Dexamethasone Inhibits Tumor Necrosis Factor-α-stimulated Gastric Epithelial Cell Migration

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Introduction

Ulcer formation is a dynamic imbalance between mucosal aggressive factors and defensive factors. When the function of defense and repairing factors is less than that of aggressive factors, mucosal injury worsens, and finally ulcer formation develops. Ulcer healing and repair requires a reconstruction of the surface epithelium and underlying connective tissue, including blood vessels and muscle layers. The early phase of mucosal repair occurs in the absence of cellular proliferation and is termed restitution or cell migration. The late phase of repair of deeper tissue damage involves cell proliferation as well as angiogenesis of granulation tissue to reconstruct the mucosal and submucosal architecture. All of these events are controlled and regulated by cytokines, growth factors, and some transcription factors that are overexpressed or activated over injured mucosa or ulcer margins.

In general, re-epithelialization, the migration of epithelial cells from the wound margin to restore epithelial continuity, is essentially an early process for gastrointestinal ulcer healing. Cell migration requires cytoskeletal rearrangement: actin microfilament assembly, stress fiber and lamellipodia formation, as well as rearrangement of focal adhesion complex attachments to the extracellular matrix. Growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF), as well as cytokines such as tumor necrosis factor-α (TNF-α).
factor (TNF)-α, interleukin (IL)-1β and IL-8, are reported to activate cell migration.²,⁷

The expression of TNF-α is increased at ulcer sites in rat stomachs; TNF-α is presumably secreted from macrophages and mononuclear cells.²,¹¹ TNF-α has been reported to indirectly stimulate cell migration, partially through the increased production of HGF and IL-8.²,¹² Our previous in vivo studies demonstrated that dexamethasone, which is a potent corticosteroid, delayed rat gastric ulcer healing by inhibiting epithelial cell proliferation and angiogenesis at the ulcer margin.¹,⁴ Our previous in vitro study also showed that dexamethasone inhibited TNF-α-stimulated gastric epithelial cell proliferation via inhibition of arachidonic acid-cyclooxygenase (COX)-2 pathway activation.¹³

In this in vitro study, we evaluated whether dexamethasone inhibited TNF-α-stimulated gastric epithelial cell migration and explored the possible mechanistic pathways. The findings further explained the mechanisms of how glucocorticoids delay gastric ulcer healing.

Methods

All study chemicals were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA) unless otherwise specified. Ten µg TNF-α (human, recombinant) was prepared in 2 mL of 0.01 M phosphate buffer saline at pH 7.4 containing 0.1% bovine serum albumin as a stock solution.

Cell culture

The RGM-1 rat gastric mucosal cells were established from normal Wistar rats and were a gift from Dr Hirofumi Matsui of Tsukuba University (RCB-0876 at Riken Cell Bank, Tsukuba, Japan). RGM-1 cells are epithelial in origin and like mucous epithelial or mucous neck cells.¹⁴,¹⁵ Ours were grown in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 100 U/mL penicillin G, 100 µg/mL streptomycin, and 20% fetal bovine serum (FBS) (Gibco BRL) in an incubator at 37°C and 5% carbon dioxide.

Cell migration

Cells were seeded in 24-well culture plates and cultured in DMEM/F-12 with 20% FBS until confluence. After confluence, monolayers of the cells were starved for 24 hours in the medium containing 1% FBS. The cells were then pretreated with mitomycin C (2 µg/mL) for 2 hours before a wound was made to inhibit cell proliferation.¹⁶,¹⁷ This dose and treatment duration of mitomycin almost abolished 10% of FBS-induced RGM-1 cell proliferation as reflected by a 96% decrease in BrdU (5-bromo-2'-deoxy-uridine) labeling compared to cells incubated with medium alone at 48 hours.¹⁰ An artificial circular wound of cell-free area 2 mm² was made in the center of the monolayer using a plastic blade.¹⁶,¹⁸ The wounded monolayer was then cultured in the medium with 1% FBS in the presence of control, or TNF-α alone (2 and 10 ng/mL), or TNF-α (10 ng/mL) with dexamethasone (10⁻⁸ M and 10⁻⁶ M), or dexamethasone alone (10⁻⁸ M and 10⁻⁶ M). The size of the cell-free area was monitored from time 0 to 48 hours using a digital image processor connected to a microscope (Nikon, Tokyo, Japan).¹⁸ The areas were calculated with an image-analyzing program (Leica, Cambridge, England).

Western blot analysis for COX-1, COX-2, EGF, and bFGF

After synchronization and pretreatment with mitomycin C as described above, cells were treated with DMEM/F-12 containing 1% FBS and 10 ng/mL TNF-α in the presence or absence of 10⁻⁶ M dexamethasone, or 10⁻⁶ M dexamethasone alone for 8 hours. Cells were then collected in radioimmunoprecipitation assay buffer for Western blot analysis. Following sonication and centrifugation, protein concentration was measured using a protein assay kit (BioRad Laboratories, Hercules, CA, USA). Proteins were separated by SDS-polyacrylamide electrophoresis gel overlaid with a 10% acrylamide stacking gel, and then transferred to Hybond C nitrocellulose membranes (Amersham International Plc., Amersham, UK). Membranes were probed with antibodies against COX-1 (1:800), COX-2 (1:500), EGF (1:500), bFGF (1:500), and β-actin (1:2,000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C and incubated for 1 hour with secondary antibodies conjugated with peroxidase. The membrane was developed using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA) and was exposed to X-ray film (Fuji Photo Film, Tokyo, Japan). Quantitation was performed using a densitometer (Scan Marker III; Microtek, Carson, NV, USA).

Measurement of PGE₂ level

After synchronization and pretreatment with mitomycin C as described above, cells were treated with 10 ng/mL TNF-α in the presence or absence of dexamethasone (10⁻⁸ M and 10⁻⁶ M), or dexamethasone (10⁻⁶ M) alone for 12 hours. Then, cells were homogenized with homogenizing buffer (0.05 M Tris-HCl...
at pH 7.4, 0.1 M NaCl, 0.001 M CaCl₂, 1 mg/mL D-glucose, 28 μM indomethacin to inhibit further PGE₂ formation) for 30 seconds. After centrifugation at 24,148 g for 15 minutes at 4°C, the supernatants were assayed using a commercially available PGE₂ enzyme-linked immunosorbent assay (Quantikine; R&D Systems Inc., Minneapolis, MN, USA). The assay procedures were in accordance with the protocol suggested in the kit. Optical densities were determined with the MRX microplate reader (Dynex Technologies Inc., Chantilly, VA, USA) at 405 nm. The amount of protein in the sample was determined by a protein assay kit, and the intracellular PGE₂ level was expressed as pg/mg protein.¹⁵

**Statistical analysis**
Analytic results are expressed as mean ± standard deviation. There were six samples in each group. Differences between the means were analyzed with the Mann-Whitney U test when appropriate. Bonferroni correction was performed to adjust for the fact that multiple comparisons were done in each experiment. A p value <0.05 was considered to be statistically significant.

**Results**

**Effects of TNF-α on cell migration in RGM-1 cells**
Treatment with TNF-α 2 ng/mL increased RGM-1 cell migration, but there was no significant difference when compared with control after 12, 24, 36 and 48 hours of treatment. However, treatment with TNF-α 10 ng/mL for 12, 24, 36 and 48 hours significantly increased cell migration when compared with control (Figure 1). Thereafter, we chose 10 ng/mL TNF-α as the working concentration for subsequent experiments in this study.

**Effects of dexamethasone on cell migration in RGM-1 cells**
Dexamethasone treatment alone (10⁻⁸ M and 10⁻⁶ M) for 12, 24, 36 and 48 hours did not have a stimulatory or inhibitory action on RGM-1 cell migration when compared with that of control (Figure 2). Therefore, there was no significant difference between the TNF-α plus dexamethasone group and the control group (Figure 3).

**Effects of TNF-α and dexamethasone on protein expression of COX-1, COX-2, EGF and bFGF**
Treatment with TNF-α 10 ng/mL for 8 hours significantly increased COX-2 expression when compared with that of the control group. In contrast, dexamethasone (10⁻⁶ M) alone did not decrease COX-2 expression when compared with the control group (Figure 4). However, the same concentrations of dexamethasone (10⁻⁶ M) significantly decreased TNF-α-stimulated COX-2 expression when compared with...
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0

β-actin

COX-2

Cell-free area (mm²) (% of control)

Time (hr)

Figure 3. Effects of dexamethasone on TNF-α stimulated RGM-1 cell migration. Cells were incubated with control, TNF-α (10 ng/mL), or TNF-α (10 ng/mL) plus dexamethasone (10⁻⁸ M or 10⁻⁶ M) for 48 hours. Sizes of the cell-free area were monitored at 0, 12, 24, 36 and 48 hours using a digital image processor connected to a microscope. Values are mean ± standard deviation for six samples per group. *p < 0.05 vs. control group; †p < 0.05 vs. TNF-α group.

β-actin

COX-2

Figure 4. Effect of TNF-α on the protein expression of COX-2 in RGM-1 cells. Cells were incubated with TNF-α 10 ng/mL in the absence or presence of dexamethasone (10⁻⁸ M and 10⁻⁶ M) for 8 hours and measured by Western blot method. Values are mean ± standard deviation of six samples per group. *p < 0.05 vs. control group; †p < 0.05 vs. TNF-α treated group.

Figure 5. Effects of TNF-α and dexamethasone on intracellular PGE₂ level. Cells were incubated with TNF-α 10 ng/mL in the absence or presence of dexamethasone (10⁻⁸ M and 10⁻⁶ M) and dexamethasone 10⁻⁶ M alone for 12 hours and measured by enzyme-linked immunosorbent assay. Values are mean ± standard deviation of six samples per group. *p < 0.05 vs. control group; †p < 0.05 vs. TNF-α treated group.

Discussion

Our study demonstrated that TNF-α increased COX-2 expression and PGE₂ formation, and stimulated rat gastric epithelial (RGM-1) cell migration. Dexamethasone inhibited TNF-α-stimulated RGM-1 cell migration, which was associated with a decrease in COX-2 expression and PGE₂ formation. These findings further explain the reasons and mechanisms for how glucocorticoids delay gastric ulcer healing.

Our previous study showed that TNF-α (2 and 10 ng/mL) and dexamethasone (10⁻⁸ M and 10⁻⁶ M
Steroid-inhibited epithelial cell migration

alone did not alter cell viability (by MTT [3-(4,5-
dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bro-
mide] reduction method) or induce RGM-1 cell
apoptosis (by DNA laddering), respectively. That
study also showed that TNF-α, which was overex-
pressed in the gastric ulcer margin, 11 played a positive
role in RGM-1 cell proliferation via activating the
arachidonic acid–COX-2 pathway, which is important
for mucosal defense and ulcer healing.1,13 The current
study showed that TNF-α at the same concentration
(10 ng/mL) significantly stimulated COX-2 expres-
sion and PGE2 formation, and increased RGM-1 cell
migration, which is the initial step in mucosal repair
before cellular proliferation. In fact, activation of the
COX-2 pathway is essential for cell migration.19,20
TNF-α also upregulated the production of HGF and
IL-8, which promoted cell migration.2,12,20

This study also revealed that dexamethasone
inhibited TNF-α-stimulated COX-2 expression and
PGE2 formation, and inhibited TNF-α-stimulated
RGM-1 cell migration. It was interesting to find that
dexamethasone alone did not have an inhibitory
action on basal RGM-1 cell migration when com-
pared with that of control, but did have an inhibitory
action on TNF-α-stimulated cell migration when com-
pared with the TNF-α-treated group. The find-
ings are in agreement with data showing that COX-2
expression and intracellular PGE2 levels were compa-
rabler between the control group and the dexametha-
sone (10⁻⁶ M) group, although COX-2 expression
and PGE2 synthesis were a little weaker in the dexam-
ethasone group than in the control group. Further
study is needed to clarify why dexamethasone alone
did not have an inhibitory action on basal RGM-1
cell migration.

The concentrations of dexamethasone (10⁻⁸ M
and 10⁻⁶ M) we used in this study are similar to the
pharmacologic concentration that is found in the
plasma of patients treated with dexamethasone.20,21
Putting together the findings of our previous studies
and the current study, we demonstrated that dexam-
ethasone inhibited EGF, bFGF, TNF-α-stimulated
RGM-1 cell proliferation, and TNF-α-stimulated RGM-
1 cell migration.13,15,22 We also found that dexam-
ethasone inhibited EGF, bFGF, and TNF-α-stimulated
COX-2 expression. These findings also support our
previous studies that showed that dexamethasone
delayed rat gastric ulcer healing via inhibition of epitel-
ial cell proliferation in ulcer margins and angiogenesis
of ulcer base.1,4

In conclusion, TNF-α increased COX-2 expres-
sion and PGE2 formation, and stimulated rat gastric
epithelial (RGM-1) cell migration. Dexamethasone
inhibited TNF-α-stimulated RGM-1 cell migration,
which was associated with a decrease in COX-2
expression and PGE2 formation.

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References

1. Luo JC, Shin VY, Liu ESL, So WHL, Ye YN, Chang FY, Cho
CH. Non-ulcerogenic dose of dexamethasone delays gastric
2. Yoo J, Lotz MM, Matthews JB. Cytokines in restitution. In:
Cho CH, Wang JY, eds. Gastrointestinal Mucosal Repair and
3. Silen W, Ito S. Mechanisms for rapid re-epithelialization of
4. Luo JC, Shin VY, Liu ESL, Ye YN, Wu WKK, So WHL, Chang
FY, et al. Dexamethasone delays ulcer healing by inhibition of epithe-
lial cell proliferation in ulcer margins and angiogenesis in rat stomachs.
5. Szabo S, Khomenko T, Gombos Z, Deng XM, Jadas MR,
Yoshida M. Review article: transcription factors and growth
factors in ulcer healing. Aliment Pharmacol Ther 2000;
14(Suppl):33–43.
6. Tarnawski A, Szabo IL, Husain SS, Soreghan B. Regeneration
of gastric mucosa during ulcer healing is triggered by growth
95:337–44.
7. Tarnawski AS. Cellular and molecular mechanisms of gastroin-
8. Bason MD, Modlin IM, Madri JA. Human enterocyte (Caco-2)
modification is modulated in vitro by extracellular matrix compo-
sition and epidermal growth factor. J Clin Invest 1992:90:
15–23.
9. Martin P. Wound healing—aiming for perfect skin regeneration.
Science 1997;276:75–81.
Nonsteroidal anti-inflammatory drugs inhibit re-epithelialization
of wounded gastric monolayers by interfering with actin, Src,
11. Takahashi S, Sigure JI, Inoue H, Tanabe T, Okabe S. Localiza-
tion of cyclooxygenase-2 and regulation of its mRNA
expression in gastric ulcers in rats. J Pharmacol Exp Ther
12. Takahashi M, Ota S, Hata Y, Mikami Y, Azuma N, Nakamura T,
Terano A, et al. Hepatocyte growth factor as a key to modulate
tumor necrosis factor-α stimulates gastric epithelial cell proliferation.
13. Luo JC, Shin VY, Yang YH, Wu WKK, Ye YN, So WHL,
Chang FY, et al. Tumor necrosis factor-α stimulates gastric
epithelial cell proliferation. J Pharmacol Exp Ther 2003;307:
692–8.


