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Immunohistochemical distribution of human epidermal growth factor in salivary gland tumours*

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Summary. Immunohistochemical identification of human epidermal growth factor (hEGF) was carried out in a total of 152 cases of salivary gland tumours, consisting 107 pleomorphic adenomas and their variants, 13 adenolymphomas and 32 adenoid cystic carcinomas. A high percentage of pleomorphic adenomas revealed markedly positive hEGF staining of the luminal surface cells of tubuloductal structures and of modified or neoplastic myoepithelial cells. Clear cells of the tumour showed various reactivities from very slight to strong. Eosinophilic epithelial cells of adenolymphoma gave a positive reaction for hEGF in all the cases, whereas most adenoid cystic adenoma lacked hEGF staining; however some cases showed positive staining of the tumour cells. The immunohistochemical detection of hEGF in most salivary gland tumours suggests this factor to be a possible new marker of salivary glands tumours, and to have a biological role in tumour proliferation.

Key words: Human epidermal growth factor – Immunohistochemistry – Salivary gland tumours

Introduction

The biologically active peptide, urogastrone (UG) was isolated and purified from human urine and its amino acid sequence has been determined. The active components of UG have been designated as β and γ UG. They are each approximately 6000 daltons and consist of a single polypeptide chain with 52(γ) or 53(β) amino acid residues (Cohen et al. 1975). It has now been accepted that UG and hEGF are the same. Biochemical identification of hEGF/UG in tissue and fluid has been done by radioimmunoassay (Starkey and Orth 1977), radioreceptor assay, and enzyme immunoassay techniques (Hayashi et al. 1985; Kurobe et al. 1986). In those quantitative methods, polyclonal hEGF/UG antiserum obtained by using highly purified hEGF/UG isolated from human urine has usually been employed (Gregory 1975; Dailey et al. 1978; Elder et al. 1978; Heitz et al. 1978; Hirata and Orth 1979a, b; Kasselberg et al. 1985; Tsukitani et al. 1987a). Recently, hEGF was synthesized by the synthetic gene technique with the use of *E. coli,* and monoclonal antibody against hEGF (Oka et al. 1985) and polyclonal hEGF antiserum generated in response to biosynthesized hEGF as an immunogen have also been employed (Gregory 1975; Heitz et al. 1978; Tsukitani et al. 1987b). The primary structure and biological activity of human epidermal growth factor (hEGF) are reported to resemble to those of mouse EGF (mEGF), which is confined to granular convoluted tubule cells in mouse submandibular glands (van Noorden et al. 1977; Barka 1980; Gresik et al. 1981; Mori et al. 1983).

Immunohistochemical localization of hEGF/UG in previous papers has been controversial: Elder et al. (1978) noted that hEGF could be detected in ductal cells of submandibular and Brunner's glands; on the contrary, Heitz et al. (1978) and Kasselberg et al. (1985) have demonstrated hEGF to be present in acinar cells in salivary glands. Also Sato et al. (1985) reported that, neoplastic intercalated duct cells obtained from human submandibular glands and grown in tissue culture release hEGF into serum-free medium. All of the above studies have been employed hEGF antiserum generated with highly purified hEGF/UG from human urine. A recent report has noted the

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detection of hEGF in ductal segments of salivary glands by the use of such monoclonal and polyclonal antibodies (Tsukitani et al. 1987b).

The present report is the first to describe the detection of hEGF, using monoclonal antibody and polyclonal anti-hEGF antiserum generated in response to biosynthetic hEGF as immunogen, in large numbers of different kinds of salivary gland tumours.

Materials and methods

A total of 152 cases of salivary gland tumours were obtained from surgery or biopsy. Some of the tumour specimens had normal glandular structures attached. The following types of neoplastic lesions were examined: pleomorphic adenomas (107 cases), adenolymphomas (13 cases), and adenoid cystic carcinomas (32 cases). The tumour materials were fixed with 10% formalin solution, and embedded in paraffin after dehydration. Specimens were cut at a 4 μm thickness for use in the detection of hEGF as well as for routine histology with haematoxylin-eosin (HE) staining.

hEGF was synthesized by the synthetic gene technique with the use of *Escherichia coli* (*E. coli*): Oka et al. (1985) reported the insertion of the synthetic gene coding for hEGF into the Hind III restriction site of secretion vector pTA 1529 and secretion and proper processing of the resultant hybrid protein by *E. coli*. This protein was used as immunogen for the production of polyclonal anti-hEGF antiserum in rabbits, and monoclonal antibodies in mice. The monoclonal antibody was obtained from ascites fluid of Balb/c mice that had been injected with a hybridoma formed by the fusion of mouse myeloma cell (P3UI) with spleen cells from a Balb/c mouse immunized with the synthesized hEGF. The detail of the procedure has been already reported (Tsukitani et al. 1987b).

1. Peroxidase anti-peroxidase (PAP) method for rabbit polyclonal anti-hEGF antiserum. The sections were immersed in 0.3% hydrogen peroxide/methanol solution for 30 min for inactivation of endogenous peroxidase, and rinsed well in phosphate-buffered saline (PBS). They were then immersed in normal swine serum (1:20) for 30 min, incubated with rabbit anti-hEGF antiserum (1:10) as first layer for 1 h, and rinsed three times in PBS, 5 min each time. The sections were next incubated with swine anti-rabbit IgG (1:20) as second layer for 30 min, and rinsed three times with PBS. Sections were finally treated with peroxidase anti-rabbit peroxidase complexes (1:100) for 30 min, rinsed with PBS, and immersed in 0.03% 3-3'diaminobenzidine hydrochloride (DAB) containing 0.005% hydrogen peroxide for 10 min.

2. Indirect method for monoclonal antibody to hEGF. Following the blocking of endogenous peroxidase and immersion of normal rabbit serum (1:20), the sections were incubated with monoclonal antibody (1:10) for 1 h. After being rinsed three times in PBS, 5 min each time, they treated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (1:20) for 30 min, and rinsed with PBS. They were then treated with the DAB solution.

3. Control method. An absorption test was carried out by the following procedure: instead of the usual immunohistochemical reagent for the first layer, polyclonal hEGF antiserum which had been preincubated overnight with hEGF was used on the sections. Negative or obviously reduced staining was obtained as a result of this procedure. Immunohistochemical reagents were applied by the dipping method for each layer. All procedures were carried out at room temperature.

Results

In normal salivary glands immunohistochemically detectable hEGF was confined to the ductal systems of salivary glands of formalin-fixed sections, and the stainings was particularly strong in the striated duct cells, and less so in the intercalated duct cells. Treatment of sections with polyclonal hEGF antibody fielded a positive reaction in blood capillary walls and smooth muscle fibers; whereas with the monoclonal antibody, these elements were usually unreactive (Fig. 1a).

Pleomorphic adenomas of the salivary gland were occasionally accompanied by obstructive changes in the otherwise normal regions of the gland. Obstructive alterations in non-neoplastic areas may have occurred as a result of tumour expansion, and the histology was essentially the same as that found in obstructive sialadenitis due to sialolithiasis, that is, there was generally acinar atrophy associated with ductal changes manifesting as degeneration or ductal dilation. hEGF staining was restricted to ductal segments, whose pathological changes were not marked (Fig. 1b). Lesions in the final stage of the disorder revealed the absence of hEGF in degenerated ductal cells, as already reported.

The histological constitution of pleomorphic adenomas varies greatly with respect to the proportions and types of proliferating neoplastic cells, which are thought to be of intercalated duct or

Table 1. hEGF distribution in normal salivary glands and pleomorphic adenomas

		hEGF staining
Normal glands	Duct cells	+
	Acinar cells	−
Pleomorphic adenomas	a) Tubuloductal structure	
	Luminal cell	+
	Outer cell	
	spindle cell	−
	dendrical cells in myxomatous area	+
	b) Glandular structure	+ − ±
	c) Solid epithelial focus	+ − ±
	d) Squamous metaplastic epithelium	− − ±

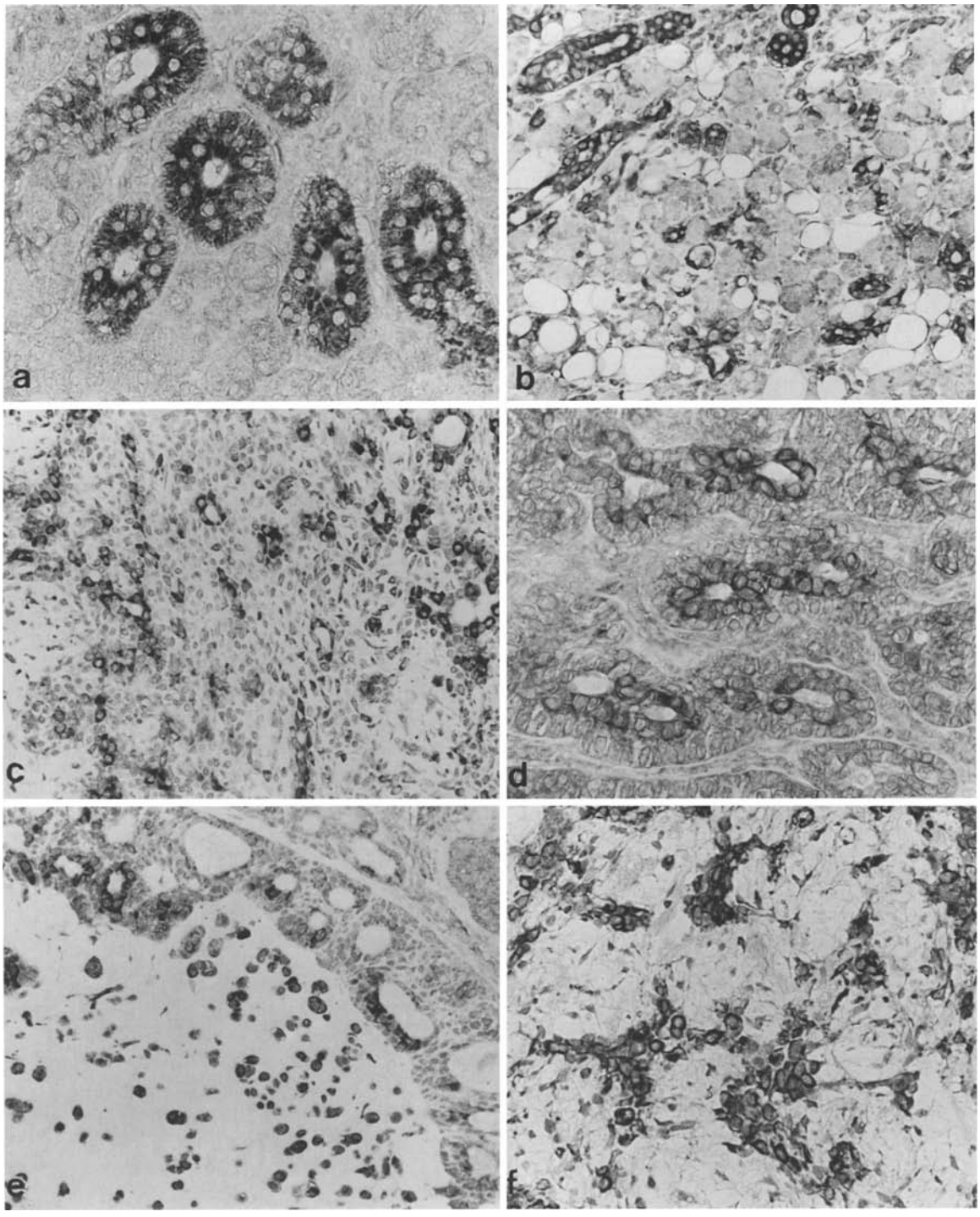

Fig. 1. a Immunohistochemical detectable hEGF in human submandibular gland. 10% formalin fixed paraffin section monoclonal hEGF antibody. hEGF staining is confined strongly to striated duct cells, weakly to intercalated duct cells (×90). **b** Obstructive lesion accompaning pleomorphic adenoma in the parotis. Acinar and ductal elements are generally atrophic, hEGF staining shows ductal segments, striated and intercalated ducts (×90). **c** Pleomorphic adenoma. hEGF staining in typical histology of pleomorphic adenoma. hEGF decoration is mainly limited in the luminal surface cells of tubular structure, but myoepithelial cells are devoid of hEGF staining (90). **d** Pleomorphic adenoma. hEGF reaction with slight levels is present in luminal side tumour cells, but it is not outer tumour cells (×180); and **f** Variant of pleomorphic adenoma. **e** hEGF deposition is confined to scattered tumour cells, and some luminal cells of the structure (×90). **f** Highly staining to hEGF is recognized in tumour cells which are grouped in hyalinous region (×180)

myoepithelial cell origin. The typical architecture of salivary pleomorphic adenomas consists of cords, nest and strands, with occasional solid foci; consisting with two types of neoplastic cells, one is luminal surface cells of tubular and duct-like structures and other is outer layered cells showing spindle or long process in shape. As for their progenitors, the former are of intercalated duct origin; and the latter, of myoepithelial origin.

The round, cuboidal, and columnar luminal surface cells of the tubular and duct-like structures, demonstrated a comparatively high concentration of hEGF; however, the flattened tumour cells gave a lower level of staining for hEGF. The outer layer tumour cells, of myoepithelial origin, showed either a slight reaction or none (Fig. 1c).

Squamous metaplasia or differentiation was also indicated in tubular and duct-like containing structures, and such neoplastic cells displayed no marked staining for hEGF.

The glandular type of pleomorphic adenoma is composed of two cellular rows in their glandular arrangement; both cells of luminal and outer layers are round or cuboidal in shape. hEGF staining was evident in luminal cells with various intensities from the strongest to slight, but it was not so prominent in the outer row of tumour cells (Fig. 1d).

The tubulo-glandular type of adenoma is mainly composed of large simple adenoma cells without accompanying modified myoepithelial cells. hEGF raction was distinct in these tumour cells; however, most of them were unreactive. In hyaline ares there were irregular tumour cell islands and these cells were polyhedral or with a plump cytoplasm. This type of tumour cell revealed more intense staining for hEGF than the other hEGF-positive cells. In rare cases of pleomorphic adenoma, some tumour cells were scattered singly or gathered throughout the hyaline region and indicated conspicuous staining with hEGF (Fig. 1e, f). These freely situated tumour cells were accompanied by luminal side cells with duct-like structure.

Many cases of pleomorphic adenoma revealed spindle-shaped cells with long anastomozing process located at the outer side of tubular and duct-like structures. This type of spindle or dendritic tumour cell showed wide variation in hEGF staining; i.e. some cells gave an almost negative reaction, whereas others displayed moderate-to-strong reactions for hEGF (Fig. 2a–c). In rare instances, these cells of myoepithelial origin, or modified myoepithelial cells, showed the highest level of hEGF staining seen in the study. Large spindle tumour cells also indicated wide variation in hEGF deposition. hEGF-positive cells were located in central areas of modified myoepithelial foci; however, outer layered tumour cells of tubular and duct-like structures, which were interconnected with spindle tumour cells (modified myoepithelial cells), did not give so prominent a reaction for hEGF. Cells having undergone chondroidal changes in hyalinous areas gave positive hEGF staining (Fig. 2d).

Myoepitheliomas or myoepithelial adenomas usually showed moderate hEGF staining, though rarely a weak reaction was noted (Fig. 2e). Clear cell adenoma or clear cell variants of pleomorphic adenoma indicated two different expressions of hEGF staining: some clear cells gave positive hEGF staining, while others were negative. These clear cell adenomas showed a mixed pattern of hEGF-positive and -negative cells, and these cells could not be indistinguished from one another by routine histological staining (Fig. 3a, b). Small tubular or duct-like structures in clear cell variants of the tumour showed strong staining for hEGF, and all the clear cells lacked hEGF. In the cases of the small cell type of clear cell adenoma, the clear cells displayed a slight hEGF reaction in their hydrolytic cytoplasm (Fig. 3c).

All specimens of adenolymphoma were strongly reactive to hEGF in their eosinophilic tumour epithelia, although some of the epithelial cells were not hEGF positive. The apical side of lymphoepithelial cells displayed abundant staining for hEGF (Fig. 3d).

Many cases of adenoid cystic carcinoma (ACC) showed slight or negative responses to hEGF staining in their tumour cells. However, there were some intensely reactive cells scattered throughout the tumour focus in some cases. Large cribriform patterns of this tumour contained small tubular structures indicating strongly positive staining for hEGF in pumped cells located at their luminal side. Small nest of ACC lesions all showed a slightly positive hEGF reaction (Fig. 3e, f).

Discussion

A similar distribution of EGF has been recognized in salivary glands of mice and humans. In the normal mouse submandibular gland, mEGF is confined to the granular convoluted tubules as one type of ductal system (van Noorden et al. 1977; Barka 1980; Mori et al. 1983) and in human salivary glands, hEGF is present mainly in striated duct and intercalated duct cells. Homologous cells of pleomorphic adenoma of salivary glands have

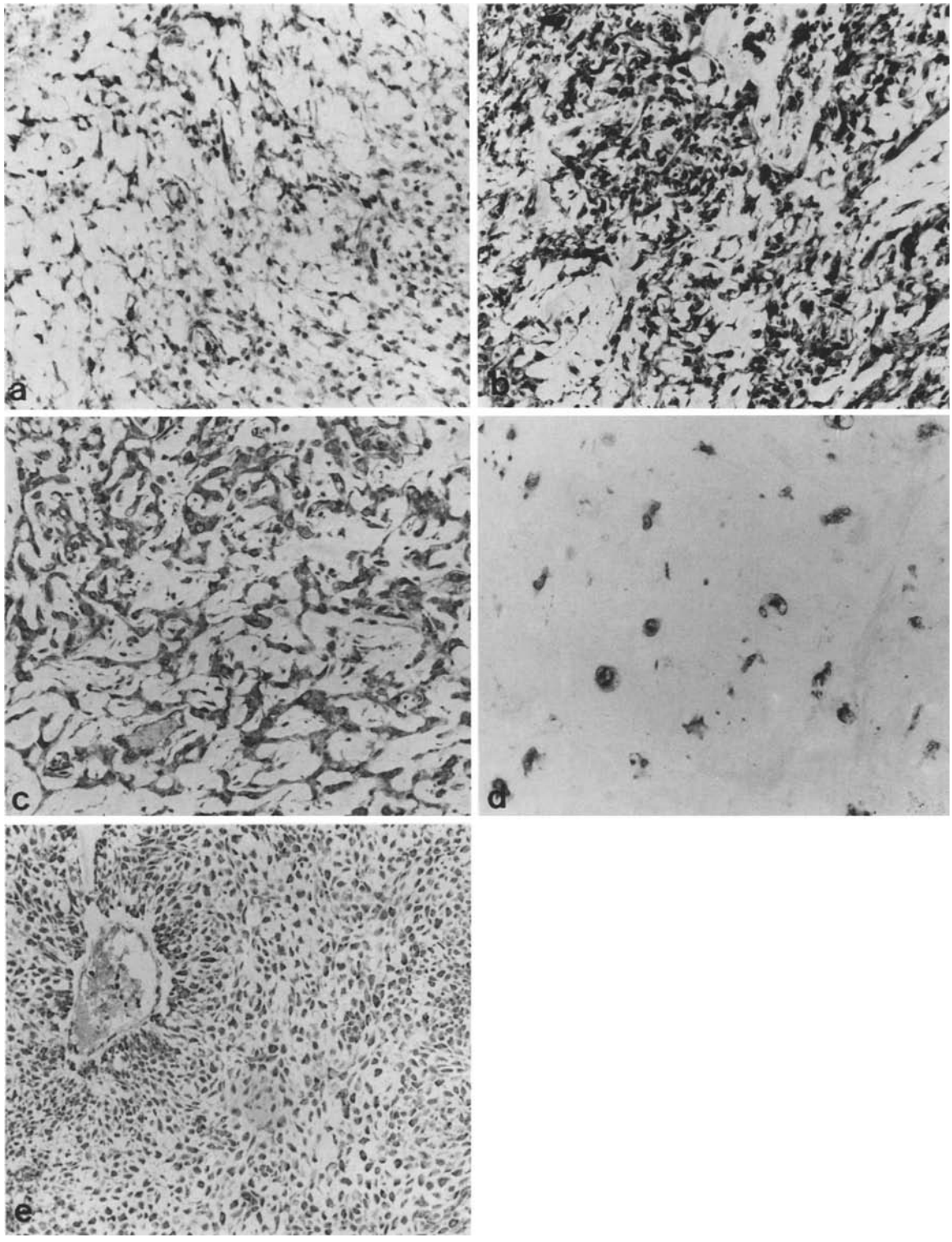

Fig. 2a–e. Modified, transformed and neoplastic myoepithelial cells. (×90) **a** Modified myoepithelial cells in pleomorphic adenoma show positive hEGF staining. **b** Transformed myoepithelial cells display the strongest hEGF staining. **c** Large spindle type of modified myoepithelial cells in pleomorphic adenoma indicates strongly positive reaction to hEGF; **d** Chondroidal changed cells. Cytoplasm of chondrocytes has contained strongly positive staining. **e** Myoepithelioma. All the myoepithelioma cells show long spingle in shap, and hEGF staining is positive to myoepithelioma cells

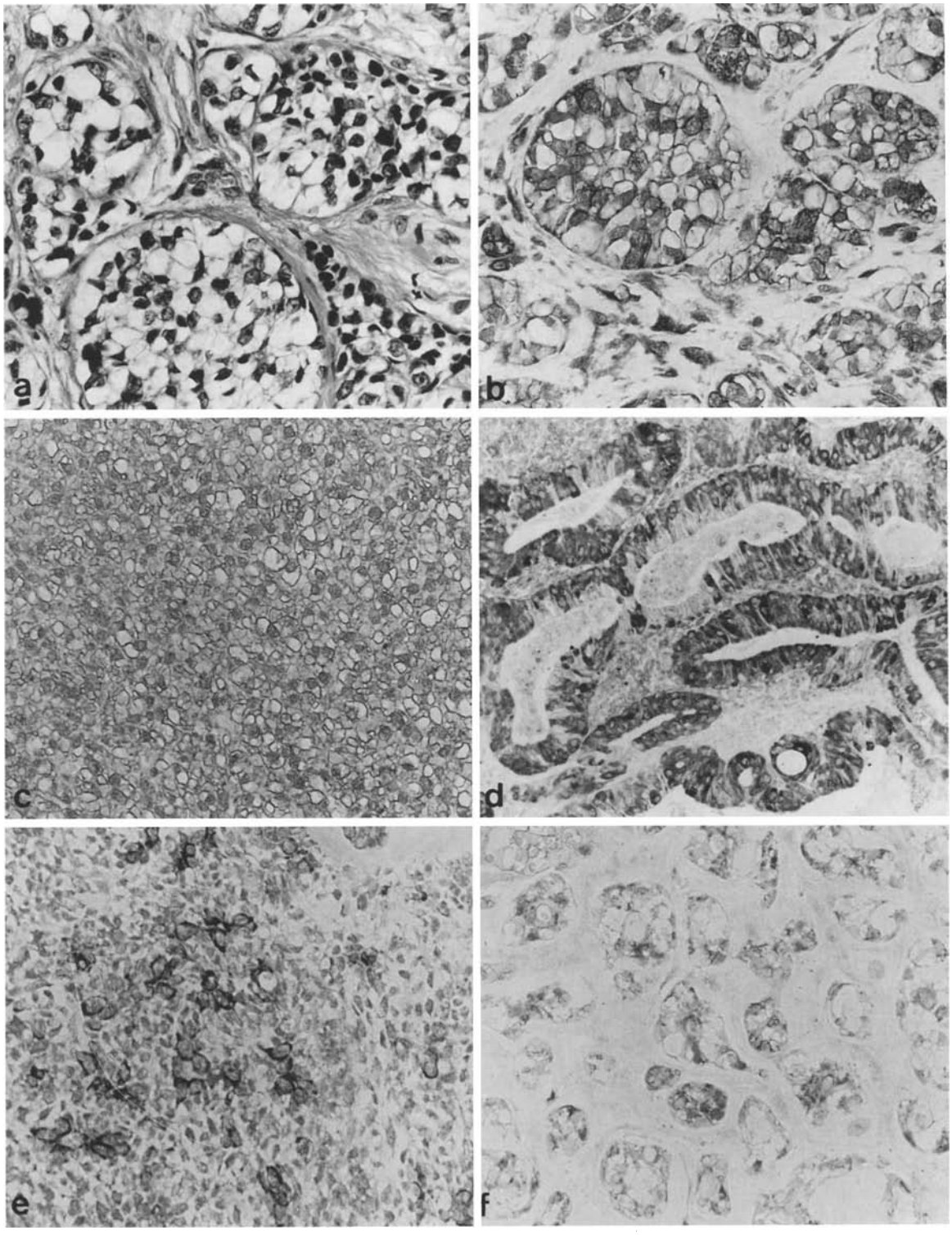

Fig. 3a, b. Clear cell adenoma or clear cell variant of pleomorphic adenoma. **a** Clear cell adenoma. HE stain (×180). **b** Serial section of Fig. 3a. Clear tumour cells show positive and negative evident to hEGF staining (×180); **c** Clear cell adenoma. Small cell type. hEGF staining is slight in whole clear tumour cells (×90). **d** Adenolymphoma. hEGF deposition is mainly restricted in eosinophilic epithelial cells, however, some epithelial cells are devoid of hEGF reaction (×90). **e, f** Adenoid cystic carcinoma (×90). **e** Large focus of adenoid cystic carcinoma display markedly positive to hEGF, which arranged micro tubular structure scattered in the foci. **f** Small focus of the tumour indicates very slight staining to hEGF

been accepted to be of two kinds: intercalated duct and myoepithelial cells (Seifert 1966; Seifert 1972; Thackrang and Lucas 1974; Dardick et al. 1983a, b). Typical pleomorphic adenomas are histologically composed of tubulo-ductal structures whose luminal surface cells may be of ductal origin and whose outer spindle cells are probably of myoepithelial origin. Immunohistochemically detectable hEGF was found in luminal cells, but not in the outer spindle cells. These findings of hEGF decoration correspond to those of normal salivary glands. Spindle or dendritic tumour cells of myoepithelial origin have shown different expression; negative or positive reactions are found. In general, those spindle tumour cells associated with luminal cells were devoid of hEGF staining, however, reactive or modified myoepithelial cells gave a comparatively high level of staining for hEGF. Neoplastic myoepithelial cells also showed a relatively high degree of staining. Why such modified or transformed myoepithelial cells contained hEGF of various concentrations is unclear. Chondroidal cells in those lesions were hEGF-positive, such finding was first notable in hEGF distribution. In this regard, the tumour foci containing modified myoepithelial cells, carried out proteoglycan synthesis, thus giving rise to the complex carbohydrates in the matrix of the hyalinous and myxomatous stroma. In tissue culture studies, fibroblasts have been shown to synthesize EGF (Kurobe et al. 1985). Modified myoepithelial cells in pleomorphic adenoma probably have their biological properties altered toward those of mesenchymal rather than epithelial cells, thus accounting for the presence of EGF in these cells.

Clear cell adenoma or the myoepithelial adenoma variant of pleomorphic adenoma showed hydropic or clear cytoplasm, and their cells are thought to be of myoepithelial origin (Hamperl 1970; Goldman and Klein 1972). Numerous studies of this type of benign salivary tumour have concluded it to be a glycogen-rich adenoma. The amount of hEGF in such clear tumour cells, or in hydropic myoepithelial cells, was found to vary over a wide range, as in the case of modified or transformed myoepithelial cells in pleomorphic adenomas. However, tubular structures found in myoepithelial adenomas were characterized by strong hEGF staining, indicating a probable ductal origin of its composite tumour cells.

The histogenesis of adenolymphoma, also called papillary cystadenoma lymphomatosum, most probably involves the ductal epithelium of the salivary gland or branchiogenic lymphoid tissues. In the present study, hEGF was concentrated in the eosinophilic epithelial cells of the tumour, and this feature suggests that the origin of tumour epithelial cells may be the ductal segments, and that the tumour cells themselves may produce hEGF.

Immunohistochemical detection of hEGF in adenoid cystic carcinomas occurred in low incidence; however, some specimens showed positive hEGF staining in a limited number of tumour cells located in pseudotubular structures in the tumour focus. The histogenesis of adenoid cystic carcinoma is also strongly suggested to involve intercalated duct and myoepithelial cells (Kleinsasser et al. 1969) as is the case for pleomorphic adenoma. Staining for hEGF in these tumours was rather slight in general, but in rare cases an abundance of hEGF was detected. This finding suggests that tubulo-ductal cells in adenoid cystic carcinoma may be of ductal origin, whereas slight or negative hEGF staining tumour cells may arise from myoepithelial cells.

In obstructive lesions, both mEGF and hEGF are reduced in or absent from degenerated ductal segments following experimental ductal ligation in the mouse submandibular gland (Takai et al. 1985; 1986) and due to obstructive sialadenitis in humans, respectively (Tsukitani et al. 1987a). The main source of biosynthesis of EGF is the ductal cell, not the myoepithelial cells, in both mouse and human salivary glands.

There are reports that experimental carcinomas of mouse submandibular glands showed decreased mEGF activity in epithelial cells transformed from ductal segments, although normal intact granular convoluted tubule cells display abundant mEGF staining (Mori et al. 1984). In submandibular gland carcinogenesis in mice, granules of convoluted tubule cells were initially reduced in number and finally disappeared and premalignant and tumour epithelial cells were devoid of mEGF staining (Tatemoto et al. 1985). But these experimental designs with EGF immunohistochemical studies are not comparatible to those human salivary gland tumours with variant histological features.

The significance of EGF production in human neoplastic tissues, both malignant and benign, is not yet understood. Recent studies on hEGF distribution have been noted in gastric cancer and Brunneroma. Tahara et al. (1986) stated that in their immunohistochemical and radioimmunoassay studies of gastric tumour tissue from patients with carcinoma of the stomach, the patients who were highly positive for hEGF had a much poorer prognosis than those who were hEGF-negative. Rüfenacht et al. (1986) first noted that "Brunneroma"

of the duodenum contains a high level of hEGF/urogastrone in the acinar and some other cells, and they suggested that the "Brunneroma" is probably not a true neoplasm but rather a hamartomatous growth. The present result suggest that hEGF is biosynthesized in luminal tumour cells and occasionally in modified myoepithelial cells in pleomorphic adenoma and that it may play some biological role particularly as a mitogen in tumour growth.

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