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Early weaning accelerates the differentiation of mucous neck cells in rat gastric mucosa: Possible role of TGFα/EGFR

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ABSTRACT

The development of the gastric mucosa is controlled by hormones, growth factors and feeding behavior. Early weaning (EW), which means the abrupt interruption of suckling, increases proliferation and differentiation in the rat gastric epithelium. Transforming growth factor α (TGFα) is secreted in the stomach, binds to the epidermal growth factor receptor (EGFR) and may control cell proliferation, differentiation and migration. Here, we investigated the influence of suckling–weaning transition on the differentiation of mucous neck cells in the stomach and its association to the expression of TGFα and EGFR. Fifteen-day-old Wistar rats were divided into two groups: suckling (control), in which pups were kept with the dam, and early weaning (EW), in which rats were separated from their mother and fed with hydrated powdered chow. TGFα and EGFR levels were increased at 18 days in EW animals compared to control ones ($p < 0.05$). Histochemical reactions with Periodic Acid-Schiff reagent+Alcian Blue or *Bandeiraea simplicifolia* II lectin were used to stain the mucous neck cells and showed an increase in this cell population throughout EW, which was more pronounced at 17 days when compared to suckling pups ($p < 0.05$). These morphological results were confirmed by RT-PCR for mucin 6. The levels of mucin 6 mRNA were higher in EW animals from the 16th to the 18th day (1–3 days post-weaning) when compared to the respective control group. Inhibition of EGFR through AG1478 administration to EW animals prevented the expansion of mucous neck cell population induced by EW ($p < 0.05$). Therefore, early weaning up regulated TGFα/EGFR expression and induced differentiation of mucous neck cells. Moreover, we showed that EGFR takes part in the maturation of this cell population. We conclude that regular suckling–weaning transition is crucial to guarantee the development of the gastric mucosa.

1. Introduction

The mucosa of the corpus region of the stomach is composed of numerous tubular glands, which are lined by different epithelial cell types. Among them there are two mucus-producing populations: surface mucous cells, which are in contact with the gastric lumen, and mucous neck cells, localized in the neck region of the gland. Mucous neck cells are small triangular cells localized between parietal cells; the nucleus is positioned at the base and cytoplasm is filled with mucin. In rats, mucous neck cells cannot be morphologically identified until the third postnatal week, but their precursors are present before this age (Ihida et al., 1988; Kataoka et al., 1990; Falk et al., 1994). These cells secrete important regulatory peptides such as trefoil factor TFF2/PS2 (Hanby et al., 1999; Ota et al., 2006), and growth factors EGF and TGFα (Alison et al., 1995). In the gastric mucosa, mucin 6 is specifically expressed by mucous neck cells (Bartman et al., 1998; Reis et al., 2000), co-localizes with TFF2 and contributes to the protective role of the secreted mucus against chemical and mechanical damage (Laine et al., 2008). Also, the mucin produced by mucous neck cells has an antibiotic effect, inhibiting the growth of *Helicobacter pylori* (Kawabuko et al., 2004).

The development and maturation of the gastrointestinal tract are coordinated by a complex interaction of hormones, growth factors, milk-born molecules, luminal microbes and genetic program (Lee and Lebenthal, 1983; Nanthakumar et al., 2005; De Andrade Sá et al., 2008). After birth, the gastric epithelial cells continue their differentiation and do not mature until end of the third postnatal week. During this period, any alteration in suckling induces immediate changes in gastric cell proliferation (Alvares and Gama, 1993; Gama and Alvares, 2000) and differentiation (Lin et al., 2001). Accordingly, if the effect of fasting on gastric mucosa is compared between pups and adult rats, two contrasting responses are found: increased epithelial cell proliferation in pups, and inhibition in adults (Alvares and Gama, 1993). Furthermore, if pups are early-weaned, the proliferative response

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to fasting reverses to the adult inhibitory pattern (Gama and Alvares, 2000), suggesting that the presence of milk in the stomach is essential to maintain the proliferation at rates that promote regular growth.

Milk contains antibodies, nutrients, hormones and growth factors (Koldovský, 1989; Xu, 1996) and these factors are not essential for survival when considered individually, even though their association has a protective effect for the gastrointestinal tract (Carver and Barness, 1996). Transforming growth factor α (TGFα) has been detected in human milk (Connolly and Rose, 1988; Okada et al., 1991), but not in rat milk (Dvorák and Koldovský, 1994). However, studies showed that high levels of epidermal growth factor (EGF) are present in milk of different species, and it might regulate the activity of TGFα in the stomach (Kelly et al., 1997; Dvorák et al., 2000; Milani and Calabró, 2001).

TGFα is a multifunctional peptide that shares 35% of homology with EGF, and both bind to the EGF receptor (EGFR) (Kumar et al., 1995). By binding to EGFR, TGFα activates the receptor and stimulates different signaling cascades (Yarden and Sliwkowski, 2001). In the gastric mucosa, TGFα is detected in surface mucous, mucous neck and parietal cells in humans and rats (Hormi and Lehy, 1994; Hormi et al., 1995; Bluth et al., 1995; Montaner et al., 1999). TGFα expression is first detected during fetal development (Hormi et al., 1995; Kelly et al., 1997) and increases throughout postnatal growth. Functionally, TGFα inhibits acid secretion (Rhodes et al., 1986), delays gastric emptying (Shinohara et al., 2001), stimulates cell proliferation, differentiation and migration (Bluth et al., 1995; Nakajima and Kuwayama, 1995), inhibits apoptosis (Kanai et al., 2001), and accelerates the repair of epithelial lesions (Milani and Calabró, 2001; Romano et al., 1992; Konturek et al., 1996).

When the influence of milk on development is studied through early-weaning model, i.e. by abruptly changing the dietary pattern, hormones and growth factors, including EGF and TGFα are not provided to the offspring. Moreover, early weaning induces alterations in the gastric mucosa as increased thickness (Gama and Alvares, 2000), high levels of ornithine decarboxylase and pepsinogen activity (Lin et al., 2001). Adding to control of cell proliferation, these parameters indicate that early weaning promotes the precocious maturation of gastric mucosa. It is possible that early weaning affects expression of growth factors that might directly or indirectly regulate all these processes.

The aim of this study was to investigate the influence of suckling–weaning transition on the maturation of the gastric mucosa. More specifically, we used the early-weaning model to study the differentiation of mucous neck cells during postnatal development of the stomach. Because TGFα is locally produced and it is one of the growth factors that may be affected by nutritional condition and by hormones, we also investigated the distribution of TGFα/EGFR in the gastric mucosa and their possible role in the differentiation of mucous neck cells.

2. Material and methods

2.1. Animals and early weaning

Wistar rats were obtained from the Animal Colony at the Department of Cell and Developmental Biology. Experiments were approved by the Ethical Committee for Animal Experiments (CEEA 124/06- ICB USP). Pregnant females were kept in isolated cages and delivery was set as day 0. Litters were culled to 8–9 pups around the 3rd day. Animals were kept at 22 °C and under 12/12 h light–dark schedule. Water was offered *ad libitum*.

At 15 days, pups were separated into two groups: control and early weaning (EW). The control group was kept with the dam until sacrifice, whereas EW animals were separated and placed in plastic cages with water and hydrated powdered chow (Nuvilab CR-1, Nuvital Nutrientes SA, Colombo, PR, Brazil) offered *ad libitum*. Because pups might not defecate and urinate, these functions were stimulated by abdominal massage twice a day. Body weight was checked throughout the experiment. At least three animals from both control and EW groups were sacrificed at 15, 16, 17 and 18 days. Pups were anesthetized with a 1:1 (v/v) mixture of Rompun (Bayer, São Paulo, Brazil) and Ketamina (Agener, São Paulo, Brazil) (0.5 ml/100 g b.wt.). The stomachs were immediately collected, opened by the lesser curvature and flushed with saline solution. Samples from the corpus region were obtained for immunohistochemistry, histochemistry and Western blot analyses.

For immunohistochemistry and histochemistry, samples were immersed either in Bouin fixative or 4% formaldehyde solution in 0.1 M phosphate buffer (pH 7.4) and embedded in paraffin wax. Samples for Western blot were obtained by scraping the mucosa of the corpus region of the stomach, which was stored in 0.01 M phenilmethane sulfonyl fluoride (PMSF) in 0.02 M Tris-buffered saline (TBS) at −80 °C.

2.2. AG1478 treatment

To test whether EGFR plays a role in mucous neck cells differentiation, we administered AG1478 (Calbiochem, San Diego, CA), which inhibits EGFR phosphorylation (Levitzki and Gazit, 1995) to early-weaned rats. After the onset of early weaning, pups were i.p. injected with 0.5% DMSO (control) or AG1478. Different doses were used as follows: in a first set of experiments, rats received one or two daily injections of AG1478 at 1200 μg/kg (AG[2400]), whereas other groups received only one daily shot at 3600 μg/kg (AG[3600]). For AG[2400] administration, treatment started in the afternoon of the 15th day with a single injection at 1200 μg/kg, and proceeded on the 16th and 17th days, with two shots at 1200 μg/kg; finally on the 18th day, they were injected with AG1478 at 1200 μg/kg again 30 min before sacrifice.

We should mention that we tested lower doses of AG1478 before deciding for the protocol above. For testing, we started with daily injections at 600 μg/kg (Kazumori et al., 2004), which then increased to 1200 and 2400 μg/kg. After observing different parameters, we defined the doses and schedules described before.

2.3. Immunohistochemistry for TGFα and EGFR

Non-serial 6 μm sections were cleared of paraffin 1 day before immunostaining procedure. Sections were rehydrated with 0.05 M phosphate-buffered saline (PBS) and the endogenous peroxidase was blocked with 0.3% hydrogen peroxide (H_2O_2) in methanol (30 min). Incubation with 10% goat serum (20 min) was used to block non-specific binding of the antibodies. Tissue sections were incubated overnight at 4 °C with the primary antibody for TGFα (6.7 μg/ml, mouse, Oncogene-Calbiochem) or EGFR (6.7 μg/ml, rabbit, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, samples were incubated with the respective biotinylated secondary antibody (13 μg/ml, Jackson ImmunoResearch Laboratories, PA), followed by the streptavidin–peroxidase complex (10 μg/ml, Jackson ImmunoResearch Laboratories). Reaction was developed by 0.05% 3,3′-diaminobenzidine (DAB) (DAKO, Carpinteria, CA) and slides were then counterstained in Mayer's hematoxylin. Negative control sections were incubated either with normal serum or without the primary antibodies.

Sections were analyzed under light microscope (Nikon, Japan) at 800 × magnification. The presence and localization of TGFα and EGFR in the corpus region of the stomach were determined and