Tumor necrosis factor-α stimulates gastric epithelial cell proliferation

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Luo, Jiing Chyuan, Vivian Yvonne Shin, Ying Hua Yang, William Ka Kei Wu, Yi Ni Ye, Wallace Hau Leung So, Full Young Chang, and Chi Hin Cho. Tumor necrosis factor-α stimulates gastric epithelial cell proliferation. Am J Physiol Gastrointest Liver Physiol 288: G32–G38, 2005. First published July 14, 2004; doi:10.1152/ajpgi.00093.2004.—TNF-α is a cytokine produced during gastric mucosal injury. We examined whether TNF-α could promote mucosal repair by stimulation of epithelial cell proliferation and explored further the underlying mechanisms in a rat gastric mucosal epithelial cell line (RGM-1). TNF-α treatment (1–10 ng/ml) for 12 or 24 h significantly increased cell proliferation but did not induce apoptosis in RGM-1 cells. TNF-α treatment significantly increased cytosolic phospholipase A₂ and cyclooxygenase-2 (COX-2) protein expression and PGE₂ level but did not affect the protein levels of EGF, basic fibroblast growth factor, and COX-1 in RGM-1 cells. The mRNA of TNF receptor (TNF-R) 2 but not of TNF-R1 was also increased. Dexamethasone dose dependently inhibited the stimulatory effect of TNF-α on cell proliferation, which was associated with a significant decrease in cellular COX-2 expression and PGE₂ level. A selective COX-2 inhibitor 3-(3-fluorophenyl)-4-[4-(methylsulfonyl)phenyl]-5,5-dimethyl-2'H-furan-2-one (DFU) by itself had no effect on basal cell proliferation but significantly reduced the stimulatory effect of TNF-α on RGM-1 cells. Combination of dexamethasone and DFU did not produce an additive effect. PGE₂ significantly reversed the depressive action of dexamethasone on cell proliferation. These results suggest that TNF-α plays a regulatory role in epithelial cell repair in the gastric mucosa via the TNF-α receptor and activation of the arachidonic acid/PG pathway.

cyclooxygenase; dexamethasone; phospholipase A₂; prostaglandin E₂

EXPERIMENTAL STUDIES have suggested that TNF-α, which is activated by Helicobacter pylori infection and by indomethacin or aspirin administration, is closely related to gastric epithelial injury and apoptosis (5, 14, 15, 20, 21, 22, 30). However, other studies (1, 29) have demonstrated that TNF-α coactivates the NF-κB transcription factor pathway, which limits apoptosis through antiapoptotic gene transcription. The expression of TNF-α is also increased at the ulcer sites in rat stomachs presumably secreted by macrophages and mononuclear cells (8, 21, 26, 32).

Cytokines and growth factors that are produced by gastric mucosal injury may regulate not only the inflammatory process but also mucosal healing by coordinating different phases of tissue repair. The early phase is characterized by rapid epithelial cell migration in the absence of proliferation, whereas the late phase involves cell division and cell proliferation to fill in the defect and restore mucosal architecture (23, 27, 32). Cytokines and growth factors can stimulate cyclooxygenase-2 (COX-2) expression in different tissues, and increased COX-2 expression has been demonstrated to be important in gastric mucosal defense and ulcer healing (2, 4, 13, 17, 26). TNF-α has been reported to indirectly stimulate cell migration through the increased production of hepatocyte growth factor and interleukin-8 (24, 32). However, the effect of TNF-α on gastric epithelial cells, especially in cell proliferation during ulcer repair, has been poorly investigated.

In this in vitro study, we examined whether TNF-α is detrimental to ulcer repair by induction of apoptosis or beneficial to ulcer healing by promotion of cell proliferation in normal gastric mucosal epithelial cell line (RGM-1). We also further explored the mechanism of action through the arachidonic acid/PG pathway, which has been shown to play a pivotal role in tissue repair in the gastrointestinal (GI) tract. In this regard, we used the COX-2 inhibitor to modulate the COX-2 enzyme activity; we also applied dexamethasone, which is known to affect the production of arachidonic acid and PG and to decrease ulcer healing in animals (11, 18, 31), to examine how it influences cell proliferation, and further on, the proliferative activity of TNF-α in RGM-1 cells.

MATERIAL AND METHODS

Reagents and drugs. All chemicals and reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless otherwise specified. The COX-2 selective inhibitor 3-(3-fluorophenyl)-4-[4-(methylsulfonyl)phenyl]-5,5-dimethyl-2'H-furan-2-one (DFU) was kindly donated by Merck (Rahway, NJ). Glucocorticoid receptor antagonist, mifepristone, and COX-2 selective inhibitor DFU were dissolved in dimethylsulfoxide (0.1 and 0.4%, respectively, as vehicle in the final concentration), PGE₂ was dissolved in pure ethanol (0.3% as a vehicle in the final concentration), and 10 μg TNF-α (human, recombinant) were prepared in 2 ml 0.01 M phosphate-buffered saline at pH 7.4 containing 0.1% bovine serum albumin as stock solution.

Cell culture. Rat gastric mucosal epithelial cell line RGM-1 (RCB-0876, Riken Cell Bank, Tsukuba, Japan) was used. Cells were grown in DMEM/F-12 medium ( Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 20% FCS (GIBCO) in an incubator at 37°C, 95% humidity, and 5% carbon dioxide. In a separate experiment, AGS (CRL-1739), a poorly differentiated human gastric adenocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 (GIBCO) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10% fetal bovine serum (GIBCO).

[3H]thymidine incorporation. [3H]thymidine incorporation assay was used to determine the amount of DNA synthesis during a...
particular period of time and was represented as cell proliferation. The method was as described previously by Tones et al. (28) with modifications. Cells were seeded in 24-well culture plates at $5 \times 10^4$ cells/ml and were allowed to grow in DMEM/F-12 medium containing 20% FCS for 24 h. Afterwards, cells were grown arrested in the serum-free medium for another 24 h to synchronize the cell cycles before any treatment. Cells were then treated with 2% FCS DMEM/F-12 medium containing either TNF-α (1, 2, 5, or 10 ng/ml), dexamethasone (10$^{-6}$ M), glucocorticoid receptor antagonist mifepristone (10$^{-6}$ M), or COX-2 selective inhibitor DFU (10$^{-4}$ M), or PGE$_2$ (10$^{-5}$ M) together with TNF-α (2 ng/ml) and dexamethasone (10$^{-8}$ M) for 24 h. After treatment, cells were incubated with 2% FCS medium containing 0.5 μCi/ml $[^{3}H]$thymidine (Amersham Biosciences, Piscataway, NJ) for 5 h at 37°C. The solution was discarded and then washed with 1 ml 0.15 M NaCl. Afterwards, 10% trichloroacetic acid was added into the wells and incubated for 15 min at room temperature. The wells were rinsed with distilled water for four times, followed by the addition of 0.5 ml of 1% SDS into each well to incubate for 15 min at 37°C. The solution was transferred into a scintillation vial and 0.5 ml 50% SDS was added to rinse the well, and it was transferred into the same vial. Water-accepting scintillation fluid (9 ml; NBCS 104, Amersham Biosciences) was added into the vial and then vortexed. Finally, the amount of DNA synthesis was measured in a liquid-scintillation counter (LS-6500 Multi-Purpose Scintillation Counter, Beckman Instruments, Pullerton, CA). Similar experiments were performed in AGS cells, with which TNF-α 2 ng/ml was incubated for 24 h.

**Determination of apoptosis.** DNA laddering was determined as described by Donovan et al. (3) with some modifications. After being seeded and cultivated for 24 h, cells were treated with 2% FCS DMEM/F-12 medium in the absence or presence of TNF-α (2 and 10 ng/ml) for 24 h as negative control and as study groups, respectively, or were treated with 2% FCS medium with staurosporin (100 nM) for 12 h as positive control. Cells were then collected by scraping the dishes with 0.01 M (pH 7.4) phosphate-buffered saline, pelleted, and disrupted in lysis buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5% Triton X-100). After RNase (final concentration 200 μg/ml, Invitrogen Life Technologies, Carlsbad, CA) was added, lysates were incubated at 37°C for 1 h and then treated with proteinase K (final concentration 200 μg/ml; Invitrogen Life Technologies) at 37°C for 1 h. DNA was precipitated by ice-cold 100% isopropanol and incubated at −20°C overnight. After centrifugation, the pellet was dissolved in Tris-boric acid-EDTA buffer (Bio-Rad Laboratories, Hercules, CA). After quantification (Amersham Biosciences), the DNA samples were separated by electrophoresis through 2.0% agarose gel. DNA was visualized by ethidium bromide staining and photographed under ultraviolet (UV) illumination (Gel Doc 1000; Bio-Rad Laboratories).

**Cell viability.** Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The number in each group ranged from four to six. Differences between the means were analyzed by Student’s t-test and one-way ANOVA when appropriate. P values of <0.05 were considered statistically significant. Every test in this study was repeated at least two times.

**RESULTS**

**Effect of TNF-α on cell proliferation.** There was no significant difference in RGM-1 cell proliferation between the TNF-α-treated groups and the control group after 3–6 h of incubation. However, TNF-α treatment at all concentrations (1, 2, 5, and 10 ng/ml) for 12 h significantly increased cell proliferation with the optimal effect occurring at 2 ng/ml (Fig. 1A). Herein, we chose 2 ng/ml TNF-α as the working concentration for subsequent experiments in this study. At this concentration of TNF-α, the mRNA level of the TNF-R2 receptor

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was time-dependently increased without affecting the TNF-R1 level (Fig. 1B), whereas in the AGS gastric cancer cell line, 2 ng/ml of TNF-α increased cell proliferation by 11.5 ± 2.0% compared with the respective control.

**Effect of TNF-α on cell apoptosis and cell viability.** There were no DNA bands visualized by UV illumination after ethidium bromide staining in the control group (2% FCS DMEM/F-12 medium) and the TNF-α-treated groups (2 and 10 ng/ml for 24 h), whereas DNA bands can be seen in the positive control group (100 nM staurosporin; data not shown). MTT tests showed that TNF-α (2 and 10 ng/ml), DFU (10^{-4} M) and dexamethasone treatments (10^{-8} and 10^{-6} M) for 24 h did not significantly change the cell viability (data not shown).

**Effect of dexamethasone and/or mifepristone on TNF-α-stimulated cell proliferation.** Dexamethasone treatment (10^{-8} and 10^{-6} M) significantly and dose dependently decreased TNF-α-stimulated RMG-1 cell proliferation (Fig. 2A). Neither dexamethasone (10^{-6} M) nor glucocorticoid receptor antagonist mifepristone (10^{-6} M) alone affected basal cell proliferation when compared with the control group (Fig. 2, A and B). However, mifepristone completely reversed the inhibitory action of dexamethasone (10^{-5} and 10^{-6} M) on TNF-α-stimulated cell proliferation (Fig. 2B).
pendently decreased COX-2 expression when compared with the TNF-α-treated group (Fig. 3A). Likewise, TNF-α treatment significantly increased the cPLA₂ expression when compared with the control group. It was slightly inhibited by dexamethasone, which by itself did not alter the cPLA₂ level (Fig. 3B). There were also no significant differences in the expressions of COX-1, bFGF, and EGF among the different treatment groups including the control, TNF-α, or dexamethasone and the TNF-α and dexamethasone-treated groups (Fig. 3, C-E).

**Effects of TNF-α and dexamethasone on PGE₂ level.** TNF-α treatment significantly increased the intracellular PGE₂ level. Again, dexamethasone treatment dose-dependently decreased the PGE₂ level in the TNF-α-treated group and reached a significant difference in the higher concentration group (10⁻⁶ M; Fig. 4).

**Effect of selective COX-2 inhibitor on TNF-α-stimulated cell proliferation.** COX-2 selective inhibitor (10⁻⁴ M DFU) alone did not affect the basal cell proliferation (Fig. 5). As with dexamethasone treatment, DFU treatment significantly reversed the stimulatory action of TNF-α on cell proliferation; the inhibitory action was less than that of dexamethasone (10⁻⁸ M), indicating that the pharmacological action of dexamethasone was more potent than DFU on cell growth. Combination of dexamethasone (10⁻⁸ M) and DFU (10⁻⁴ M) did not have an additive effect in the inhibition of TNF-α-stimulated cell proliferation when compared with the individual drug-treated group (Fig. 5).

**Effect of PGE₂ on the inhibitory action of dexamethasone on cell proliferation by TNF-α.** Again, dexamethasone significantly reduced the stimulatory action of TNF-α on thymidine
ever, PGE2 treatment alone at the same concentration had no action of dexamethasone on cell proliferation (Fig. 6). How-  
with these agents for 24 h significantly reversed the inhibitory difference between the Dexa-alone group and the control group. There was no significant difference between the DFU-alone group and the control group. **

0.05 when compared with the TNF- 

Fig. 5. Effect of Dexa and/or COX-2 selective inhibitor [3-(3-fluorophenyl)-4-[4-(methylsulfonyl)phenyl]-5,5dimethyl-2-furan-2-one} (DFU) on [3H]thymi- 

dine incorporation in rat gastric epithelial cell line treated with TNF-α and Dexa. Cells were incubated with 2 ng/ml TNF-α in the absence or presence of 10^{-8} M Dexa and 10^{-6} M Dexa alone for 24 h and measured by an enzyme-linked immunosorbent assay kit. Values are means ± SE of 6 samples per group. ***P < 0.01 when compared with the control group. †P < 0.05 when compared with the TNF-α-treated group. There was no significant difference between the Dexa-alone group and the control group.

Fig. 6. Effect of PGE2 on [3H]thymidine incorporation in rat gastric epithelial cell line treated with TNF-α and Dexa. Cells were incubated with 2 ng/ml TNF-α in the absence or presence of 10^{-8} M Dexa with or without 10^{-8} M PGE2 for 24 h, and the amount of DNA synthesis was measured. Values are means ± SE of 4 samples per group. ***P < 0.001 when compared with the control group. ††P < 0.01 when compared with the TNF-α-treated group. ###P < 0.01 when compared with the group treated with TNF-α and Dexa.

DISCUSSION

In the present study, we demonstrated for the first time that TNF-α treatment as low as 1–2 ng/ml significantly stimulated rat gastric epithelial cell proliferation but did not induce apoptosis. The latter finding is consistent with Naito et al. (15), who showed that TNF-α treatment (10–100 ng/ml) for the same period of time (24 h) did not significantly induce cell apoptosis in gastric epithelial cells. In contrast, previous reports (20, 21) showed that TNF-α, which was activated by Helicobacter pylori infection and nonsteroidal anti-inflammatory drug administration, caused gastric mucosal damage and gastric epithelial cell apoptosis. These dichotomous findings may be due to the different cell lines used in this study (rat normal gastric epithelial cell line) and other reports (human gastric epithelial cancer cell line MKN45) (20) and different study models (in vitro study in our study vs. in vivo rat study in other reports) (21). Furthermore, TNF-α treatment markedly increased the COX-2 but not the COX-1 expression and also stimulated PGE2 formation in gastric epithelial cells. These findings were consistent with Takahashi et al. (26), showing that TNF-α significantly stimulated COX-2 expression at the gastric ulcer site, which may be associated with the process of ulcer repair in the stomach. All of these findings are interesting in the exploration of differential roles of TNF-α in facing gastric mucosal injury. On one hand, it is closely related to tissue inflammation and damage; on the other hand, it is intimately associated with tissue repair and reconstruction. The mechanisms involved in the latter action is not yet defined and constituted the major objective in the present study. It is interesting to note that TNF-α also stimulates AGS gastric cancer cell proliferation, although to a lesser degree than nontransformed cells (11 vs. 50%). A causal relationship between chronic inflammation and the risk of malignancy has been implicated in a previous report (10), and this is supported by the present study. In this context, TNF-α could play a
PGE2 is found to stimulate gastric epithelial cell proliferation.

Concerning the mechanism on tissue repair, TNF-α only increased TNF-R2 receptor expression in RGM-1 cells (Fig. 1B); this receptor is likely to participate in the promotion of gastric epithelial cell growth. In fact, a similar conclusion has been reached in a study of an intestinal cell line from the mouse (7). COX is the rate-limiting enzyme for PGE2 formation. PGE2 is found to stimulate gastric epithelial cell proliferation in vitro and in vivo (11, 16). In the current study, TNF-α treatment significantly increased the protein expressions of cPLA2, COX-2, and PGE2 production in RGM-1 cells, whereas the expressions of EGF and bFGF, which were shown to be important in ulcer healing (9, 12), were not significantly affected by TNF-α treatment. Furthermore, the inhibitory action of dexamethasone on cell proliferation stimulated by TNF-α was associated with the decrease of COX-2 expression and PGE2 formation without influence on EGF and bFGF expressions. PGE2 administration significantly reversed the suppressive action of dexamethasone on gastric cell growth. All these findings supported the phenomenon that TNF-α significantly stimulated gastric epithelial cell proliferation primarily via a direct stimulatory action on cPLA2 and COX-2 expressions followed by PGE2 formation but not through the growth factors studied in this report. Our preliminary study also showed that using the same concentration of TNF-α did not affect the protein expression of NF-κB (p65) and I-κB in RGM cells (data not shown). However, the action of TNF-α on the protein expression and the activity of NF-κB remains unknown. Nevertheless, with the current findings, it is likely that the action of TNF-α on cell proliferation is via a NF-κB-independent pathway that may be operational in vivo (25).

The direct involvement of cPLA2 and COX-2 in the stimulatory action of TNF-α on cell proliferation was further studied. Our study demonstrated that the glucocorticoid-receptor antagonist mifepristone significantly and completely reversed the inhibitory action of dexamethasone on TNF-α-stimulated cell proliferation, suggesting that the pharmacological action of dexamethasone was glucocorticoid-receptor mediated. Glucocorticoids indeed can induce annexin 1 (lipocortin 1) synthesis and suppress arachidonic acid release via antagonizing PLA2 activity followed by decrease of COX-2 expression and PG synthesis (11, 18, 31). COX-2 selective inhibitor, on the other hand, can directly inhibit COX-2 activity and PG levels in cells. The present study revealed that the COX-2 selective inhibitor DFU significantly decreased TNF-α-stimulated cell proliferation similar to that of dexamethasone (Fig. 5). Furthermore, the inhibitory action of dexamethasone was blocked by PGE2 pretreatment (Fig. 6), implicating that the pharmacological action of the drug was largely mediated through the inhibition of PG synthesis to decrease cell proliferation. Interestingly, combination of dexamethasone and the COX-2 selective inhibitor did not produce an additive effect in the inhibition of cell growth in the TNF-α-treated cells, suggesting that PG could be the ultimate target for both drugs on cell proliferation. However, they only partially reversed the stimulatory action of TNF-α on [3H]thymidine incorporation in RGM-1 cells, suggesting that other factors could also contribute to the stimulatory action of TNF-α on gastric epithelial cells. It was also shown that both DFU and dexamethasone did not affect cell viability, indicating that the inhibitory action on cell growth by these drugs was not due to the cytotoxic effect. Moreover, the present findings are also in accord with our previous in vivo study in which the nonulcerogenic doses of dexamethasone can delay ulcer healing, which is mediated, in part, through the inhibitory action on cell proliferation in the gastric mucosa (11). Nevertheless, all of these findings suggest that the arachidonic acid-PG pathway plays a key role in cell repair during ulcer healing. It also contributes partly in the TNF-α-associated cell proliferation in gastric epithelial cells.

In conclusion, TNF-α in the gastric mucosa can be activated by mucosal injury and stimulate epithelial cell proliferation. This action is, at least in part, via the release of arachidonic acid by activation of cPLA2 followed by COX-2 expression and finally PGE2 formation in gastric epithelial cells. TNF-α produced during ulceration not only participates in inflammatory but also could play a modulatory role in ulcer repair in the stomach.

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