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In vivo effects of TGFβ1 on the growth of gastric epithelium in suckling rats

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Abstract

As the content of Transforming Growth Factor-β (TGFβ) wanes in the milk of lactating rat, an increase in TGFβ is observed in the gastric epithelia concomitant with differentiation of the glands upon weaning. Whereas TGFβ has been shown to inhibit the proliferation of gastrointestinal cells *in vitro*, its functional significance and mechanisms of action have not been studied *in vivo*. Therefore, we administered TGFβ1 (1 ng/g body wt.) to 14-day-old rats in which the gastric epithelium was induced to proliferate by fasting, and determined the involvement of signaling through Smads and the impact on epithelial cell proliferation and apoptosis. After the gavage, we observed the progressive increase of active TGFβ1 while TβRII-receptor remained constant in the gastric mucosa. By immunohistochemistry, we showed Smad2/3 increase at 60 min ($p<0.05$) and Smad2 phosphorylation/activation and translocation to the nucleus most prominently between 0 and 30 min after treatment ($p<0.05$). Importantly, TGFβ1 inhibited cell proliferation ($p<0.05$), which was estimated by BrDU pulse-labeling 12 h after gavage. Lower proliferation was reflected by increased $p27^{kip1}$ at 2 h ($p<0.05$). Also, TGFβ1 increased apoptosis as measured by M30 labeling at 60 and 180 min ($p<0.001$), and by morphological features at 12 h ($p<0.05$). In addition, we observed higher levels of activated caspase 3 (17 kDa) from 0 to 30 min. Altogether, these data indicate a direct effect of TGFβ1 signaling through Smads on both inhibiting proliferation, through alteration of cycle proteins, and inducing apoptosis of gastric epithelial cells *in vivo*. Further, the studies suggest a potential role for both milk and tissue-expressed TGFβ1 in gastric growth during postnatal development.
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Keywords: TGFβ1; Smads; p27; Cell proliferation; Apoptosis; Stomach

1. Introduction

The growth of the gastrointestinal tract is coordinated by a complex interaction of hormones, growth factors, milk-borne molecules, and genetic program [1]. During the first three postnatal weeks of rat development, any disturbance in suckling induces immediate changes in gastric cell proliferation [2,3] and differentiation [4]. Accordingly, if the effect of fasting on gastric mucosa is compared between pups and adult rats, two opposite responses are found: increase of epithelial cell proliferation in the pups, and inhibition in the adults [2]. Furthermore, if pups are early-weaned, the proliferative response to fasting reverses to the adult pattern [3], suggesting that the presence of milk in the stomach is essential to maintain the proliferation at rates that promote regular growth.

Transforming Growth Factor-β (TGFβ) was detected in murine milk, either when TGFβ1 (112 to 236 pM on the 1st postpartum week) [5] or TGFβ2 were studied (128 to 216 pM) [5] (157 ng/ml on the 10th postpartum day) [6]. The expression of both TGFβ and TGFβ-signaling receptors, TβRI and TβRII, increases in the different cell types of the gastric gland upon weaning [7]. In addition, as the concentration of TGFβ wanes in milk [6], it increases in the gastric tissue [7], which might indicate the existence of an imprinted timing for the expression of TGFβ and receptors throughout postnatal development. Interestingly, fasting changes the distribution of TGFβ in gastric cells in suckling rats [8], suggesting that the presence of milk in the stomach can be an epigenetic factor of control in the synthesis of TGFβ.

TGFβ belongs to a large family of peptides [9] and regulates a remarkable number of cellular processes in the gastrointestinal

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tract including proliferation [10,11], differentiation [7,12], migration [13], and apoptosis [14]. Upon activation, TGFβ binds to the transmembrane receptor TβRII, which phosphorylates TβRI, and induces the formation of a complex [15] that directly phosphorylates proteins of the Smads family, specifically Smad2 and/or Smad3 [16,17]. Phosphorylated Smad2/3 dissociates from the receptor, combines with Smad4, and the complex translocates to the nucleus to activate TGFβ-regulated genes [17].

TGFβ arrests the epithelial cell cycle at late G1 [18–20]. Following treatment, p15 binds to cyclin D-Cdk4 and Cdk6, displacing $p27^{kip1}$ from this complex. p27 is a *k*inase *i*nhibitor *p*rotein (KIP) from a family of *Cdk i*nhibitors (Cki) which are differently required for cell cycle arrest [21–24]. Accordingly, $p27^{kip1}$ (p27) plays a critical role in G1/S transition by inhibiting the activity of cyclin E-Cdk2 complex [21,22,25]. p27 levels are regulated through translational and degradation controls [26–28], which can be influenced by TGFβ [22,24]. Moreover, it has been demonstrated that p27 deficiency and *H. pylori* infection cooperate *in vivo* in gastric cancer development [29].

Although the inhibitory role of TGFβ on the gut epithelium has been mainly studied in established cell lines and primary cultures [10,11,30], the mechanisms involved in TGFβ action have not been determined *in vivo*. Also, the functional significance of TGFβ in milk and its progressive distribution in the gastric gland throughout postnatal development has not been investigated yet. Because fasting can be used to induce hyperproliferation in the stomach at suckling phase [2], and such state represents a challenge to the inhibitory function of TGFβ, fasting was used as a condition to test TGFβ effect in the gastric epithelium. Therefore, the current study was aimed at defining the effect of TGFβ *in vivo* by simulating TGFβ presence in gastric content by exogenous gavage. Specifically, we focused on the TGFβ-mediated activation of the Smads signaling cascade and its effect on cell proliferation, including the regulation of p27 level, and apoptosis in gastric epithelium of suckling rats.

2. Materials and methods

2.1. TGFβ1 treatment

Lyophilized TGFβ1 (R&D Systems, Minneapolis, MN) was reconstituted according to manufacturer's directions into a 1μg/ml stock solution in 4 mM HCl containing 0.1% BSA. For all experiments TGFβ1 was further diluted in a vehicle containing 0.9% saline and 0.2 mg/ml BSA.

Wistar rats from the Department of Cell and Developmental Biology Animal Colony (ICB USP) were used according to the Procedures of the Animal Ethics Committee (protocol approved 027/2004). Litters were kept at 22 °C under 12/12 h light/dark schedules. Water was offered *ad libitum* and was weekly supplemented with a 0.09% multivitamin complex (Vitagold, Tortuga, São Paulo, Brazil).

Fasting was used to induce cell proliferation in the gastric mucosa [2] as a challenge to the inhibitory effect of TGFβ1. To restrict food intake, 14-day-old rats were separated from the dams and placed in aluminum cages with removable bottom to avoid coprofagy. Thirty nanograms of TGFβ1 were administered by gavage (1 ng/g body wt.) in a single dose 90 min after the beginning of fasting treatment, which was maintained for 12 h. Such schedule was planned to allow TGFβ1 function concomitant with progressive fasting and dose was calculated based on the studies of Playford et al. [31] who considered colostrum and milk sources of TGFβ. After the indicated time periods of TGFβ1 treatment, rats were anesthetized with a 1:1 mixture of xilazine (Bayer, São Paulo, Brazil) and ketamine (Parke-Davis, São Paulo, Brazil) (0.25 ml/100 g body wt.) and the stomachs were immediately collected for experimental procedures. All the studies were performed in the corpus region of the stomach.

2.2. Bromodeoxyuridine (BrDU) labeling and DNA synthesis index (SI) determination

We used BrDU pulse-labeling for 1 h to estimate DNA synthesis index 12 h after TGFβ1 gavage. As previously shown [32], a period of 12 h is long enough to allow the changes in cell cycle control, specifically in G1/S transition, to be reflected on the number of proliferating cells. Therefore, animals that had been treated with vehicle containing 0.9% saline and 0.2 mg/ml BSA (control group, $n=5$) or TGFβ1 ($n=7$) were injected intraperitoneally with BrDU (100 mg/kg body wt., Sigma, St Louis, MO) at 8:00 a.m. and sacrificed 1 h later. Immunohistochemistry for BrDU was used to determine DNA synthesis index (SI) for epithelial cells inside the proliferative compartment that occupies the whole gland in the corpus region of the stomach in suckling rats [2]. Positive nuclei were quantified from a total of 2500 cells per animal [33] under light microscope (Nikon, Japan) (800× using 8× integrative eyepiece, Zeiss, Germany). Only randomly selected fields containing longitudinally sectioned gastric glands were considered. The SI was determined as the number of BrDU positive nuclei/total cells × 100.

2.3. Immunohistochemistry

This procedure was used to detect BrDU, Smad2/3 and Smad2P. For Smad2/3 and Smad2P, we used time-course experiments to evaluate the kinetics variation. To that, samples ($n=3$ for each period) were collected at 0, 10, 30, 60, 120, 180 min and 12 h after treatment with TGFβ1. For immunohistochemistry, the stomachs were excised, rinsed with 0.9% saline solution, fixed in 10% formalin and embedded in paraffin wax. Non-serial 6 μm sections were placed on slides coated with poly-L-lysine or 3-aminopropyltriethoxy-silane (only sections for Smads immunolabeling) (Sigma). Sections were deparaffinized, rehydrated and peroxidase activity was blocked with 0.15% H_2O_2 (Sigma) in methanol. Nonspecific binding was blocked with 5% goat or rabbit serum (Jackson Laboratories, West Groove, PA), and antigen retrieval was performed with 0.1% pepsin (Merck, Darmstadt, Germany) in 0.1 N HCl for BrDU, and with 10 mM citric acid pH 6.0 for Smads (microwave for 5 min at high power followed by 3 × 3 min at medium power). Tissue sections were incubated overnight at

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