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Phenotypic expression of human epidermal growth factor in foetal submandibular gland and pleomorphic adenoma of salivary gland

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Summary. The phenotypic expression of the human epidermal growth factor (EGF) was investigated immunohistochemically in human foetal submandibular glands from the 5th to 10th month of gestation, adult normal submandibular glands and 48 cases of pleomorphic adenomas. In foetal submandibular glands, both the terminal buds and primary ducts at the intermediate stage of gestation were positive for EGF, and in particular, the outer layer cells of primary ducts showed strong EGF-immunoreactivity. EGF-positive cells decreased as the gestational stage advanced and only ductal cells were weakly positive for EGF at the terminal stage of gestation. In the adult normal submandibular gland, weak immunoreactivity for EGF was restricted to ductal cells. However, 41 (86%) of the 48 pleomorphic adenomas had EGF-positive cells which were distributed among the ductal, chondroid and myxoid portion. No EGF-immunoreactivity was detected in the solid portion of pleomorphic adenomas. These results suggest that EGF may play an important role in the growth and differentiation of foetal cells as well as the proliferation of tumour cells in pleomorphic adenomas.

Key words: Human epidermal growth factor – Foetal submandibular gland – Pleomorphic adenoma – Immunohistochemistry

Introduction

Mouse epidermal growth factor (EGF) which was first isolated from the male submandibular gland (Cohen 1962), is a polypeptide containing 53 amino acids (Cohen and Savage 1974; Cohen and Tailar 1974). Human β-urogastrone, a potent inhibitor of gastric acid secretion (Gregory 1975), was subsequently isolated from human urine and is probably identical to human EGF (Cohen and Carpenter 1975; Starkey et al. 1975). The actions of EGF are mediated through its specific cell surface receptor, a glycoprotein, with a molecular weight of 170 kDa, which is homologous in some respects to the avian erythroblastosis virus v-*erb* B transforming protein (Cohen et al. 1982; Downward et al. 1984). EGF has been known to stimulate the proliferation and differentiation of a great variety of cells in vitro and in vivo (Carpenter and Cohen 1979; Yeh et al. 1981). However, the site of production of EGF has not been fully elucidated. In mice, a large quantity of EGF has been shown to be produced in the granular convoluted tubule cells in the submandibular gland (Gresik and Barka 1977; Noorden et al. 1977). A significant amount of mouse EGF has also been detected in milk (Cohen and Taylor 1974) and a small quantity has been found in the kidney, stomach and parotid gland (Cohen and Savage 1974). Many reports have dealt with the localization of human EGF producing cells with the discovery of many discrepancies. Elder et al. (1978) have confirmed the presence of immunoreactive human urogastrone/EGF-containing cells in the submandibular gland and Brunner's gland of the duodenum. Immunoreactive human EGF has been also found in the saliva, sweat, milk, plasma and urine (Starkey and Orth 1977; Hirata and Orth 1979a,b; Dailey et al. 1978). Recently, we have demonstrated that human EGF producing cells are scattered in various tissues including the thyroid gland, renal tubule and salivary gland duct (Sumiyoshi et al. 1986). However, the phenotypic expression and biological significance of EGF in foetal submandibular gland

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and salivary gland tumour are still unknown. In the present study, we examined the expression of EGF in various developmental stages of the foetal submandibular gland and pleomorphic adenomas of the salivary gland. We used the anti-human EGF antibody immunohistochemically in order to clarify the biological role of EGF, during the development of the salivary gland and, in the proliferation of the salivary gland tumour.

Materials and methods

In total, 19 cases of human foetal submandibular glands (4 cases in the 5th month of gestation, 5 cases in the 6th, 2 cases in the 7th, 3 cases in the 8th and 5 cases in the 10th), 20 cases of adult normal submandibular glands and 48 cases of pleomorphic adenomas were examined. All of these tissues were fixed in 10% neutral formalin and embedded in paraffin.

The anti-human EGF antibody, which was developed by injecting highly purified human EGF, prepared from a genetic-engineered E. coli host, into New Zealand white rabbits, was kindly supplied by Wakunaga Pharm. Co. (Hiroshima, Japan). Anti-human EGF rabbit serum purified by human EGF affinity chromatography has been shown to be human EGF specific, and to have no cross reactivity with other peptides including mouse EGF, human transforming growth factor α, platelet derived growth factor, fibroblast growth factor, secretin, insulin, glucagon, cholecytokinin and endorphin (Tahara et al. 1986a).

A modification of the immunoglobulin enzyme bridge technique was employed, as described by Hsu et al. (1981). Deparaffinized sections (4.5 μm) were treated consecutively for more than 30 min with: (1) anti-EGF antiserum at room temperature, (2) biotinylated anti-rabbit IgG goat antiserum (diluted 1:100, Vector Laboratories, Inc., USA), and (3) avidin DH-biotinylated horseradish peroxidase complex (diluted 1:50, Vector Laboratories, Inc., USA). We then stained with peroxidase for 10–20 min using a solution of 30 mg of 3,3′-diaminobenzidine-tetrahydrochloride in 100 ml of 50 mM Tris-HCl (pH 7.6) containing 0.0001% H_2O_2. The sections were counterstained with 3% methylgreen. The specificity of the reaction was determined by the method of Sternberger: 1) anti-EGF antiserum was absorbed at 4° C for 24 h with human EGF; 2) non-immune rabbit serum was used, at the same time as the first rabbit serum was used, as the first layer; and 3) 3,3′-diaminobenzidine-tetrachloride on H_2O_2 was omitted from the incubation mixture for the peroxidase reaction. The EGF-immunoreactivity in the tissue was graded as follows: Tissue with more than 50% of cells having immunoreactivity was graded + + +, 25–50% + +, less than 25% +, and negative −.

Results

The results of immunohistochemical staining of EGF in foetal and adult submandibular glands are summarized in Table 1. At the 5th month of gestation, the structure of the submandibular gland was morphologically still immature and the constituent cells were only recognized as cell clusters, that is, they were composed of terminal buds and primary ducts only (Fig. 1a). At the 7th month of gestation, myoepithelial cells were detected around the ducts and immature acini (Fig. 1c), and at the 10th month, most of the submandibular glands were fully differentiated (Fig. 1e). Immunohistochemically, EGF-positive cells were detected in foetal submandibular glands throughout the gestational stage. At the 5th month of gestation, most of the constituent cells of both terminal buds and primary ducts were positive for EGF, and in particular, the outer layer cells of primary ducts showed strong immunoreactivity for EGF (Fig. 1b). At the 7th month of gestation, EGF-immunoreactive cells decreased, and the ductal cells of the terminal tubules were positive for EGF (Fig. 1d). However, at the terminal stage of gestation, weak EGF-immunoreactivity was restricted to ductal cells (Fig. 1f). Weak immunoreactivity for EGF was also detected in ductal cells in one-half of the adult normal glands (Fig. 2a).

Table 1. Localization of EGF-immunoreactive cells in foetal and adult submandibular glands

	Localization	EGF-immunoreactivity[a]	
Foetus	5–6 month	Terminal buds	+ +
		Primary ducts	+ + +
	7–8 month	Ductal cells	+ ~ + +
	10 month	Ductal cells	+
Adult		Ductal cells	+

[a] EGF-immunoreactivity in tissue was graded + to + + + as described in "Materials and methods"

Table 2 shows the incidence of EGF-immunoreactivity in the tumour cells of pleomorphic adenomas. Out of the 48 pleomorphic adenomas, 41 (86.8%) showed EGF-immunoreactivity (Fig. 2b–d). EGF-positive cells in pleomorphic adenomas were widely distributed in both the epithelial and mesenchymal components including the ductal, chondroid and myxoid portion, but no EGF-immunoreactivity was detected in the solid portion, which consists of cell masses, such as, fusiform, polygonal, and stellate cells that could not be distinguished as either ductal or myoepithelial cells (Thackray and Lucas 1974).

Discussion

Immunohistochemical studies using the anti-urogastrone antibody have revealed the presence of EGF in the human submandibular gland. Many reports were made in which urogastrone immunoreactivity was found in both acinic cells and ductal cells (Heitz et al. 1978; Poulsen et al. 1986; Kasselberg et al. 1985). In the present study, using anti-

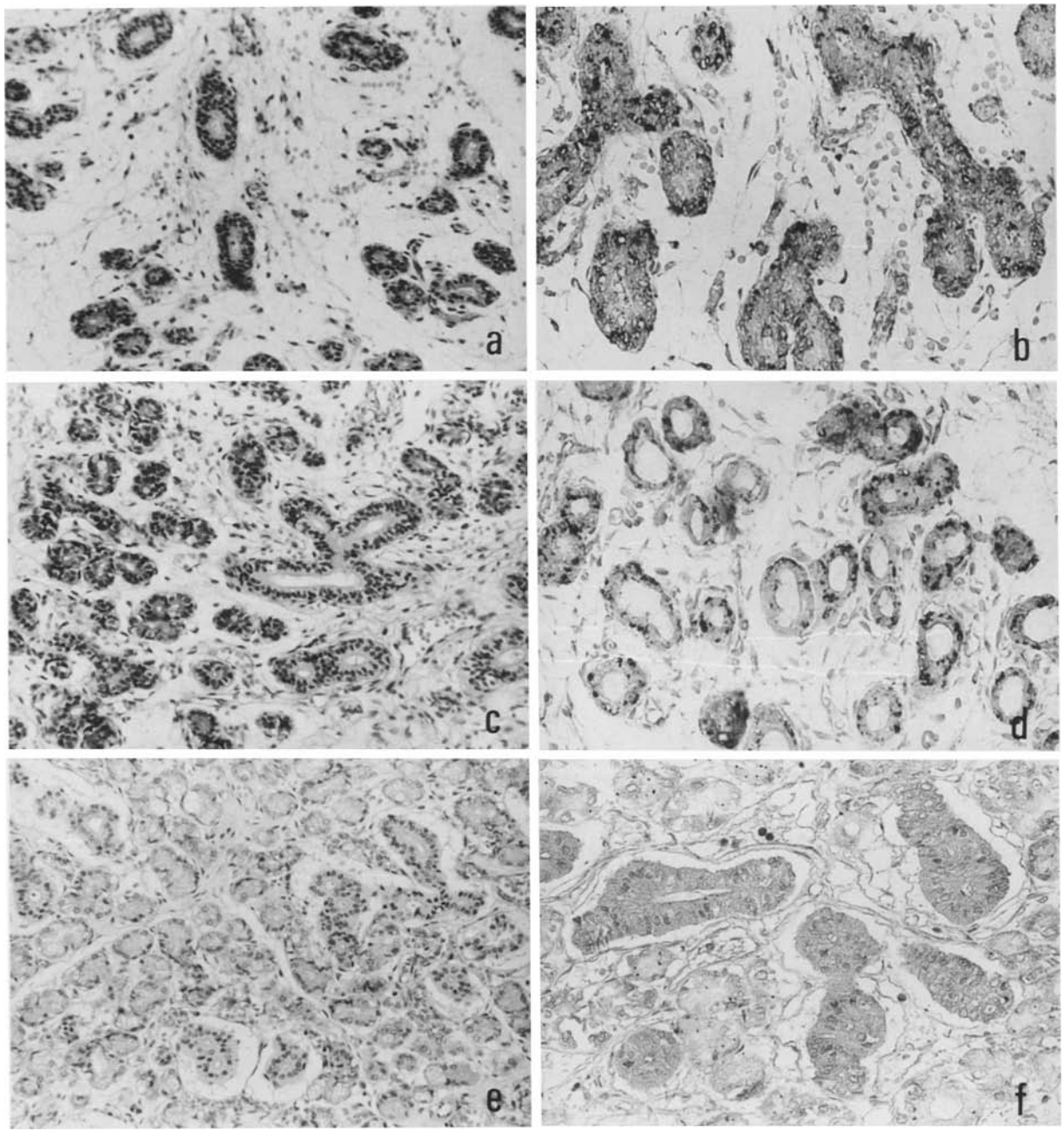

Fig. 1a–f. Foetal submandibular gland. **a, b** 5th month of gestation: All of the constituent cells, especially the outer layer cells of primary ducts, show strong EGF-immunoreactivity (**a**, H&E staining ×180; **b** immunostaining for EGF ×280). **c, d** 7th month of gestation: Some of the ductal cells of terminal tubule are positive for EGF (**c**, H&E staining ×180; **d**, immunostaining for EGF ×280). **e, f** 10th month of gestation: Weak EGF-positive cells are restricted to ductal cells (**e**, H&E staining ×180; **f**, immunostaining for EGF ×280)

human EGF antibody, the presence of EGF was restricted to ductal cells, but not to acini of the submandibular gland. These findings are divergent from those of 3 previous reports. We reasoned that this discrepancy may be caused by the difference of antibodies. Anti-urogastrone antibody was made against urogastrone purified from human urine and has the possibility of cross-reaction with human tissues; whereas the anti-human EGF antibody used in this study cannot, because human EGF was derived from genetic-engineered E. coli. Sumiyoshi et al. (1986) have examined the tissue content of EGF by radioimmunoassay (RIA) as well as the localization of EGF immunohistochem-

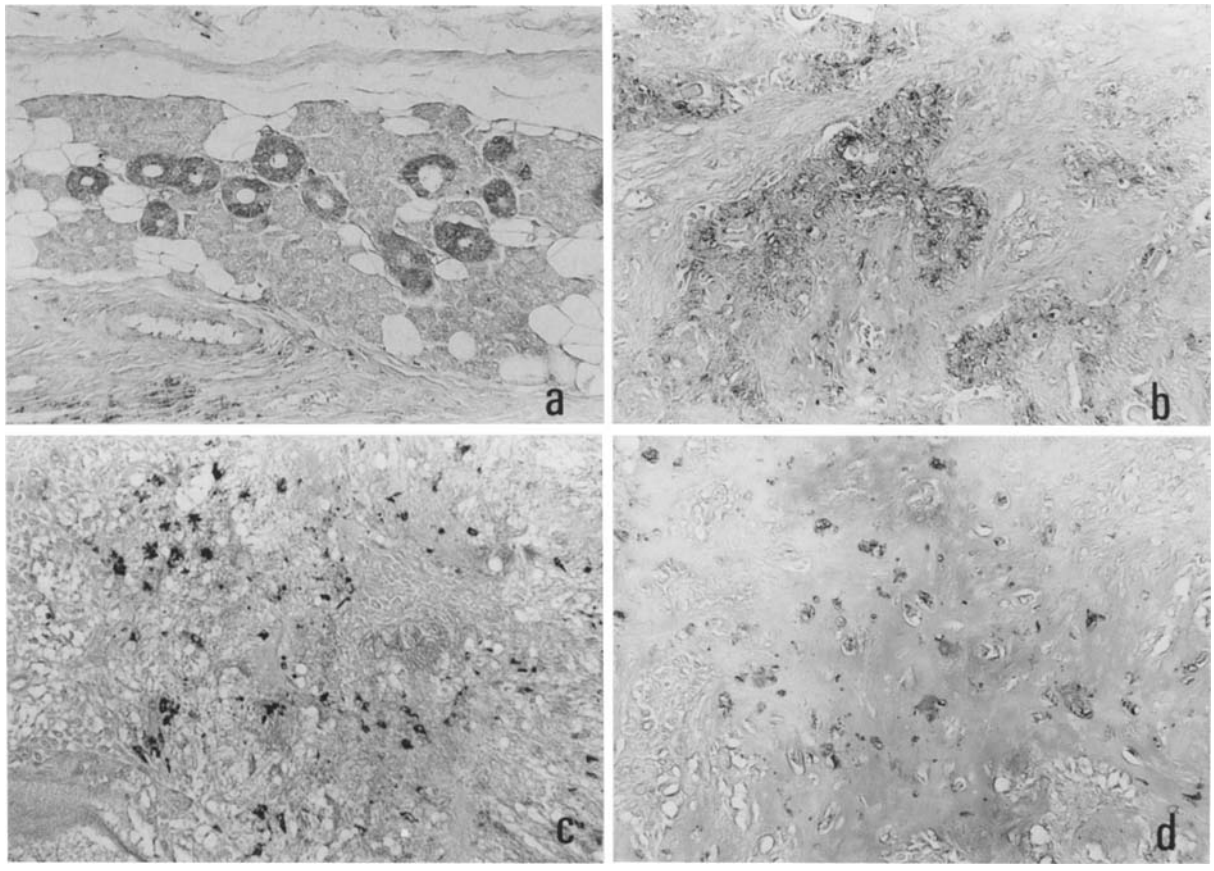

Fig. 2. **a** Adult normal submandibular gland: Weak EGF-immunoreactivity is restricted to ductal cells (immunostaining for EGF ×280). **b** Pleomorphic adenoma: EGF-positive cells are detected in ductal portion (immunostaining for EGF ×240). **c** Pleomorphic adenoma: Most of the cells in myxoid portion have EGF-immunoreactivity (immunostaining for EGF ×280). **d** Pleomorphic adenoma: EGF-immunoreactivity is also recognized in chondroid portion (immunostaining for EGF ×240)

Table 2. Incidence of cases with EGF-immunoreactivity in each portion of pleomorphic adenoma

	Number of cases	Cases with EGF-immunoreactivity	
		Number	Incidence %
Pleomorphic adenoma	48	41 (16)[a]	85.4 (33.3)[a]
Ductal portion	47[b]	39 (14)	83.0 (30.0)
Chondroid portion	25[b]	15 (10)	60.0 (40.0)
Myxoid portion	46[b]	23 (11)	50.0 (47.8)
Solid portion[c]	33[b]	0 (0)	0 (0)

[a] Number of cases or incidence of cases show + + or + + + EGF-immunoreactivity as described in "Materials and methods"

[b] Number of cases having ductal, chondroid, myxoid and solid portion in 48 pleomorphic adenomas, respectively

[c] Solid portion is the area of cell masses which consists of fusiform, polygonal, and stellate cells that cannot be distinguished as either ductal or myoepithelial cells

ically. In his report, a large amount of EGF has been found in the thyroid gland 7.1 ± 1.0 ng/g wet weight ($n=8$, Mean $\pm$ SD), the kidney 4.5 ± 0.8 ng/g ($n=6$), the salivary gland 3.7 ± 0.9 ng/g ($n=9$) and a small quantity in various other tissues. EGF-positive cells were detected immunohistochemically in ductal cells and not in acinic cells. The result of Sumiyoshi et al. support our findings; moreover, these results aided us in our examination of the expression of EGF in foetal tissues. Strong EGF-immunoreactivity was detected at the intermediate stage. At the terminal stage of gestation, when the structure of submandibular glands was almost mature, the expression of EGF was confined to ductal cells. It is interesting to note that most of the cells constructing terminal buds and primary ducts show strong immunoreactivity for EGF at the intermediate stage of gestation, especially in the outer layer cells of primary ducts. At this stage, foetal cells show the most active proliferation and differentiation of the salivary gland. We have previously confirmed that functional

markers of the foetal submandibular gland such as keratin, S-100 protein and secretory component could not be detected at the intermediate stage of gestation, but were recognized at the terminal stage (Yamahara et al. 1987). Therefore, it is likely that EGF produced by foetal cells at the intermediate stage of gestation participates in proliferation, and initiates differentiation of foetal cells themselves in an "autocrine or paracrine" manner.

There has been no report on the expression of EGF in human salivary gland tumours, but in this study, more than 80% of the pleomorphic adenomas were positive for EGF. The expression of EGF was widely detected in both epithelial and mesenchymal tissues, for example, in the ductal, chondroid and myxoid portion. EGF expression in tumour cells of pleomorphic adenomas as well as in foetal cells at the intermediate stage of gestation, was stronger than that in the normal cells of adult tissue. This strong expression of EGF in tumour tissue might be explained by the reversion of tumour cells to foetal expression; moreover, EGF-immunoreactive cells were detected in differentiated areas (the ductal, chondroid and myxoid portion), whereas no EGF-immunoreactivity was observed in the solid portion consisting of undifferentiated cells. Our results indicate that EGF in pleomorphic adenoma may play an important role in differentiation rather than proliferation of tumour cells. However, there is a possibility that EGF produced by tumour cells may be concerned with proliferation and progression of malignant tumours. In fact, we have demonstrated that EGF is produced in 20–30% of advanced gastric carcinomas and is closely related with the malignant behavior of carcinomas (Tahara et al. 1986a). Before these studies can be advanced, there is a need to clarify the relationship between the expression of EGF in tumour cells and growth activity of tumour in the salivary gland.

Interest has been recently focussed on the interaction between growth factors and oncogenes in tumour growth and carcinogenesis. Many reports indicate that signal transduction of growth factors is regulated by *ras* p21. For instance, EGF stimulation of DNA synthesis is blocked by the antibody to p21 (Mulcahy et al. 1985) and EGF enhances the guanine nucleotide binding activity of c-*ras* p21 (Kamata and Feraminsco 1984). In gastric carcinomas, we have reported that c-Ha-*ras* p21 as well as EGF is closely related to the depth of tumour invasion, metastatic potential and prognosis (Tahara et al. 1986b). More recently, Hayashi et al. (1987) have demonstrated the expression of Ha-*ras* p21 in salivary gland tumours. The expression of EGF in a salivary gland tumour might enhance the expression of *ras* gene or vice versa; moreover, EGF and other growth factors share the ability to induce transcription of c-*fos* and c-*myc* gene (Muller et al. 1984; Persson and Leder 1984; Curran et al. 1984). In order to clarify the mechanism of tumour progression induced by EGF, the relationship between the expression of oncogenes and EGF should be studied in the salivary gland.

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