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Differential distribution of transforming growth factor beta and receptors in the hyper or hypoproliferative gastric mucosa of developing and adult rats

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Summary. Transforming growth factor ß (TGFß) isoforms are known for their antiproliferative effect on epithelial cells *in vitro*, but the role of each isoform *in vivo* is poorly understood, mainly when non-pathological conditions are considered. We correlated the presence and distribution of isoforms and receptors to physiological changes in gastric cell proliferation in developing and adult rats. We used fasting to induce either the hyper (14-day-old pups) or hypoproliferation (60-day-old rats) of the gastric epithelium. In 14-d-old pups fasting reduced only TGFß3 labelling in the gland. Conversely, in 60-d-old rats there was an increase of TGFß1 and TGFß3 immunolabelled cells. Receptors were detected at both ages. Therefore, the changes induced by fasting in the constitutive TGFß expression can be correlated to the differential epithelial proliferation in the stomach of developing and adult rats. These results suggest that one of the functional roles of TGFß *in vivo* is to locally regulate cell proliferation.

Key words; TGFß, TßRI, TßRIL, Gastric mucosa, Rat, Cell proliferation

Introduction

Previous studies provided evidence that members of the TGFß family are growth-inhibiting factors for epithelial cells (Barnard and Coffey, 1994), including those isolated from the gastric mucosa (Nakajima and Kuwayama, 1993; Rokutan et al., 1998). However, the potent *in vivo* functions of each isoform have been discussed mainly in knockout animals and usually correlated to pathological conditions (Shull et al., 1992; Kulkarni et al., 1993). Different reports showed that TGFß2 is associated with inhibition of epithelial proliferation in the intestinal immunopathology (Mowat et al., 1996), whereas TGFß1 induces apoptosis in gastric cancer cells (Yamamoto et al., 1996). Dünker et al. (2002) observed that apoptosis is reduced in the intestinal epithelium of TGFß2$^{+/-}$ and TGFß3$^{+/-}$ heterozygous mice, suggesting that both isoforms take part in cell death control. The same study showed that while TGFß2 is detected in endocrine cells, TGFß3 is predominantly found in intestinal goblet cells, supporting the idea that there is also a functional distribution (Dünker et al., 2002).

In vivo gastric glands are complex, with several cell types: mucous neck cells, parietal and chief cells, besides undifferentiated and endocrine cells. There is strong evidence that the pattern of TGFß expression in the stomach is complex and depends on the many cell populations of the gland. In the normal human gastric mucosa, while parietal cells and some surface mucous cells exhibit TGFß1 immunoreactivity, TGFß2 labelling is seen exclusively in the cytoplasm of chief cells, and TGFß3 is found in all secretory cells (Naef et al., 1997). In gastric cancer TGFß1, ß2 and ß3 isoforms colocalize in different cell types (Naef et al., 1997) and the overexpression of TGFß1 and ß3 mRNAs (Naef et al., 1997) is possibly counterbalanced by the absence of functional receptors (Yamamoto et al., 1996; Oliveira et al., 1998), as shown in other tumors (Parekh et al., 2002; Biswas et al., 2004).

During embryonic development, expression of TGFßs follows a spatial and temporal distribution that parallels the morphogenetic alterations in tissues (Pelton et al., 1991). We have recently described the ontogenic expression of TGFß and receptors in the gastric mucosa of rats throughout the first month of life (de Andrade Sá et al., 2003) and we showed that the immunolabelling of secretory cells is concomitant with the differentiation

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sequence in the stomach epithelium (de Andrade Sá et al., 2003). The specific localization of each isoform and receptor can be related to autocrine and paracrine interactions, but isolated effects on the proliferation and differentiation of gastric epithelial cells have not been elucidated.

It is known that the dietary condition is an important regulatory factor for gastric development (Gama and Alvares, 2000) and that fasting stimulates cell division in suckling animals, leading to hyperproliferation (Alvares 1992; Alvares and Gama, 1993). In contrast, it reduces the process in the gastric mucosa of adult rats (Hunt, 1957; Alvares 1987), creating a hypoproliferative state.

Because the proliferation of the gastric epithelium can be modulated by feeding condition, without any pathological interference, it offers a reliable paradigm to compare the normal states of hypo and hyperproliferation. In the present study, we evaluated the presence and distribution of TGFß isoforms and receptors TßRI and TßRII in the gastric epithelia of suckling and adult rats, submitted or not to fasting. We aimed further to determine a functional role for TGFß i*n vivo.*

Material and Methods

Animals

Wistar rats from the Cell and Developmental Biology Department Animal Colony were used according to the Procedures of the Animal Ethic Committee (CEEA 69/2001 ICBUSP). Rats were mated and pregnant females were kept in isolated cages until the time of birth. Animals were kept at 22°C and under 12/12 hr light dark schedule. Water offered ad libitum was weekly supplemented with a multivitamin complex (Vitagold, Tortuga, São Paulo, Brazil). The delivery was set as day 0. Litters were culled to 8 pups around the 3rd day and were weaned on the 21st day.

In order to induce hyper or hypoproliferation in the gastric epithelium (Alvares and Gama, 1993) and try to correlate the changes with TGFß and receptors levels, animals were fasted as follows: at 13 days pups were separated from their mothers for 16 h, whereas at 59 days rats were fasted for 30 h. They were all placed in alluminum cages with removable bottom to avoid coprophagy. Control animals were kept under regular feeding. At least three animals were used for each age and condition.

Animals were killed at 14 and 60 days after birth with a body weight range of 25.2±2.3g and 200±30g, respectively. They were anesthetized with a 1:1 (v/v) mixture of Rompun, (Bayer, São Paulo, Brazil) and Ketalar (Parke-Davis, São Paulo, Brazil) (0.5 ml/ 100g b.wt.). The stomachs were opened through the lesser/greater curvature (14/60 days, respectively), stretched on a cork piece and fixed in 4% paraformaldeyde solution in 0.1M phosphate buffer (pH 7.4) for no more than 8 h.

Immunohistochemistry

Semi- serial sections of five µm were placed on poly-l-lysine (Sigma, MO) coated slides and cleared of paraffin at least one day before immunohistochemical procedure. The protocol was used according to de Andrade Sá et al. (2003). Samples were rehydrated in 5 mM Tris buffered saline (TBS) containing 0.1% (v/v) Triton X-100 at room temperature (RT) for 10 min, washed in TBS for 5 min and incubated with 0.3 % (v/v) hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity. Slides were subsequently washed in water and TBS with 0.1% (w/v) BSA (10 min), and then non-specific binding was blocked with either 5% (v/v) fetal bovine serum (TGFß1 reaction) or 5% (v/v) goat serum (other antigens reactions) at RT for 30 min. Tissue sections were incubated overnight at 4°C with one of the following antibodies (Santa Cruz Biotech, CA): goat anti-TGFß1 (10µg/ml; sc-146G), rabbit anti-TGFß2 (10µg/ml; sc-90), rabbit anti-TGFß3 (5µg/ml; a generous gift from Dr Leslie Gold, NYU), rabbit anti-TßRI (20mg/ml; sc-399) and rabbit anti- TßRII (20µg/ml, sc-220). Samples were washed in TBS/BSA and incubated for 2hr at RT with biotinylated secondary antibodies: anti-goat IgG (16µg/ml, Dako, Denmark) for TGFß1 reaction and anti-rabbit IgG (11µg/ml, Jackson Labs, PA) for the other antigens. After washing in TBS/BSA, the sections were treated with streptavidin-peroxidase complex (10µg/ml, Jackson Labs, PA) for 1 hr at RT. Peroxidase activity was developed by Fast DAB® (Sigma, MO) in 50 mM Tris-HCl (pH 7.4) for 30 min and slides were then counterstained in Mayer's Hematoxylin. Control sections were incubated either with normal serum according to the reaction (goat or rabbit) or without the primary antibodies.

Researchers blindly analyzed the slides under light microscope (x800, Nikon, Japan,). Semi-serial sections were used to avoid the observation of the same cells. The presence and general distribution of labelling was compared between the groups.

Results

The present study demonstrates that at 14 and 60 days, specific changes in protein expression of TGFß isoforms and receptors in the gastric mucosa are concomitant to the previously demonstrated (Alvares, 1992; Alvares and Gama, 1993) increase and decrease of cell proliferation induced by fasting.

14-day-old rats

At 14 days surface mucous cells cover the mucosa and gastric pits, and the gland is filled with differentiating cells such as pre-chief, pre-neck and parietal cells (Karam and Leblond, 1992). Through immunohistochemistry we observed that many surface mucous cells were labelled for the TGFß isoforms and

receptors with a differential distribution between fed and fasted animals. Figure 1 shows that immunostaining seems to be more concentrated in the apex after fasting, indicating an upward movement of the TGFß1 inside the cell (Fig. 1A,B). The same inversion was noted for the other isoforms and receptors. Though the intensity was not recorded, we found that it was strong and uniform in the surface mucosa.

In the gastric gland, a weak signal was observed in the apical membrane of scattered cells when the isoforms, TßRI and TßRII, were evaluated. Fasting induced a decrease of TGFß3 immunolabelling (Fig.

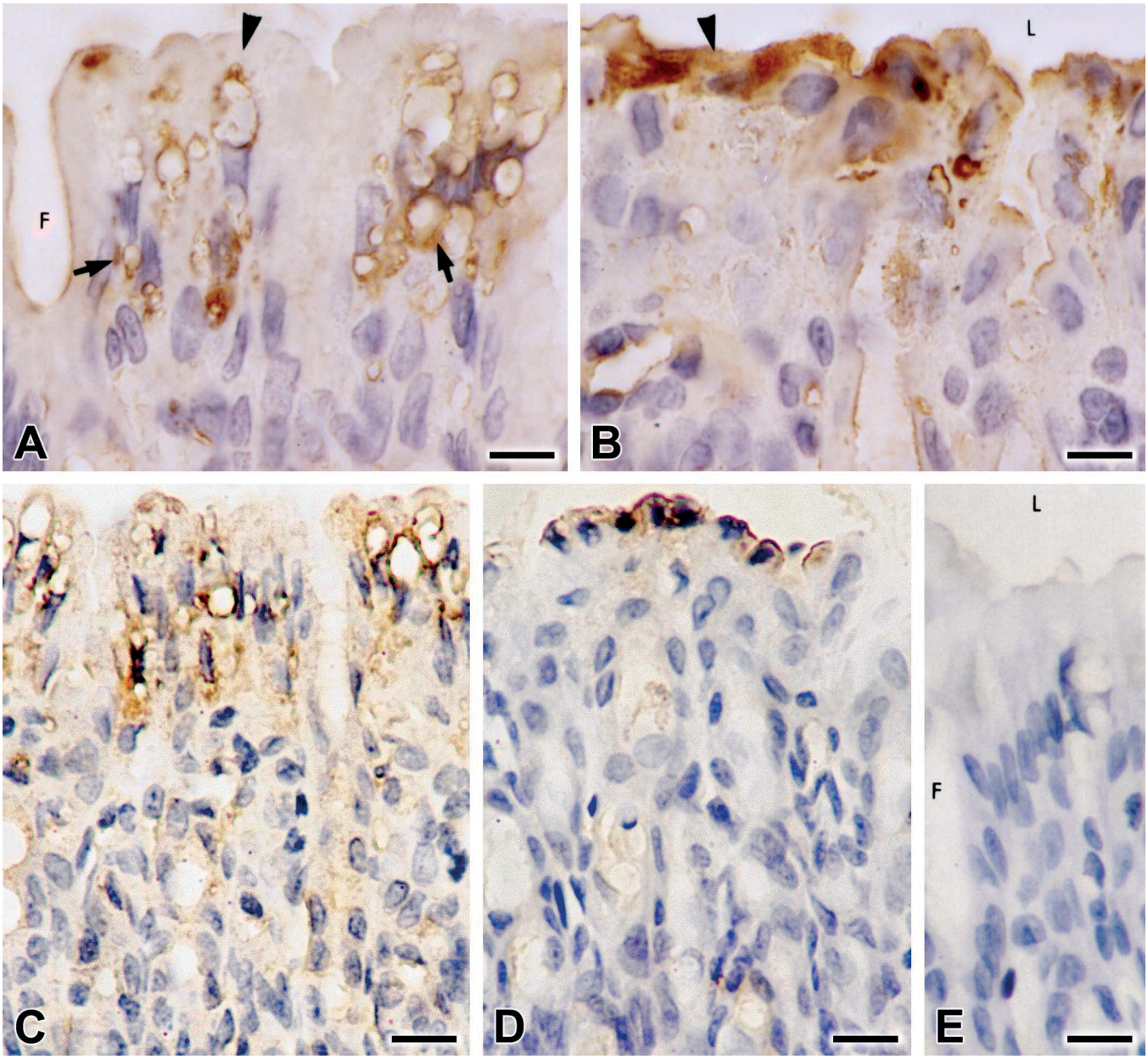

Fig. 1. Immunostaining for transforming growth factor beta (TGFß) isoforms in the gastric mucosa of 14-day-old rats. **A and B.** Gastric surface and foveolar (or pit) cells immunolabelled for TGFß1 at the basal (arrows) and apical cytoplasm (arrowheads). Positivity moves from the base of the cells (arrow) in fed rats **(A)** to the apex (arrowhead) after fasting **(B)**. **C and D.** Immunolabelling for TGFß3 in fed **(C)** and fasted rats **(D)**. Observe the reduction of immunostaining in the glands after treatment. **E.** Negative control for TGFß3 reaction. F and L indicate the foveolar (or pit) region and the gastric lumen, respectively. Light microscopy for immunohistochemistry with polyclonal antibodies (see Methods for details). Slides were counterstained with Mayer's Hematoxylin. Scale bars: A, B, 8.3 µm; C-E, 12.5 µm

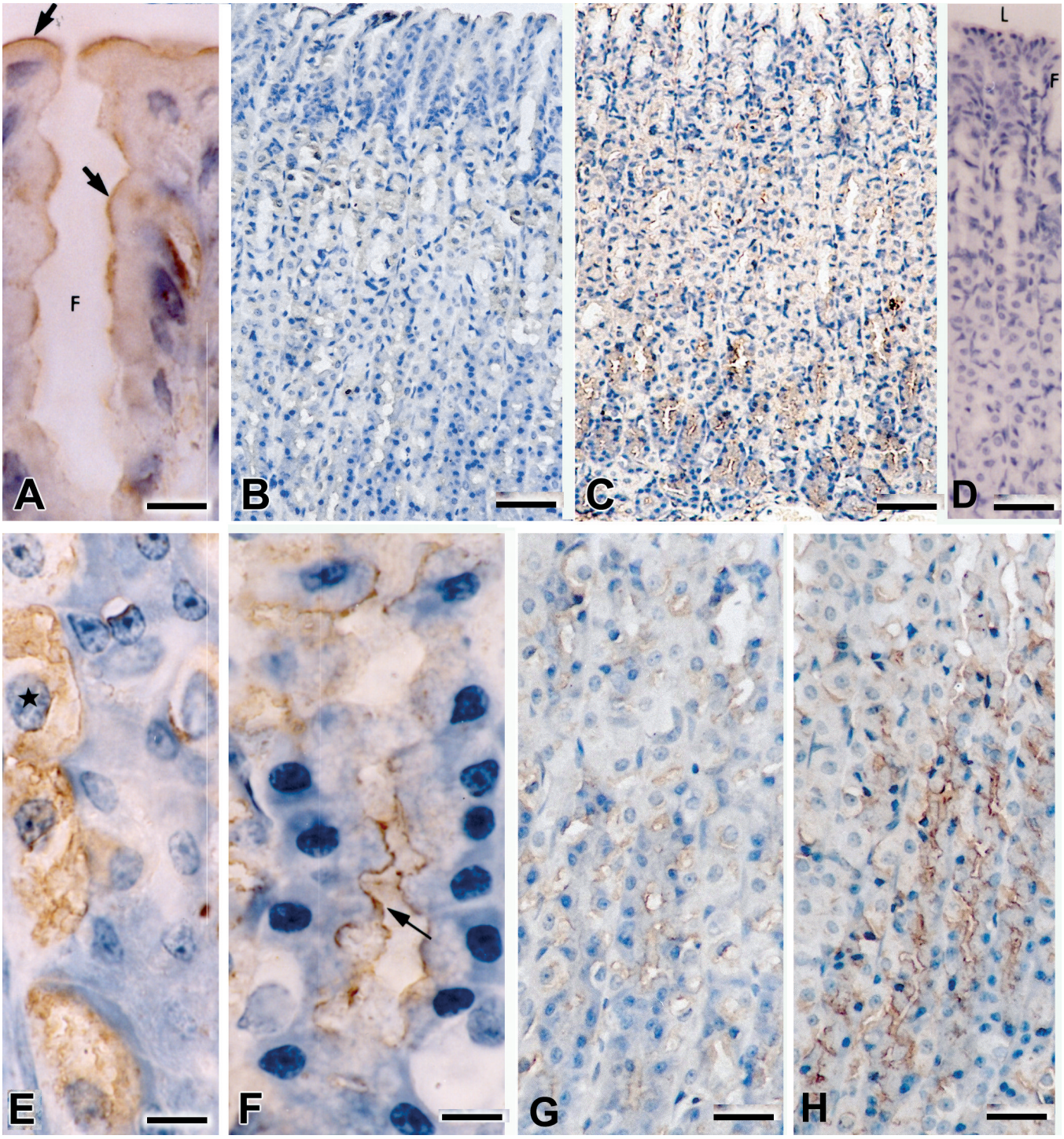

Fig. 2. Immunostaining for transforming growth factor beta (TGFß) isoforms and receptors in the gastric mucosa of 60-day-old rats. **A.** Surface mucous cells (arrows) immunolabelled for TGFß1 in fasted rats. **B and C.** Immunolabelling for TGFß1 in fed **(B)** and fasted rats **(C)**. Observe the increase of TGFß1 distribution in the gastric gland after treatment. **D.** Negative control for TGFß3 reaction. **E.** TßRI positive cells in the neck region of the gland in fed rat (asterisk for parietal cells). **F.** Chief cells immunostained for TßRII (arrow indicates labelling at the membrane) after fasting. **G and H.** immunolabelling for TGFß3 in fed **(G)** and fasted rats **(H)**. Note the increase of TGFß3 immunostaining in the gastric gland after treatment. F and L indicate the foveolar (or pit) region and the gastric lumen, respectively. Light microscopy for immunohistochemistry with polyclonal antibodies (for details see Methods). Slides were counterstained with Mayer's Hematoxylin. Scale bars: A, E, F, 8.3 µm; B-D, 50 µm; G, H, 25 µm

1C,D) and did not change TGFß1, ß2, and receptors. Negative control sections for all reactions were completely devoid of any signal (Fig. 1E).

60-day-old rats

At 60 days gastric glands are deep, the isthmus, neck and basal regions are identified and filled with differentiated mucous, parietal and chief cells (Karam and Leblond, 1992). We observed that the surface mucous cells were less reactive than in 14-day-old rats. Sometimes only the apical membrane was weakly labelled or few cells showed basal reaction (Fig. 2A).

We observed that gland cells were strongly immunolabelled for all isoforms and receptors. Parietal cells presented intense staining in the cytoplasm and the membrane of canaliculi could be sporadically inferred as reactive (Fig. 2E). Chief cells were also immunolabelled especially at the apical membrane (Fig. 2F) with varying intensities. Connective cells were also positive but were not considered in this study.

Fasting treatment increased the immunostaining for TGFß1 (Fig. 2B,C) and TGFß3 (Fig. 2G,H) and did not induce any change of TGFß2. Fasting did not alter the presence of TßRI (Fig. 2E) and TßRII (Fig. 2F), and both were regularly distributed in the gastric mucosa.

The distribution of the reactions among the different cell types seemed to be influenced by feeding condition. We observed that labelling of parietal cells in the isthmus/neck region was slightly more intense in the fed group (Fig. 2B). However, after fasting the population of chief cells were notably more reactive (Fig. 2C). These results show that fasting not only enhanced the expression of TGFß, but also affected its distribution in the gastric mucosa. Negative control sections for all reactions were completely devoid of any signal (Fig. 2D).

Discussion

The current study reports the *in vivo* profile of the three mammalian TGFß isoforms and receptors TßRI and TßRII in the gastric mucosa of 14- and 60-day-old rats, which represent different phases of stomach growth and maturation and show antagonistic proliferative responses towards fasting.

Previously, different studies described that the TGFßs spread along the gland with growth (Pelton et al., 1991; Naef et al., 1997; de Andrade Sá et al., 2003). Our results support this idea, because we found that at 14 days most immunostaining was observed in surface mucous cells whereas at 60 days the whole mucosa was labelled. We verified that fasting did not modify the ontogenic pattern, but induced specific changes in the distribution of isoforms and receptors. At 14 days we recorded a movement of TGFßs from the basal cytoplasm to the apex of surface mucous cells. De Andrade Sá et al. (2003) suggest that at 14 days the presence of peptides at the base of the cell might generate a crosstalk with the adjacent tissue, which would be important for the paracrine action of TGFß on gastric cells. Fasting only disturbed the localization of the isoforms and we can speculate that such change might be related to the absence of milk in the gastric lumen and to the effort of the cell to secrete and replace the TGFßs present in milk (Letterio et al., 1994; Penttila et al., 1998). However, the localization of TGFßs was not completely inverted and we still detected it at the basal cytoplasm after fasting, indicating that the interaction of the epithelium with connective tissue was not abolished. At 60 days surface mucous cells did not represent the major immunolabelled population and intracellular alterations were not seen.

The distribution of TGFß isoforms and receptors in the gastric mucosa of adult rats is more complex to analyze, because the cell types that cover the glands are immunostained with distinct patterns. We observed that the parietal cells from isthmus/neck region were the major cell type expressing TGFß1 in fed animals, whereas after fasting chief cells represented the main population. These results corroborate the study of Fujisawa et al. (2004) who used fasting as a standard for experiments with rats and observed that TGFß1 concentrates on chief cells. Other reports identified and localized the peptides in the gastric mucosa (Tanigawa et al., 2005). Naef et al. (1997) showed that human parietal cells were labelled for TGFß1 and ß3, but not for TGFß2 which in turn, was expressed exclusively by chief cells. De Andrade Sá et al. (2003) described that in 30-day-old rats, TGFß1 and ß3 were concentrated in the chief cells, whereas TGFß2 appeared mostly in parietals. They suggested that despite the specific functional features of the human and rat stomachs, the expression of TGFß and receptors is restricted to areas that are not committed to cell growth but most likely differentiating. Our findings are consistent with this premise, especially when we consider the hypothesis introduced by Fujisawa et al. (2004) about the mutual control between parietal and chief cell populations that would be dependent on TGFß maturation, paracrine action and EGFR signaling.

The endogenous expression of TGFß is one of the few known pathways to inhibit epithelial cell proliferation (Gold, 1999; Massagué et al., 2000; Parekh et al., 2002). We have shown that milk-borne molecules and dietary conditions are extremely important in the control of gastric postnatal growth (Gama and Alvares, 1996, 1998, 2000), but it is clear that the gland microenvironment is essential to keep the balance between cell proliferation and differentiation (Basque et al., 2002). We used fasting, a physiological and local interference, to stimulate and inhibit epithelial cell division (Alvares, 1987, 1992) and to evaluate the tissue expression of TGFß and receptors. Our results showed low levels of TGFß3 in parallel to epithelial hyperproliferation at 14 days, whereas increased TGFß1 and TGFß3 expression was recorded with the hypoproliferative condition at 60 days. This is the first evidence of endogenous regulation of the constitutive

expression of TGFß and receptors in non-pathological gastric mucosa concomitant with cell proliferation response. These findings are in agreement with the effects of TGFß described in vitro for gastric and intestinal cells (Barnard et al., 1989; Rokutan et al., 1998; McKaig et al., 1999; Tsutsumi et al., 2002). In gastric cancers the immunostaining for all three TGFß isoforms is intense and TGFß1 and ß3 mRNAs levels are also increased (Naef et al., 1997; Gold, 1999), but mutations in TßRII may prevent the inhibitory effect and inactivate the cascade (Park et al., 1994; Oliveira et al., 1998). However, some gastric cancer cells have functional receptors (Chang et al., 1997; Gold, 1999; Li et al., 2005), but have altered elements in Smad signalling pathway or in responsive genes (Kang et al., 1999; Li et al., 2002; Hu et al., 2005). Our results suggest that the equilibrium of TßRII is essential to control gastric cell proliferation and that the mucosa is responsive to TGFß either in developing or in adult rats. Therefore, besides the potential role on differentiation and maturation, TGFß1 and TGFß3 seem to be related to cell division. Moreover, during postnatal development, regulation may depend on TGFß3, whereas in adults, TGFß1 and TGFß3 can be the key for control. We showed that changes in the constitutive TGFß expression induced by fasting can be correlated to the differential epithelial proliferation in the stomach of developing and adult rats. These results suggest that one of the functional roles of TGFß in vivo is to locally regulate cell proliferation.

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