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Immunohistochemical localization of epidermal growth factor (EGF) and EGF receptor in human oral mucosa and its malignancy

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Summary. The immunohistochemical localizations of human epidermal growth factor (hEGF) and EGF receptor (EGFr) in oral tissues, including normal mucosa, leukoplakia and squamous cell carcinoma were examined by the use of monoclonal antibodies to hEGF and EGFr. In normal mucosa and leukoplakia, immunostaining of hEGF was limited to an underlying layer of connective tissue near the epithelium. The intensity of extracellular staining appeared to increase with the degree of epithelial malignancy and was eventually most striking in the stroma of invasive carcinoma. The epithelial cells in normal mucosa, leukoplakia, and squamous cell carcinoma showed negligible immunoreactivity for hEGF. Expression of EGFr appeared to be associated with the proliferative activity of cells and/or epithelial malignancy. In normal mucosa, anti-EGFr monoclonal antibody reacted only with the basal cell layer. In all sections of leukoplakia, the positive cells for EGFr were found in the prickle cell layer in addition to the basal cell layer. Most tumour cells in squamous cell carcinoma were strongly positive for EGFr. These findings indicate increased expression of hEGF and EGFr with malignancy. The characteristic localization of extracellular hEGF in the underlying connective tissue and in stroma of oral mucosal tumours suggests a possible epithelial-mesenchymal interaction in hEGF secretion.

Key words: Epidermal growth factor – EGF receptor – Oral mucosa – Leukoplakia – Oral cancer

Introduction

Epidermal growth factor (EGF), a single-chain polypeptide consisting of 53 amino acids, was first isolated from the male mouse submandibular gland (Cohen 1962). Human EGF (hEGF), subsequently isolated from human urine, is considered to be identical to human β-urogastron, a potent inhibitor of stimulated gastric acid secretion (Cohen and Carpenter 1975; Gregory 1975). hEGF has been found in human extracellular fluids, urine, saliva and milk (Starkey and Orth 1977; Dailey et al. 1978; Hirata and Orth 1979). Recent immunohistochemical studies suggest widespread distribution of hEGF in human tissues, such as the salivary gland, Brunner's glands of the duodenum, kidney and a variety of endocrine organs (Elder et al. 1978; Heitz et al. 1978; Kasselberg et al. 1985; Mori et al. 1989). However, the presence of hEGF in the oral mucosa has not yet been demonstrated.

EGF is a potent mitogen and initiates cellular responses by binding to specific receptors on the surface of target cells. EGF receptor (EGFr), a transmembrane glycoprotein of 170 kDa, is closely related to the oncogene product of avian erythroblastosis virus (v-erb-B) (Downward 1984). Several investigators have postulated that EGF exerts widespread biological effects on the growth and differentiation of various cell types and tissues. Changes in the expression of EGF and EGFr might be observed in some fundamental processes, including early embryogenesis, cellular differentiation and neoplastic transformation. While in vitro studies have provided valuable information concerning the effect of EGF and EGFr actions, their relevance to the in vivo system is only beginning to be examined. To understand the nature of EGF and EGFr actions in vivo, it is essential to be able to identify these proteins in target tissues.

Leukoplakia is a term that has been used for many years to indicate a white patch or plaque occurring on the surface of a mucous membrane. There is little doubt that a certain percentage of cases of oral leukoplakia will undergo transformation into squamous cell carcinoma (Bánoczy and Csiba 1976). Biopsy materials of lesions diagnosed clinically as leukoplakia may include various degrees of epithelial dysplasia, carcinoma in situ or even early invasive carcinoma. Therefore, oral leukoplakia is a useful lesion for studying the carcinogenesis of oral epithelium.

In this study, we have investigated the immunohisto-

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chemical localizations of hEGF and EGFr in the normal oral mucosa, oral leukoplakia and squamous cell carcinoma.

Materials and methods

Biopsy specimens were taken from 41 patients with oral leukoplakia and 6 patients with squamous cell carcinoma. Normal oral mucosa was obtained from 9 healthy volunteers without any oral mucosal lesions. Surgically removed specimens were immediately frozen in OCT compound (Lab-Tek, Illinois, Calif., USA) and stored at −80° C until use. These specimens were cut at a thickness of 4 μm and examined histopathologically and immunohistochemically.

The sections of lesions clinically diagnosed as leukoplakia showed simple hyperkeratosis and epithelial dysplasia. Epithelial dysplasia was diagnosed and graded according to the criteria of Bánoczy and Csiba (1976), who have suggested that histological changes occurring in epithelial dysplasia comprise (1) irregular epithelial stratification, (2) hyperplasia of the basal layer, (3) drop-shaped rete pegs, (4) increased number of mitotic figures, (5) increased nuclear-cytoplasmic ratio, (6) loss of polarity of the basal cells, (7) nuclear polymorphism, (8) nuclear hyperchromatism, (9) enlarged nucleoli, (10) keratinization of single cells or cell groups in the prickle-cell layer, and (11) loss of intercellular adherence. The grade of the dysplasia was divided into three groups: mild, moderate and severe, representing two, two to four, and five or more of the above-listed changes in the lesion, respectively.

Immunohistochemical staining was done by the ABC technique using an avidin-biotin-alkaline phosphatase kit (Vector Laboratories, Burlingame, Calif., USA). Mouse anti-hEGF monoclonal antibody (Wakunaga Pharma, Hiroshima, Japan) and mouse anti-human EGFr monoclonal antibody (Oncogene Science, Manhasset, N.Y., USA) were diluted 1:100 and 1:20, respectively, with phosphate buffered saline (PBS, pH 7.4) containing 0.1% bovine serum albumin (BSA). Serial cryostat sections were air-dried and fixed in acetone at 4° C for 10 min. To reduce non-specific binding, the sections were pre-incubated for 20 min in PBS containing 1% normal horse serum and 0.1% BSA. After washing with PBS, the sections were incubated with the indicated primary antibody for 30 min in a humidified chamber at room temperature, washed extensively and then incubated with biotinylated horse anti-mouse IgG complex for 30 min. After washing with PBS, the sections were stained with alkaline phosphatase kit I (producing a red-coloured precipitate at the sites where the enzyme is localized in the tissue section) and counterstained with 1% methyl green.

The mouse monoclonal antibody against recombinant hEGF has been well characterized in human various tissues and organs by Mori et al. (1987, 1989). Controls included replacing the primary antibody with normal mouse IgG. To test the specificity of the antibody for hEGF, we used monoclonal antibody that had been pre-incubated with a 20-fold molar excess of hEGF (Earth Chemical Company, Hyogo, Japan) for 2 h at room temperature before this mixture was applied to the section for hEGF. The specificity of the mouse anti-EGFr monoclonal antibody was examined by Western blot analysis of a human carcinoma cell line, A431. Briefly, the membrane of A431 cells were solubilized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.5% NP-40 and 0.2 mM phenylmethylsulphonyl fluoride, and centrifuged at 100000 *g*. The soluble proteins (20 μg) were loaded onto 7.5% SDS-polyacrylamide gel, and transferred to immobilon transfer membrane. Thereafter, the membrane was stained with the monoclonal antibody or normal mouse IgG, horseradish peroxidase conjugated anti-mouse IgG, and diaminobenzidine. Figure 1 shows a single band of 170 kDa stained with anti-EGFr monoclonal antibody, suggesting a specific reaction with EGFr.

Results

Table 1 summarizes the data on the immunohistochemical localization of hEGF on human oral mucosa tissues. In all cases, including normal mucosa and leukoplakia, staining with anti-hEGF monoclonal antibody showed a similar pattern of distribution; hEGF was mainly localized in an underyling layer of connective tissue near the epithelium (Fig. 2a–c). The staining intensity appeared to increase with the degree of epithelial malignancy: it was faint in normal mucosa, and stronger in leukoplakia with severe epithelial dysplasia than in leukopakia with simple hyperkeratosis and with mild or moderate dysplasia. This staining of extracellular hEGF was more striking in invasive squamous cell carcinomas: very strong staining with anti-EGF antibody was found throughout the surrounding stroma (Fig. 2g). Negligible immunostaining was observed in epithelium with and without dysplasia, and parenchyma of carcinomas. Vascular endothelial cells were positive for hEGF.

The distribution of EGFr-positive cells is summarized in Table 2. In normal mucosa, anti-EGFr monoclonal antibody reacted with only epithelial cells of basal cell

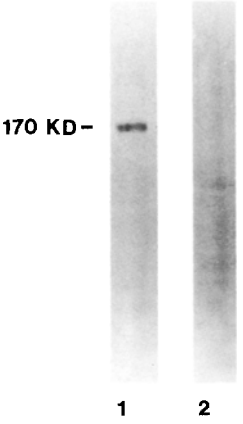

Fig. 1. Specificity of anti-epidermal growth factor receptor (EGFr) antibody in Western blotting. A 170 kDa membrane protein of A431 is reacted with the anti-EGFr monoclonal antibody (*lane 1*), but not with normal mouse IgG (*lane 2*)

Table 1. Localization of human epidermal growth factor in oral mucosa and its lesions

Histopathological classification	No. of cases	Location	
		Epithelial cells	Connective tissue
Normal mucosa	9	−	−
Leukoplakia			
Simple hyperkeratosis	5	−	+
Mild dysplasia	11	−	+
Moderate dysplasia	16	−	+
Severe dysplasia	9	−	++
Squamous cell carcinoma	6	−	++

Staining pattern: −, no staining; +, moderate staining; ++, strong staining

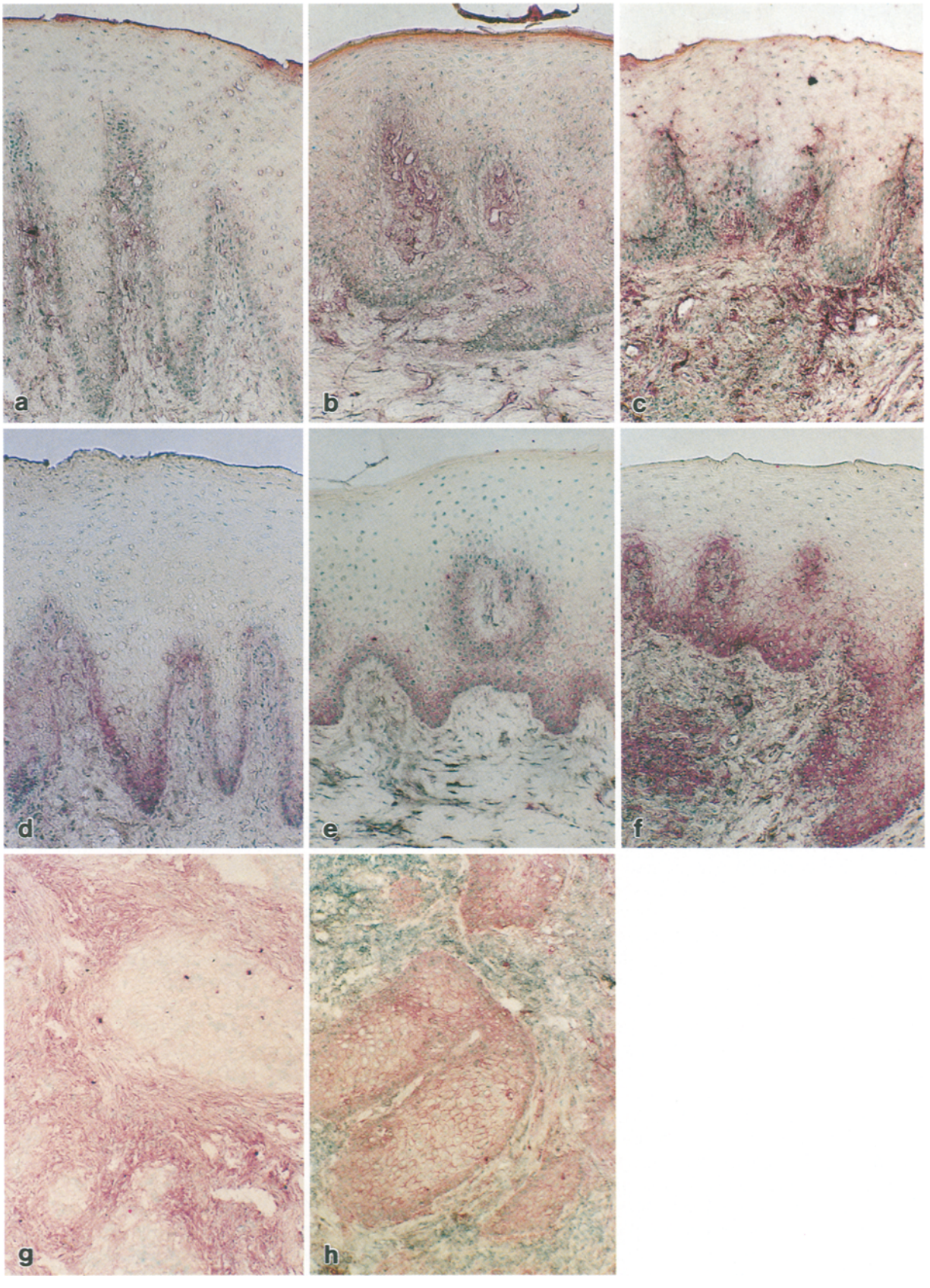

Fig. 2a–h. Localizations of epidermal growth factor (EGF) and EGF receptors (EGFr) immunoreactivity in oral mucosal tissue and its lesions. Materials include normal mucosa (**a**, **d**), leukoplakia with mild (**b**, **e**) or severe (**c**, **f**) dysplasia, and oral squamous cell carcinoma (**g**, **h**). They were stained with anti-EGF antibody (**a–c**, and **g**) or anti-EGFr antibody (**d–f**, and **h**). In all cases, the connective tissue near the epithelium is characteristically stained with anti-EGF antibody (**a–c**). The intensity of the extracellular staining is negligible in normal mucosa and appears to increase with increasing epithelial malignancy. Very strong staining for EGF is found throughout the surrounding of squamous cell carcinoma (**g**). In normal mucosa, EGFr-positive cells are found in basal cell layer of epithelium (**d**). In leukoplakia with mild or severe dysplasia (**e–f**), positive cells for EGFr are found in not only in the basal cell layer but also in the prickle cell layer. Most carcinoma cells are positive for EGFr (**h**). All photomicrographs, × 50

Table 2. EGF receptor expression in oral mucosa and its lesions

Histopathological classification	No. of cases	Location				
		Epithelial cells				Connective tissue
		Basal	Prickle	Granular	Horny	
Normal mucosa	9	+	−	−	−	−
Leukoplakia						
Simple hyperkeratosis	5	++	+	−	−	−
Mild dysplasia	11	++	+	−	−	−
Moderate dysplasia	16	++	+	−	−	−
Severe dysplasia	8	++	+	−	−	−
Squamous cell carcinoma	6		++			−

Staining pattern: −, no staining; +, moderate staining; ++, strong staining

layer (Fig. 2d). In all cases of leukoplakia, the positive cells for EGFr were found in prickle cell layer in addition to basal layer (Fig. 2e–f). The immunostaining reactivity of EGFr appeared to decrease gradually with the differentiation of keratinocytes, and the granular cell and horny cell layers were not stained by the antibody. In squamous cell carcinomas, most tumour cells were positive for EGFr except for a few cells with cancer pearl formation due to keratinization (Fig. 2h).

Discussion

The mucous membrane is a two-layered structure with an architecture basically similar to that of skin. Its surface is oral stratified squamous epithelium, which is supported by an underlying layer of connective tissue, the lamina propria. The two are separated by a basement membrane. Supporting connective tissue may influence the growth and differentiation of oral epithelial cells. Our immunohistochemical studies have demonstrated that hEGF is mainly located in a limited area of connective tissue near the epithelium and that the intensity of staining with anti-hEGF antibody is stronger in squamous cell carcinoma and leukoplakia with severe epithelial dysplasia when compared with normal mucosa and with leukoplakia with simple hyperkeratosis and mild or moderate dysplasia. Expression of EGFr appeared to be associated with proliferative activity of epithelial cells and/or epithelial malignancy. In the normal mucosa, anti-EGFr antibody reacted only with the basal cell layer of the epithelium. In all cases of leukoplakia, the positive cells for EGFr were found in the prickle cell layer in addition to the basal cell layer. Most tumour cells of squamous cell carcinomas were positive for EGFr.

Overexpression of EGFr has been shown in a variety of neoplastic cell lines (Ullrich et al. 1984; Libermann et al. 1985) and in squamous carcinomas of various origin (Hendler and Ozanne 1984; Neal et al. 1985; Mydlo et al. 1989). Tahara et al. (1986) demonstrated that immunoreactive hEGF was significantly higher in advanced gastric carcinomas than in early invasive carcinomas. An increased co-expression of hEGF or transforming growth factor α and EGFr with malignancy was demonstrated in malignant human tumours (Perosio and Brooks 1989; Mydlo et al. 1989). These reports support an autocrine hypothesis: by simultaneously producing or co-expressing both a growth factor and its receptors, a tumour may stimulate its own growth (Sporn and Todaro 1980). The current study indicated negligible immunoreactivity for hEGF in epithelial cells with and without malignancy and does not support this hypothesis.

It is noteworthy that the most characteristic finding reported here is the presence of extracellular hEGF. Its staining reactivity is closely related with epithelial malignancy. Many immunohistochemical studies have demonstrated the presence of intracellular hEGF in various epithelial cells (Elder et al. 1978; Heitz et al. 1978; Kasselberg et al. 1985; Mori et al. 1989), while extracellular distribution of hEGF has been reported in limited areas such as the interstitial tissue of the renal medulla (Kasselberg et al. 1985). The detection of hEGF in urine, plasma, saliva and milk (Starkey and Orth 1977; Dailey et al. 1978; Hirata and Orth 1979) suggests secretion of hEGF from certain cells. It is unlikely that the anti-hEGF antibody used in this study reacts exclusively with extracellular hEGF because its reactivity was found in the cytoplasm of acinar and ductal cells, but not in connective tissue when human submandibular glands were stained (our preliminary experiment and Mori et al. 1989). Moreover, this reactivity could not be found if the tissues were stained with the monoclonal antibody after absorption with EGF. Thus, the extracellular staining of hEGF in the oral mucosal tissue is specific and substantial. The source of extracellular hEGF is not known. Epithelial cells, underlying mesenchymal cells, or both are candidates for hEGF-producing cells. Since extracellular hEGF is always located in the underlying layer of connective tissues or stroma of tumour tissues, it seems likely that hEGF was secreted mainly from mesenchymal cells and accumulated in limited areas near epithelial cells. In vitro studies have suggested that human fibroblasts participate in the production of hEGF (Kurobe et al. 1985). An increased intensity of staining for hEGF with epithelial malignancy suggests secretion

of hEGF, probably by mesenchymal cells, is regulated by an epithelial-mesenchymal interaction. Accumulated hEGF may exert an important biological effect on growth of epithelial cells because epithelial cells positive for EGFr were always present near areas where hEGF was accumulated. Based on these results we speculate that there may be a paracrine mechanism behind the growth of oral epithelium in vivo, although other mechanisms are not excluded. To prove this hypothesis, it should be determined what types of cells in oral mucosa are involved in the production of hEGF, whether hEGF influences growth of oral epidermoid carcinoma cells, and whether hEGF secretion is induced by a mixed culture of mesenchymal cells and carcinoma cells. Studies addressing these possibilities are now in progress.

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