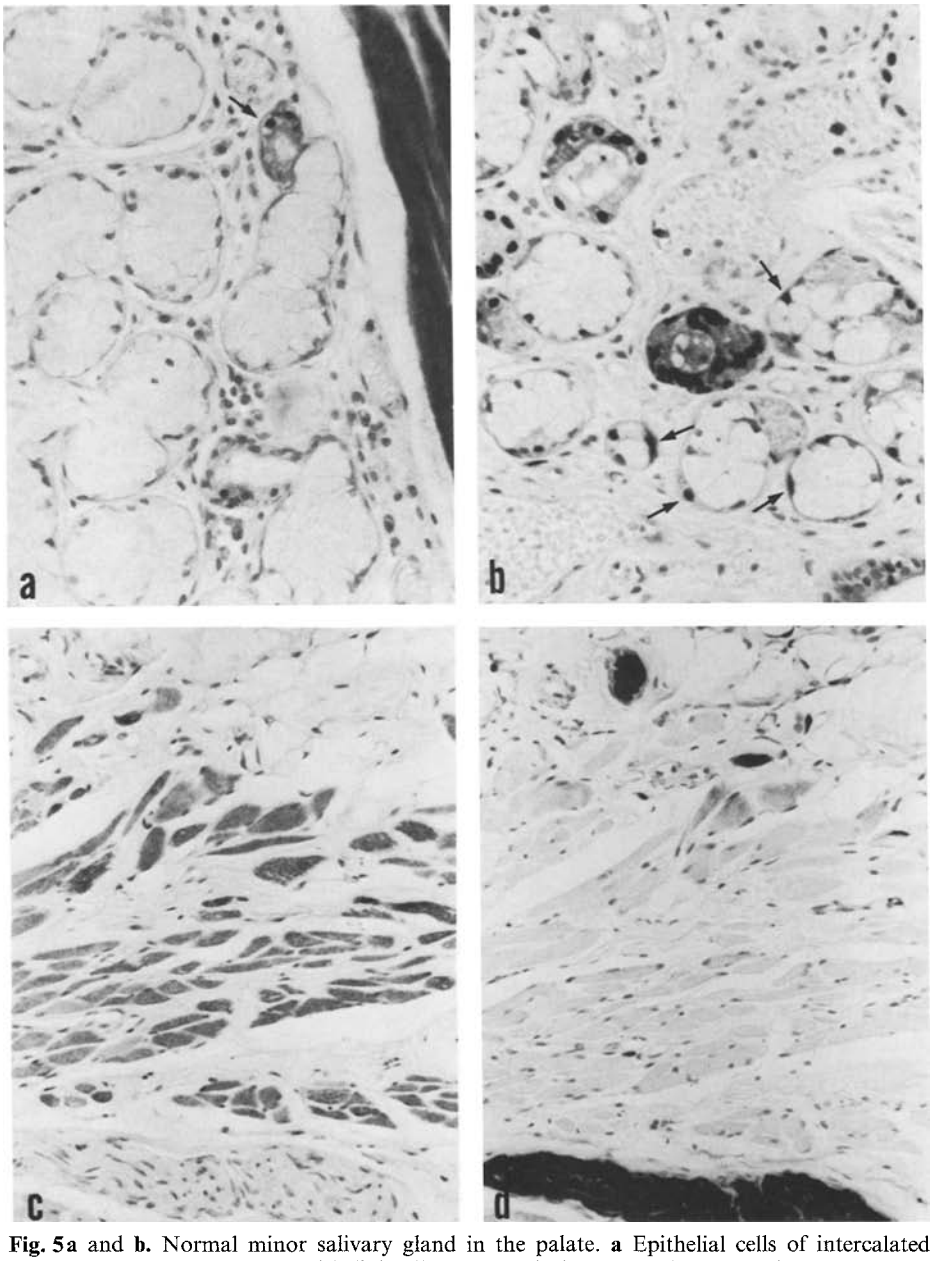

Fig. 5a and **b.** Normal minor salivary gland in the palate. **a** Epithelial cells of intercalated duct (*arrow*) and a few myoepithelial cells are weakly immunostained with S-100 α subunit antibody. Skeletal muscle cells (*top right*) are intensely positive. **b** Myoepithelial cells (*arrow*) and duct lining cells of intercalated duct are positive for β subunit. **a** and **b** Immunoperoxidase and haematoxylin, $\times 230$. **c** Skeletal muscle cells are positive for α subunit, but Schwann cells (*bottom*) and fat cells (*top*) remain negative. **d** Schwann cells and fat cells are immunostained with β subunit antibody, but muscle cells (*center*) are negative. **c** and **d** Immunoperoxidase and haematoxylin, $\times 180$

S-100 protein was found in pleomorphic adenoma, adenolymphoma, tubular adenoma, adenoid cystic carcinoma, a part of acinic cell tumour, adenocarcinoma and carcinoma in pleomorphic adenoma. Mucoepidermoid tumour did not contain S-100 protein. Although the distribution of α and β subunits had many similarities, the immunoreactivity for the α subunit was less intense than for the β subunit. The reaction products of the α subunit antibody were granular and mainly located in the cytoplasm. β subunit antibody immunostained both the nuclei and cytoplasm of tumour cells. The pattern of immunostaining with conventional antiserum was almost identical with that observed with the β subunit antibody. This suggests that the antigenicity of the β subunit may predominate over that of the α subunit and play a major role in the immunized rabbits to produce antiboedies against S-100 protein.

With respect to the implications of immunohistochemical localization of S-100 protein in salivary gland tumours, the applicability to histogenetic and diagnostic assessment of tumours should be considered. It is reasonable to assume, on the basis of the normal distribution of S-100 protein in salivary glands, that tumours arising from myoepithelial cells may contain S-100 protein. Indeed, the results presented here as well as those of others (Nakajima et al. 1982; Nakazato et al. 1982b; Hara et al. 1983) agree with this assumption. In addition to the tumour cells of myoepithelial linage, S-100 protein was also found in duct lining cells of tubular adenoma, adenoid cystic carcinoma and adenocarcinoma. This observation, on the basis of the presence of S-100 protein in duct lining cells of the intercalated ducts, seems to give additional evidence of the origin of these tumours from the intercalated ducts.

S-100b protein has been reported to be found in normal and neoplastic myoepithelial cells, with immunohistochemical and immunoelectron microscopic studies, using monospecific anti-S-100b antibody (Hara et al. 1983). However, epithelial cells of the intercalated ducts and acinar cells were negative for S-100b protein. The difference in the staining pattern of Hara and this study may be attributed to the difference of immunohistochemical method used. While the sections were incubated overnight with the first antiserum at 4° C in our method, Hara et al. incubated sections for only 30 min. Since prolonged incubation with the first antiserum significantly increases the sensitivity of immunoperoxidase staining (unpublished observation), our method seems to make the weak immunoreactivity of the intercalated duct cells and serous acinar cells to S-100 protein antibodies visible.

The distribution of the S-100 subunits in adenolymphoma correlates well with that of normal lymph nodes in which the α subunit-positive cells are found in the germinal center and the β subunit immunostaining is observed in the dendritic reticulum cells of the thymus dependent area (Takahashi et al. 1984).

There were remarkable differences in the immunostaining of α and β subunits in the stromal tissue. Schwann cells and fat cells were positive for the β subunit and negative for the α subunit. In contrast, striated muscle cells were positive for the α subunit and negative for the β subunit. It

is possible that the S-100 protein in striated muscle is an α homodimer comprised of two α subunits (S-100ao). There has, however been no report on the distribution of S-100 protein in striated muscle. As conventional rabbit antiserum against S-100 protein reacts weakly with the S-100ao protein; and the immunostaining of striated muscle cells with rabbit antiserum is very weak, it seems possible that this staining reaction was considered to be negative by earlier workers.

The GFAP-immunoreactivity was found in benign and malignant pleomorphic adenomas. It is necessary to resolve whether the GFAP-related antigen of pleomorphic adenoma is identical to the GFAP of glial cells. The GFAP-related antigen contained 6 to 7 polypeptide bands with molecular weights of 35,000 to 51,000 daltons. This immunoblot pattern was almost identical with that of human GFAP isolated from spinal cord according to the method of Dahl and Bignami (1975). Tumour cells of pleomorphic adenoma contain intermediate-sized filaments in the cytomplasm (Mills and Cooper 1981) and are positively immunostained with anti-keratin and anti-vimentin antibodies (Caselitz et al. 1981). Nevertheless, five classes of intermediate filaments have been shown to share a common antigenic determinant (Pruss et al. 1981). However, the GFAP antiserum used in this study specifically immunostains glial cells but never reacts with neurons, epithelial cells, mesenchymal cells or muscle cells (Yamaguchi 1980; Nakazato et al. 1982b). Thus it is not probable that the keratin type or vimentin type of intermediate filaments in the adenoma cells cross-reacted with the GFAP antibody. It is suggested that the GFAP-related antigen of the pleomorphic adenomas is identical with the GFAP of glial filaments. The lower molecular weight polypeptides of the GFAP-related antigen may be degradation products of 51,000 dalton polypeptide. This possibly occurs during tissue processing for biochemical analysis, because the human GFAP purified from normal or pathological brain has been reported to be prone to automatic degradation producing several low molecular weight components (Dahl and Bignami 1975).

The presence of a glial type of antigen in pleomorphic adenomas might be related to the peculiar differentiating potential of tumour cells of the myoepithelial cell linage. The immunohistochemical demonstration of GFAP gives us an another criterion for the diagnosis of salivary gland tumours, and is especially useful for the differential diagnosis of benign and malignant pleomorphic adenomas from other types of tumours.

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