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Epidermal growth factor and transforming growth factor-α mRNA in rat small intestine: in situ hybridization study

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Abstract The expression of epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) mRNA in the small intestine of suckling and adult rats was examined by in situ hybridization. EGF mRNA was found mainly in the intestinal crypts in adult rats. Adult rats also exhibited a considerably stronger signal for EGF mRNA in comparison to suckling rats, where the signal was very low or absent. In contrast to EGF, very strong expression of TGF-α mRNA was observed in the small intestine of both adult and suckling rats. These data suggest the differences between the expression of EGF and TGF-α in the developing small intestine.

Key words: Epidermal growth factor; Gastrointestinal tract; Submandibular gland; Transforming growth factor-α; In situ hybridization

1. Introduction

The gastrointestinal epithelium undergoes a continuing process of cell proliferation, differentiation and maturation. Rapidly proliferating crypt enterocytes migrate from the crypts to the villus tip, losing their proliferative capacity and increasing their differentiation capability. The mechanisms that regulate and coordinate this rapid turnover are not clear, but a number of observations suggests that hormones and peptide growth factors are an essential part of this regulatory system. Two growth factors that are thought to play an important role in this process are epidermal growth factor (EGF) and transforming growth factor-α (TGF-α); mitogenic peptides that share 33% structural homology [1] and bind to the same cell surface TGF-α/EGF receptor [2,3].

EGF has been detected in many tissues and body fluids, including salivary and gastrointestinal secretions, colostrum and milk, and amniotic fluid. It has been shown that EGF has widespread effects on growth, cell proliferation and maturation of a variety of tissues [4], including fetal, neonatal and adult gastrointestinal epithelium [5–11], as well as on gastric acid secretion [12]. EGF was first isolated from the submandibular glands (SMG) of the adult male mouse by Cohen [13], and its amino acid sequence was determined by Savage et al. [14]. Although EGF gene expression has been detected in various organs, the highest level of expression of EGF mRNA has been found in the male SMG and kidney [15]. Localization of EGF mRNA transcripts in these organs from rodents has been demonstrated using in situ hybridization [15–20].

TGF-α was initially considered an embryonic growth factor [21,22], but it is now accepted as an integral regulator of growth in normal tissues [23,24]. Localization of TGF-α mRNA using in situ hybridization has been reported in rodent maternal decidua and fetal tissues [25–27]. Both TGF-α peptide and TGF-α mRNA have also been found in the human fetal and adult gastrointestinal tract [28–31] and in the human fetal pancreas [32].

In rodents, EGF and TGF-α peptides have been reported in the gastrointestinal tract of adult, as well as in suckling animals [33–36]. Immunohistochemical studies have detected EGF peptide in the Brunner's gland of the duodenum and the Paneth cells of the small intestine of adult rats [37], whereas immunoreactive TGF-α has been localized in the villus but not in the intestinal crypts [35,38]. An abundance of TGF-α mRNA was found in the gastrointestinal tract of adult rodents, whereas EGF mRNA was not detected in the gastrointestinal tract of either adults [30,35,39] or sucklings [40]. Recently, Miettinen reported the presence of EGF mRNA in the developing human fetal gastrointestinal tract using ribonuclease protection assay and polymerase chain reaction analysis [41].

Based on these observations, we decided to test whether developing rat gastrointestinal tissue itself has the capability to produce EGF and TGF-α peptides. The main goal of this study was to clarify the presence of EGF mRNA and TGF-α mRNA in the developing rat small intestine. In order to detect these transcripts, we initially established a sensitive in situ hybridization technique for detection of EGF mRNA in rat male submandibular glands. This technique was then used for detection and localization of EGF and TGF-α mRNA in the small intestine of suckling as well as adult male rats. Some of the results were recently presented at the American Gastroenterological Association meeting [42].

2. Materials and methods

2.1. Tissue preparation

Sprague–Dawley male rats bred in our colony were used in this experiment. Rats had unlimited access to drinking water and a standard laboratory diet (Lab Blox, TechLab, Indianapolis, IN). Ten adult 70- to 80-day-old male rats (450–520 g) and fifteen suckling 12-day-old male rats (31–35 g), each group originally from five different litters, were

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killed by decapitation and the duodenum, jejunum (first third of the jejunoileum) and submandibular glands quickly removed. The organs were embedded in OCT compound (Tissue Tek II, Miles, Elkhart, IN), snap-frozen in liquid nitrogen and stored at −70°C. 8-μm thick sections were cut with a cryostat and mounted on glass slides coated with poly-L-lysine (Sigma Co., St. Louis, MO).

2.2. Probes

A 784 bp *ApaI/SpeI* fragment of mouse EGF cDNA inserted into plasmid pGEM-5Z and a 925 bp *EcoRI* fragment of human TGF-α cDNA inserted into plasmid pGEM-1 were kindly provided by Dr. R. Coffey, of Vanderbilt University, Nashville, TN. The pGEM-5Z plasmid was linearized either by using *SalI* restriction endonuclease to generate the appropriate template for EGF antisense RNA probe, or by using *ApaI* restriction endonuclease for the EGF sense RNA probe. The pGEM-1 plasmid was linearized either by using *HindIII* to generate a template for the TGF-α antisense RNA probe or by using *PvuII* for the TGF-α sense RNA probe. All restriction endonucleases were from Boehringer-Mannheim, Indianapolis, IN. A non-radioactive technique was used for labeling the RNA probes. Digoxigenin-labeled (DIG-labeled) UTP was included in the nucleotide mixture and RNA probes were generated by in vitro transcription from linearized cDNA templates using the RNA Labeling Kit (Boehringer-Mannheim) according to the standard manufacturer's protocol. T7 RNA polymerase was used to synthesize EGF or TGF-α antisense RNA probes, while SP6 RNA polymerase gave EGF or TGF-α sense RNA probes. Digoxigenin incorporation was measured by dot blot analysis of serial dilutions of the probes. The amount of RNA transcribed was determined by comparing serial dilutions with a standard RNA solution.

2.3. In situ hybridization

All treatments with sections, prehybridization, hybridization and washing steps were derived from the protocols of Salido et al. [17] and Couwenhoven et al. [19]. Recently, Viaene and Baert [43] used a similar protocol for non-isotopic in situ hybridization in human esophageal mucosa. All solutions were prepared with diethylpyrocarbonate-treated water. Tissue sections from suckling and adult rats were assayed simultaneously under the same reaction conditions. The cryosections were first treated with 1 μg/ml of proteinase K in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, at 37°C for 7 min. After rinsing with phosphate buffered saline (PBS), pH 7.4, containing 2 μg/ml of glycine, the sections were post-fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 5 min. The slides were then rinsed with PBS containing 2 μg/ml glycine and with 0.2 × SSC (1 × SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0). After 5 min incubation in 0.1 M triethanolamine (TEA) buffer (pH 8.0), the sections were treated in TEA buffer with 0.25% (v/v) acetic anhydride for 10 min and then washed in 2 × SSC for 10 min. After dehydration through an ascending ethanol series, the hybridization buffer containing 50% formamide, 0.75 M NaCl, 25 mM EDTA, 1 × Denhardt's solution, 0.1 M dithiothreitol (DTT), 0.2% sodium dodecyl sulfate (SDS), and 100 μg/ml yeast transfer RNA, with the addition of heat denatured herring sperm DNA at 300 μg/ml, was applied to each section and slides were prehybridized at 37°C for 2 h. All chemicals were purchased from Sigma Co. After prehybridization, the hybridization buffer was removed and 25 μl of hybridization buffer (without herring sperm DNA) containing 50 ng/μl EGF, or TGF-α DIG-labeled antisense or sense RNA probes (for control sections) were added to each section, which were then coverslipped and the slides incubated in a moist chamber at 45–48°C overnight. After hybridization coverslips were gently removed by immersion in 4 × SSC. The slides were incubated for 30 min at 37°C with 50 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA to digest any unhybridized probe. The slides were then washed with 2 × SSC for 30 min at room temperature, 0.2 × SSC for 15 min at 42°C, 0.1 × SSC for 15 min at 42°C, and 0.1 × SSC for 10 min at room temperature. Hybridized probes were immunologically detected with the alkaline phosphatase-coupled anti-digoxigenin antibody, by using the Nucleic Acid Detection Kit (Boehringer-Mannheim) according to the standard manufacturer's protocol. The stable color precipitates were developed by the reaction of alkaline phosphatase with Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate and levamisole. Levamisole was included to inhibit the activity of endogenous phosphatases. The sections were then counterstained with hematoxylin and permanently covered with coverslips using ACCU Mount 60 Mounting Medium (Baxter, Scientific Products, McGaw Park, IL) and stored in the dark.

3. Results

3.1. EGF

To determine that the in situ hybridization technique was specific and provided results consistent with those in the literature, we performed in situ hybridization on sections of the submandibular glands from the adult male rats. The sections that hybridized with the DIG-labeled EGF antisense RNA probe demonstrated an intensely positive signal in the granular convoluted tubule cells, whereas hybridization with the EGF sense (control) probe resulted in only a background level over these cells (figures not shown). After these verification experiments, in situ hybridization was performed on small intestinal sections from adult and suckling rats.

In adult male rats, when the EGF antisense RNA probe was applied to sections of the duodenum and jejunum, an intense positive staining was evident in the cytoplasm of the intestinal crypt cells (Fig. 1A). The enterocytes of the lower half of the villi also gave a positive signal for the presence of EGF mRNA. No staining was observed in the upper part of the villi or in the lamina propria. The sense probe yielded only a uniform background signal (Fig. 1B).

Previous studies have implicated a role for exogenous milk-borne EGF in growth and development of the intestine [33,34]. To determine the potential contribution of endogenous EGF, we performed in situ hybridization for EGF mRNA in 12-day-old suckling male rats. Tissues from duodenum or jejunum of suckling rats usually showed no staining throughout all sections. In some cases (one from five animals), a low expression of EGF mRNA was observed in the intestinal crypt enterocytes (Fig. 2A); however, no signal was detected in the cells of the villi. Hybridization with the sense EGF probe resulted in only uniform background labeling (Fig. 2B).

3.2. TGF-α

To determine the site of expression of the TGF-α mRNA, in situ hybridization for TGF-α mRNA was performed on the

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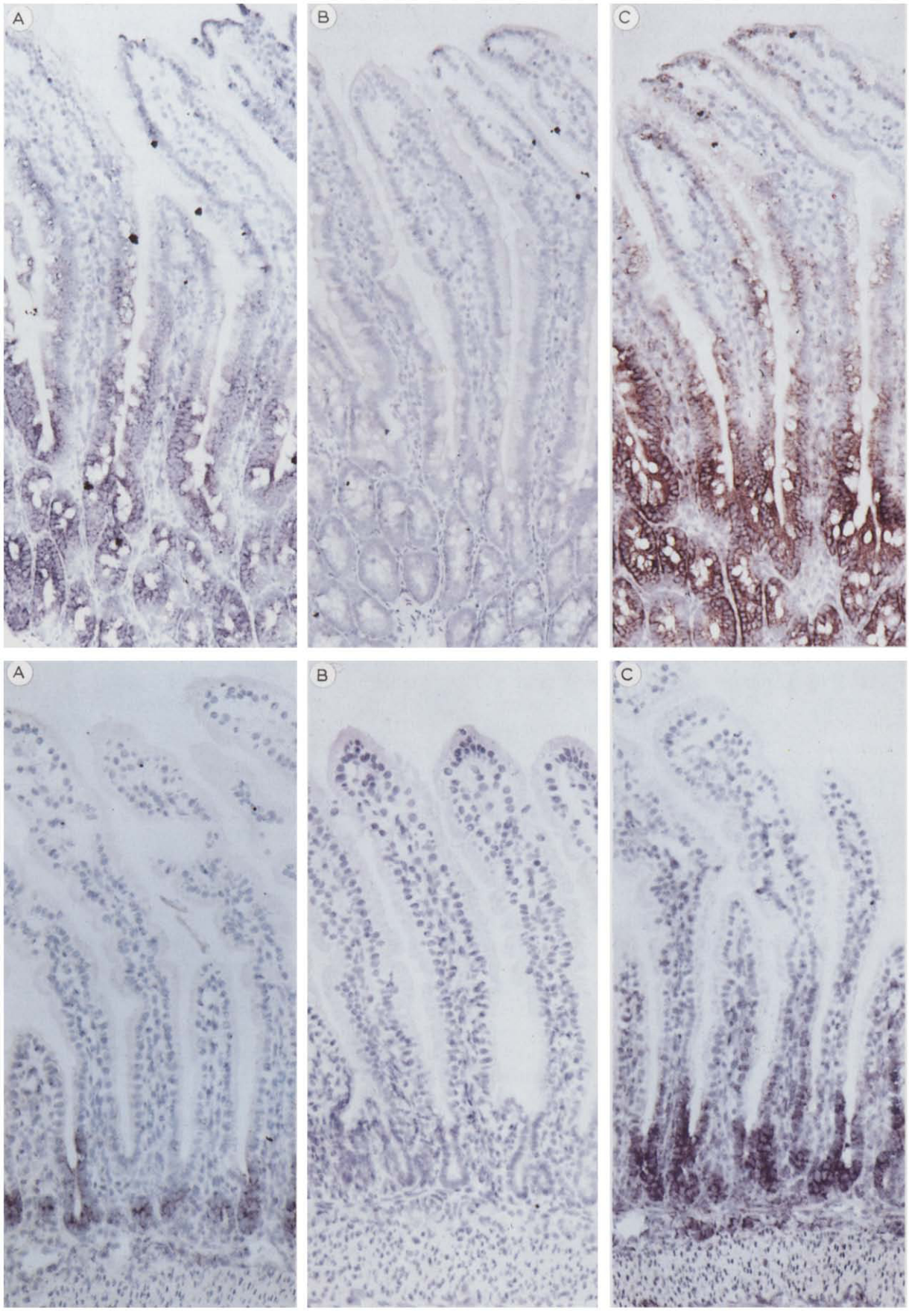
Fig. 1. In situ hybridization of EGF and TGF-α mRNA with the DIG-labeled RNA probes in the adult male rat jejunum. (A) Strong positive signal for the presence of EGF mRNA is in the crypt cells. Enterocytes of the lower half of the villi also exhibit a positive signal (×140). (B) Hybridization with the EGF sense probe yields only a uniform background signal (×140). (C) The expression of TGF-α mRNA. Very strong staining appears in the crypt cells and in the enterocytes of the lower two-thirds of the villi. The tips of the villi exhibit a weak staining (×140).

Fig. 2. In situ hybridization of EGF and TGF-α mRNA with the DIG-labeled RNA probes in rat jejunum from suckling (12-day-old) male rat. (A) In some rats (one out of five) a low positive signal for the presence of EGF mRNA appears in crypt cells. The enterocytes of the villi are not stained (×180). (B) Control EGF sense probe shows only a uniform background signal (×180). (C) Strong positive staining for the presence of TGF-α mRNA is present in the intestinal crypt cells (×180).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same sections as for EGF. Application of the DIG-labeled TGF-α antisense RNA probe to the adult male rat duodenum and jejunum resulted in very strong staining throughout the intestinal crypts (Fig. 1C). In addition, a very strong signal was also observed in enterocytes of the lower two-thirds of the villi with reduced staining intensity to the tips of the villi. Enterocytes at the villus tip exhibited weaker, but still well detectable staining (not shown). The sense probe gave only a uniform background signal which was similar to that seen with the EGF sense probe (Fig. 1B).

Previous studies have indicated the presence of TGF-α mRNA in adult rat intestine [30,35,39]. We have previously shown that TGF-α peptide is absent in rat milk [36], but the peptide is detectable throughout the gastrointestinal tract of suckling rats. To determine the possible sources of TGF-α in the developing gastrointestinal tract, we also performed in situ hybridization using the TGF-α probe in the small intestine of suckling rats. These experiments showed an intensely positive signal for the presence of TGF-α transcripts in the crypt cells of duodenum and jejunum from suckling rats (Fig. 2C). Incubation with the sense probe resulted in only uniform low background labeling.

4. Discussion

Our studies using the in situ hybridization technique with non-radioactive DIG-labeled RNA probes clearly show the expression of both EGF and TGF-α transcripts in the crypt enterocytes of adult male rats. The presence of TGF-α mRNA was detected in the enterocytes of the whole villi, whereas the expression of EGF mRNA was observed only in the enterocytes from the lower part of the villi. The intensity of staining suggested much higher abundance of TGF-α mRNA in comparison with EGF mRNA. In suckling male rats, crypt cells gave an intense signal for TGF-α mRNA, whereas the EGF mRNA signal was either very low or, more often, no signal was observed at all.

EGF and TGF-α have long been regarded as likely modulators of gastrointestinal epithelial growth and differentiation [6–11,28,29,39,44–46]. The structural similarity and functional resemblance of these two growth factors have suggested similar origins and fates in the gastrointestinal tract. However, previous reports from our laboratory have shown significant differences between these two growth factors. Both peptides are present in the gastrointestinal tract of suckling and adult rats, but EGF, which is present in milk from several mammalian species including rat milk [47], belongs to the group of milk-borne biologically active peptides [48]. EGF levels in the gastrointestinal tract of sucklings are significantly higher than in adults [33] and are dependent on EGF intake [34]. Interestingly, suckling rats fasted overnight or hand fed for 3 days with EGF-free rat milk substitute kept some EGF content in the small intestine (about 15% of the initial level) [49]. This could either be interpreted as an effect of adrenal stimulation or be the result of local production of EGF in the small intestinal tissue.

On the other hand, TGF-α peptide has not been found in rat milk, and its small intestinal levels are similar in both suckling and adult rats [36]. Barnard et al. [35] studied the distribution of TGF-α mRNA and TGF-α peptide in the small intestine of adult rats. They found that TGF-α mRNA was evenly expressed along the jejunal crypt-villus axis, whereas TGF-α immunoreactivity was detected only along the villus. The authors postulated that TGF-α produced in the jejunal epithelium may not only stimulate cell proliferation, but also affect the migration of enterocytes to the top of the villus. Alison et al. [38] recently reported a similar pattern of staining with TGF-α antibodies in adult rat jejunum; a strong signal was detected in the surface of villus, but the crypt cells were totally unstained. The exact locus, as well as the biological function, of TGF-α within the small intestine, are not completely known, but the strong signal for TGF-α mRNA was detected in IEC-6 cells, a cell line thought to be derived from rat intestinal crypt cells [35,39]. While the expression of TGF-α mRNA in the gastrointestinal tract of adult rats was repeatedly reported [30,35,39]; at the same time EGF mRNA was not detectable. In these studies, Northern blot was used for detection of mRNA transcripts in the enterocyte fractions eluted from the small intestine. Miettinen [41] has shown that the expression of TGF-α mRNA in the human fetal small intestine was more abundant than EGF mRNA. The presence of EGF mRNA was detectable only by using RNase protection assay or polymerase chain reaction analysis, the sensitivity of Northern blot analysis was insufficient to detect any EGF transcripts. It was also speculated that EGF mRNA has not been previously detected in the gastrointestinal tract because of the insensitivity of the methods used. Other findings of interest were that in the jejunum, exogenous EGF peptide up-regulated TGF-α mRNA expression 3-fold, whereas exogenous TGF-α reduced its own mRNA by 40% [41]. Similar results were observed with the rat intestinal cell line IEC-6, where the addition of EGF peptide to culture medium significantly increased TGF-α mRNA expression [50].

In the present study, a sensitive method that was specific for the in situ cytological localization of EGF mRNA and TGF-α mRNA in the small intestine of suckling and adult rats was used. EGF mRNA transcripts were detected mainly in intestinal crypt cells. The variations seen in the intensity of staining also permit comparisons of the abundance of the message for EGF between suckling and adult rats. Duodenum and jejunum from adult rats exhibited a significantly stronger signal in comparison with sucklings, where a very low or no signal was observed. These observations invite speculation that milk-borne EGF reduces EGF mRNA expression in the gastrointestinal tract in suckling rats.

In contrast to EGF, strong expression of TGF-α mRNA was observed in the small intestine of both suckling and adult rats. These findings support our previous results where we found that TGF-α peptide is present in similar concentrations in the small intestinal mucosa from suckling and adult rats, and overnight fasting did not significantly affect these levels in sucklings [36]. Moreover, in suckling rats, 90% of immunoreactive TGF-α from the total content of intestinal lumen and mucosa was found in the mucosa, suggesting intestinal tissue as a site of TGF-α peptide synthesis. On the other hand, mucosal EGF peptide in suckling rats represents only 25–60% of its total intestinal content [33,34].

This study demonstrates the expression of EGF and TGF-α mRNA in the small intestine of adult rats. It is noteworthy that in sucklings, a high content of EGF in rat milk corresponds to very low expression of intestinal EGF mRNA, whereas the absence of TGF-α in rat milk is accompanied by high concentrations of TGF-α mRNA in the same tissue.

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