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Localization of epidermal growth factor/transforming growth factor-α receptor in the human gastric mucosa

An immunohistochemical and in situ hybridization study

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Abstract. Current evidence indicates that epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) play a pivotal role in the maintenance of gastric mucosal integrity, via binding to a common cell-surface receptor (EGF/TGF-α receptor). We examined the distribution and cellular sites of synthesis of EGF/TGF-α receptor in normal human gastric mucosa by immunohistochemical and in situ hybridization techniques. Intense EGF/TGF-α receptor immunoreactivity was observed in the basal cytoplasm and along basolateral membranes of mucus neck cells, foveolar columnar cells, and surface epithelial cells facing the gastric lumen. Parietal cells and mucus-secreting pyloric gland cells displayed a distinct basolateral immunostaining, whereas the luminal membrane was unstained. Immunoreactivity was also noted in spindle-shaped cells of the lamina propria and in smooth muscle cells of the muscularis mucosae and muscularis propria. In situ hybridization revealed EGF/TGF-α receptor RNA transcripts in all cell types displaying positive immunoreaction. These results suggest a physiological role for EFG/TGF-α in the regulation of multiple gastric functions. The receptor distribution at the luminal aspect of the gastric mucosa provides the anatomical basis for a possible interaction of gastric juice EGF (or TGF-α) with cells of the mucosal surface, whereas the expression of EGF/TFG-α receptor in cells which are not in direct contact with the gastric lumen is consistent with blood-mediated or paracrine/autocrine mechanisms of EGF/TGF-α action on these cells.

Key words: Epidermal growth factor – Transforming growth factor-α – Receptor – Gastric mucosa – In situ hybridization

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Introduction

Epidermal growth factor (EGF), a 6-kDa pleiotrophic polypeptide, was originally isolated from male mouse submandibular glands on the basis of its ability to stimulate precocious incisor eruption and eyelid opening in newborn mice (Cohen 1962). EGF was subsequently purified from human urine, and found to be structurally related to human β-urogastrone, a potent inhibitor of stimulated gastric acid secretion (Cohen and Carpenter 1975; Gregory 1975; Starkey et al. 1975). EGF shares 35% sequence homology and a nearly identical spectrum of biological activities with transforming growth factor-α (TGF-α), a 50-aminoacid peptide first found in culture fluids from various oncogenically transformed cells (De Larco et al. 1978; Marquardt et al. 1984; Derynck 1988). EGF and TGF-α have a similar ability to promote proliferation and differentiation of a variety of epithelial and mesenchymal cells under both in vitro and in vivo conditions (Derynck 1988; Carpenter and Cohen 1990). They interact with target cells through a common cell-surface membrane receptor (EGF/TGF-α receptor), a 170-kDa transmembrane glycoprotein with tyrosine-specific protein kinase activity (Cohen et al. 1982; Ullrich et al. 1984).

Although many of their biochemical properties have been clarified, the exact physiological role of EGF and TGF-α in the gastrointestinal tract remains uncertain in many instances. TGF-α mRNA expression and protein production has recently been shown in normal gastric mucosa (Beauchamp et al. 1989; Bennet et al. 1989; Malden et al. 1989). Expression of EGF mRNA, however, has never been demonstrated in the human stomach (Beauchamp et al. 1989; Kajikawa et al. 1991) but substantial amounts of immunoreactive EGF are present in biological fluids (serum, saliva, gastric juice) that can reach the gastric mucosa (Gregory et al. 1979; Oka and Orth 1983; Konturek et al. 1989; Orsini et al. 1991). Both EGF and TGF-α are acid-stable proteins (Cohen 1962; Taylor et al. 1972; Derynck 1988) that stimulate epithelial cell migration (Blay and Brown 1985; Barran-

don and Green 1987), promote cell proliferation (Johnson and Guthrie 1980; Dembinski et al. 1982; Derynck 1988; Carpenter and Cohen 1990; Takagi et al. 1992), and suppress acid production (Konturek et al. 1984; Finke et al. 1985; Dembinski et al. 1986; Rhodes et al. 1986; Lewis et al. 1990). Removal of submandibular glands (the major source of intraluminal EGF) in rats reduces the rate of tritiated thymidine incorporation into gastric mucosa as well as its resistance to various damaging factors (Skinner and Tepperman 1981; Tepperman and Soper 1990; Konturek et al. 1991), whereas topically applied EGF in non-antisecretory amounts prevents experimentally induced mucosal lesions (Konturek et al. 1981, 1990) and accelerates chronic gastric ulcer healing (Skov Olsen et al. 1986; Konturek et al. 1988). All together, these observations suggest that EGF and TGF-α may play a pivotal role in the maintenance of gastric mucosal integrity and participate in the reparative events following acute and chronic gastric mucosal injury (Konturek 1988; Calabrò et al. 1992; Konturek et al. 1992; Polk et al. 1992).

The exact definition of the cellular distribution of EGF/TGF-α receptor is crucial to the evaluation of this proposed role. Therefore, the purpose of this study was to analyse the precise distribution and localize the cellular sites of synthesis of EGF/TGF-α receptor in normal human gastric mucosa by in situ hybridization and immunohistochemistry with a specific monoclonal antibody.

Materials and methods

Biopsy specimens of fundic and antral mucosa were taken from ten subjects (four male, six female; mean age 47, range 22–74 years) undergoing endoscopy for upper gastrointestinal tract symptoms, but without any evidence of macroscopic abnormalities. All subjects had past and family histories negative for peptic ulcer, were not receiving any medication, and were free from known endocrine, renal or other serious diseases. Additional samples were collected from four patients (three male, one female; mean age 52, range 38-63 years) who underwent total gastrectomy for gastric cancer, at least 8 cm away from the edge of the neoplasm, and were used only after histological examination showed normal mucosa. Tissue from mature placenta was used as positive control (Hock and Hollemberg, 1980). Informed consent was obtained from all patients prior to endoscopy or surgery. Samples were immediately snap frozen and stored in liquid nitrogen. For in situ hybridization, cryostat sections (4–7 μm thick) were dried on a hot plate at 80° C, fixed in 4% paraformaldehyde/phosphate buffered saline (PBS), pH 7.4 for 20 min, and washed three times in PBS (Milani et al. 1989). For immunohistochemistry, frozen sections (7 μm) were collected onto celan slides and dried overnight at room temperature. After fixation with acetone and chloroform (30 min each), sections were air-dried and incubated with the primary antibody.

Immunostaining was performed with a commercially available mouse monoclonal antibody to human EGF/TGF-α receptor (EGF receptor Ab-1, IgG_{2a}, clone 528; Oncogene Science, Manhasset, N.Y., USA), at a dilution of 1:2–1:50. This antibody was produced by fusion of NS-1 mouse myeloma cells and spleen cells of mice immunized with partially purified EGF receptor from human A431 epidermal carcinoma cells. It inhibits EGF binding to its receptor and is an antagonist of in vivo EGF-stimulated tyrosine kinase activity (Kawamoto et al. 1983; Gill et al. 1984). The alkaline phosphatase anti-alkaline phosphatase (APAAP) method (Cordell et al. 1984) was used for immunohistochemical localization of EGF/TGF-α receptor immunoreactivity. All incubation steps were carried out at room temperature, in a humidified chamber, for 30 min. Each step was followed by two washes (5 min each) in TRIS-hydrochloric acid (HCl) buffered saline (TBS; pH 7.5). A rabbit antiserum directed against mouse IgG (Dakopatts, Copenhagen, Denmark) diluted 1:20 was used as linking antibody. After incubation with monoclonal APAAP complexes (Dakopatts), diluted 1:50 in TBS, the slides were developed for alkaline phosphatase with a mixture of naphtol AS-BI phosphate and new fucsin. Levamisole (Sigma, Munich, Germany) was added to the development solution in order to block endogenous alkaline phosphatase activity. As endogenous placental alkaline phosphatase could not be fully inhibited by levamisole, the peroxidase antiperoxidase (PAP) method was employed for immunostaining of placental sections, using a monoclonal PAP immune complex (Dakopatts) diluted 1:50 in PBS. Endogenous peroxidase was blocked by 1% hydrogen peroxide/methanol, and 3,3′-diaminobenzidine tetrahydrochloride (Sigma) was used as chromogen. Finally, some sections were weakly counterstained with Mayer's haemalum, and coverslipped using Kayser's gelatin. Negative controls were performed by omitting the primary or secondary antibodies, and employing non-immune mouse serum as first layer. Sections from the same tissues were also stained with haematoxylin and eosin (H & E).

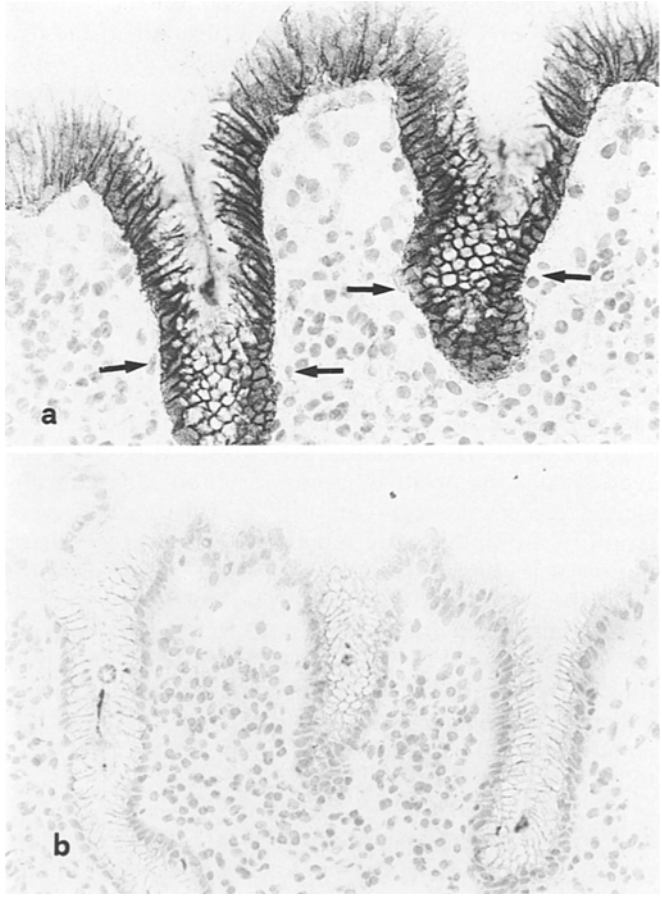

Fig. 1a, b. Immunostaining of epidermal growth factor/transforming growth factor-α (EGF/TGF-α) receptor on normal gastric mucosa of the antrum. **a** EGF/TGF-α receptor immunoreactivity is observed in the basal part of the cytoplasm and along basolateral membranes of mucus-secreting cells lining surface epithelium and pits, and in mucus neck cells of the proliferative zone (*arrows*). APAAP, ×260. **b** Negative control (non-immune mouse serum). APAAP, ×200

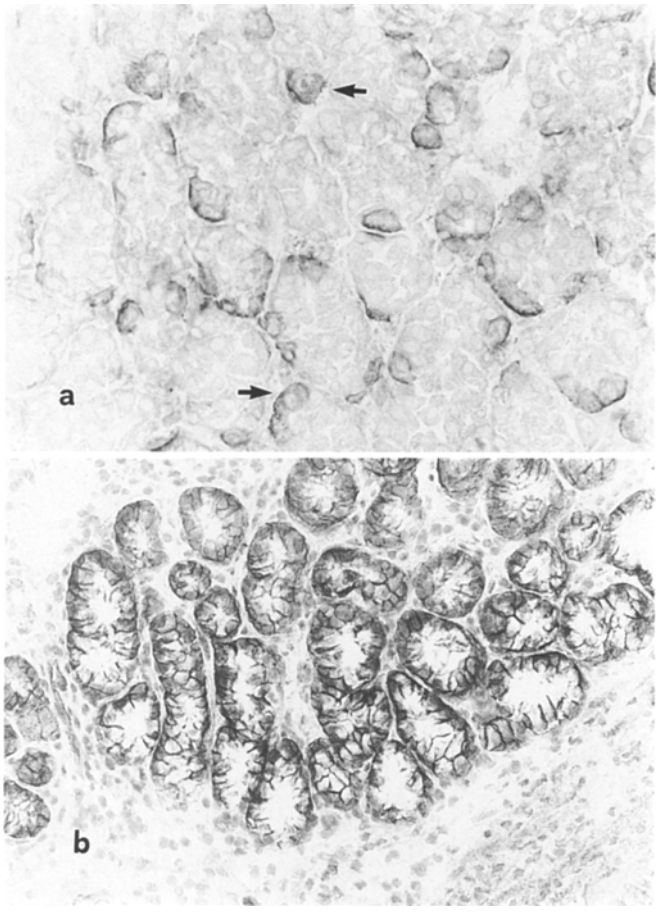

Fig. 2a, b. Immunostaining of EGF/TGF-α receptor on normal gastric mucosa. **a** Parietal cells display a definite linear staining along basolateral membranes sometimes associated with a weak cytoplasmic reaction (*arrows*). APAAP, ×260 (no counterstaining). **b** Pyloric gland cells show evident staining of basal cytoplasms and basolateral membranes. APAAP, ×200

Anti-sense (complementary to mRNA), and sense (anti-complementary, negative control) RNA probes specific for EGF/TGF-α receptor mRNA were obtained by run-off transcription, with SP6 or T7 RNA-polymerase respectively, of the 900 bp *Eco*RI/*Hin*dIII cDNA fragment of human epidermal growth factor receptor (plasmid pHER-A64-1, ATCC 57347; Ullrich et al. 1984), subcloned into the appropriate restriction sites of the plasmid pGEM1 (Promega Biotec, Heidelberg, Germany). Linearized plasmids were used for probe labelling employing 60 μCi of [^{35}S]-uridine-5′-(α-thio)-triphosphate (1250 Ci/mmol, New England Nuclear, Dreieich, Germany), as described previously (Milani et al. 1989). RNA probes were stored at −80° C and used within 2 days. The specific activity routinely obtained was $1.2–1.4 \times 10^9$ cpm/μg.

Pre-hybridization, hybridization, removal of non-specifically bound probe by RNase A digestion, and further washing procedures were performed for positive and negative strand RNA probes as described in detail elsewhere (Milani et al. 1989). Briefly, air-dried sections were treated in 0.2 M hydrochloric acid, digested with 0.125 mg/ml pronase/PBS (Boehringer Mannheim) for 10 min at room temperature, rinsed in 0.1 mol/l glycine/PBS, fixed for 20 min in ice-cold 4% paraformaldehyde/PBS, and acetylated with acetic anhydride diluted 1:400 in 0.1 mol/l triethanolamine, pH 8.0, for 10 min. After dehydration with graded ethanols, sections were briefly air-dried prior to hybridization. A volume of 25 μl of hybridization mixture [50% formamide, 10% dextran sulphate, 10 mmol/l dithiothreitol, 0.1 mol/l TRIS-HCl pH 7.5,

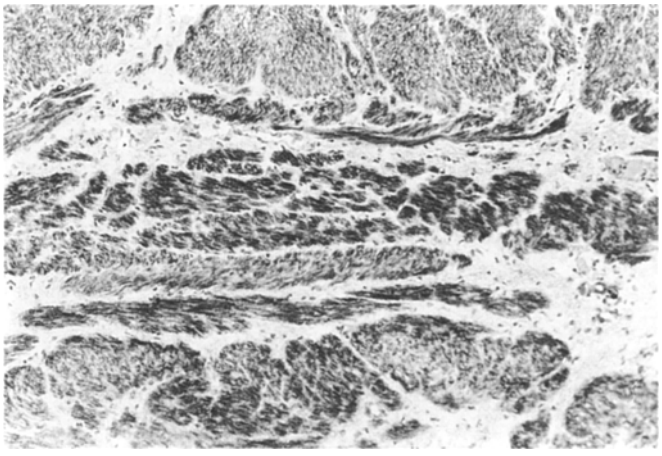

Fig. 3. Immunostaining of EGF/TGF-α receptor on muscular layers of human stomach. Strong reaction is particularly evident in smooth muscle cells of the external layer of muscularis propria. APAAP, ×200

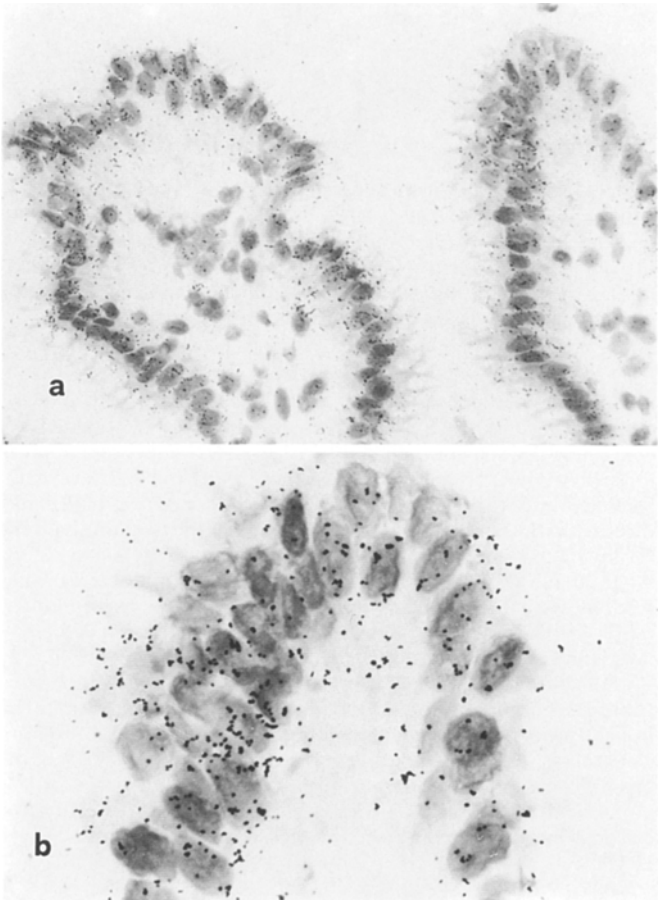

Fig. 4a, b. In situ hybridization of EGF/TGF-α receptor mRNA with antisense [^{35}]S-labelled RNA probe in normal gastric mucosa (exposure time 30 days). **a** Moderate amounts of EGF/TGF-α receptor gene transcripts are expressed by mucus-secreting cells of the surface epithelium and pits. (×400). **b** Detail of surface epithelial cells. ×1000

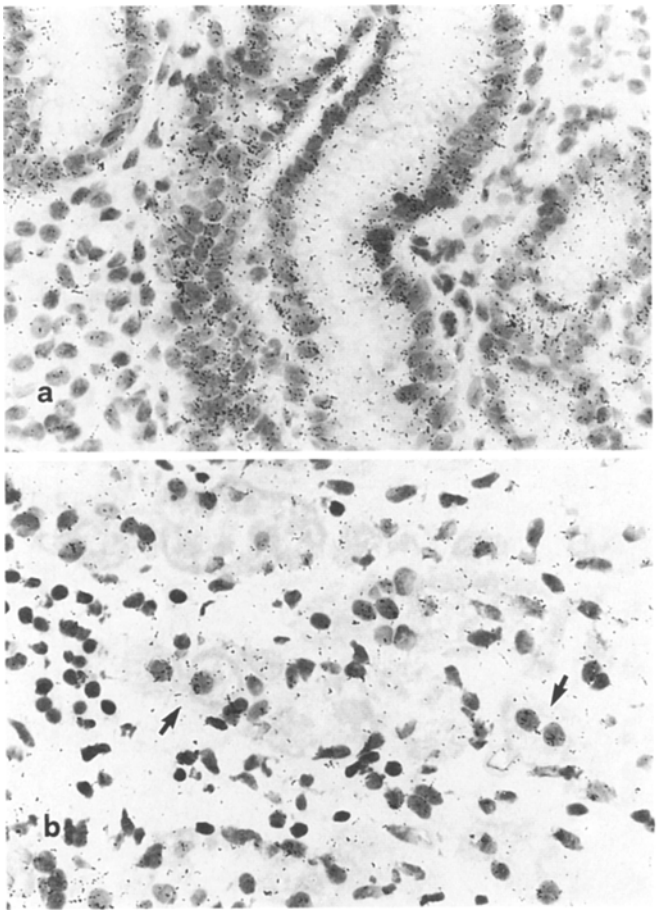

Fig. 5a, b. In situ hybridization of EGF/TGF-α receptor mRNA with antisense [^{35}]S-labelled RNA probe in normal gastric mucosa (exposure time 30 days; ×400). **a** Foveolar and mucus neck cells of proliferative zone display high levels of EGF/TGF-α receptor mRNA expression. **b** Parietal cells of fundic glands display small amounts of EGF/TGF-α receptor mRNA transcripts (*arrows*)

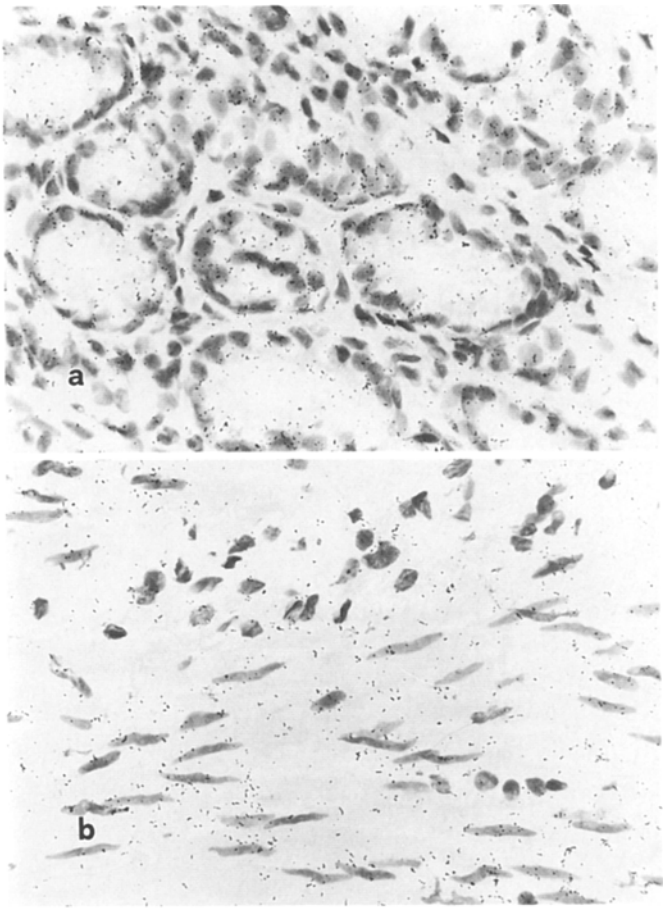

Fig. 6a, b. In situ hybridization of EGF/TGF-α receptor mRNA with antisense [^{35}]S-labelled RNA probe in normal stomach (exposure time 30 days, ×400). Mucus-secreting cells of pyloric gland cells (**a**), and smooth muscle cells of the muscularis propria (**b**) express moderate amounts of EGF/TGF-α receptor mRNA transcripts

0.1 mol/l sodium phosphate, 0.3 mol/l sodium chloride (NaCl), 50 mmol/l EDTA, 1 × Denhardt's solution, 0.2 mg/ml yeast tRNA] containing 200000 cpm of [^{35}S]-labelled RNA probe were applied to each section. Hybridization was continued for 18 h at 50° C. Sections were washed for 4 h at 52° C in hybridization buffer and digested with 20 µg/ml RNase A in TES buffer (0.1 mol/l TRIS-HCl pH 7.5, 1 mmol/l EDTA, 0.5 mol/l NaCl) for 30 min at 37° C. After 30 min washing at 37° C in TES buffer without the enzyme, sections were further rinsed in 2× saline-sodium citrate buffer (SSC) and 0.1 × SSC for 15 min each, dehydrated in graded ethanols, and air-dried prior to the autoradiographic procedure.

For autoradiography, dried slides were coated with Ilford G5 nuclear emulsion (Ilford, Mobberley, UK), dried for 2 h, stored in light-proof boxes containing desiccant, and exposed at 4° C for 20–30 days. Exposed slides were developed in Kodak D19 developer (Kodak, Hemel Hempstead, UK) for 2.5 min, rinsed in 1% acetic acid and fixed in Kodak fixer for 3 min. After extensive washing in tap water, the slides were counterstained with Harris H & E.

Results

A slight cellular infiltrate of the lamina propria, confined to the pit or foveolar regions of the stomach (mild superficial gastritis), was observed by routine histological examination in all surgical samples and in biopsy specimens from 2 patients undergoing endoscopy. The distribution and level of EGF/TGF-α receptor immunoreactivity and mRNA expression in these cases, however, did not differ from those seen in gastric mucosa free of inflammatory cells.

In normal gastric mucosa, strong EGF/TGF-α receptor immunoreactivity was observed in the mucus-secreting cells of the surface epithelium and pits and in mucus neck cells of the proliferative zone. Positive immunoreaction was particularly intense in the basal part of the cytoplasm and along basolateral cell membranes (Fig. 1a). A definite linear staining decorated the basolateral membranes of parietal cells (Fig. 2a) and mucus-secreting pyloric gland cells (Fig. 2b), whereas the luminal surface membrane was not stained. Also, faint cytoplasmic EGF/TGF-α receptor immunoreactivity was noted in some parietal (Fig. 2a) and spindle-shaped cells of the lamina propria (not shown). Oxyntic gland cells other than parietal cells occasionally displayed a weak reaction, that was, however, insufficient to allow confirmation of positivity. EGF/TGF-α receptor immunoreactivity was also evident in smooth muscle cells of the

muscularis mucosae (not shown) and the muscularis propria of tissue specimens from patients operated on for gastric cancer (Fig. 3).

In term placenta, strong immunostaining was localized to the entire surface of the syncytiotrophoblast in contact with the maternal blood. A weak but distinct reaction was observed at the basal plasma membrane of the syncytium, in some cytotrophoblast cells, and in stromal cells of chorionic villi (not shown). Control sections processed with non-immune mouse serum and by omission of the primary or secondary antibodies invariably gave negative reactions (Fig. 1b).

In situ hybridization revealed EGF/TGF-α receptor mRNA in all cell types showing positive immunostaining, namely mucus-secreting cells of the surface epithelium and pits (Fig. 4a, b), mucus neck cells (Fig. 5a), parietal cells (Fig. 5b), pyloric gland cells (Fig. 6a), and smooth muscle cells of the muscularis mucosae and muscularis propria (Fig. 6b). Particularly intense labelling was observed in the lower part of the foveolae and proliferative zone (Fig. 5a). No evident relationship was found between the staining intensity and autoradiographic signal. High levels of EGF/TGF-α receptor RNA expression were found in the syncytiotrophoblast covering the chorionic villi and, to a lesser extent, in some cytotrophoblast cells (not shown). No specific labelling was observed in control sections hybridized with the sense (anti-complementary) EGF/TGF-α receptor probe.

Discussion

Over the past decade a wealth of evidence has accumulated indicating the importance of EGF in maintenance of gastric mucosal integrity (Konturek et al. 1988; Calabrò et al. 1992). Clinical studies have reported reduced EGF concentrations in saliva and gastric juice of patients with active ulcers (Ohmura et al. 1987; Calabrò et al. 1990); however, the role of luminal EGF deficiency in the development or in the healing process of chronic peptic ulcer is poorly understood. In particular, the existence of membrane receptors for EGF in cells facing the lumen of the stomach, a necessary prerequisite to hypothesize any interaction between gastric juice EGF and mucosal cells, have never been demonstrated in man. Adding further complexity to this scenario, TGF-α mRNA expression and protein production have been demonstrated in normal gastric mucosa (Beauchamp et al. 1989; Bennet et al. 1989; Malden et al. 1989), suggesting possible paracrine/autocrine regulatory effects on gastric functions, and raising the questions as to which is the physiological ligand involved in phenomena initially attributed to EGF. In this context, it is crucial to define pathways and mechanisms by which EGF and TGF-α exert their effects on gastric mucosa.

The results of this study, which is the first simultaneously investigating EGF/TGF-α receptor expression in the human stomach at both RNA and protein level, demonstrate that EGF/TGF-α receptor is widely distributed in normal gastric mucosa, and that it is expressed in cell types endowed with different physiological functions. Indeed, in addition to the parietal acid-secreting cells, receptors for EGF/TGF-α were found in surface epithelial cells secreting mucus and bicarbonate, in mucus neck cells of the proliferative zone, and in the mucus-secreting pyloric gland cells of the antrum. EGF/TGF-α receptor expression was also noted in spindle-shaped mesenchymal cells among glands, and in smooth muscle cells of the muscularis mucosae and muscularis propria. The specificity of these findings is strengthened by the cellular co-localization of immunohistochemical staining and autoradiographic signal for EGF/TGF-α receptor.

The presence of abundant EGF/TGF-α receptors in isolated gastric glands of guinea pig and mucosal homogenates of human gastric mucosa (Forgue-Lafitte et al. 1984; Pfeiffer et al. 1990) has been established previously by specific binding of [^{125}-I]-EGF. Moreover, evidence has been provided that the rat gastric mucosa possesses a functional EGF/TGF-α receptor, based on its ability to undergo autophosphorylation in the presence of EGF (Slomiany et al. 1990). In addition, expression of EGF/TGF-α receptor messenger RNA has been reported in normal gastric mucosa of various species (Beauchamp et al. 1989; Bennet et al. 1989; Malden et al. 1989). However, these studies are unable to reveal the exact localization of EGF/TGF-α receptor in complex epithelia composed of multiple cell types, such as gastric mucosa. In the rat stomach, receptors for EGF/TGF-α have been demonstrated immunohistochemically at the luminal surface of scattered mucus neck cells of the proliferative zone (Lee et al. 1991) and, in addition, in some parietal cells (Tarnawski et al. 1992).

In man, data on EGF/TGF-α receptor distribution in gastric mucosa are scanty and mainly derive from studies investigating the biological significance of its expression in gastric carcinoma. Receptors for EGF/TGF-α have been shown by immunohistology on parietal cells of apparently normal gastric mucosa adjacent to cancer nests (Sakai et al. 1986) and localized at the ultrastructural level to the outer membrane except in the apical portion (Mori et al. 1987). Our immunohistochemical findings confirm the presence of EGF/TGF-α receptor on parietal cells, in agreement with the well-known inhibitory effect of EGF and TGF-α on gastric acid secretion (Konturek et al. 1984; Finke et al. 1985; Dembinski et al. 1986; Rhodes et al. 1986; Lewis et al. 1990). The basolateral localization of EGF/TGF-α receptor implies a role for circulating rather than intraluminal EGF in the control of acid secretion. However, locally produced TGF-α may also exert an important regulatory function via paracrine/autocrine mechanisms, as supported by TGF-α mRNA expression in enriched parietal cell fractions from guinea pig gastric mucosa (Beauchamp et al. 1989). Consistent with this hypothesis, EGF and TGF-α inhibit histamine-stimulated acid secretion from isolated guinea pig gastric mucosa when delivered to the serosal but not luminal surface (Finke et al. 1985; Rhodes et al. 1986), whereas orally administered EGF does not change gastric acid secretion, irrespective of the dose used (Dembinski et al. 1982). The concept of endocrine or paracrine/autocrine mechanisms of action of EGF/TGF-α is strengthened by the basolateral receptor distri-

bution in mesenchymal and epithelial pyloric gland cells which are not in direct contact with the gastric lumen.

The most important finding of the present study, however, is the demonstration of EGF/TGF-α receptors in mucus neck cells of the regenerative zone and in mucus-secreting epithelial cells facing the gastric lumen. The presence of dense binding sites in the luminal aspect of the canine gastric mucosa has recently been demonstrated by tissue section autoradiography (Sottili et al. 1992). EGF/TGF-α receptor localization in lateral cell membranes at this level could represent a potential site of action of intraluminal EGF/TGF-α. Accordingly, luminal administration of EGF has been shown to stimulate gastrointestinal mucosal cell proliferation (Dembinski et al. 1982; Ulshen et al. 1986) and mucus elaboration (Sarosiek et al. 1988). Although the trophic role of intraluminal EGF has been questioned (Goodlad et al. 1987), indirect evidence favouring this route of action comes from the observation that hypersecretion of submandibular saliva elicits trophic response in the small intestine, whereas stimulants of salivary secretion do not increase intestinal growth when administered to sialoadenectomized animals (Li et al. 1983).

Finally, of particular interest appears to be the expression of EGF/TGF-α in smooth muscle cells of the muscularis mucosae and muscularis propria. Great attention has recently been focused on EGF as a regulator of smooth muscle contractility and metabolic activities (Itoh et al. 1988; Muramatsu et al. 1988; Nanney et al. 1988) and distinct receptors for EGF/TGF-α have been shown in gastric circular and longitudinal smooth muscle from the guinea pig (Muramatsu et al. 1988; Hollemberg et al. 1989).

In conclusion, our data demonstrate that EGF/TGF-α receptor is present in several gastric mucosal cell types and provide an anatomical basis for the well-known effects of EGF and TGF-α on gastric acid secretion, mucosal cell renewal, mucus production, and smooth muscle cell contraction. The peculiar distribution of EGF receptor on epithelial cells facing the gastric lumen supports a physiological role for gastric juice EGF or TGF-α in the maintenance of mucosal integrity. This may provide a rational basis for oral EGF administration in pathological conditions characterized by reduced luminal EGF concentrations (Calabrò et al. 1990).

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