Differential distribution of transforming growth factor-α immunohistochemistry within whole gastric mucosa in rats

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Transforming growth factor-α (TGF-α) plays an important role in both proliferation and differentiation of mucosal cells at the gastrointestinal level, including stomach, where it is constitutively produced. This study evaluated the immunohistochemical distribution of TGF-α within whole gastric mucosa in rats, through the examination of seriate sections. Each stomach was opened along the greater curvature, pinned upon a cork plate, fixed in formalin and cut in 2-mm parallel strips which were sequentially superimposed on a glass slide. Sections were immunostained for TGF-α and pictures were taken from three areas: greater and lesser curvature; mucosa lying between the two curvatures. The sections were graded on the basis of the intensity of TGF-α staining, which was scored as follows: 0) no staining; 1) weakly positive; 2) intensely positive. The percent number of immunopositive cells and a mean intensity were calculated. Gastric mucosa showed a marked immunopositivity to TGF-α, mainly in parietal cells whose cytoplasm displayed moderate to intense staining. Positive cells (and the mean intensity) of total mucosa were 15.7±6.1% (1.13±0.42). However, they were not uniformly distributed, being 26.3±1.9% (1.67±0.24) in the mucosa lying between the two curvatures, 12.4±2.5% (1.52±0.22) along the lesser curvature and 8.3±2.1% (0.31±0.17) along the greater curvature. These results show that parietal cells of rat gastric mucosa exhibit immunoreactivity to TGF-α. Considering the gastroprotective effects of this factor, its non-homogeneous distribution within different areas may be of importance in understanding the lesion pattern of gastric damage after the administration of noxious agents.

Key words: immunohistochemistry, parietal cell, rat, stomach, transforming growth factor α.

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The gastric mucosa is one of the most rapidly proliferating tissues. Under normal conditions, cells renew by the progenitor neck cells and migrate to replace the exfoliated or damaged surface epithelium (Konturek, 1989). This process is greatly enhanced after acute damage as a consequence of the overexpression of certain growth factors at ulcer margin (Konturek et al., 1995).

Several growth factors, including transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), are important in this regulation. TGF-α and EGF play prominent roles in proliferation and differentiation of mucosal cells of the gastrointestinal tract, including stomach (Konturek et al., 1992). In particular, TGF-α mediates this function under both normal conditions and after acute injury, while EGF predominantly exerts its actions during healing of chronic ulcer (Jones et al., 1999). Furthermore, TGF-α shows a variety of biological activities in gastric mucosa, such as inhibition of gastric acid secretion (Brzozowski et al., 1991), protection against ulcerogenic factors (Romano et al., 1994) and stimulation of gastric mucus production (Sarostek et al., 1988).

TGF-α and EGF exhibit a significant molecular homology (Shum et al., 1994) and both act on gastric mucosa through a direct action on a common cell-surface membrane receptor (Waterfield, 1989), named EGF receptor (EGF-R). Hansson et al. (1990) demonstrated the presence of EGF-Rs on mucosal cells of the proliferative zone, where TGF-α and EGF exert their main effects. In further studies, the EGF-R was detected also on parietal cells, where it mediates the inhibitory effects of EGF and TGF-α on gastric acid secretion (Tarnawski et al., 1991).

Previous studies on samples of human gastric mucosa, obtained from normal adult subjects, showed that TGF-α is expressed ubiquitously: cells covering the luminal surface as well as those lining the gastric pits showed a cytoplasmic staining and

parietal cells consistently exhibited stronger immunoreactivity than chief cells (Thomas et al., 1992; Nasim et al., 1992). Several studies which focused their attention on biological functions of TGF-α, especially those concerning gastroprotection, were carried out on rats. However, at present data concerning the localization of TGF-α and its receptor in the upper digestive tract of the rat are lacking and discordant. For example, Livingstone et al. (1994) and Hormi et al. (1995) found a different distribution of TGF-α immunopositivity between fundic (cardiac) and antral mucosa in rat stomach, these data being criticized by Montaner et al. (1999). In any case, in all these studies the immunolocalization of TGF-α in the rat gastroduodenal area was evaluated only on the basis of the examination of a few gastric mucosal samples (Livingstone et al., 1994; Montaner et al., 1999), then an accurate determination of distribution and localization of TGF-α in the whole gastric mucosa are not available.

For these reasons, the aim of this study was to investigate the immunohistochemical distribution of TGF-α in normal rat gastric mucosa through the examination of seriate histological sections of whole stomachs by using a histomorphometric approach (Natale et al., 2001).

Materials and Methods

Animals

Albino male Wistar rats, 200-220 g body weight, were used throughout the study. They were fed standard laboratory chow and tap water *ad libitum* and were not used for at least one week after their delivery to the laboratory. The animals were housed, six in a cage, in temperature-controlled rooms on a 12-hour light cycle at 22-24°C and 50-60% humidity. Their care and handling were in accordance with the provisions of the European Community (EC) Council Directive 86-609 recognized and adopted by the Italian Government. Twenty-four hours before the experiments, animals were maintained in single cages and were deprived of food. Free access to water *ad libitum* was allowed until one hour before the beginning of the experiments.

Tissue collection and preparation

At the time of the experiment, the animals were euthanized by cervical dislocation and their stomachs were rapidly removed and processed for the histomorphometric evaluation of TGF-α immunohistochemistry following the procedure described by Natale et al. (2001). Each stomach was opened along the greater curvature, gently washed with saline (154 mM NaCl), pinned upon a cork plate with the mucosal surface turned upwards, and fixed in 10% formalin buffered with phosphate for 24 h at 4°C. The glandular portion of the stomach was then dissected into 2-mm-wide parallel strips, perpendicular to the lesser curvature. The strips obtained from each stomach were sequentially placed on a glass slide and oriented with the side of each strip distal to the pylorus upwards. In order to maintain this arrangement, a solution of melted 3% agar was gently poured over the strips and quickly cooled at 4°C to induce solidification. Then, the agar block was removed from the glass slide, introduced into a tissue embedding cassette for histology, and finally dehydrated and embedded in paraffin wax (Vogel Histo-Comp, Giessen, Germany). Three micrometer thick paraffin sections were cut using the microtome HM 330 Microm (Heidelberg, Germany) and processed for immunohistochemistry of TGF-α. Three sections of each gastric strips were examined for each rat, leading to a more accurate histological reading.

TGF-α immunohistochemistry

After preparation of tissue sections, slides were deparaffinized, incubated for 10 min in 1% hydrogen peroxide diluted in methanol to quench endogenous peroxidase activity, and washed in 0.01 M PBS, twice for 5 min each. Then the slides were incubated for 1 h at room temperature in blocking serum to avoid non specific protein binding and subsequently overnight with goat polyclonal anti TGF-α (Santa Cruz Biotechnology, California, USA) at 2 µg/mL in PBS containing 0.1% sodium azide and 0.2% gelatin.

TGF-α antibody cross-reacted with mouse, rat and human protein and was not cross-reactive with EGF. After three changes of PBS for 5 min each, the sections were incubated for 1 h with biotinylated secondary antibody (1:100 goat ABC Staining System, Santa Cruz Biotechnology, California, USA).

The slides were washed in PBS twice for 5 min, incubated for 30 min with avidin and biotinylated horseradish peroxidase (AB reagents). These sections were incubated for 3 min with peroxidase substrate containing 2.5 mg/100 mL 3-3′-diaminoben-

zidine tetrahydrochloride (DAB) chromogen dissolved in distilled water. As negative control for immunostaining, 0.01 M PBS was used instead of primary antibody. None of these control sections showed immunoreactivity. Rat pancreas was used as positive control. The slides were counterstained with Mayer's haematoxylin, dehydrated using graded ethanol solutions, cleared in xylene, mounted by mounting medium and covered with a glass coverslip. All sections were examined under a light microscopy equipped with a photographic apparatus (Leitz Diaplan, Wetzlar, Germany).

The analysis of immunointensity of the tissue sections was carried out by two independent observers (GL and GN). For each stomach, six pictures were taken from the following three areas: greater and lesser curvature; mucosa lying between the two curvatures (Figure 1). The sections were graded on the basis of the intensity of TGF-α staining, which was scored as follows: 0) no staining; 1) weakly positive; 2) intensely positive. For each picture, data were expressed as the percent number of immunopositive cells out of the total number of counted cells and a mean intensity was calculated.

Statistics

Results are given as mean ± standard error of the mean (S.E.M.). The statistical significance of data was evaluated by Student's t test and *p* values lower than 0.05 were considered significant. *n* indicates the number of animals. All statistical procedures were performed by means of personal computer programs (InStat 2.01 for Apple Macintosh).

Results

Rat gastric mucosa showed a significant immunopositivity to TGF-α. In particular, the immunolabelling was detectable throughout the gastric glands, mainly in parietal cells where the cytoplasm displayed a weak to strong homogeneous staining (Figure 2A). In some cases, the cytoplamic staining was more intense in the subnuclear region (Figure 2A and 2B). The observation of seriate sections did not show a significant difference in the distribution of TGF-α immunopositivity along gastric glands: the immunopositive parietal cells can appear mainly concentrated in the upper and in the middle parts as well as in the lower part of gastric glands. Some superficial and gastric pit cells also appeared stained, whereas chief cells resulted immunonegative (Figure 2A). In all cases, no nuclear staining was observed. The overall number of positive cells and the mean intensity of the staining in the total gastric mucosa was 15.7±6.1% and 1.13±0.42, respectively (Table 1).

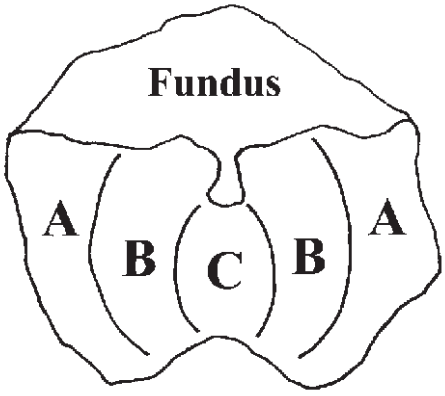

Figure 1. Schematic drawing representing the rat stomach opened along the greater curvature with the mucosal surface turned upwards. The fundus, also called forestomach, is characterized by a stratified squamous epithelium without glands. The glandular part of gastric mucosa was examined around the greater curvature (A), between the two curvatures (B) and around the lesser curvature (C).

However, both TGF-α immunopositivity and mean intensity were not uniformly distributed within the whole gastric mucosa (Table 1). Indeed, when examining the seriate gastric strips, a different immunoreactivity was found, moving from a curvature to the other. The highest number of immunopositive cells was counted in the mucosa lying between the two curvatures (Figure 2A). In this area, the immunostaining also appeared more intense (Table 1). By contrast, the mucosa lying around the greater curvature, although rich in parietal cells, exhibited a lower number of immunopositive cells as well as a weak staining intensity (Figure 2C and Table 1). The thin mucosa around the lesser curvature, which lacks of parietal cells, showed a significant staining only at the level of superficial cells and of some cells covering the long gastric pits (Figure 2D and Table 1).

Discussion

The present results showed that under basal conditions rat gastric mucosa exhibited a marked TGF-α immunoreactivity. These data are in agreement with previous studies which reported an immuno-

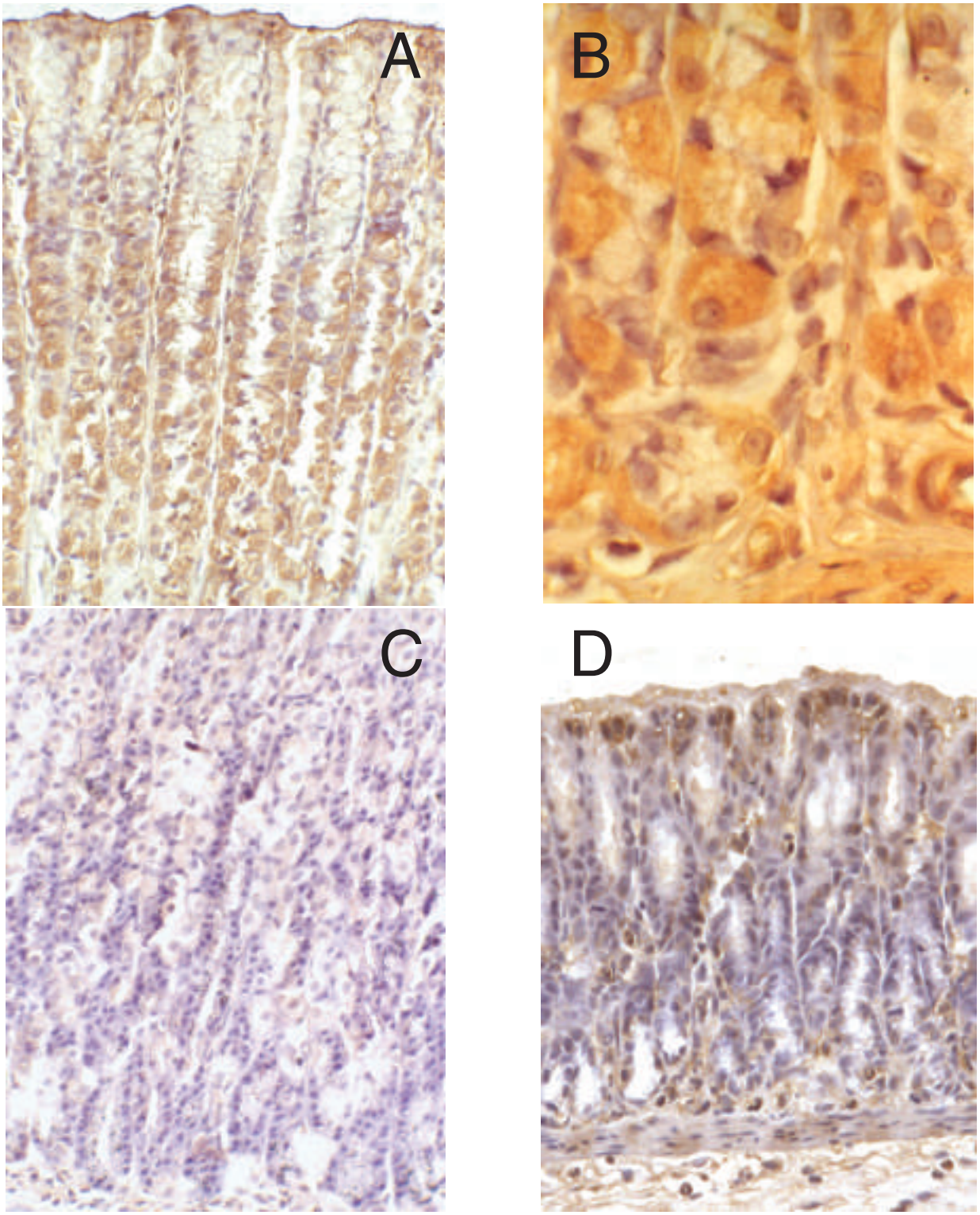

Figure 2. (A) Normal rat gastric mucosa lying between the two curvatures: TGF-α immunoreactivity is marked and is mainly evident in parietal cells along gastric glands. Magnification: x280. (B) Fundus of a normal rat gastric gland from mucosa lying between the two curvatures: TGF-α immunoreactivity is markedly present in the cytoplasm of parietal cells. Magnification: x1100. (C) Normal rat gastric mucosa close to the greater curvature: TGF-α immunoreactivity is weak or absent. Magnification: x280. (D) Normal rat gastric mucosa close to the lesser curvature: TGF-α immunoreactivity is present in superficial cells and some gastric pit cells. Magnification: x280.

Table 1. Immunopositivity to TGF-α in the rat gastric mucosa.

	No. of immunopositive cells (percentage)	*Immunostaining intensity*
Mucosa around the greater curvature	8.3±2.1(*)	0.31±0.17(*)
Mucosa around the lesser curvature	12.4±2.5(*)	1.52±0.22
Mucosa lying between the two curvatures	26.3±1.9	1.67±0.24
Total mucosa	15.7±6.1	1.13±0.42

Data are given as mean ± standard error of the mean (S.E.M.). n=10. (*) Significant difference from mucosa lying between the two curvatures: p<0.05.

positivity to TGF-α in gastric mucosa from normal rats (Livingstone et al., 1994; Montaner et al., 1999) and humans (Thomas et al., 1992; Nasim et al., 1992; Abe et al., 1997). Accordingly, TGF-α mRNA, but not EGF mRNA, was shown to be expressed in the normal stomach from several species (Beauchamp et al., 1989; Malden et al., 1989). In fact, unlike EGF, TGF-α has been demonstrated to be synthesized in the normal gastric mucosa. By contrast, EGF is produced in salivary glands and reaches gastric lumen with saliva (Konturek et al., 1989). The significant presence of TGF-α within rat gastric mucosa confirms its importance in the regulation of several processes, including cell renewal, mucus secretion and gastric acid output. Accordingly, the administration of both EGF and TGF-α results in gastroprotection against experimentally induced mucosal damage (Konturek, 1989; Romano et al., 1994).

In the present study, TGF-α immunoreactivity was estimated with a histomorphometric approach which allows the analysis of whole stomachs through the examination of seriate histological sections (Natale et al., 2001). Thanks to this method, the quantification of TGF-α immunopositivity does not simply result from the evaluation of limited samples of gastric mucosa, but more precisely it rather reflects the overall staining present in the whole stomach.

According to this methodological approach, in the present study the immunoreactivity of this growth factor did not appear uniformly distributed within gastric mucosa. A different pattern of distribution of TGF-α immunopositivity, along the fundic-antral axis within normal gastric mucosa, was also reported in adults (Livingstone et al., 1994) as well as in developing rats (Hormi et al., 1995). In particular, Livingstone et al. (1994) found a more even localization in distal, antral crypts, with respect to proximal, cardiac mucosa where TGF-α immunoreactivity was concentrated in the differentiated compartment from mid crypts towards the lumen. This was interpreted as a broadening of the zone of differentiation in distal mucosa, resulting in a slower mucosal turnover. Montaner et al. (1999) underlined the possibility that in both the above-mentioned studies the oxyntic mucosa was confused with the cardiac or fundic mucosa which has no glands.

The present histomorphometric analysis allowed to detect a particular pattern of TGF-α immunopositivity in the rat stomach, suggesting a different axis of distribution, from the greater to the lesser curvature. In all gastric mucosa, several superficial cells, as well as gastric pit cells, appeared immunostained. The major immunopositivity was found in parietal cells. In the gastric mucosa lying between the two curvatures, the cytoplasm of oxyntic cells appeared intensely positive independently from their position along gastric glands. By contrast, parietal cells were poorly immunostained in the mucosa lying along the greater curvature. A consistent percentage of immunostained cells was also found along the lesser curvature, even if in this part of the stomach parietal cells are scarce or absent. This is due to the fact that this thinner mucosa with deeper pits contains a higher proportion of positive superficial and pit cells. This is in agreement with the data of Montaner et al. (1999) and Livingstone et al. (1994) who found immunopositivity to TGF-α also in rat antral mucosa.

In our experiments, TGF-α staining was mainly evident in the cytoplasm of parietal cells. A noticeable immunopositivity for this growth factor was also found in acid-secreting cells in rats (Livingston et al., 1994; Montaner et al., 1999) as well as in humans (Thomas et al., 1992). The content of TGF-α in gastric mucosa was found to be significantly greater than the concentration of EGF, making TGF-α the natural agonist for the EGF-R located on parietal cells (Cartlidge & Elder 1989). Furthermore, the levels of TGF-α and EGF receptor mRNA are abundant in parietal cells, supporting the hypothesis that TGF-α acts as an autocrine factor regulating oxynthic cell function (Beauchamp et al., 1989). In particular, TGF-α seems to act by means of autocrine and/or paracrine mechanisms, where it is produced by the parietal cell and feeds back to inhibit acid secretion by that or adjacent parietal cells (Wang et al., 1993). In support of this

action, electron microscopic studies evidenced EGF-R immunoreactivity at the level of basolateral surface of gastric parietal cells (Mori et al., 1987).

The present findings seem to indicate that, under normal conditions, TGF-α acts at different levels within gastric wall. In this respect, it should be noted that the thinner mucosa along the lesser curvature can require a higher cell turnover to prevent the onset of mucosal damage. For the same reasons, considering the gastroprotective and ulcer healing effects of TGF-α, the present data may be of importance in understanding the lesion pattern of gastric damage after the application of noxious agents.

In conclusion, our findings confirm that TGF-α is significantly present in rat gastric mucosa, at the level of superficial epithelium, gastric pits and parietal cells where it mediates several important functions, including the regulation of acid secretion and mucosal renewal. When interpreted in light of the described actions of TGF-α, these data provide morphological evidence that local production of this growth factor is differentiated within gastric wall and might contribute to the explanation of the lesion pattern when gastric mucosa is damaged by noxious agents.

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