Accelerate of wound healing in gastric ulcers by local injection of neutralising antibody to transforming growth factor $\beta_1$

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Abstract

**Background**—Application of neutralising antibodies (NAs) to transforming growth factor $\beta_1$ (TGF$\beta_1$) improves wound healing in experimental glomerulonephritis and dermal incision wounds. TGF$\beta_1$ has been detected in the stomach, but despite the fact that this cytokine plays a central part in wound healing no information is available to determine if modulation of the TGF$\beta_1$ profile influences the healing of gastric ulcers. This study examines gastric ulcer healing in the rat after local injection of NAs to TGF$\beta_1$.

**Method**—Chronic gastric ulcers were induced in Wistar rats by the application of 100% acetic acid to the serosal surface of the stomach. Immediately after ulcer induction and on day 2, NAs to TGF$\beta_1$ (50 $\mu$g), TGF$\beta_1$ (50 ng), saline or control antibodies (IgG; 50 $\mu$g) were locally injected into the subserosa. Controls received no subserosal injections. Animals were killed on day 5 or 11, the ulcer area was measured planimetrically, sections were embedded in paraffin wax, and stained with trichrome or haematoxylin and eosin. Depth of residual ulcer was assessed on day 11 by a scale of 0–3, the percentage of connective tissue was determined by a semiquantitative matrix score and granulocytes and macrophages in the ulcer bed were also assessed.

**Results**—The application of NAs to TGF$\beta_1$ led to a significant acceleration of gastric ulcer healing on day 11 (0–6 (SD 0–8) vs 3–7 (SD 2–6) mm$^2$), a reduction in macrophages (23–7 (SD 22–6) vs 38 (26) per 40× power field) and granulocytes (8–5 (SD 5–6) vs 20 (10) per 40× power field), fewer histological residual ulcers (mean 1 (SD 0–9) vs 2 (1–1)), a reduced matrix score, and a regenerative healing pattern. Excessive scarring was seen in the TGF$\beta_1$ treated group.

**Conclusion**—Further treatment of gastric ulcers may induce a new treatment modality by local injection of NA to TGF$\beta_1$ in an attempt to accelerate and improve ulcer healing.

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Keywords: gastric ulcer, wound healing, TGF$\beta_1$, neutralising antibodies to TGF$\beta_1$, quality of wound healing.

Fetal wounds heal with a reduced inflammatory and cytokine response and complete restitution of the normal tissue architecture. In contrast healing of adult wounds does not necessarily lead to complete restitution of normal structure and function. Studies in humans and animals have shown that pathological accumulation of extracellular matrix is a central biological feature of poor healing and poor preservation of the tissue architecture in interstitial lung fibrosis, glomerulosclerosis, scarring of skin wounds, and healing of gastric ulcers. Transforming growth factor $\beta_1$ (TGF$\beta_1$) has been shown to act as a molecular switch that turns the repair process on and off by exerting a variety of effects on the extracellular matrix. Data obtained from studies in animals and humans show that TGF$\beta_1$ plays a central part among cytokines in the stimulation of matrix production, inhibition of matrix degradation, and modulation of matrix receptors to increase cell adhesion to the matrix.

In animal studies it has been shown that several growth factors applied to gastric ulcers, such as epidermal growth factor, TGF$\alpha$, platelet derived growth factor, and bFGF, accelerate the healing process by mechanisms that are not completely understood.

TGF$\beta_1$ has been detected in the rat stomach, but no information is available at present, as to whether TGF$\beta_1$ has any influence on the healing of gastric ulcers. In experimental glomerulonephritis and dermal incision wounds, systemic application of neutralising antibodies (NAs) to TGF$\beta_1$ improved the healing process and reduced the deposition of extracellular matrix. Application of TGF$\beta_1$ to skin wounds in rats resulted in an increase in collagen synthesis within the wound, and accelerated rate of healing as with NAs. In this study we report that locally applied NAs to TGF$\beta_1$ in an experimental model of chronic gastric ulcer leads to an acceleration of ulcer healing, a reduction in extracellular matrix deposition, and an improvement in the restoration of tissue architecture.

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. Each group of animals contained 9–10 rats. The results were pooled for statistical analysis.

**Experimental design**

Two series of experiments were performed. In
the first series 50 rats were divided into five
groups and treated with local anti-transform-
ing growth factor therapy or controls (as
described below), were killed after day 5, and
the ulcer size was measured. In the second
series 50 rats were divided also into the same
groups and given the same treatments, but
were killed on day 11. Tissues were removed
and the ulcer size was measured and the ulcer
area or scar was embedded in paraffin wax.

Local anti-transforming growth factor therapy
Immediately after the induction of ulcers
(during laparotomy) four of five groups, each
containing 10 animals, received local (in
the area of application of acetic acid) sub-
serosal application of either 50 μg NA
to TGFβ1 (AB-101-NA, R and D Systems,
Minneapolis, USA), 50 ng TGFβ1 (BDP1,
British Biotechnology, Oxford), 50 μg
chicken IgG-control (AB101-C, R and D
Systems, Minneapolis, USA), or saline
(0-9%). The fifth group received no sub-
serosal application.

On day 2 a laparotomy was performed and
the NAs or control antibodies (and saline)
were again injected locally into the ulcer area.
These subserosal injections comprised the
respective substance in phosphate buffered
saline at a volume of 100 μl, and were applied
just beside the ulcer on the same wall of the
stomach. In one group, only a laparotomy was
performed (sham operation).

Specificity of NAs to TGFβ1
The specificity of the neutralising antibody
against TGFβ1 has been tested in direct
ELISA and western blot analysis by R and D
Systems. This antibody shows 10% cross reac-
tivity with TGFβ2 and <5% cross reactivity
with TGFβ3 and TGFβ3.

The concentration of neutralising antibody
required to yield one half maximal inhibition
of TGFβ1, when TGFβ1 is present to yield 100% activity, was tested in a neutralising bioassay
using the murine T helper cell line, HT-2 cells.
Three μg/ml of antibody will neutralise 50% of
the biological activity caused by 0-25 ng/ml
of TGFβ1. Each batch of neutralising antibody
to TGFβ1 has been tested in this bioassay by
R and D Systems.

Determination of ulcer size and depth
The area of the ulceration was measured plani-
metrically by a person who was blinded to the
origin of the coded specimens, using a com-
puter planimeter (Morphomat 10, Opton,
Germany), and results expressed in square mil-
limetres. The sections were embedded in
paraffin wax and stained with haematoxylin
and eosin. In addition to measuring the surface
area of the ulcer in millimetres, a scale of 0-3
was used to measure the depth of the residual
ulcer on day 11 (0, complete healing of the
ulcer; 1, superficial erosion; 2, deep ulcer
extending into the muscularis; and 3, penetrat-
ing or perforating ulcer).

Morphometric analysis
For the histological examination the ulcers
were embedded in paraffin wax and stained
with haematoxylin and eosin or trichrome.

Matrix score
On day 11 the area of dense connective tissue
fibre material in the gastric ulcer bed in the
trichrome stained sections was assessed by a
semiquantitative score. This was expressed as
the percentage of submucosal area covered by
dense connective tissue fibre material. Two
submucosal areas beneath the ulcer and two
submucosal areas under normal mucosa were
selected (100 point raster; with a x100 object-
ive) by two independent observers. The
results were pooled for statistical analysis. The
results obtained in normal submucosa (same
slides as healing ulcers) in the five different
groups were used as control values.

Morphometric analysis of macrophages and
granulocytes
On day 11 granulocytes were stained by leuco-
cyte esterase, while macrophages were identi-
fied in the haematoxylin and eosin stain and
were counted per low power x40 objective
field in the ulcer bed.

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

Results
The mean ulcer size (mm2) of animals treated
with NAs to TGFβ1, was reduced slightly on
days 5 (mean 7.1 (SD 3.2)) and greatly on day
11 (mean 0.6 (SD 0.84)) (Fig 1A and B). Sur-
prisingly, treatment with TGFβ1 alone
delayed slightly ulcer size on day 5, but also
reduced the ulcer area significantly (p<0.05)
on day 11 (1.7 (SD 1.6)) v controls (3.7 (SD
2.6)). Treatment with TGFβ1, however, reduced
the ulcer size to a smaller extent than

Eleven days after ulcer induction, histo-
logical assessment of residual ulcers by a score
established by Szabo et al12 did show that the
number of rats with histological residual ulcer
was smaller only in the NA treated group
(mean 1 (SD 0.9) v controls 2 (SD 1.1)). The
mean score of gastric ulcers in the sham
treatment (mean 2.1 (SD 1.0)), chicken IgG
(mean 2 (SD 1.1)) or NaCl treated group (2.2
(SD 1.2)) showed almost no completely healed
ulcers. In the group of animals treated with
TGFβ1 (mean 1.7 (SD 1.0)) only two deep
penetrating ulcers were seen. In the NA group
(mean 1.0 (SD 0.9)), there were only three
with residual ulcers and seven had completely
healed ulcers or superficial erosions.

NA treated wounds had fewer macrophages
(mean 23.7 (SD 22.6) v 38 (SD 26) in the
controls) per low power field (×40 objective)
and fewer neutrophil granulocytes on day 11 (8·5 (SD 5·6) v 20 (SD 10)) per low power field (×40 objective) than the controls. The number of macrophages and granulocytes in the ulcers treated with TGFβ1 did not differ significantly from those in the controls.

Histological examination (trichrome and haematoxylin and eosin stain) of the connective tissue compartment showed that ulcers treated with NAs had a more regenerative pattern of normal gastric mucosal architecture. Collagen fibre orientation in the gastric mucosa showed an almost normal regenerative pattern. A semiquantitative assessment of connective tissue fibres in the ulcer bed was made using a semiquantitative matrix score. The matrix score (mean of NA treated group 19 v 26 in the TGFβ1 treated group, and a mean of 23 in the control groups v 13 in normal submucosa) showed a less dense distribution of extracellular matrix in the submucosa of the ulcer bed in the NA treated group (Fig 2). The difference of the matrix scores between the NA to TGFβ1 treated group and the TGFβ1 treated group is statistically significant (p<0·05). Apart from the matrix score the ulcers treated with TGFβ1 showed an orientation of collagen similar to that seen in scar tissue (Fig 3).

Discussion

Our experiments show that immediately after induction of gastric ulcers local application of TGFβ1 or NAs against TGFβ1 affects the rate of ulcer healing. Both lead to an acceleration of healing, but with different histological features – excessive scarring in TGFβ1 treated ulcers versus a more regenerative healing pattern in NA treated ulcers.

The production and deposition of extracellular matrix is a key factor in tissue repair.5 TGFβ1 induces the formation of extracellular matrix components in a very complex way. It

Figure 1: (A) Effect of treatment with NAs to TGFβ1, TGFβ3, or controls on the healing of chronic gastric ulcers on day 5 after induction of the ulceration (n=9–10 each group). Results are expressed as means (SD). Error bars=1 SD. (B) Effect of treatment with NAs to TGFβ1, TGFβ3, or controls on the healing of chronic gastric ulcers on day 11 after induction of the ulceration (n=9–10 each group). Results are expressed as means (SD). Asterisk shows significant (p<0·05) decrease below the control value. Error bars=1 SD.

Figure 2: Gastric ulcer treated with NAs to TGFβ1. Regeneration of normal tissue architecture. Newly formed muscularis propria (arrows; mp) and small submucosa (sm) (original magnification ×25). Bar 100 μm.

Figure 3: Gastric ulcer treated with TGFβ1 (trichrome stain). Scarring in the submucosa (sm) (original magnification ×25). Bar 100 μm.
promotes extracellular matrix deposition not only by stimulation of extracellular matrix protein synthesis, but also by inhibition of protease synthesis, stimulation of protease inhibitor synthesis, and an increased synthesis of cell adhesion receptors, which function to bind a wide variety of extracellular matrix components.

In gastroduodenal ulcer disease poor healing with submucosal scarring has been implicated as a factor of local ulcer recurrence.7 Shiann et al have shown that different therapeutic treatments may lead to different patterns of histological maturity.7 Tarnawski et al found that treatment with Maalox led to better submucosal healing and improved the overall quality of ulcer healing in contrast with treatment with H2 blockers.15

There is now evidence that overproduction of TGFβ1 may lead to scarring.6 7 16 In evolutionary terms, it seems that adult wounds may be optimised for speed of healing and this may result in an excessive TGFβ1 release. In response to wound damage TGFβ1 is released by platelets. TGFβ1 induces local cells to produce extracellular matrix and more TGFβ1. A potential regenerative response may be over-run by a cytokine surplus leading to scar formation.2 5 6

Evidence of a failure in the regulation of TGFβ1 has been implicated in experimental glomerulonephritis and disorders associated with interstitial fibrosis.3 5 Systemic application of TGFβ1 NAs prevented autoinduction of TGFβ1-mRNA, and limited the inflammatory response in experimental glomerulonephritis, and decreased the deposition of extracellular matrix.3 In contrast with adult wounds with an excess of cytokines, cytokine profiles in fetal wounds are reduced.1 Here we show that lowering the growth factor concentrations of TGFβ1 in a chronic gastric ulcer model leads to faster healing with considerably diminished scarring and infiltration of the ulcer base with fewer macrophages and granulocytes.

In contrast, local application of TGFβ1 resulted in massive scar formation. Recently, Mustoe et al showed that treatment of partial thickness gastric serosal incisions with a single dose of TGFβ1 accelerated the healing of the wounds.16 In our experiments, application of TGFβ1 accelerated the overall healing rate compared with the control groups.

This finding may be explained by the hypothesis that during the process of evolution adult wounds may have been optimised for speed of healing under adverse conditions. This may have resulted in excessive inflammatory infiltrates and an excess of cytokines during wound healing in the adults.5 Shah et al mimicked this fetal wound situation in the healing adult rat skin wound by local injection of an anti-TGFβ1 NA to reduce TGFβ1 concentrations. This resulted in diminished scarring in adult wounds and almost normal dermal architecture. Injection of TGFβ1 alone had the opposite effect.

Macrophages and activated platelets are the principal source of TGFβ1 in the adult wound. In the foetus, wounds heal with very little inflammation, and the reduced concentration of TGFβ1 may reflect the absence or minimal macrophage infiltrates in those wounds.6 15

Our study showed a reduction in the number of macrophages in wounds treated with NAs. But in wounds treated with TGFβ1 there was no significant difference in the number of macrophages compared with controls.

For ulcer relapse, excessive deposition of scarring tissue has been implicated as a risk factor.4 Here we show that lowering the active growth factor concentration of TGFβ1 by local application of NA at the ulcer site accelerates ulcer healing and improves the arrangement of the tissue architecture of the healing ulcer.

Our findings suggest that an anti-TGFβ1 therapy modality in gastric ulcer disease may be a novel means of stimulating and improving ulcer healing.

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Acceleration of wound healing in gastric ulcers by local injection of neutralising antibody to transforming growth factor beta 1.

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