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Expression of epidermal growth factor in human tissues

Immunohistochemical and biochemical analysis

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Summary. The expression of epidermal growth factor (EGF) was examined on various human tissues by radioimmunoassay, immunohistochemistry and Northern blot analysis. Immunoreactive EGF was found in most of the human tissues by radioimmunoassay at various levels. Large quantities of EGF were detected in the kidney and thyroid gland. Immunohistochemically, EGF immunoreactivity was detected mainly in the epithelial cells of the lung, stomach, duodenum, pancreas, kidney, pituitary gland, thyroid gland, mammary gland, ovary, uterus and placenta. Weakly EGF-positive cells were also found in the adrenal gland. The results of EGF-immunostaining were not always consistent with the data from radioimmunoassay. We consider that the amount of EGF measured by radioimmunoassay reflects the density of EGF-positive cells in the tissues and the concentration of EGF in individual EGF-positive cells. Furthermore, EGF mRNA was expressed in the salivary gland, thyroid gland, mammary gland and kidney. It is thus evident that EGF is produced by a variety of human tissues. The kidney expressed exceptionally high levels of EGF mRNA which was about one-tenth of the expression in mouse submandibular gland, suggesting that most of EGF in the urine is produced and secreted by the epithelial cells of renal tubules.

Key words: Epidermal growth factor – Human tissues – Radioimmunoassay – Immunohistochemistry – Northern blotting

Introduction

Epidermal growth factor (EGF) is a pleiotrophic polypeptide of 53 amino acids which was first purified from male mouse submandibular gland (Cohen 1962; Cohen and Taylar 1974). Human urogastrone, a potent inhibitor of gastric acid secretion, was subsequently isolated from human urine and is shown to be identical to human EGF (Cohen and Carpenter 1975; Gregory 1975; Starkey et al. 1975). EGF stimulates proliferation and differentiation of a great variety of cell types in vivo and in vitro (Carpenter 1981; Al-Nafussi 1982; Dembinski and Johnson 1985; Yasui et al. 1988c). EGF acts through its specific cell surface receptor which is a glycoprotein with a molecular weight of 170 kDa (Cohen et al. 1982; Ullrich et al. 1984). EGF receptor has tyrosine specific protein kinase activity and shows autophosphorylation as well as phosphorylation of target molecules in response to EGF (Ushiro and Cohen 1984; Hunter 1984). EGF receptor is encoded by c-erbB proto-oncogene, which is a cellular counterpart of v-erbB oncogene of avian erythroblastosis virus (Downward et al. 1984). Although many of its biochemical properties have been clarified, the site of production of human EGF has not been fully elucidated. A large quantity of mouse EGF is produced in the granular convoluted tubule cells in the submandibular gland (Gresik and Barka 1977; Noorden et al. 1977). Human EGF has been found in the saliva, sweat, milk, plasma and urine (Starkey and Orth 1977; Dailey et al. 1978; Hirata and Orth 1979b; Hirata et al. 1980). Several studies dealing with the localization of human EGF-producing cells by radioimmunoassay or immunohistochemistry demonstrated that certain levels of EGF were detected in a variety of tissues including the kidney, thyroid gland, salivary gland and duodenum (Hirata and Orth 1979a; Kasselberg et al. 1985; Stahlman et al. 1989). No comparative study between radioimmunoassay and immunohistochemistry has been reported. In addition, EGF contents in human tissues reported previously (Starkey and Orth 1977; Hirata and Orth 1979a, b) are too low to account for the large amounts of EGF excreted in the urine (microgram quantities). Thus, the identification of the main source of EGF in the urine has not been made. Furthermore, even though both assays show EGF immunoreactivity, it is still possible that EGF is produced by different cells and temporarily stored in the cells or bound to the cells.

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Table 1. Quantitation of epidermal growth factor (EGF) in human tissues by radioimmunoassay

Tissue	EGF content (ng/g wet weight)
1. Circulatory system	
Heart (11)[a]	2.19±0.26[b]
Aorta (4)	2.52±0.99
2. Respiratory system	
Trachea (5)	0.38±0.22
Lung (8)	2.03±0.33
3. Digestive organ	
Tongue (3)	0.73±0.35
Salivary gland (9)	3.72±0.92
Esophagus (5)	1.37±0.28
Stomach, fundus (14)	2.26±0.43
antrum (13)	1.90±0.49
Duodenum (6)	0.67±0.18
Jejunum (4)	1.12±0.49
Ileum (4)	3.31±1.75
Caecum (3)	3.53±0.65
Appendix (3)	Not detectable
Colon (13)	1.19±0.38
Rectum (4)	1.21±0.47
Liver (6)	1.73±0.64
Gallbladder (7)	0.75±0.25
Pancreas (7)	1.38±0.29
4. Urinary organ	
Kidney (6)	4.51±0.78
Ureter (2)	1.63
Urinary bladder (2)	0.58
5. Endocrine organ	
Pituitary gland	Not examined
Thyroid gland (8)	7.14±0.96
Arenal gland (2)	3.74
6. Reproductive organ	
Testis (2)	1.14
Prostate (4)	3.05±1.63
Ovary (2)	1.87
Uterus (6)	0.82±0.17
Vagina (1)	0.56
Mammary gland	Not examined
7. Hematopoietic system	
Bone marrow (2)	2.86
Lymph node (3)	1.45±0.26
Spleen (7)	2.58±0.23
Thymus	Not examined
8. Others	
Bone (2)	3.00
Muscle, striated (4)	1.74±0.56
smooth (34)	1.72±0.23
Skin (2)	1.00
Brain (1)	0.68
Placenta (2)	1.02

[a] Number of samples examined
[b] Mean±SE

This paper presents our comparative study on the quantity and localization of EGF by radioimmunoassay and immunohistochemistry in several human tissues. Moreover, expression of human EGF mRNA was studied by Northern blot analysis.

Materials and methods

Human tissues from 41 different sites were obtained by surgical operations or autopsies which were performed within 1 h after death at Hiroshima University Hospital. They were fixed in 10% neutral formalin and embedded in paraffin. Portions of them were frozen in liquid nitrogen immediately after removal and stored at −80° C for radioimmunoassay. For Northern blot analysis, only tissue samples obtained by surgical operations were used. All the tissues were histologically confirmed as showing no pathological changes. Clinical data showed no endocrine disorder in any of the patients from whom the samples were taken.

The EGF content in human tissues was measured by the double antibody method using anti-human EGF antibody and anti-rabbit IgG goat serum as described (Tahara et al. 1986). Affinity-purified anti-human EGF antibody was kindly provided by Wakunaga (Hiroshima). This antibody is human EGF specific and has no cross-reactivity with other peptides, including mouse EGF, fibroblast growth factor, platelet-derived growth factor, epithelial cell growth supplement, secretin, insulin, glucagon, cholecystokinin and endorphin (Tahara et al. 1986). Standard assay condition was as follows. Each tissue (about 1.0 g wet weight) was homogenized in five volumes of 1 M acetic acid and extraction was repeated twice. A mixture of the first and second supernatants was lyophilized and dissolved in 1 ml phosphate-buffered saline (PBS). Aliquots (100 µl) were incubated at 4° C in 800 µl PBS containing 10 pg of [^{125}I]human EGF (2100 mCi/µmol) and anti-EGF antibody at a dilution of 1:10000. EGF was labelled with Na^{125}I (Amersham, England) by the chloramine T method. After incubation overnight, 100 µl anti-rabbit IgG goat serum (diluted 1:10; MBI, Nagoya, Japan), 100 µl polyethylene glycol and 100 µl normal rabbit serum (diluted 1:100) as a carrier were added, followed by incubation for 6 h at 4° C. The reactants were then centrifuged and the precipitates were counted in an Aloka γ-autoscintillation

Table 2. Immunohistochemical localization of EGF in human tissues

Tissue	EGF content (ng/g wet weight)	EGF immunoreactivity
Lung	2.03±0.33[a]	Bronchial epithelial cells
Salivary gland	3.72±0.92	Ductal cells
Stomach (antrum)	1.90±0.49	Epithelial cells in pyloric glands
Duodenum	0.67±0.18	Epithelial cells in Brunner's gland
Pancreas	1.38±0.29	Acinar cells
Kidney	4.51±0.78	Epithelial cells of renal tubules
Pituitary gland	Not examined	Endocrine cells in anterior lobe
Thyroid gland	7.14±0.96	Follicular epithelial cells
Adrenal gland	3.74	Medulla cells
Bone marrow	2.86	Megakaryocytes
Mammary gland	Not examined	Glandular and ductal cells
Ovary	1.87	Follicle cells
Uterus	0.82±0.17	Epithelial cells of endometrial gland
Placenta	1.02	Epithelial cells

[a] Mean±SE

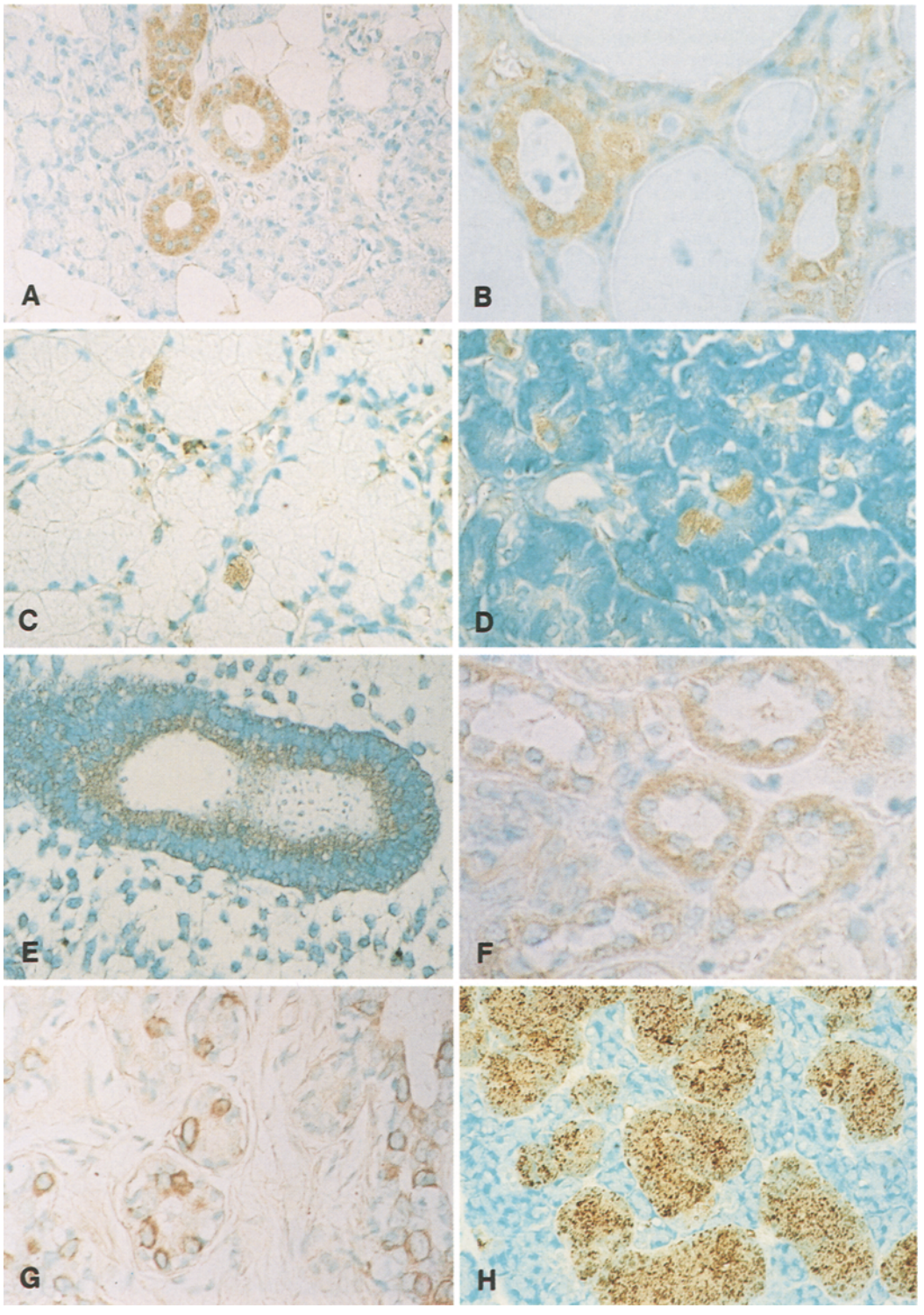

Fig. 1. Immunohistochemical detection of epidermal growth factor (EGF) in several human tissues. **A** Salivary gland. EGF immunoreactivity is restricted to ductal cells. ×410. **B** Thyroid gland. Follicular epithelium shows EGF immunoreactivity. ×325. **C** Brunner's gland of the duodenum. ×650. A small number of epithelial cells contain EGF immunoreactivity. **D** Pancreas. EGF immunoreactivity is observed in some acinar cells. ×650. **E** Endometrium of the uterus. All epithelial cells of endometrial gland show EGF immunoreactivity. ×650. **F** Cortex of the kidney. EGF immunoreactivity is observed in the epithelial cells of renal tubules. ×820 **G** Mammary gland. Many epithelial cells of the mammary gland show immunoreactivity to EGF. ×325. **H** Mouse submandibular gland. The granular convoluted tubule cells are strongly positive to EGF. ×165

counter (Tokyo, Japan). All assays were done in triplicate. More than 90% of exogenous EGF was consistently recovered in this assay. The limit of detection in this assay was about 100 pg/g EGF.

For immunohistochemistry a modification of the immunoglobulin enzyme bridge technique (ABC method) was used as described previously (Yasui et al. 1988a). Deparaffinized tissue sections (4 μm) were immersed in methanol containing 0.03% hydrogen peroxide for 20 min to block the endogenous peroxidase activity and then incubated with non-immunized goat serum (diluted 1:20) for 30 min to block the non-specific antibody binding sites. The sections were treated consecutively for more than 30 min with: (1) anti-EGF antibody (diluted 1:20) at room temperature; (2) biotinylated anti-rabbit IgG goat serum (diluted 1:100, Vector, Burlingame, Calif.); and (3) avidin DH-biotinylated horseradish peroxidase complex (Vecstatin ABC kit, Vector). Peroxidase staining was performed for 10–20 min using a solution of 30 mg 3,3′-diaminobenzidine-tetrahydrochloride (DAB) in 100 ml of 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with 3% methyl green. The specificity of the reaction was determined as follows: (1) anti-EGF antibody was absorbed at 4° C overnight with excess EGF; (2) non-immune rabbit serum was used in the primary reaction.

For Northern blot analysis RNA were extracted by the guanidium isothionate/caesium chloride method (Maniatis et al. 1984). Ten micrograms of poly(A)$^+$ selected RNA was electrophoresed on 1% agarose/formaldehyde gel and blotted onto Zeta-probe nylon filter membrane (Bio-Rad, Richmond, California). Filters were baked for 2 h at 80° C under vacuum. After prehybridization, hybridization was performed at 42° C for 12–15 h using ^{32}P-labelled human EGF cDNA probe (Yoshida et al. 1990). Hybridization solution contained 0.1 M PIPES-NaOH (pH 6.8), 0.65 M NaCl, 5× Denhardt's solution (1× Denhardt's solution contains 0.02% w/v each of bovine serum albumin, Ficoll, and polyvinyl pyrolidine), 0.1% sodium dodecyl sulphate (SDS), 50% deionized formamide, 10% dextran sulphate and 100 μg/ml salmon sperm DNA. Filters were washed twice with 0.1× SSC-0.1% SDS (1× SSC consists of 0.15 M NaCl and 15 mM sodium citrate) for 30 min at room temperature, followed by two washes in 0.1× SSC-0.1% SDS for 60 min at 65° C and a rinse in 0.1× SSC. The filters were autoradiographed with Kodak XAR-5 films.

The 1.9 kb human EGF cDNA insert from phEGF15 was kindly provided by Dr. Graeme I. Bell (Bell et al. 1986). β-Actin probe was purchased from Oncor (Gaithersburg, Md).

Results

The amounts of EGF measured by radioimmunoassay in various human tissues are shown in Table 1. Immunoreactive EGF was detected in all the tissues examined except for the appendix. Large quantities of EGF were found in the kidney (4.51±0.78 ng/g wet weight; mean±SE) and thyroid gland (7.14±0.96 ng/g). A relatively large quantity of EGF (3.72±0.92 ng/g) was detected in the salivary gland where the major portion of mouse EGF is found (Cohen and Savage 1974; Gresik and Barka 1977). More than 3 ng/g of EGF was found in the ileum, caecum, adrenal gland and prostate.

The results of immunostaining of EGF in various human tissues are summarized in Table 2. The salivary gland, kidney and thyroid which showed large quantities of EGF (more than 3 ng/g) by radioimmunoassay contained EGF-immunoreactive cells (Fig. 1A, B, F). The epithelial cells in the ducts, renal tubules and follicles were positive to EGF. Weakly EGF-positive cells were also found in the medulla of adrenal gland. EGF immunoreactivity was detected in the bronchial epithelium of the lung and megakaryocytes in the bone marrow, both of which showed certain levels of EGF (about 2 ng/g) in radioimmunoassay. Only a small number of EGF-positive cells were detected in the stomach, duodenum and pancreas, which showed small quantities of EGF by radioimmunoassay (Fig. 1C, D). The epithelial cells of the mammary gland, uterus and placenta also showed EGF immunoreactivity (Fig. 1E, G). However, some tissues such as the ileum, caecum and prostate, which showed more than 3 ng/g of EGF, did not reveal EGF immunoreactivity in any cells. Thus, the results of EGF immunostaining were not necessarily consistent with the levels of EGF measured by radioimmunoassay.

The expression of EGF mRNA in human tissues is shown in Fig. 2. EGF mRNA was detected as about 5.0 kb transcript in the salivary gland, thyroid gland and

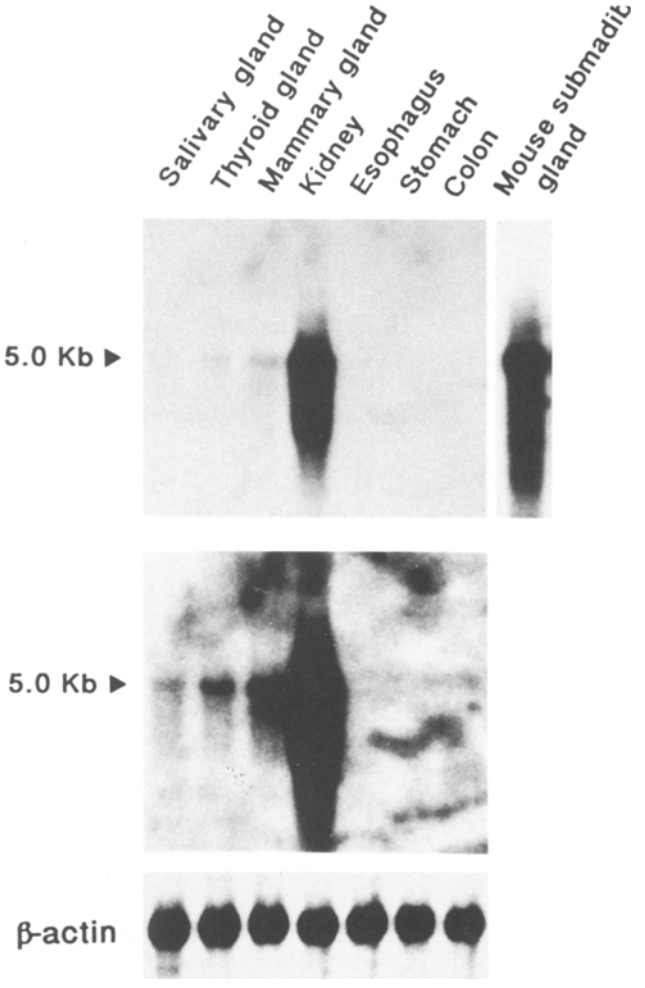

Fig. 2. Northern blot analysis of EGF mRNA on several human tissues. RNA was extracted from several human tissues obtained by surgical operation and 10 μg of poly(A)$^+$ selected RNA was subjected to Northern blot analysis as described in Materials and methods. Autoradiographic exposure time was 6 h (*upper panel*) and 24 h (*lower panel*) at −80° C. In case of mouse submandibular gland, 1 μg of the RNA was subjected to Northern blot analysis under exactly the same conditions as the other tissues. We used β-actin probe as an internal control

kidney, which had a large amount of EGF measured by radioimmunoassay. The kidney expressed extremely high levels of EGF mRNA, which was about one-tenth of the expression in mouse submandibular gland. (In Fig. 2, we applied 10 μg and 1 μg of RNA from the kidney and mouse submandibular gland, respectively.) EGF mRNA expression was also detected in the mammary gland. Expression of EGF mRNA was not detected in the mucosa of the oesophagus, colon or both the antrum (data not shown) or fundus of the stomach.

Discussion

We first examined the quantities of EGF in various human tissues by radioimmunoassay and found that all the tissues examined except for the appendix showed varying levels of EGF. More than 3 ng/g EGF was found in the thyroid gland, salivary gland, ileum, caecum, adrenal gland, kidney and prostate, among which EGF-immunoreactive cells were immunohistochemically observed in the thyroid gland, salivary gland and kidney. Weakly immunoreactive cells to EGF were also found in the adrenal medulla. However, the ileum, caecum and prostate did not show any EGF-positive cells by immunohistochemistry. The discrepancy may be due to low concentration of EGF in individual cells, although the amount of EGF was more than 3 ng/g by radioimmunoassay. However, in the present study, we cannot exclude the possibility that the EGF immunoreactivity detected by radioimmunoassay might be due to contamination with mesenchymal components such as connective tissue or endothelial cells, which have also been shown to be EGF-immunoreactive. It is also possible that radioimmunoassay might be influenced by an EGF-like peptide co-extracting with EGF but showing different tissue distribution. However, EGF-positive cells were detected in the lung, stomach, duodenum, pancreas, ovary, etc. whose EGF content was lower than 3 ng/g. As shown in Fig. 1, only a small number of EGF-immunoreactive cells were observed in these tissues (for example, the duodenum and pancreas). Therefore, it is likely that the quantities of EGF measured by radioimmunoassay also reflect the density of EGF-positive cells in individual tissues.

Even though both radioimmunoassay and immunohistochemistry showed EGF immunoreactivity, it is still possible that EGF is produced by different cells in other tissues and temporarily stored in the cells. It is also probable that EGF is not produced by the cells but is bound to EGF receptor of the cells, showing EGF immunoreactivity in these assays. We then examined EGF mRNA expression in the tissues in order to confirm the production of EGF by the tissues. Expression of EGF mRNA was detected in the thyroid gland, salivary gland and kidney, which showed a high level of EGF immunoreactivity. EGF mRNA was also expressed in the mammary gland, where EGF-immunoreactive cells were found in the glands and ducts. It seems to be consistent with the fact that milk contains a significant amount of EGF (Hirata and Orth 1979b; Pesonen et al. 1987). Therefore, in these tissues, EGF is evidently produced by the tissues. Nevertheless expression of EGF mRNA was not detected in the oesophagus, stomach and colon, where a certain level of EGF immunoreactivity was detected by radioimmunoassay. Two possible explanations of this discrepancy can be made. One is that the small amount of EGF mRNA in these tissues could not be detected by the sensitivity of Northern blot analysis we performed. The second is that EGF immunoreactivity in these tissues did not result from the production of EGF by the tissues. It is well known that the epithelial cells of the oesophagus, stomach and colon possess EGF receptor and the proliferation of these mucosae is stimulated by EGF (Yeh and Scheving 1981; Yasui et al. 1988b, c; Yoshida et al. 1990). We might examine the possibility that EGF is produced by different tissues and is bound to the cell surface receptor temporarily. However, this possibility seemed unlikely because the immunohistochemical analysis showed that EGF immunoreactivity was mainly found in the cytoplasm of the cells.

The most interesting finding observed in the present study was that the kidney expressed an extremely high level of EGF mRNA, which was about one-tenth of the expression in mouse submandibular gland. Since mouse submandibular gland contains exceptionally large amounts of EGF (up to 1.6 mg/g wet weight) (Cohen and Savage 1974; Hirata and Orth 1979a), the expected EGF content in the human kidney can be roughly estimated to be 150 μg/g wet weight according to the ratio of mRNA expression in the kidney to that in mouse submandibular gland. A fairly large amount of EGF (about 50 μg/day) is excreted into the urine (Starkey and Orth 1977; Dailey et al. 1978). However, only 4.5 ng/g wet weight of EGF was detected in the kidney by radioimmunoassay. According to available data on the stability of tissue EGF, EGF in tissues is stable at room temperature for at least 24 h (Hirata and Orth 1979a). Therefore, it is unlikely that the period between surgical removal or death and freezing (10 min to 1 h) accounts for the small quantities of EGF in the present study. EGF immunoreactivity was observed in the epithelial cells of renal tubules. These findings overall strongly suggest that large amounts of EGF are produced by epithelial cells of renal tubules and secreted into the urine as well as the plasma immediately without stockpiling in the kidney. Furthermore, it is possible that, like the submandibular gland in mice, the kidney may be a main source of EGF in human plasma. In situ hybridization would provide more information on the sites of EGF production.

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