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SUBSEROSAL APPLICATION OF TRANSFORMING GROWTH FACTOR-β1 IN RATS WITH CHRONIC GASTRIC ULCERS: EFFECT ON GASTRIC ULCER HEALING AND BLOOD FLOW

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Transforming growth factor β1 (TGF-β1) has been shown to play a central role in wound healing. This peptide has been detected in the stomach, but no information is available at present whether TGF-β1 influences the healing of gastric ulcers and whether the mucosal expression of TGF-β1 changes in the course of this healing. In this study, gastric ulcers were induced by serosal application of acetic acid and TGF-β1 or vehicle saline was injected twice into the subserosa around the ulcer area, once immediately after ulcer induction and two days later. Local application of TGF-β1 led to significant acceleration of gastric ulcer healing. Gastric blood flow at the ulcer margin was significantly higher than that in the ulcer crater but no significant difference was found in this flow between studied groups. Immunohistochemistry showed that the expression of TGF-β1 reached the peak at day 2 and then declined in the course of healing. We conclude that TGF-β1 accelerates ulcer healing possibly by increasing the formation of granulation tissue and cell migration probably mediated by locally expressed TGF-β1 but the healing effects of TGF-β1 do not depend on the vascular factor.

Key words: *gastric ulcer, wound healing, TGF*-β1, gastric blood flow

INTRODUCTION

Transforming growth factor β1 (TGF-β1) is a multifunctional cytokine that regulates a number of different cellular processes involved in wound repair (1). Alpha granules of platelets contain a high concentration of TGF-β1 (2). At the site of tissue injury TGF-β1 is released from platelets into the surrounding tissues. It is one of the first cytokines responsibile for the recruitment of

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inflammatory cells in the repair process. Once present, these cells actively produce TGF-β1 (2). Various studies on skin wounds have shown that direct and systemic application of TGF-β1 to incisional wounds and ulcer in rabbits improved the rate of repair and the rate of formation of granulation tissue (3—5). Moreover, injection of TGF-β1 into intact skin induced an angiogenic and fibrotic response mimicking wound healing. Furthermore, increased blood flow in the granulation tissue around the wound was also found to be enhanced by TGF-β1 (6). When injected locally, TGF-β1 induced the formation of granulation tissue and angiogenesis and accelerated wound healing (4).

In mammals there are three isoforms of TGFβ, which have a high sequence homology in common (2). TGFβ isoforms are secreted as inactive precursor molecules. They are composed of a signal peptide, a latent associated protein (LAP), and a bioactive mature region, forming a dimeric complex. Only the mature form can bind to TGFβ-receptors (1).

TGF-β1 has been detected in most rat tissues and also in the rat stomach (7), but no information is available at present as to whether local availability of TGF-β1 has any influence on the healing of gastric ulcers.

In the present study we show that in an experimental model of chronic gastric ulcer, locally injected TGF-β1 leads to an acceleration of ulcer healing but not to enhancement of gastric blood flow and angiogenesis at the ulcer area.

MATERIALS AND METHODS

Animal model

In all the experiments, chronic gastric ulcers were induced in male Wistar rats weighing 150-180 g by the method of acetic acid application to the serosa described elsewhere (8). Each group of animals contained 9-10 rats. The results were pooled for statistical analysis.

Experimental design

Thirty rats were divided into three groups and treated with locally injected TGF-β1 or vehicle (saline) (as described below). On day 11 they were anesthetized with ether to determine gastric blood flow as described previously (9), the ulcer size was measured and the ulcer area was excised, fixed in formalin and embedded in paraffin.

In a second series of experiments, chronic gastric ulcers were induced in 12 rats. Animals were killed at day 2, 4, 6, and 8. Mucosal biopsies were taken and fixed in 10% formalin and embedded in paraffin and processed for immunocytochemistry.

Local TGF-β1 therapy

Immediately after the induction of ulcers (during laparotomy) two out of 3 groups, each containing 10 animals, received local (in the area of previous application of acetic acid) subserosal

injection of either 50 ng TGF-β1 (BDP1, British Biotechnology, Oxford) or saline (0.9%). The third group of rats was submitted to laparotomy but did not receive any subserosal injection.

On day two a laparotomy was performed and the TGF-β1 or saline was again injected locally into the ulcer area. These subserosal injections comprised of TGF-β1 in phosphate-buffered saline in a volume of 100 μl and were applied just around the ulcer on the same wall of the stomach. In one group, only a laparotomy was performed (sham-operation).

Measurement of gastric mucosal blood flow

In all groups of rats treated (sham-operated or locally injected with saline, or with TGF-β1 itself) the gastric blood flow (GBF) was determined using laser Doppler flowmetry (Laserflo, BPM403B, Vasamedics Inc., St. Paul, MN, USA) as described previously (9). The 24 h fasted rats were anesthetized with ether, the abdomen was opened and gastric contents were gently evacuated to the exterior through the cut in the forestomach. The optical flow probe was placed gently on the mucosa in the oxyntic gland urea to monitor GBF in the ulcer crater, the ulcer margin and the intact oxyntic mucosa distant from the ulcer area. The area of laser emission of the probe was 1 mm^2. Since the depth of the measurement by laser flowmetry is about 6 mm, the technique used determined the total blood flow of the stomach wall. The GBF was measured in three adjacent sites of the mid portion of the anterior wall of the intact oxyntic part of the stomach. The mean values of three recordings were calculated and they were relatively constant in the fasted rats. The values of GBF in the ulcer bed and ulcer margin were expressed as percent change from those recorded in this intact oxyntic mucosa.

Determination of ulcer size

The area of the ulceration was measured planimetrically by a person who was blinded to the origin of the coded specimens, using a computerizing planimeter (Morphomat 10, Opton, Berlin, Germany) and results expressed in mm^2. The sections were embedded in paraffin and stained with hematoxilin and eosin (H&E).

Morphometric analysis

For the histological examination the ulcers were embedded in paraffin and stained with H & E. Morphometric analysis of angiogenesis in the ulcer crater and ulcer margin was performed using the paraffin sections stained with H&E. Histological examination of coded specimen was performed by an observer unaware of the treatment used. Quantitative assessment of microvessel profiles in the ulcer bed and ulcer margin was performed with a 40 × objective and expressed as the mean number of microvessels per microscopical field. Mean values were calculated from at least three fields at the ulcer base and three fields at the ulcer edge.

Immunohistochemistry

For immunohistochemistry, serial sections obtained from these paraffin blocks were dewaxed, rehydrated, pre-treated in citrate buffer (pH 6) in a microwave oven (3 × 5 min) and incubated with specific chicken polyclonal antibodies against TGF-β1 (1:100; AB101-NA; R&D Systems, Minneapolis, USA) using the ABC method.

Controls omitting the primary antibody and controls with purified chicken IgGs (AB 101-C, R&D Systems, Minneapolis, USA) were also performed. Coded specimens were independently assessed by two observers.

Statistical analysis

For statistical analysis the nonparametric Mann-Whitney U and Kruskal-Wallis tests for unpaired comparisons were applied where appropriate.

RESULTS

The ulcer area of TGF-β1 treated ulcers was 1.7 (SD 0.5) mm^2 versus 5.5 (SD 1.4) mm^2 in sham-treated and 3.5 (SD O.9) mm^2 in saline-treated control ulcers (*Fig. 1*). Histological assessment of sham and saline-treated controls revealed a ulcer crater consisting of necrotic debris surrounded by inflammatory cells (*Fig. 2*).

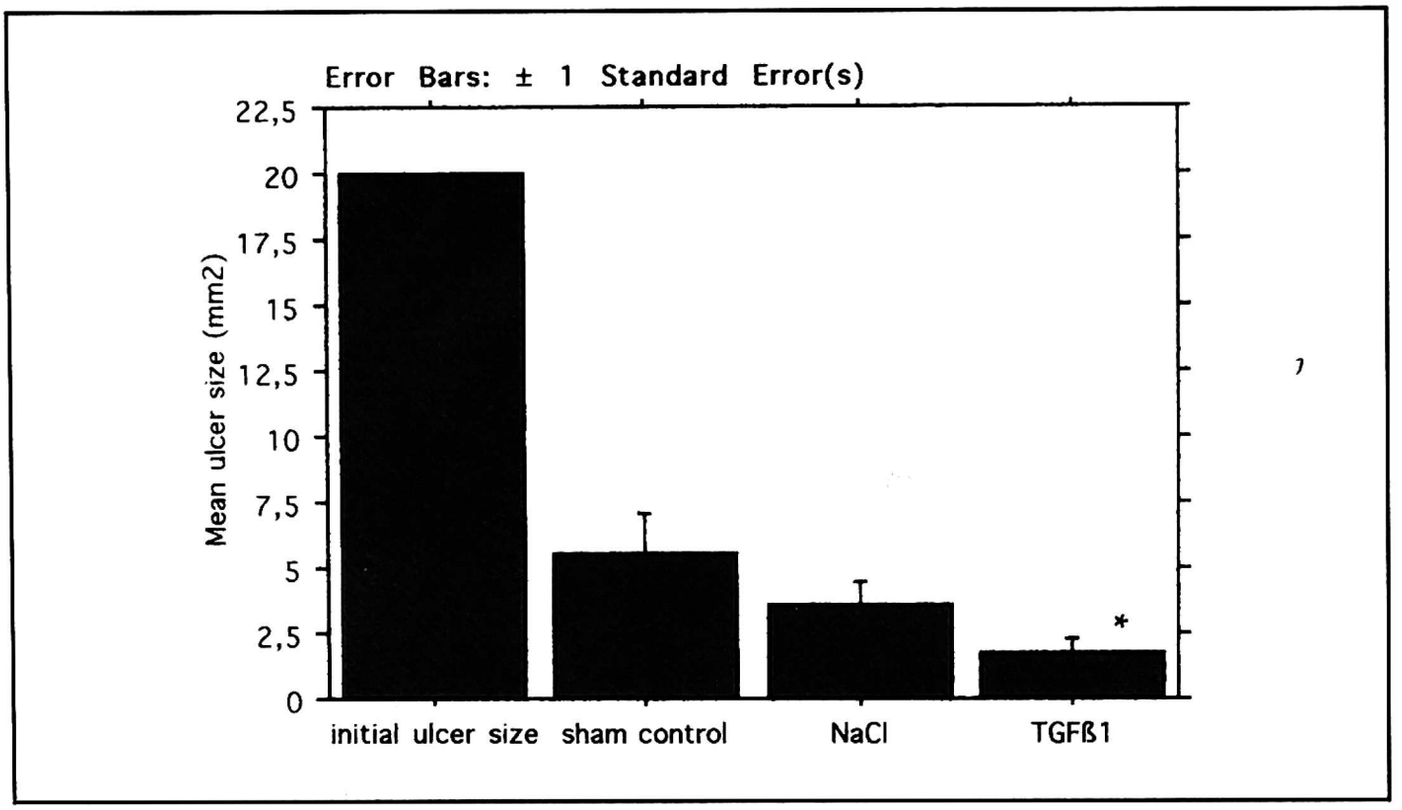

Fig. 1. Effect of treatment with TGF-β1 or control saline on the healing of chronic gastric ulcers on day 11 after induction of the ulceration (N = 8—10 in each group). Results are expressed as means ± standard error. Asterix indicates significant ($p<0.05$) decrease below the control value.

In TGFβ1-treated ulcers there was almost complete healing with only a small residual crater (*Fig. 3*). In the ulcer bed a dense granulation tissue with infiltrates of inflammatory cells was observed. Two of 10 TGF-β1-treated ulcers were completely healed and did show a densely scarred submucosa.

Gastric blood flow (of normal GBF) was higher at the ulcer margin (mean of all groups 82.4 [SD 5.7] than at the ulcer bed (mean of all groups 73.9 [SD 5.8]). There was no statistically significant difference in the blood flow between studied groups of rats (*Fig. 4*).

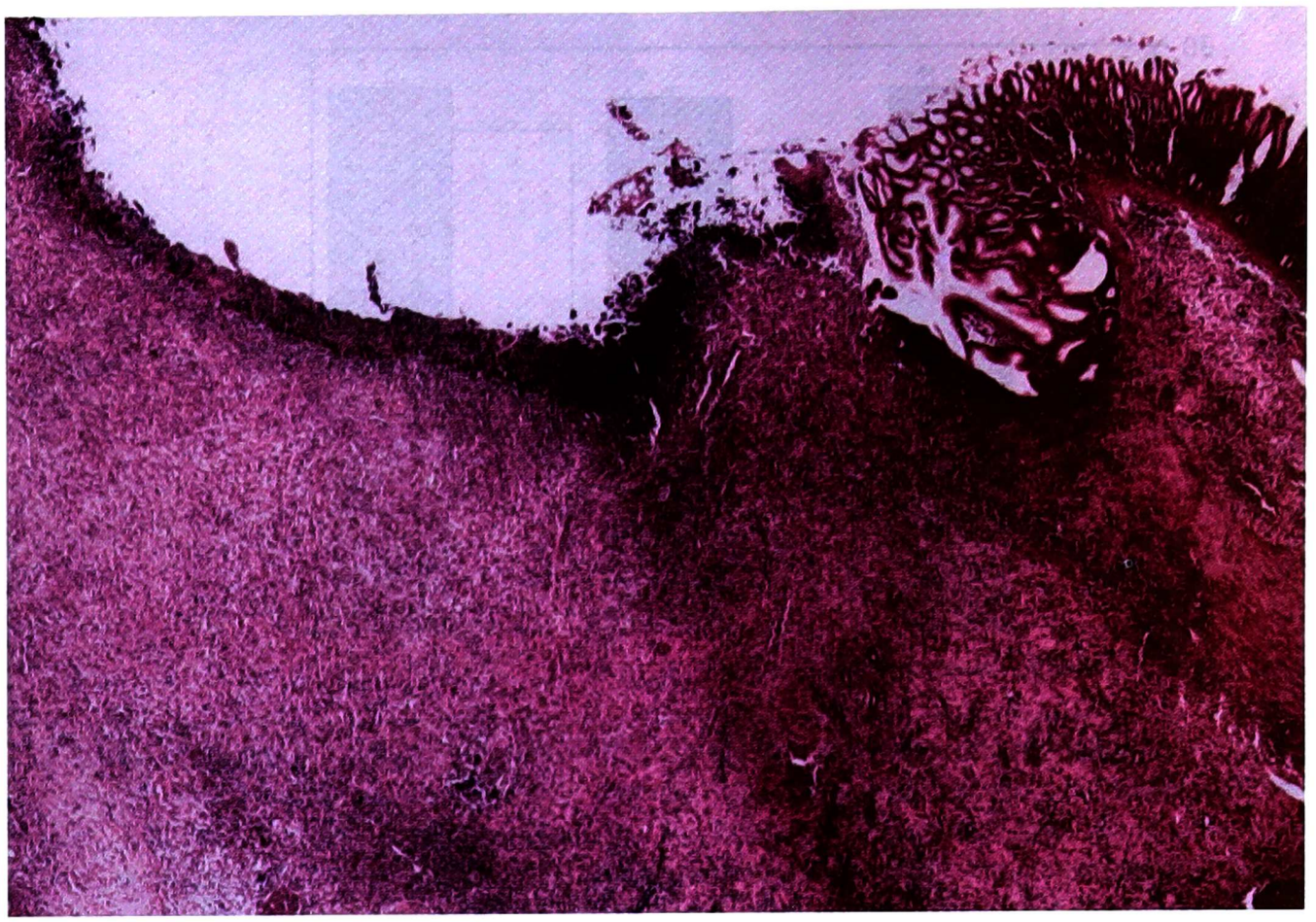

Fig. 2. Sham-control. Ulcer edge and ulcer base 11 days after ulcer induction (original magnification × 37).

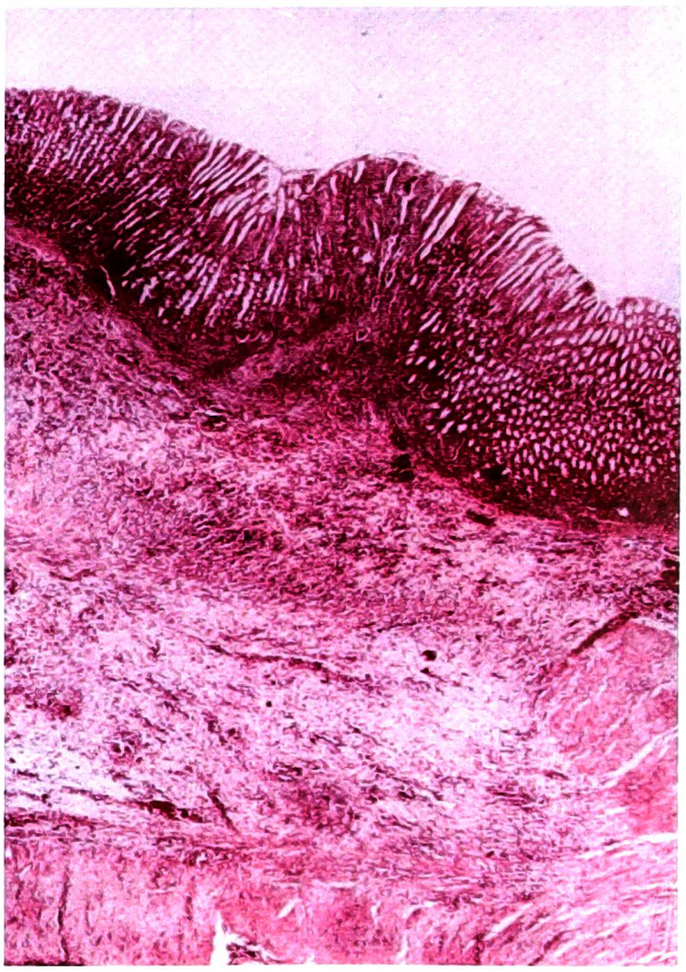

Fig. 3. TGF-β1 treated ulcer. Ulcer scar with dense granulation tissue 11 days after ulcer induction (original magnification × 37).

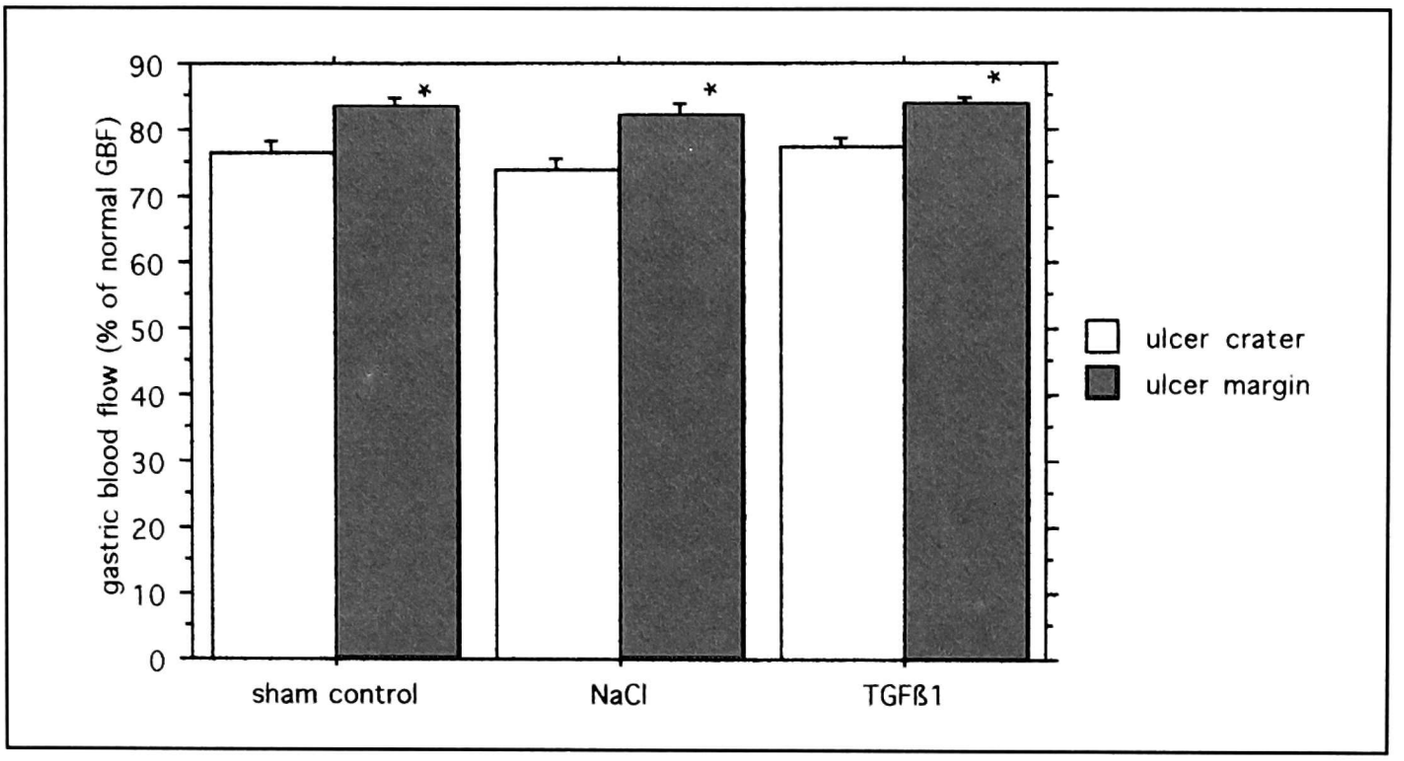

Fig. 4. Gastric blood flow at the ulcer margin is significantly higher than at the ulcer crater. There is no significant difference in blood flow between studied groups. Results are expressed as means ± standard error of gastric blood flow in normal adjacent mucosa. Asterix indicates significant ($p<0.05$) increase above the value recorded at the ulcer crater.

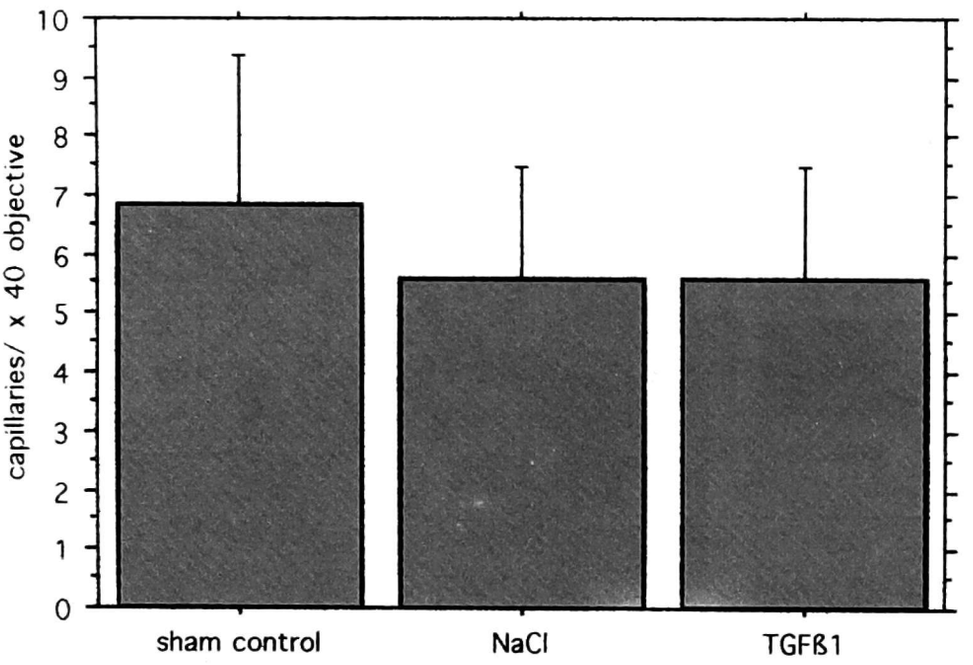

Fig. 5. Mean number of capillaries (±SD) in the ulcer edge and ulcer bed. No significant difference was found between studied groups.

Morphometric analysis of blood vessels at the ulcer edge and the ulcer bed did not show any significant difference between TGF-β1-treated animals and control groups (*Fig. 5*).

Immunohistochemistry of gastric ulcers treated with saline or sham-operated on day 11 did show that migrating epithelial cells were negative for TGF-β1. Immunostaining for TGF-β1 was observed in epithelial cells at the ulcer edge and this was associated with the presence of extracellular matrix at the ulcer bed (*Figs 6 & 7*). Fibroblasts and inflammatory cells in the ulcer base showed immunoreactivity for TGF-β1.

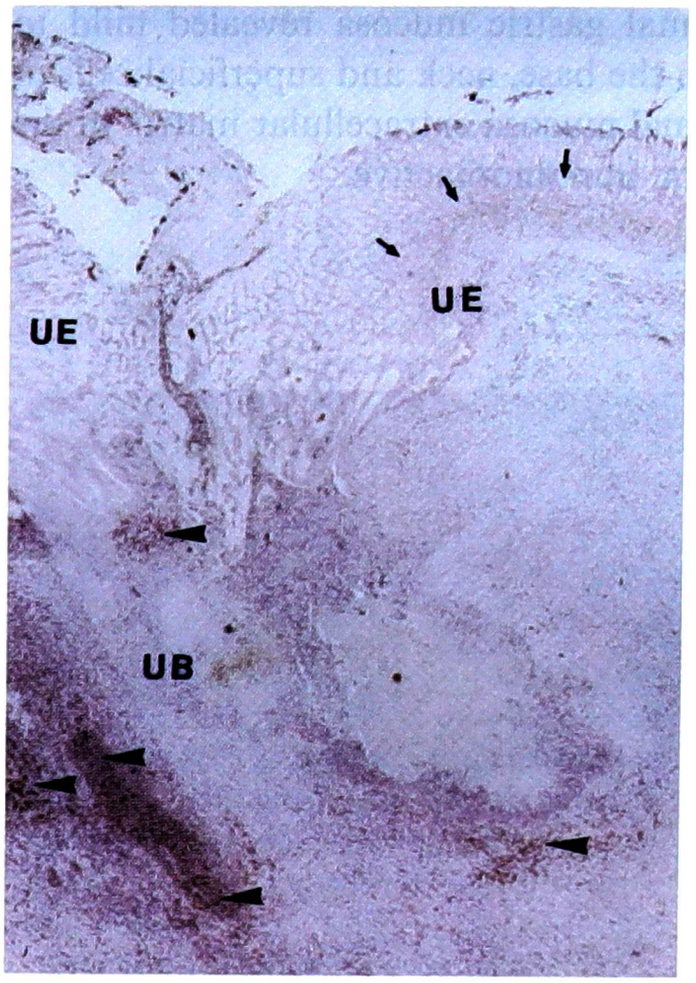

Fig. 6. Immunoreactivity for TGF-β1 treated ulcer at 11 days after ulcer induction. Mild immunostaining of basal epithelial cells of the gastric gland at the ulcer edge (UE, small arrows). Patchy areas of TGF-β1 immunoreactivity in the ulcer base (UB) may be related to injected TGF-β1 (large arrows) (original magnification × 37).

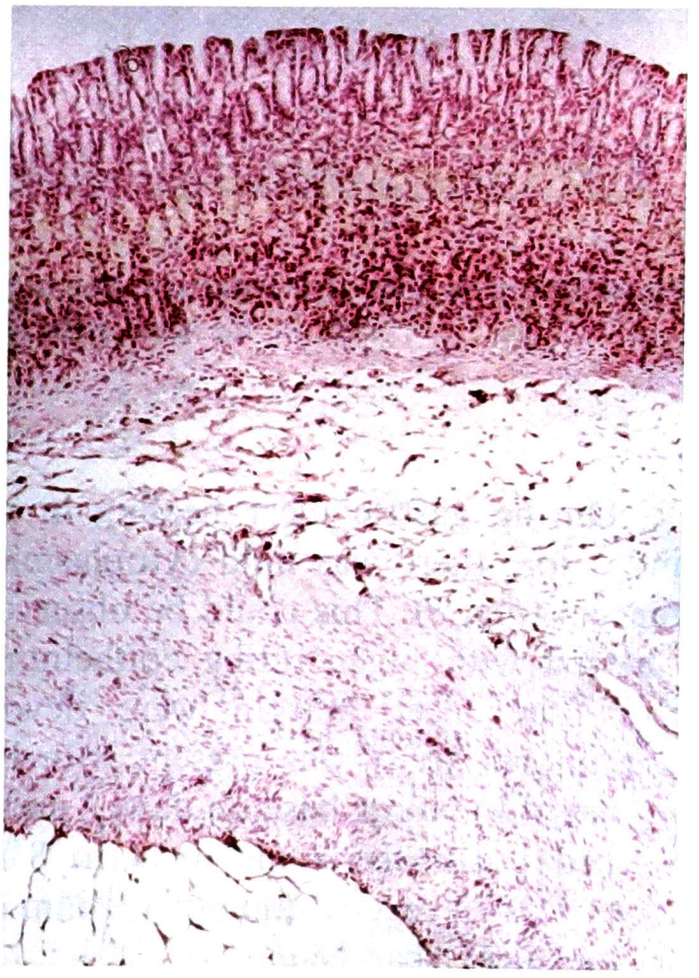

Fig. 7. Normal mucosa. Control section with purified chicken IgG (original magnification × 94).

In contrast, immunostaining of normal gastric mucosa revealed mild to moderate intensity of TGF-β1 staining in the base, neck and superficial cells of gastric glands. In control sections of normal mucosa extracellular matrix in the lamina propria and submucosa was not immunoreactive.

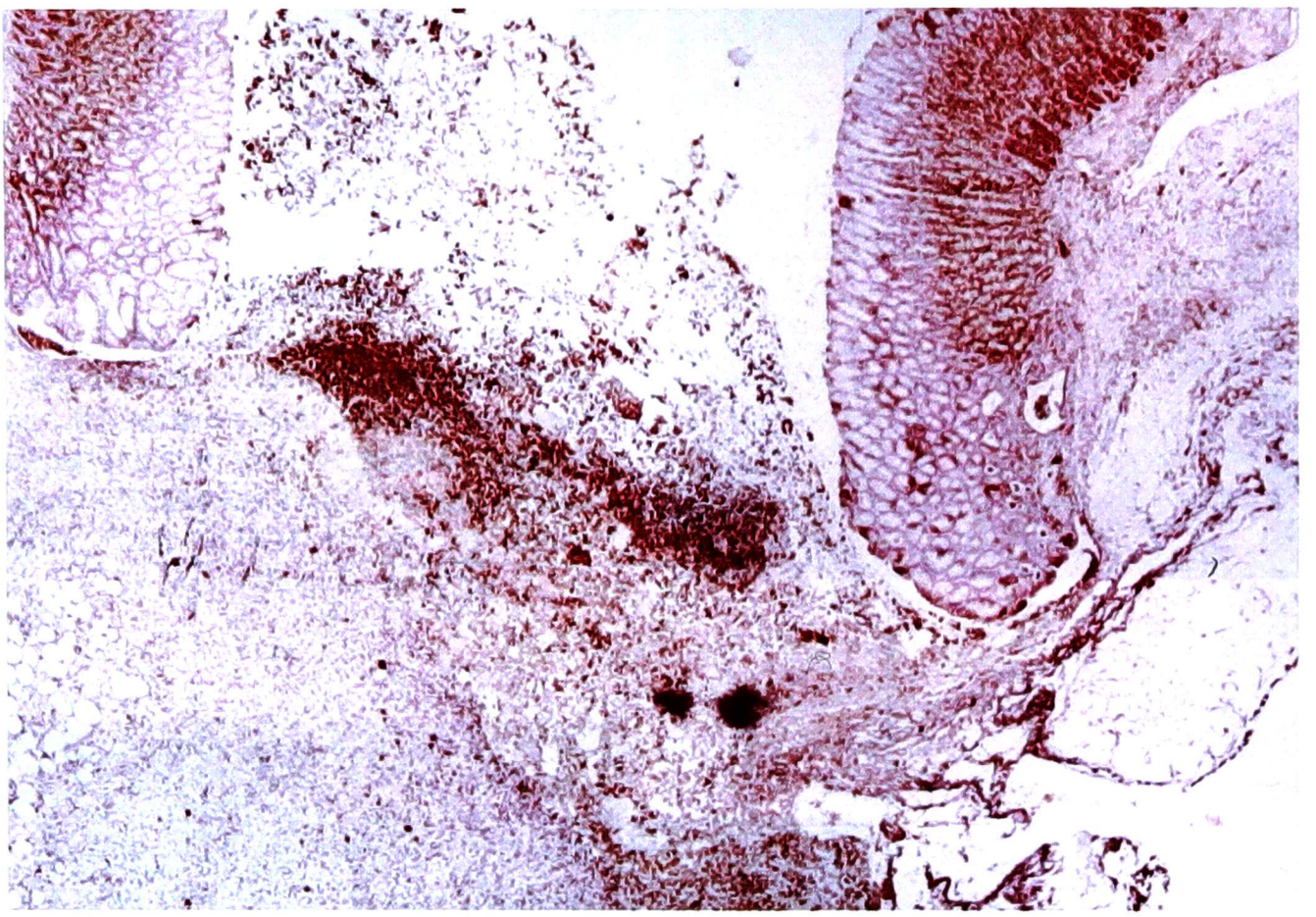

Fig. 8. Two days after ulcer induction. Immunoreactivity for TGF-β1 in the clot and in epithelial cells at the ulcer edge (original magnification ×37).

During gastric ulcer healing in untreated animals two days after ulcer induction, there was mild cytoplasmic staining of epithelial cells at the ulcer margin (*Fig. 8*). This could be observed until the ulcer was almost completely covered with regenerating epithelium. Migrating epithelial cells originating from the ulcer margin were devoid of TGF-β1 (*Fig. 9*). A band of immunoreactivity was present with inflammatory cells within the superficial region of the ulcer base on days 2 and 4. Extracellular matrix in the ulcer base stained positively from day 4 to 8 (*Fig. 10*). Ulcer completely healed and covered with regenerating epithelium did still show TGF-β1 immunoreactivity in the ulcer scar (*Table 1*).

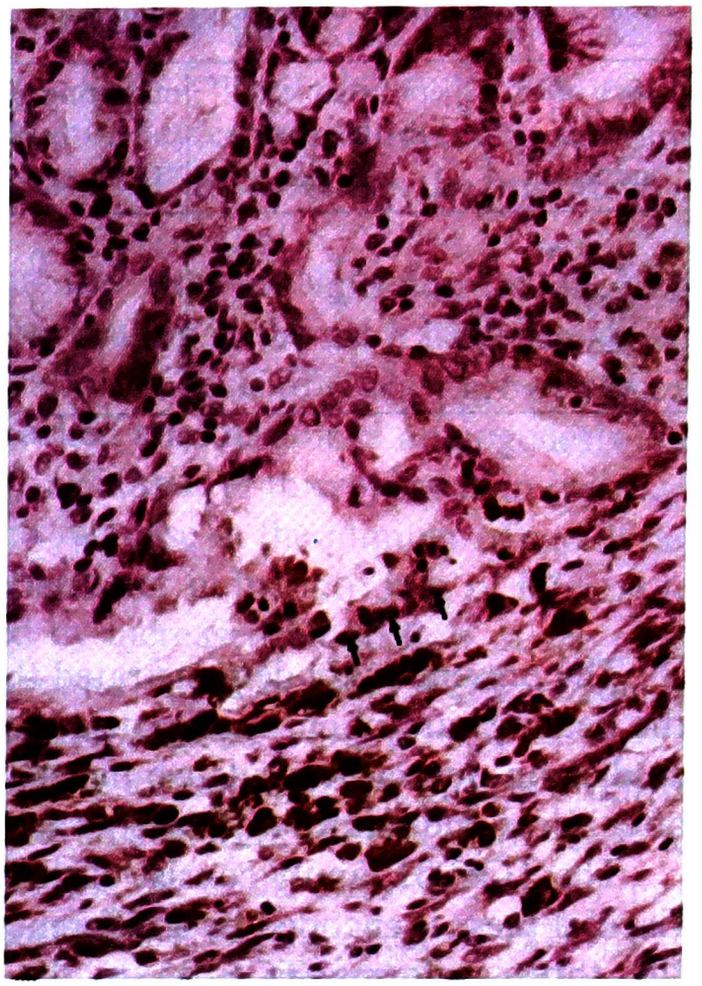

Fig. 9. Two days after ulcer induction. The migrating epithelium (arrows) is devoid of TGF-β1 immunoreactivity. Immediately below there is immunoreactivity associated with inflammatory cells but not with fibroblasts (original magnification × 375).

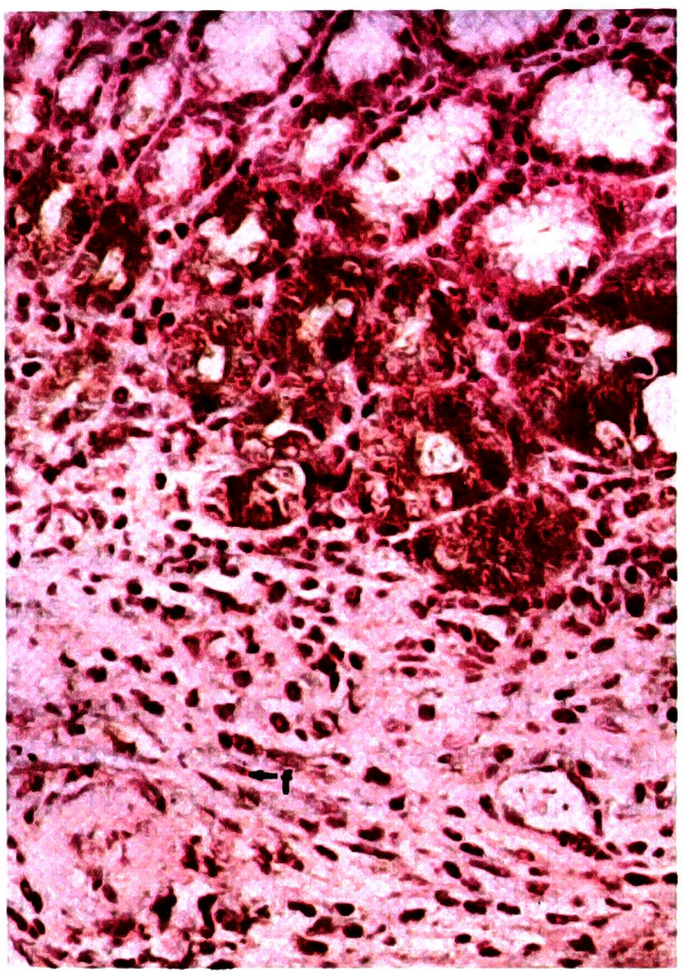

Fig. 10. Four days after ulcer induction. Diffuse cytoplasmic staining for TGF-β1 in epithelial cells at the base of the ulcer margin. TGF-β1 immunostaining is associated with the extracellular matrix, inflammatory cells but not fibroblasts (f) (original magnification × 375).

Table 1. Distribution of TGF-β1 immunoreactivity during healing of chronic gastric ulcers.

days after ulcer induction	epithelial cells at the ulcer margin	migrating epithelial cells	clot at the ulcer base	extracellular matrix at the ulcer base
day 2	+	0	+ +	
day 4	+	0	+ +	+
day 6	+	0	+ +	+
day 8	+		+ +	

DISCUSSION

The present study demonstrated that local application of TGF-β1 increases the healing rate of chronic gastric ulcers in the rat. In accordance with other studies on dermal wounds we injected TGF-β1 in the ulcer area immediately after ulcer induction with acetic acid and 48 h later.

The TGF-β1 family consist of three isoforms that were found in mammals. Each of these is encoded by a different gene and has an unique promotor (2). Of the three isoforms TGF-β1 is the most abundant in all tissues of the gastrointestinal tract of the rat. TGF-β1 has been detected in the stomach and duodenum (7,10).

The expression of TGF-β1 is induced by wounding, and is regulated by means of AP-1 and Egr-1 transcriptional elements (5,11). AP-1 sites mediate autoinduction of TGF-β1. Application of different TGFβ isoforms to wounds leads to the expression of TGF-β1, but not TGF-β2 or TGF-β3 (1,12). Studies on dermal wounds have shown that enhanced expression of TGF-β1 is due to increased synthesis or activation of existing stores of peptides. In our model, TGF-β1 immunoreactivity was detectable at the margin and crater of healing ulcer, where inflammatory cells and extracellular matrix stained positive for TGF-β1. It is of interest that TGF-β1 immunoreactivity was absent in migrating surface epithelial cells. These cells may have a supply of TGF-β1 from the blood clot at the ulcer base.

Possibly more important than transcriptional regulation might be the activation of TGF-β1 secreted by monocytes or released by platelets and sequestered by the extracellular matrix (13).

After tissue injury, a complex consisting of TGF-β1, its latency associated protein (LAP) and the latent TGF-β1 binding protein is released into the serum. A smaller complex consists of noncovalently associated TGF-β1 and LAP, and is retained in the clot at the ulcer base (14).

In our study, we injected TGF-β1 into the submucosa at the margin of the ulcer. We injected relatively large amounts of TGF-β1 (50 ng) into the

subserosa at two sites immediately after ulcer induction, to provide an enhanced pool of TGF-β1 at the ulcer site. Only femtomolar concentrations of TGF-β1 are needed for the chemotaxis of monocytes, lymphocytes, neutrophils and fibroblasts. It has also been shown that TGF-β1 increases the migration of epithelial cells in organ culture.

TGF-β1 stimulates angiogenesis at the sites of infection *in vivo*. In a different animal model of duodenal ulcer healing in the rat it has been shown by Szabo et al (15) that the topical application of basic fibroblast growth factor (bFGF), a growth factor that leads to marked stimulation of angiogenesis and ulcer healing, also led to an increase in the number of microvessels in the ulcer scar. In our study there was no significant increase in the number of capillaries between the groups studied at day 11 after ulcer induction. It is of interest to note that ulcer healing was accompanied by increased blood flow at the ulcer margin, suggesting that the blood flow around the ulcer plays a crucial role in the healing process as has been suggested previously (9).

There was, however, no significant difference between the groups studied. Our data suggest that, despite the fact that local injection or TGF-β1 leads to angiogenesis, the effect of more rapid ulcer healing in the examined model may not depend upon the vascular factor. Most studies on the effect of TGF-β1 in wound healing involved of a single topical application of TGF-β1 at the time of wound induction. Studies in a rabbit ear dermal ulcer model showed that the application of TGF-β1 24 hours after wounding did not have any effect of ulcer healing (3). This might suggest that in the early stages of ulcer healing there is a particular cell population that might be optimally stimulated by TGF-β1 (1).

It has been shown that systemic administration of TGF-β1 even 24 hours before induction of lesions enhances repair of the wound (1,4). This might suggest that circulating monocytes, and possibly tissue fibroblasts, are potential targets of systemic application of TGF-β1.

In conclusion we have provided evidence that local application of TGF-β1 to chronic gastric ulcers leads to more rapid ulcer healing that is not dependent upon the vascular factor such as gastric blood flow and angiogenesis.

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