EGFR is involved in control of gastric cell proliferation through activation of MAPK and Src signalling pathways in early-weaned rats

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Abstract

Objectives: Early weaning (EW) increases proliferation of the gastric epithelium in parallel with higher expression of transforming growth factor alpha and its receptor epidermal growth factor receptor (EGFR). The primary objective of the present study was to examine involvement of EGFR signalling in regulating mucosal cell proliferation during the early weaning period.

Materials and methods: Fifteen-day-old rats were split into two groups: suckling (control) and EW, in which pups were separated from the dam. Animals were killed daily until the 18th day, 3 days after onset of treatment. To investigate the role of EGFR in proliferation control, EW pups were injected with AG1478, an EGFR inhibitor; signalling molecules, proliferative indices and cell cycle-related proteins were evaluated.

Results: EW increased ERK1/2 and Src phosphorylation at 17 days, but p-Akt levels were unchanged. Moreover, at 17 days, AG1478 administration impaired ERK phosphorylation, whereas p-Src and p-Akt were not altered. AG1478 treatment reduced mitotic and DNA synthesis indices, which were determined on HE-stained and BrdU-labelled sections. Finally, AG1478 injection decreased p21 levels in the gastric mucosa at 17 days, while no changes were detected in p27, cyclin E, CDK2, cyclin D1 and CDK4 concentrations.

Conclusions: EGFR is part of the mechanism that regulates cell proliferation in rat gastric mucosa during early weaning. We suggest that such responses

might depend on activation of MAPK and/or Src signalling pathways and regulation of p21 levels.

Introduction

Growth and maturation of the gastrointestinal tract involve balance of cell proliferation, migration, differentiation and death, and all these processes are coordinated by a complex interaction of hormones, growth factors, milk-borne molecules, luminal microbes and genetic programmes (1,2).

Complete maturation of the gastric epithelium takes place during the first 3 weeks of post-natal life, which coincides with dietary change from milk to solid food (3). During this period, disturbances in suckling induce immediate responses of gastric cell proliferation (4,5) and differentiation (6,7). Accordingly, when pups are submitted to fasting, cell proliferation is stimulated, whereas the opposite effect is observed in adult rats (4). Furthermore, if pups are weaned early, the proliferative response to fasting reverses to the adult pattern (5) and differentiation is accelerated (7), suggesting that presence of milk in the stomach is essential to maintain epithelial proliferation at rates that promote regular growth.

Milk contains antibodies, nutrients, hormones and growth factors (8–10) and although they are not crucial for survival when individually considered, their association has a protective role on the gastrointestinal tract (11). Early weaning can be used as an experimental model to remove milk-borne molecules from feeding, abruptly changing dietary pattern. When pups are weaned early, several alterations are observed in the gut. Permeability of the intestinal barrier is increased (12) and expression of small intestinal alkaline phosphatase expression, an enterocyte differentiation marker, is reduced (13). In the stomach, early weaning increases incidence of gastric erosion (14), induces activity of the ornithine decarboxylase enzyme (6), stimulates gastric epithelial cell proliferation (5) and accelerates differentiation of mucous neck cells

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(7), as mentioned above. In addition, we have previously shown that early weaning elevates expression of transforming growth factor α (TGF α) and its receptor, epidermal growth factor receptor (EGFR), in the rat gastric mucosa (7). TGF α has been detected in human milk (15), but not in rat milk (16). However, EGF present in milk of different species might regulate activity of TGF α in the stomach (17,18). EGF and TGF α bind to the same receptor and have similar biological activity, but despite the presence of both peptides in the gastrointestinal tract, TGF α is more widely distributed and may be the main ligand for EGFR in this mucosa (19). TGF α is a multifunctional peptide, which stimulates cell proliferation, differentiation and migration (20–22), inhibits apoptosis (23), delays gastric emptying (24), inhibits acid secretion (25,26) and accelerates repair of lesions (27,28). In the gastric mucosa, TGF α and EGFR are detected in surface mucous, mucous neck and parietal cells (7,29,30).

TGF α and other ligands bind to EGFR and trigger different signalling cascades such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and Src kinase pathways (31,32). After stimulation of these molecules, transcription factors are activated to culminate in different responses such as cell proliferation, differentiation, migration and death (32,33).

Cell cycle progression depends on function of cyclins and cyclin-dependent kinases (CDKs), which form complexes regulated by CDK-inhibitory (CKI) proteins such as p21^{waf1} and p27^{kip1} (hereafter referred to as p21 and p27). Among CDK–cyclin complexes, CDK4–cyclin D is induced during G₁ phase, whereas G₁–S transition is controlled by CDK2–cyclin E (34,35).

Early weaning stimulates cell proliferation and up-regulates EGFR expression in the gastric mucosa of rats, but there is no evidence of association between these two events. To explore this issue, we studied how EGFR could be involved in increased gastric cell proliferation, and subsequently, which intracellular pathways could be part of this mechanism. Following this hypothesis, we first evaluated signalling cascades in the gastric epithelium throughout early weaning and then we investigated effects of EGFR inhibition on the same pathways, levels of cell cycle-related proteins and ultimate function on cell proliferation.

Materials and methods

Animals and early weaning

Wistar rats were obtained from the Animal Colony at the Department of Cell and Developmental Biology (ICB USP, Institute of Biomedical Sciences, University of São Paulo, Brazil). Experiments were approved by the Ethical

Committee on Animal Experimentation (CEEPA protocol number 124/2006). Animals were kept at 22 °C and under 12 h light cycle. Water was offered *ad libitum*. Pregnant females were kept in isolated cages and delivery was set as day 0. Litters were culled to 8–9 pups around the third day. At 15 days, pups were separated into two groups: suckling control and early weaning (EW). The suckling group was kept with the dam until killing, whereas EW animals were placed in plastic cages with water and hydrated powdered chow (Nuvilab CR-1; Nuvital Nutrientes SA, Colombo, Brazil) *ad libitum*. As pups might not defecate and urinate, these functions were stimulated by abdominal massage twice a day. Body weight was checked throughout the experiment.

Stomach collection

At least three animals from both suckling and EW groups were killed at 15, 16, 17 and 18 days. Pups were anaesthetized with a 1:1 (v/v) mixture of xylazine (Rompun; Bayer, São Paulo, Brazil) and ketamine (Ketamina; Agener, São Paulo, Brazil) (0.5 ml/100 g body weight). Stomachs were immediately excised, opened at the small curvature, flushed with 0.9% saline solution and submitted either to mucosal scraping or to fixation in 10% formaldehyde.

Cell culture

AG1478 (Calbiochem, San Diego, CA, USA) is a tyrosine kinase inhibitor that inhibits EGFR phosphorylation (36). Before using AG1478 *in vivo*, we first evaluated its efficiency *in vitro* on normal rat intestinal epithelial cells. AG1478 was diluted in 0.5% DMSO and kept at –20 °C. IEC-6 cells [CRL-1592; American Type Culture Collection (ATCC), Manassas, VA, USA] were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum, insulin (0.1 U/ml), and 1% antibiotics. Cells were separated into three different groups: control; control stimulated with EGF (60 ng/ml for 15 min); and treated with 1 μ M AG1478 (30 min), followed by EGF stimulation (60 ng/ml for 15 min). After incubation, cells were washed in phosphate-buffered saline (PBS) and harvested for protein extraction.

AG1478 administration

To investigate the role of EGFR in gastric cell signalling and proliferation *in vivo*, we administered AG1478 to early-weaned rats. After onset of EW, pups were daily injected intraperitoneally with vehicle (0.5% DMSO) as control (EW) or AG1478 at 3600 μ g/kg (AG1478), as

described previously (7). Animals received the last injection 30 min before being killed.

Western blot analysis

Gastric mucosa of the corpus region was scraped and stored in 10 mM PMSF (Merck, Darmstadt, Germany) in 0.02 M Tris-buffered saline (TBS) at -80°C . Protein was extracted using RIPA lysis buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate in 50 mM Tris-HCl, pH 7.5) containing protease inhibitors (1 mM PMSF, 0.45 mg/ml benzamidine, 1 mM leupeptin and 1 mM aprotinin) (Sigma Chemical, St Louis, MO, USA). Protein concentration was determined using the Bradford method (37) and 30 μg of total protein was separated on a 5–20% gradient SDS-PAGE. Samples were electroblotted on to nitrocellulose membranes (Hybond-ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and incubated overnight at 4°C with rabbit polyclonal antibodies to signalling proteins ERK1/2 (1:5000), phospho-Akt (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phospho-Src (1:300; Cell Signaling Technology, Boston, MA, USA), and to cell cycle proteins cyclin E, CDK2, cyclin D1 (1:200), CDK4 (1:300), p27 (1:100) (Santa Cruz Biotechnology) and p21 (1:100; Abcam, Cambridge, MA, USA). Monoclonal antibodies were used against phospho-ERK1/2 (1:5000, Santa Cruz Biotechnology) and β -actin (1:10000, Sigma Chemical). Reactions were developed with ECL Kit (GE Healthcare) and signals were registered on X-ray films (MXG-Plus; Kodak, São Paulo, Brazil). Densitometry was performed using IMAGE J (1.37v Software, NIH Public Domain, USA).

Cell proliferation analyses

Epithelial cell proliferation was evaluated in the gastric mucosa of the corpus region of stomach. Non-serial 6 μm paraffin sections were submitted either to immunohistochemistry for BrdU or staining with haematoxylin and eosin (H&E), which were respectively used to determine DNA synthesis (SI) and mitotic indices (MI).

SI was detected in gastric sections obtained from 18-day-old rats treated or not treated with AG1478 and injected intraperitoneally with BrdU (100 mg/kg body weight) 1 h before killing. After clearance of the paraffin wax, sections were rehydrated with 0.05 M PBS; endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol (30 min) and non-specific binding was blocked by using 10% goat serum (20 min). Tissue sections were incubated overnight at 4°C with monoclonal anti-BrdU (1:50; GE Healthcare). After washing, peroxidase-conjugated antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added, and the

reaction was developed by 0.05% 3,3'-diaminobenzidine (DAB) (Dako, Carpinteria, CA, USA) in PBS containing 0.15% H_2O_2 . Slides were counterstained in Mayer's haematoxylin and negative controls were incubated with normal serum to replace the primary antibody.

MI was determined in H&E-stained histological sections and both SI and MI were obtained using light microscopy (Nikon, Tokyo, Japan) at 800 $\times$ magnification using an 8 $\times$ integrative eyepiece with ocular grid (Zeiss, Oberkochen, Germany). SI was estimated by counting around 2500 epithelial cells as BrdU-immunolabelled or non-labelled, along the proliferative compartment. Only longitudinal sections were considered. SI was determined for each animal as BrdU-labelled cells/total epithelial cells $\times 100$. MI was similarly obtained by counting mitotic and interphase cells, following the same criteria. MI was represented as mitotic cells/total epithelial cells $\times 100$.

Statistical analyses

Results were analysed using Student's *t*-test or ANOVA followed by Tukey's test (GraphPad Prism 5.01; GraphPad Software, Inc., La Jolla, CA, USA) to evaluate differences between groups. Significance level was set at $P < 0.05$.

Results

Effect of early weaning on cell signalling pathways activated by EGFR

Suckling (control) and early-weaned animals at 15, 16, 17 and 18 days were used to study the influence of early weaning on MAPK, Src and PI3K signalling pathways, which can be differentially activated by EGFR (32,33). We evaluated phosphorylation of ERK1/2, Src and Akt. Levels of p-Akt initially decreased then increased on the 18th day, of both suckling and EW animals (Fig. 1a). Src activation was different from that of Akt, and was significantly higher in EW pups compared to those of the suckling group, on the 17th day ($P < 0.05$) (Fig. 1b). Finally, we observed that phosphorylation of ERK1/2 increased every day and was significantly higher in early-weaned animals compared to the suckling control group at 17 days (Fig. 1c) ($P < 0.05$).

AG1478 inhibited EGFR signalling in gastric epithelial cells in vivo

As mentioned earlier, we first tested efficiency of AG1478 to inhibit EGFR activity *in vitro*. We found that IEC-6 cells stimulated with EGF had higher levels of ERK1/2 phosphorylation compared to controls (Fig. 2). However,

Figure 1. Effect of early weaning on cell signalling pathways activated by EGFR in the gastric mucosa of suckling (S) or early-weaned (EW) rats at 15, 16, 17 and 18 days post mortem. Representative immunoblots and respective densitometries are shown for (a) p-Akt, (b) p-Src and (c) p-ERK1/2. Thirty micrograms of total protein was loaded into each lane and each band is representative of one animal. β -actin and ERK1/2 were used as loading controls for p-Akt and p-Src, and p-ERK1/2, respectively. Relative densitometry as ratio of loading control is shown as fold change of the 15-day-old group and is presented by bars as mean $\pm$ SD. (n) indicates number of rats used in each condition. * $P < 0.05$ when compared to the suckling control group at the same age. # $P < 0.05$ compared to previous day at identical conditions.

when these cells were pre-treated with AG1478, activation of ERK 1/2 was not increased (Fig. 2), indicating that AG1478 addition prevented phosphorylation of ERK1/2 stimulated by EGF.

As efficiency of AG1478 was confirmed *in vitro*, we administered this tyrosine kinase inhibitor *in vivo* throughout early weaning and collected gastric mucosa from 17-day-old rats, which represent 2 days after onset of treatment. This age had been chosen as we had observed that it is crucial

for cell signalling control during early weaning (Fig. 1). We did not observe any change in Akt and Src activation (Fig. 3). However, levels of p-ERK1/2 decreased in pups treated with AG1478 when compared to the control group injected with vehicle ($P < 0.05$) (Fig. 3).

Cell proliferation in 18-day-old early-weaned animals after AG1478 treatment

Early weaning stimulated proliferation of gastric epithelial cells (5). To evaluate whether EGFR had a role in this process, we estimated mitoses and DNA synthesis in 18-day-old animals submitted to early weaning and treated with AG1478. DNA synthesis index decreased after AG1478 administration compared to the control group ($P < 0.05$) (Fig. 4a,b,f). MI was similarly inhibited after AG1478 treatment ($P < 0.05$) (Fig. 4c,e,g).

Effect of AG1478 on cell cycle-related proteins

After finding that AG1478 decreased p-ERK1/2 levels and inhibited gastric cell proliferation in early-weaned pups, we investigated which cell cycle-related proteins

Figure 2. Effect of AG1478 on ERK1/2 activation *in vitro*. IEC-6 cells were separated into three groups: control, control stimulated with EGF (60 ng/ml, 15 min), and pre-treated with 1 μ M AG1478 (30 min) then stimulated with EGF (60 ng/ml, 15 min). Representative immunoblots for p-ERK1/2. ERK1/2 used as loading control.

Figure 3. Effect of AG1478 on cell signalling pathways activated by EGFR in gastric mucosa of 17-day-old rats submitted to early weaning and treated daily with vehicle or AG1478. Representative immunoblots and respective densitometries shown for p-Akt, p-Src and p-ERK1/2. Thirty micrograms of total protein was loaded into each lane and each band is representative of one animal. β -actin and ERK1/2 were used as loading controls for p-Akt and p-Src, and p-ERK1/2, respectively. Relative densitometry as ratio of loading control is shown as fold change of the control group (EW) and is presented with bars, mean $\pm$ SD. (n) = 4 animals at each condition. * P < 0.05 comparing AG1478 to EW control.

could be influenced by this tyrphostin. Accordingly, we studied concentrations of cyclin E, CDK2, cyclin D1, CDK4, p21 and p27. We observed that whereas p21 levels were lower in AG1478-treated animals when compared to the control group (P < 0.05) (Fig. 5), the other protein levels did not change (Fig. 5).

Discussion

Early weaning is a stressful condition caused by maternal separation and breast milk deprivation, and there are several consequences to the gastrointestinal tract, such as increased gastric epithelial cell proliferation and EGFR expression. In the present study, we investigated participation of EGFR in cell proliferation stimulus, as well as cell signalling pathways that could be involved in this event and levels of cell cycle-related proteins.

It is known that early weaning augments incidence of gastric erosion (14), induces activity of the enzyme ornithine decarboxylase (6), stimulates epithelial gastric cell proliferation (5) and mucous neck cell differentiation (7). Presence of increased levels of TGF α and EGFR in the gastric mucosa of early-weaned animals (7) prompted us to investigate EGFR-stimulated signalling pathways that could be involved in gastric cell proliferation and maturation. EGFR acts by stimulating different intracellular pathways (31,32), among which we studied MAPK, PI3K-Akt and Src cascades throughout early weaning. We observed increases in ERK1/2 and Src phosphorylation in early-weaned rats at 17 days, indicating higher activation of Ras-MAPK and Src pathways. Ras leads to phosphorylation of MAPKs such as ERK 1 and 2, which translocate to the nucleus and phosphorylate transcription factors. After

EGFR activation, increase in ERK phosphorylation triggers several cell responses. The most common action associated with this pathway is a stimulus for cell proliferation (28,38), but ERKs can also prevent apoptosis (39), stimulate cell migration (40), differentiation (41) and goblet cell mucin secretion (42). Src is a protein tyrosine kinase that controls cell proliferation, differentiation and migration (43). In context, this increases EGFR-stimulated proliferation of fibroblasts and tumour cells (44,45). Src is phosphorylated by EGFR, but can be stimulated by other receptors, such as GPCRs, then transactivating EGFR (46), inducing gastric epithelial restitution (28).

The main difficulty in studying cell signalling *in vivo* is that EGFR does not use any exclusive pathway; all the cascades activated by this receptor can be stimulated by other growth factors also. Unresponsiveness of certain signalling molecules can be a consequence of: (i) lack of involvement of EGFR transduction, (ii) activation by other receptors counterbalancing EGFR-stimulated signalling and (iii) exact moment of response may not be precisely determined. Thus, increased levels of p-ERK and p-Src in 17-day-old EW rats might indicate that both Ras-MAPK and Src signalling pathways could be involved in gastric cell proliferation and differentiation (33,45,47). Moreover, we did not observe variation in p-Akt levels comparing control and EW rats, but we cannot completely rule out participation of PI3K cascade in modifications taking place throughout early weaning.

Diversity of biological responses to EGFR-associated activation of Ras-MAPK and Src pathways indicates that both cascades could be stimulating gastric cell proliferation and/or mucous neck cell differentiation. However, when early-weaned animals were injected with AG1478, only ERK phosphorylation was inhibited, suggesting that this pathway is certainly involved in cell proliferation and differentiation. Although we did not observe inhibition of p-Src after AG1478, it does not mean that this molecule is not associated with those cell processes in early-weaned animals. As mentioned previously, activation of Src can be upstream or downstream of EGFR phosphorylation (28,46). Thus, activation of Src may be upstream of EGFR stimulation in our model, and AG1478 injection would not in this case interfere with p-Src levels. Alternatively, as our treatment is long, it is possible that our sampling time did not coincide with the moment when cell signalling molecules were regulated.

Cell proliferation can be regulated by many factors. TGF α , EGF and IGFs stimulate gastric cell proliferation (21,22,48), while TGF β 1 administered to suckling rats has the opposite effect (1). Previously, it was shown that early weaning stimulates cell proliferation (5) and increases EGFR expression (7). Currently, besides evaluating the signalling pathways involved in this response, we aimed to

Figure 4. Cell proliferation in the gastric mucosa of 18-day-old rats submitted to early weaning and treated daily with vehicle or AG1478. (a, b) BrdU-immunolabelled cells (brown) distributed in glands (G) of early-weaned (EW) and AG1478-treated pups (AG1478) respectively. (c) negative control section. Sections were counterstained with Mayer's haematoxylin. (d, e) mitotic cells observed in glands of EW and AG1478 groups respectively. Sections stained with haematoxylin and eosin. (f) DNA synthetic index (SI as percentage) in gastric epithelium of each condition. (g) mitotic index (MI as percentage). Both SI and MI are represented by bars as mean $\pm$ SD. (n) = 3 animals at each condition. * P < 0.05 comparing AG1478 to EW control group. Bars: (a–c), 20 μ m, (d, e), 10 μ m.

study the association of EGFR with proliferation of the gastric epithelium. Using AG1478, we noted involvement of the receptor in cell proliferation. After injecting AG1478 in early-weaned animals, we evaluated DNA synthesis and mitotic indices at 18 days and found them to be lower compared to the control group. These results show that EGFR inhibition impaired cell proliferation stimulus triggered by early weaning, suggesting a role for EGFR in this event. Similar to early weaning, which increases gastric cell proliferation (5), small bowel resection (SBR) has a stimulatory effect on enterocyte proliferation, accompanied by an increase in EGFR expression and activation (49). Inhibition of EGFR activity reduces enterocyte

proliferation in mice submitted to SBR, suggesting a function for this receptor in intestinal adaptation (50). Although early weaning is not a surgical condition, these results are in accordance with our findings.

Cell cycle control depends on interaction between cyclins, CDKs and CKIs. p21 and p27 belong to a group of molecules capable of inhibiting CDKs, acting as anti-proliferative molecules (51,52). In this study, we found low levels of p21 after AG1478 injection, which could be considered an odd event as cell proliferation also decreased. However, the role of CKIs has been re-discussed recently. p21 and p27 can be pro-proliferative molecules in certain situations and increase levels and activity of cyclin D/

Figure 5. Effect of AG1478 on cell cycle-related proteins in the gastric mucosa of 17-day-old rats submitted to early weaning and treated daily with vehicle or AG1478. Representative immunoblots and respective densitometries shown for p21, p27, cyclin D1, cyclin E, CDK2 and CDK4. Thirty micrograms of total protein was loaded into each lane and each band is representative of one animal. β -actin was used as loading control. Relative densitometry as ratio of β -actin is shown as fold change of the control group (EW) and is presented by bars as mean $\pm$ SD. (n) = 4 animals at each condition. * P < 0.05 when comparing AG1478 with EW control.

CDK complexes in the cytoplasm, which phosphorylate Rb and promote progression through G_1 phase (53,54).

The process of aging is associated with increased cell proliferation in the gastric and colonic mucosa, which is followed by increased EGFR expression (55,56) and decreased levels of p21 (57,58), suggesting an inverse relationship between the peptides. Nevertheless, Sheng *et al.* (59) have shown that EGFR activation induces proliferation of intestinal cells and increases p21 levels. *In vivo*, EGFR stimulus and increased p21 levels are important for intestinal adaptation after SBR, an intervention that increases cell proliferation (50,60). When p21-deficient mice are submitted to SBR, the pro-proliferative effect is not observed, suggesting that this CKI is essential for EGFR-stimulated proliferation (59). EGFR inhibition *in vitro* decreases cell proliferation and increases p27 levels (61–63). However, the action of EGFR on p21 is not clear, since diverse responses have been reported (59,62–64). These studies suggest that p27 is still mainly associated with inhibition of cell proliferation when related to EGFR, but p21 can act differently after EGFR-stimulated cell proliferation. In this study, we demonstrated that EGFR inhibition decreases levels of p21, suggesting that this CKI could be positively regulated by EGFR.

We conclude that early weaning induces phosphorylation of MAPK and Src pathways in the gastric mucosa, and these signalling cascades may be involved in cell proliferation stimulus through activation of EGFR. We suggest that there is an association between suckling–weaning transition and EGFR, and their interaction is part of the regulatory mechanism of gastric cell proliferation and therefore, of cell proliferation in the stomach.

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