Transforming Growth Factor α Protection against Drug-induced Injury to the Rat Gastric Mucosa In Vivo

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Abstract

This study was designed to determine whether transforming growth factor α (TGFα) protects rat gastric mucosa against ethanol- and aspirin-induced injury. Systemic administration of TGFα dose-dependently decreased 100% ethanol-induced gastric mucosal injury; a dose of 50 μg/kg delivered intraperitoneally 15 min before ethanol decreased macroscopic mucosal injury by > 90%. At the microscopic level, TGFα prevented deep gastric necrotic lesions and reduced disruption of surface epithelium. Pretreatment with orogastric TGFα (200 μg/kg) only partially (40%) decreased macroscopic ethanol damage. Intraperitoneal administration of TGFα at a dose of 10 μg/kg, which does not significantly inhibit gastric acid secretion, decreased aspirin-induced macroscopic damage by > 80%. TGFα protection does not seem to be mediated by prostaglandin, glutathione, or ornithine decarboxylase-related events, as evidenced by lack of influence of the inhibition of their production. Pretreatment with the sulfhydryl blocking agent *N*-ethylmaleimide partially abolished (40%) the protective effect of TGFα. In addition, systemic administration of TGFα resulted in a twofold increase in tyrosine phosphorylation of phospholipase C-gamma 1 and in a time- and dose-dependent increase in levels of immunoreactive insoluble gastric mucin; these events occurred in a time frame consistent with their participation in the protective effect of TGFα. (*J. Clin. Invest.* 1992. 90:2409–2421.) Key words: ethanol • aspirin • prostaglandin • sulfhydryl • transforming growth factor α

Introduction

Epidermal growth factor (EGF)[1] protects gastric mucosa against a variety of ulcerogens (1–4). Transforming growth factor alpha (TGFα) is a polypeptide that shares with EGF structural homology (35%) (5, 6), a common membrane receptor, i.e., TGFα/EGF receptor (TGFα/EGFr) (7–9), and a qualitatively similar spectrum of biological activities (10). TGFα mRNA, but not EGF mRNA, has been shown to be expressed in the normal gastric mucosa from several species, including man (11). Also, TGFα mRNA and protein have been demonstrated to increase following acute injury to the rat stomach (12). Whether TGFα is protective to the gastric mucosa has not been studied. The present series of experiments was designed to determine whether systemic or orogastric administration of recombinant human TGFα afforded the rat gastric mucosa protection against an acid-independent form of acute injury (100% ethanol, EtOH) or an acid-dependent form of injury (aspirin, ASA). In the latter case, TGFα was administered intraperitoneally at a dose that was determined not to reduce gastric acidity significantly. In addition, we explored the mechanisms by which such protection may be conferred.

Methods

Male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 180–220 g were used. Rats were housed individually in wire mesh cages to avoid coprophagy and fasted 24 h with ad lib. access to drinking water before the experiments.

Induction of gastric mucosal damage

EtOH-induced gastric mucosal damage was accomplished by the orogastric administration of 1 ml of 100% EtOH. Rats were killed 1 h later by cervical dislocation. Aspirin-induced gastric mucosal damage was accomplished by the orogastric administration of acetylsalicylic acid (Sigma Chemical Co., St. Louis, MO) (200 mg/kg body wt) suspended in 1 ml of 0.15 N HCl with the addition of two drops of Tween 80 (Sigma Chemical Co.) per 10 ml to keep ASA in a homogenous suspension. Rats were killed by cervical dislocation 4 h after ASA administration.

Assessment of damage

Gross. Excised stomachs were opened along the greater curvature and rinsed in saline. The degree of gastric mucosal damage was evaluated by using a computerized image analysis system (IM4–152; Analytical Imaging Concepts, Inc., Irvine, CA). The damage surface area was expressed as the percentage of the total glandular area.

Microscopic. The mucosal surface of each stomach was examined for the presence of hemorrhagic and necrotic lesions. Six tissue blocks were taken from each stomach from the areas of normal-appearing gastric mucosa. Specimens were fixed in buffered formalin and stained with hematoxylin and eosin plus periodic acid-Schiff. Coded mucosal specimens were evaluated qualitatively under light microscopy by an investigator (M. Romano) unaware of the treatment. In addition, the extent of deep histological necrosis (defined as necrotic lesions penetrating into the mucosa deeper than 0.2 mm) was quantitated morphometrically in a blinded fashion with the aid of planar morphometry computer software (Southern Micro Instruments, Inc., Atlanta, GA),

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1. *Abbreviations used in this paper:* ASA, acetylsalicylic acid; DFMO, difluoromethylorinthine; EGF, epidermal growth factor; EtOH, ethanol; INDO, indomethacin; NEM, *N*-ethylmaleimide; ODC, ornithine decarboxylase; PLC, phospholipase C; TGFα, transforming growth factor α; TGFα/EGFr, TGFα/EGF receptor.

by measuring the length of mucosal strips and the length of necrotic lesions for each strip. Similarly, the disruption of continuity of the surface epithelium was quantitated by measuring the length of the mucosal strip and the length of mucosa devoid of the superficial epithelial layer. Results are expressed as a percentage of total mucosal strip length for each studied strip.

Scanning electron microscopy

Gastric mucosal specimens were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. After a fixation period of 18 h, samples were dehydrated in a graded series of EtOH solutions and critically point dried by liquid CO_2 substitution. Samples were mounted on aluminum stubs using silver paste and coated with palladium gold. Specimens were then examined and photographed on a Hitachi 500 scanning electron microscope at a voltage of 20 kV.

Prostaglandin E_2 assay

Gastric juice. After a 24-h fast, rats were anesthetized with pentobarbital (50 mg/kg body wt intraperitoneally [i.p.]), the abdomen was opened, and a polyethylene catheter was introduced into the stomach through a small duodenal incision. The esophagus and the pylorus were ligated (the latter over the catheter). The stomach was washed three times with 2 ml of normal saline, and 1.5 ml of normal saline then was instilled. TGFα (200 μg/kg) or normal saline (control) was administered i.p. and samples of the gastric contents (0.3–0.5 ml) were obtained 15, 30, and 60 min later. Samples were buffered with PBS pH 7.4, and frozen at −70°C until assayed. Later, samples were thawed, [$^4H_2$]PGE_2 internal standard added, and PGE_2 quantified after extraction and purification using negative ion chemical ionization gas chromatography/mass spectrometry (13). Results are expressed as picograms per milliliter.

Gastric mucosa. The ex vivo generation of PGE_2 was determined according to described methods (14, 15). The animals were killed by cervical dislocation 30 min after i.p. administration of normal saline (control) or TGFα (200 μg/kg). The stomachs were dissected out, opened along the greater curvature, rinsed in saline, and laid flat on absorbent paper to blot excess water. A portion of the corpus mucosa was peeled off, placed in a microfuge tube, weighed (~ 150 mg), and chopped with fine scissors for 1 min in 1 ml of 0.1 M phosphate buffer, pH 7.4. The mixture was centrifuged (Centrifuge 5415 C; Eppendorf Inc., Fremont, CA) at 14,000 rpm for 30 s, the supernatant was discarded, and 0.5 ml of buffer was added. The pellet was dislodged and the tube vortexed for 1 min at room temperature. Indomethacin (INDO) (Sigma Chemical Co.) 50 μg in 25 μl of 1% $NaHCO_3$, was added to stop arachidonate metabolite generation. The mixture was centrifuged at 14,000 rpm for 30 s and the supernatant frozen for PGE_2 determination as described above. Results are expressed as nanograms per gram tissue.

Glutathione assay

Total GSH concentration was measured with the glutathione reductase-5,5′-dithiobis-(2-nitrobenzoic acid) recirculating assay of Tietze (16). In brief, animals were killed by cervical dislocation. The stomach was opened along the greater curvature and the gastric mucosa was scraped and weighed (~ 30 mg). Tissue samples were homogenized in 10% TCA with a homogenizer (Polytron; Brinkmann Instruments, Inc., Westbury, NY). The homogenates were centrifuged at 1,500 *g* for 15 min at 4°C and an aliquot of the supernatant was neutralized with 0.3 M NaH_2PO_4. Neutralized samples were diluted (1:10) with 0.125 M Na_3PO_4, 6.3 mM EDTA, pH 7.5 (stock buffer); thereafter, 150 μl of 0.3 mM NADPH (Boehringer Mannheim Biochemicals, Indianapolis, IN), 100 μl of 6 mM dithiobis-(2-nitrobenzoic acid) (Sigma Chemical Co.), an aliquot of the sample, and stock buffer to give a final vol of 1 ml were added to cuvettes and the reaction was started by adding 10 μl of glutathione reductase (~ 50 U/ml) (Sigma Chemical Co.). The absorbance was monitored at 412 nm. The glutathione content of the aliquot assayed was determined by comparison with known amounts of glutathione (Sigma Chemical Co.). Results are expressed as nanomoles per gram tissue.

Gastric secretion

Under ether anesthesia, the pylorus was ligated and the abdomen was closed. TGFα (1–100 μg/kg) or normal saline was then injected i.p. 1 h after treatment, the animals were killed by cervical dislocation, the esophagus was ligated, and the stomach dissected out. Gastric juice was collected in graduated test tubes, its volume measured to the nearest 0.1 ml, and acid concentration determined by titration with 0.01 N NaOH to pH 7. The values are expressed in milliequivalents per liter (meq/liter) (acid concentration) and microequivalents per hour (μeq/h) (acid output).

Determination of tyrosine phosphorylation of phospholipase C-gamma 1 (PLC-γ1)

The method has been described previously (17). Scraped gastric mucosa was ground in a Polytron homogenizer (Brinkman Instruments, Inc.) on ice in hypotonic buffer (20 mM Hepes, pH 7.4, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, and 1 μg/ml aprotinin, pepstatin, and leupeptin), centrifuged at 1,000 *g* for 10 min at 4°C, and the pellet discarded. A membrane fraction was obtained by centrifuging the supernatant at 100,000 *g* for 30 min at 4°C. The particulate membrane fraction was solubilized in a buffer containing 1% Triton X-100, 50 mM Hepes, pH 7.5, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na orthovanadate, 1 mM PMSF, and 1 μg/ml aprotinin, pepstatin, and leupeptin. The supernatant cytosolic fractions from the ultracentrifugation were concentrated by lyophilization, reconstituted in the same buffer, and stored at −80°C until further use. For Western blots, membrane and cytosolic fractions (170 μg) from gastric mucosa were subjected to 7% SDS-PAGE, transferred to nitrocellulose, and then incubated with a 1:500 dilution of the PLC-γ1 antiserum followed by ^{125}I-donkey anti-rabbit IgG (~ 200,000 cpm/ml; Amersham Corp., Arlington Heights, IL). The immunodetected PLC-γ1 bands were visualized by autoradiography. For phosphotyrosine immunoprecipitation, a known amount of cytosolic protein (2 mg) from tissue extracts in a buffer containing phosphatase and protease inhibitors (see above), was absorbed onto 200 μl of a Sepharose-linked antiphosphotyrosine (monoclonal 1G2) bead matrix (18) for 2–4 h at 4°C with rocking as described (19). After washing, the specifically absorbed phosphotyrosine proteins were eluted with 20 mM phenylphosphate, electrophoresed, and subjected to PLC-γ1 immunoblot. To assess the specificity of the antiphosphotyrosine matrix, the tissue cytosols were immunoprecipitated with the antiphosphotyrosine matrix in the presence of excess phenylphosphate or phosphotyrosine before elution and PLC-γ1 immunoblot. Protein was determined using the method described by Bradford (20).

Determination of ornithine decarboxylase (ODC) activity

Rat gastric mucosa was scraped away from the underlying smooth muscle with a glass slide. The tissues so obtained were then assayed for ODC activity and total protein. ODC assay was as described by Pegg and McGill (21). Tissues were placed in homogenization buffer (50 mM Tris-HCl, 100 μM EDTA, 2.5 mM DTT, and 50 μM pyridoxal phosphate), homogenized, sonicated, and centrifuged at 30,000 *g* at 4°C for 20 min. A 400-μl aliquot of the supernatant was then incubated for 60 min at 37°C with homogenization buffer supplemented with 0.2 mM L-ornithine and 0.2 mM pyridoxal phosphate and with L-[^{14}C]ornithine (0.25 μCi). The liberated $^{14}CO_2$ from the decarboxylation of ornithine was trapped on a piece of filter paper impregnated with 100 μl of hyamine hydroxide, which was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of 0.1 ml of 30% TCA; after an additional 30–60 min to collect residual radiolabeled CO_2, the filters were added to scintillation fluid and counted in a liquid scintillation counter. Aliquots of the 30,000 *g* supernatant were assayed for total protein, using the method described by Bradford (20). Results are expressed as picomoles $^{14}CO_2$ released per hour per milligram protein (pmol/h per mg).

Determination of gastric insoluble mucin

Adherent (insoluble) gastric mucin was gently removed from the gastric mucosa using a glass slide. PBS (1 ml) then was added and samples were stored frozen (−70°C) until assayed. Insoluble mucin was determined by an ELISA as described previously (22). In brief, 100 μl of mucin-containing sample was mixed with 100 μl of gastric mucin antibody in an Eppendorf tube (Brinkmann Instruments, Inc., Westbury, NY) and allowed to bind to equilibrium overnight at 37°C. At the same time, purified gastric mucin was added to each well of a 96-well plate (500 ng/well) and allowed to bind overnight. The next day the 96-well plate was rinsed with PBS three times and 300 μl of 0.5% crystalline grade BSA was added to each well and incubated for 1 h at 37°C. Each well was then washed with a "wash buffer" (PBS containing 0.05% Tween 20) three times and 75 μl of mucin-antibody mixture was added to the 96-well plate and incubated for 1 h at 37°C. Each well was rinsed again with wash buffer (three times) and 100 μl of biotinylated goat-anti-rabbit antibody (at a dilution of 1:2,000 in PBS containing 0.05% Tween 20) was added to each well and incubated for 1 h. Each well was rinsed again three times and incubated with 200 μl of streptavidin-horseradish peroxidase at a concentration of 625 ng/ml in PBS-Tween, pH 6.5 for 1 h. Each well was rinsed five times with wash buffer and color was developed using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (0.01 g/25 ml) in 100 mM sodium citrate buffer, pH 4.2 plus 75 μl of 30% H_2O_2/25 ml buffer (added immediately before use) for 5 min in the dark. The antibody binding was determined by reaching the color developed at 405 nm with a Titertek multiscan plate reader (Flow Laboratories, McLean, VA). Insoluble gastric mucin was expressed as nanograms per milliliter.

Experimental design

The experimental design is described in detail in the figure legends.

Statistical analysis

Results are expressed as mean±SEM. Significance of differences was assessed by Student's *t* test or analysis of variance followed by Duncan multiple range test (23) as appropriate. Differences were considered statistically significant if P was < 0.05. Data which were expressed as percentage of control were analyzed before being normalized versus control.

Results

Effect of TGFα on EtOH- or ASA-induced damage. Orogastric administration of 100% EtOH is a well-characterized animal model that yields a reproducible degree of gastric mucosal injury (24, 25). This experimental model was selected because induction of mucosal injury is independent of luminal acidity (24, 26). As such, this model is able to test the gastric mucosal protective ability of the drug studied. Pretreatment with TGFα (200 μg/kg intraperitoneally [i.p.]) provided nearly complete protection against gross mucosal injury to the rat glandular stomach (Fig. 1). TGFα administered i.p. at doses between 25 and 200 μg/kg dose-dependently decreased gastric mucosal injury induced by EtOH ($r = -0.88$, $P < 0.05$) (Fig. 2), as quantitated by computerized image analysis. TGFα (200 μg/kg) decreased EtOH injury by more than 90% either when administered as a single pretreatment or when administered in four equally divided injections (Fig. 2). Pretreatment with TGFα (25 μg/kg or 50 μg/kg) exerted a higher protective effect than that achieved by the same total dose given in repeated injections before and after EtOH (78 and 91%) protection vs 27 and 63% protection, respectively (Fig. 2).

Unlike EtOH injury, ASA-induced gastric mucosal damage is acid dependent (27), i.e., the higher the acidity, the greater the damage. To standardize the effect of acid inhibition on TGFα protection, we determined a dose of TGFα that did not significantly affect gastric secretion. Also, ASA was suspended in HCl 0.15 N (pH = 1.02). Fig. 3 shows a dose-dependent inhibition of gastric acid secretion by i.p. TGFα ($r = -0.86$, $P < 0.05$). At an i.p. dose of TGFα (10 μg/kg), which did not significantly affect gastric secretion (Fig. 3), there was 84.5% reduction in the damage induced by ASA (200 mg/kg-0.15 N HCl) ($P < 0.001$, Fig. 4). Repeated i.p. administration of TGFα 10 μg/kg (30 min before and 1, 2, and 3 h after ASA) was not more effective than a single TGFα injection in preventing ASA injury (82.8% protection vs 84.5% protection, respectively, Fig. 4).

Kinetic and morphometric studies of TGFα's protective effect. The EtOH-induced damage model was used in more detailed studies of the protective effect of TGFα. Fig. 5 shows the effect of orogastric administration of TGFα on EtOH-induced injury. TGFα (50 μg/kg) given 30 min before EtOH did not afford significant protection (Fig. 5). TGFα (200 mg/kg) decreased EtOH injury by only 40% ($P < 0.05$) (Fig. 5). Thus, in all subsequent studies, TGFα was administered i.p. The time-course of TGFα protection against EtOH injury is shown in Fig. 6. The protective effect was still significant (50% decrease in EtOH injury, $P < 0.01$) 6 h after the administration of TGFα (200 μg/kg) (Fig. 6).

Fig. 7, *A–C*, are representative photomicrographs that display the protective effect of TGFα on EtOH-induced injury. The typical histological appearance of normal gastric mucosa is shown in Fig. 7 *A*. EtOH induced complete disruption of the superficial epithelium and caused necrosis in the upper region of the gastric glands (Fig. 7 *B*). In contrast, the gastric mucosa appeared to be well preserved if rats were pretreated with TGFα (200 μg/kg i.p.) 30 min before EtOH challenge (Fig. 7 *C*). These sections were taken from areas that appeared grossly normal. The extent of disrupted surface epithelium and deep mucosal necrosis was quantified by planar morphometry in a blinded fashion and expressed as percentage of total mucosal strip length (Fig. 8; see Methods). Surface epithelial disruption was partially reduced by TGFα (22.4%±0.7 vs 47.1%±5.2 in controls, $P < 0.01$) (Fig. 8). In addition, pretreatment with TGFα significantly reduced EtOH-induced deep necrosis (2.9%±vs13.2%±1.9 in controls, $P < 0.01$).

Fig. 9 (*A–C*) shows the scanning electron microscopy of normal rat gastric mucosa (Fig. 9 *A*) and of EtOH-injured mucosa from saline-pretreated (Fig. 9 *B*) and TGFα-pretreated rats (Fig. 9 *C*). Once again, samples were taken from areas which appeared normal upon gross examination. In control animals, EtOH caused severe disruption of the surface epithelium that resulted in formation of areas of denuded lamina propria (Fig. 9 *B*). Pretreatment with TGFα (200 μg/kg) partially prevented EtOH injury to the superficial epithelium (Fig. 9 *C*).

Effect of systemic TGFα on tyrosine phosphorylation of PLC-γ1. We considered whether the protection afforded by TGFα was mediated through activation of the TGFα/EGFr in the gastric mucosa. We studied the effect of systemic TGFα on tyrosine phosphorylation of PLC-γ1, a putative substrate for the TGFα/EGFr (28, 29). Cytosolic and membrane fractions of scraped gastric mucosa were examined for PLC-γ1 by Western blot analysis. The cytosolic fractions contained the majority of PLC-γ1 (Fig. 10 *A*) (data related to membrane fractions are not shown). To detect the tyrosine phosphorylation species of PLC-γ1, 2 mg of cytosolic protein was absorbed onto phos-

Figure 1. Macroscopic appearance of rat gastric mucosa after orogastric administration of 100% EtOH in normals saline (*NS*)- and TGFα-pretreated animals. TGFα (200 μg/kg) or normal saline was administered i.p. 30 min before EtOH.

photyrosine-Ab matrix, eluted with phenylphosphate, and PLC-γ1 examined by Western blot analysis. TGFα (200 μg/kg) caused a time-dependent increase in tyrosine phosphorylation of PLC-γ1 in the gastric mucosa (Fig. 10 *B*). At 15 min, there was a twofold increase in tyrosine phosphorylation of PLC-γ1, as assessed by measurement of relative optical density (data not shown). These findings were confirmed in two additional experiments (data not shown). Thus systemic administration of TGFα results in a biochemical event in the gastric mucosa linked to postreceptor signaling that occurs in a time frame consistent with its participation in TGFα-mediated gastric protection.

Role of endogenous prostaglandin in TGFα protection. We next examined possible mechanisms by which TGFα might exert its protective effect. Prostaglandins are considered to play a role in the ability of the gastric mucosa to resist different forms of injury (24). TGFα (200 μg/kg) did not increase PGE_2 release into the gastric juice nor did it increase gastric tissue levels of PGE_2 (Fig. 11 *A*), as assessed by mass spectrometry. Also, TGFα was not able to counteract INDO-induced depletion of PGE_2 in the gastric mucosa (Fig. 11 *B*). To further explore the role of endogenous prostaglandins in TGFα protection, we studied whether pretreatment with INDO (5 mg/kg), a concentration which decreases PGE_2 content of the rat gastric

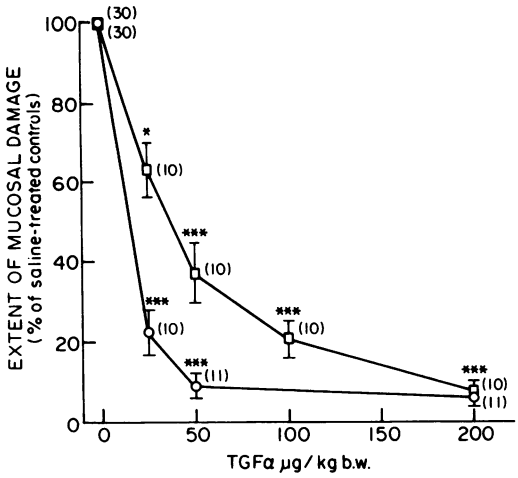

Figure 2. Effect of systemic administration of TGFα on EtOH-induced rat gastric mucosal injury as quantitatively assessed by computerized image analysis. Rats were pretreated with i.p. injection of TGFα (25–200 μg/kg) or normal saline and, after 15 min, were given orogastric 100% EtOH (*circles*). In another series of experiments the total dose of TGFα was divided into four equal doses which were administered i.p. 15 min before and 15, 30, and 45 min after orogastric 100% EtOH (*squares*). Animals were killed 60 min after EtOH. The damaged area in the control groups is 22.9%±2.1 of the total glandular area. Mean±SE of (n) rats per each study group. □, Divided doses; ○, single dose. $^{*}P < 0.05$ vs control; $^{***}P < 0.001$ vs control.

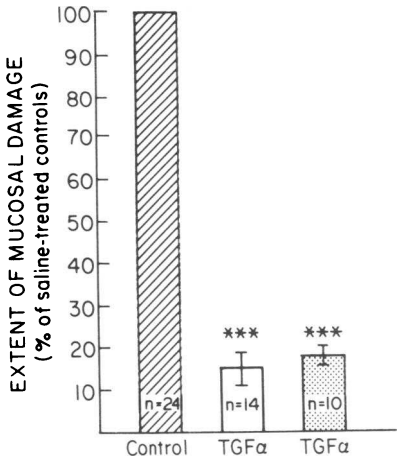

Figure 4. Effect of TGFα on acidified aspirin (ASA)-induced damage to the rat gastric mucosa. Gross mucosal injury was quantitated by computerized image analysis 4 h after ASA (200 mg/kg) in rats pretreated with i.p. injection of TGFα (10 μg/kg) or normal saline 30 min before ASA. In another series of experiments, rats were treated with TGFα (10 μg/kg) or normal saline 30 min before and 1, 2, and 3 h after ASA administration. Mean±SE of (n) rats per each study group. □, TGFα 10 μg/kg body wt; □, TGFα 10 μg/kg body wt × 4; $^{***}P < 0.001$ vs control.

mucosa by > 70% (Fig. 11 *B*), was able to prevent TGFα-induced protection. INDO caused a slight increase in EtOH-induced injury, but this did not reach statistical significance (Fig. 12). Pretreatment with INDO did not abolish the protective effect of TGFα (Fig. 12).

Role of ornithine decarboxylase activity in TGFα protection. Recently, an increase in ODC activity has been shown to be associated with the ability of EGF to protect rat gastric mucosa against stress-induced injury (30). TGFα (100 μg/kg) administered i.p. increased ODC activity in the rat gastric mucosa from 14.7±2.7 to 22.0±4.0 pmol/h per mg. The ODC inhibitor difluoromethylorinthine (DFMO), at a concentration that decreased basal ODC activity by 65% ($P < 0.01$) and counteracted TGFα-stimulated increase in ODC activity (51% inhibition, $P < 0.01$), did not prevent the protective effect of TGFα (Fig. 13). Thus TGFα-induced gastric mucosal protection appears to be mediated by nonprostaglandin and non-ODC related signaling pathways.

Role of endogenous sulfhydryls in TGFα protection. Sulfhydryl compounds also have been demonstrated to be involved in the protection of the gastric mucosa against various forms of injury (31–33). TGFα did not stimulate glutathione synthesis in the stomach (Fig. 14 *A*). To evaluate further the effect of TGFα on glutathione metabolism, we evaluated whether TGFα prevented the depletion of gastric glutathione induced by phorone, an electrophilic agent which acts through glutathione-*S*-transferases (34). Phorone (50–250 mg/kg) decreased gastric glutathione tissue levels in a dose-dependent manner ($r = -0.97$, $P < 0.05$), (data not shown). Pretreatment with TGFα (200 μg/kg) did not prevent the glutathione-depleting effect of phorone (125 mg/kg) (Fig. 14 *B*).

To assess further the role of endogenous sulfhydryl compounds in TGFα-mediated protection, we studied whether the sulfhydryl group alkylator *N*-ethylmaleimide (NEM) counteracted TGFα's protective effect. Fig. 15 shows that NEM (10 mg/kg), a concentration which did not significantly increase EtOH injury to the gastric mucosa, decreased the protective effect of TGFα by 40%. NEM (20 mg/kg) abrogated the protective effect of TGFα. However, NEM at this concentration

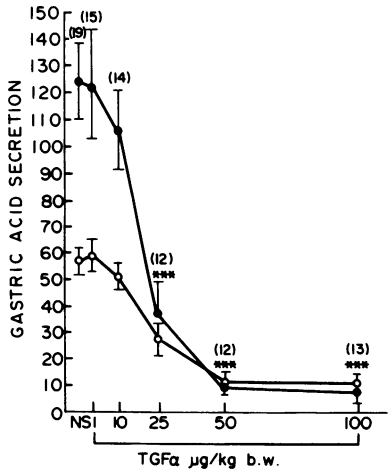

Figure 3. Effect of TGFα on gastric acid secretion. Gastric acid secretion was measured in pylorus-ligated rats 1 h after i.p. administration of TGFα (1–100 μg/kg) or normal saline. Mean±SE of (n) rats per each study group. ●, Acid output (μeq/h); ○, acid concentration (meq/liter); $^{***}P < 0.001$ vs NS.

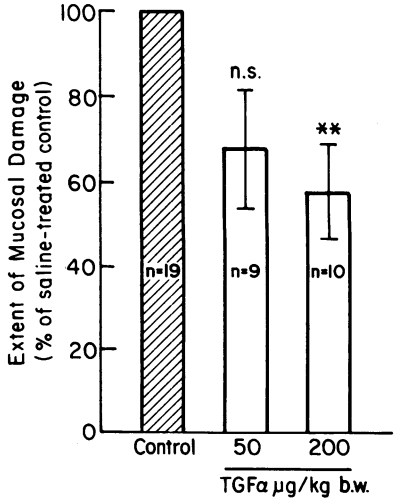

Figure 5. Effect of orogastric administration of TGFα on EtOH-induced injury. TGFα (50 and 200 μg/kg) or normal saline was administered orogastrically 30 min before EtOH. The damaged area in the control group is 17.0%±4.0 of the total glandular area. Mean±SE of (n) rats per each study group. n.s., not significant vs control; $^{**}P < 0.01$ vs control.

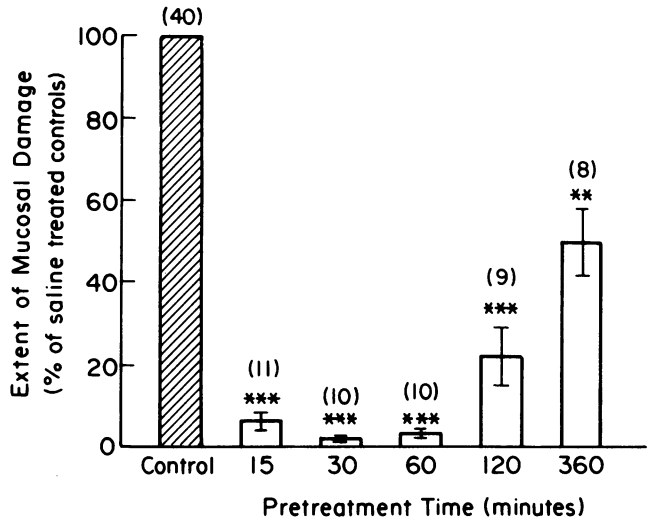

Figure 6. Time sequence of TGFα protection against EtOH injury. TGFα (200 μg/kg) was administered i.p. 15 min to 6 h before EtOH administration. The damaged area in the control group is 25.9%±4.0 of the total glandular area. Mean±SE of (*n*) rats per each study group. $^{**}P < 0.001$ vs control.

significantly affected EtOH-induced mucosal injury by increasing the damage to the glandular area from 20.4%±2.1 to 34.7%±6.4 ($P < 0.05$) (Fig. 15). NEM (10 and 20 mg/kg) did not affect glutathione tissue levels (data not shown).

Effect of TGFα on gastric insoluble (adherent) mucin. We also examined the effect of TGFα on levels of gastric mucin. In these studies, the gastric mucosa was scraped lightly with a glass slide at the indicated times, and mucin levels determined by a reverse ELISA with an antibody that recognizes biologically active, insoluble gastric mucin (22). The gastric mucosa was intact microscopically after light scraping of both TGFα and normal saline-treated rats (data not shown). TGFα (100 μg/kg) time-dependently increased gastric insoluble mucin (Fig. 16 *A*). At 15 and 30 min from TGFα administration, there was a 7.3-fold ($P < 0.001$ vs control) and 14.5-fold ($P < 0.001$ vs control) increase in adherent mucus, respectively (Fig. 16 *A*). TGFα (1–100 μg/kg) increased gastric adherent mucin in a dose-dependent manner ($r = 0.987$, $P < 0.05$) (Fig. 16 *B*).

Discussion

EGF protects gastric mucosa against damage induced by ASA (1, 2), cysteamine (3), EtOH (4, 35–37), and stress (30). EGF is localized mainly in submandibular salivary glands and in Brunner's glands (38, 39). Induction of a novel EGF-secreting cell lineage has been shown adjacent to ulcerated human gastrointestinal mucosa (40); however, it is controversial whether EGF is expressed in the normal gastric mucosa (11, 38, 39, 41–43). TGFα is a 50-amino acid polypeptide (44) which shares with EGF structural homology (5, 6), a common receptor (7–9) and a nearly identical spectrum of biological activity (10). In particular, both EGF and TGFα stimulate proliferation of gastric epithelial cells in vitro (45) and inhibit gastric acid secretion in vitro (46–48) and in vivo (49, 50). Unlike EGF, TGFα has been demonstrated to be expressed in the normal gastric mucosa of different species, including man and rat (11, 43, 51). Also, immunoreactive TGFα is present in the normal human stomach in significantly higher levels than immunoreactive EGF (43). Recently, TGFα mRNA and protein have been shown to increase after acute gastric injury in the rat (12), thus suggesting a role for TGFα in gastric mucosal repair. Whether TGFα is able to prevent different forms of injury to the gastric mucosa has not been studied. Therefore, we evaluated the effect of TGFα on EtOH- or ASA-induced damage to the rat gastric mucosa.

Systemic administration of TGFα prevented EtOH injury in a dose-dependent manner and decreased EtOH-induced gross mucosal damage by > 90% (Fig. 2). A single pretreatment with systemic TGFα was as effective as repeated administration (Fig. 2). At the microscopic level, pretreatment with TGFα prevented by > 75% deep gastric mucosal necrosis and reduced by > 50% disruption of surface epithelium induced by EtOH (Fig. 8). We also evaluated whether orogastric TGFα exerted any protective effect against EtOH injury. TGFα (50 μg/kg), a dose that affords a > 90% protection when administered systemically, did not significantly prevent EtOH injury. At an oral dose of 200 μg/kg, TGFα provided only 40% protection to the rat gastric mucosa against EtOH-induced damage (Fig. 5). It remains controversial whether the TGFα/EGFr, which has been demonstrated to be located on the basolateral membrane of target cells, is also expressed on the cell surface (52–54). Systemic EGF inhibits gastric acid secretion in rats while orogastric EGF does not (55). Likewise, serosal, but not luminal EGF, inhibits acid secretion from guinea pig gastric mucosa mounted in Ussing chambers (46). Moreover, it is well known that acidic pH impairs the binding of TGFα to its receptor (8, 56). Therefore, we speculate that the partial protection obtained with orogastric administration of TGFα reflects a systemic effect secondary to the absorption of TGFα with subsequent delivery to TGFα/EGFr located on the basolateral membrane.

ASA-induced gastric mucosal damage is dependent on the pH of gastric contents (the more acid, the more damage) (27), whereas EtOH injury is not (24, 26). Therefore, to rule out the influence of gastric acid inhibition on protection, we used a concentration of TGFα (10 μg/kg) that did not significantly affect gastric secretion (Fig. 3). Moreover, ASA was suspended in 0.15 N HCl (pH = 1.02). TGFα (10 μg/kg) decreased acidified ASA-induced damage by > 80% (Fig. 4). Repeated administration of TGFα (10 μg/kg) (30 min before and 1, 2, and 3 h after ASA) did not show any significant improvement in gastric mucosal protection as compared to a single injection of TGFα (10 μg/kg) 30 min before ASA administration. Therefore, TGFα is able to protect the gastric mucosa from a necrotizing agent such as EtOH whose damaging effect is acid independent and, in a non-antisecretory dose, from ASA-induced injury that is acid-dependent. Thus, TGFα may be considered a true gastric mucosal protective agent.

We did not directly compare the gastric mucosal protective ability of TGFα and EGF. However, previous studies have shown that mouse EGF (100 μg/kg) given orogastrically or parenterally only partly reduces the mucosal damage caused by absolute EtOH (35, 37). Also, recombinant human EGF (30 μg/kg) has recently been shown not to exert any protective effect when given 6 h before 50% EtOH (4). On the contrary, in our study, a 50% protective effect against EtOH injury was still seen 6 h after i.p. administration of TGFα (Fig. 6). These previous studies, if corroborated, suggest that TGFα, even though acting through the same receptor as EGF, seems more efficient that EGF in gastric mucosal protection. This is not altogether

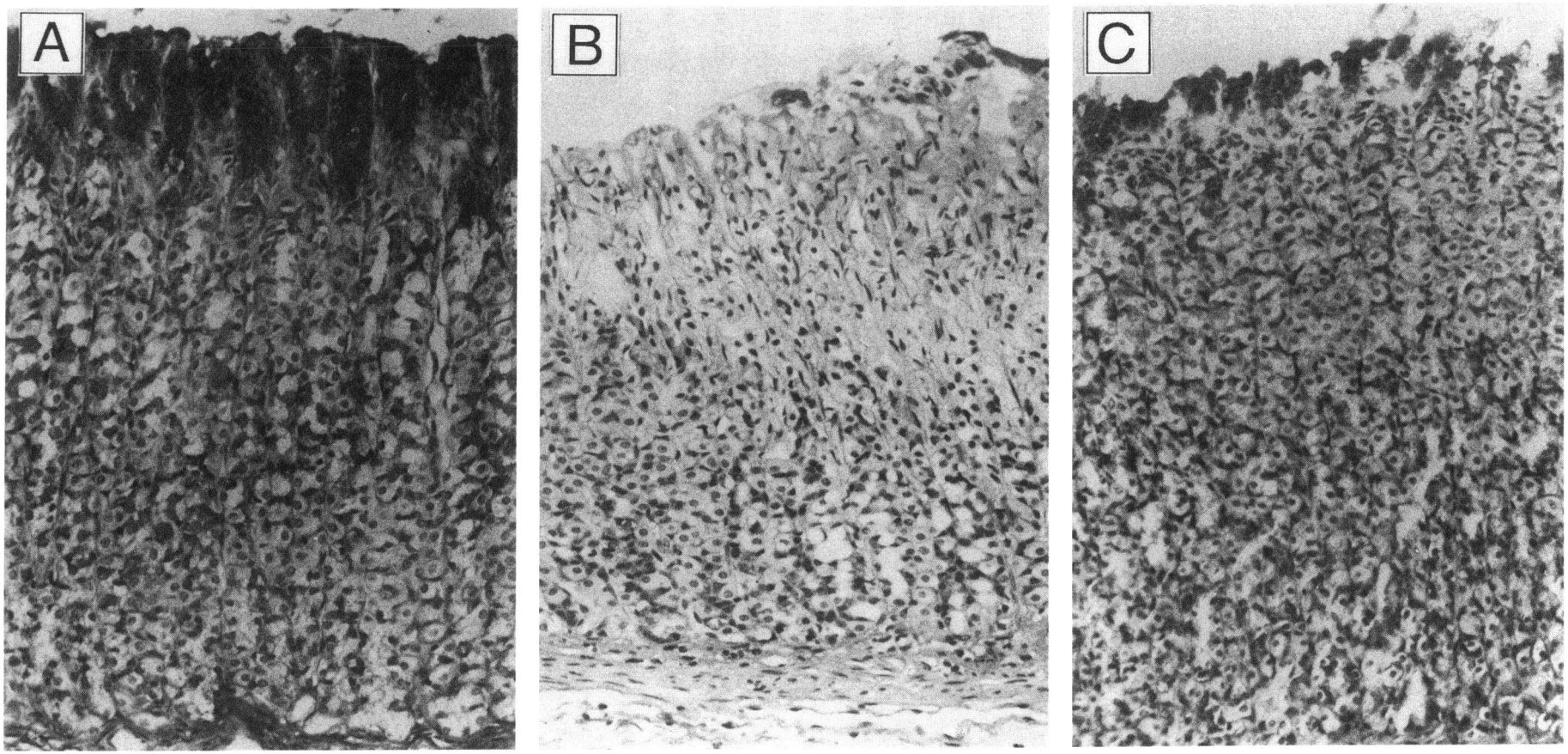

Figure 7. (*A–C*) Histological effect of TGFα on EtOH injury. *A* represents normal rat gastric mucosa. EtOH induced disruption of the superficial epithelium and caused deep necrotic damage (*B*). Pretreatment with TGFα (200 μg/kg) partially preserved surface cells from EtOH injury and prevented deep glandular necrosis (*C*). Specimens were taken from areas which appeared normal upon gross examination. Sections were stained with hematoxylin and eosin and periodic acid-Schiff. ×66.

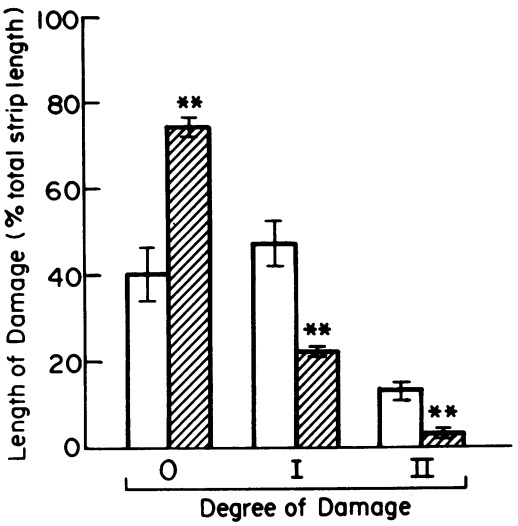

Figure 8. Quantitation of deep gastric mucosal necrosis and disruption of gastric surface epithelium in TGFα-pretreated and control rats after EtOH administration. Rats were given 100% EtOH 30 min after i.p. administration of TGFα (200 μg/kg) or normal saline (control). Results are expressed as percentage of total mucosal strip length and represent the mean±SE of six rats per each study group. 0, no damage; I, disruption of surface epithelium; II, deep necrosis (> 0.2 mm). Specimens were from macroscopically noninjured gastric mucosa. Total mucosal strip length was 9.5±0.4 mm in controls and 9.7±0.8 mm in TGFα-pretreated animals. □, Normal saline; ▩, TGFα 200 μg/kg body wt; $^{**}P < 0.01$ vs normal saline.

surprising, since several quantitative differences have been observed in the biological activity of TGFα and EGF (10). For example, TGFα has been shown to be more potent than EGF in inducing angiogenesis in hamster cheek pouches (58). Also, TGFα is more effective than EGF in increasing regional blood flow in the anesthetized dog (59) and in stimulating osteoclast precursor cells (60). These quantitative differences may, in part, be explained by differential processing of the ligand-receptor complexes (61). Thus far, no distinct TGFα receptor has been identified. However, to examine the comparative protective effects of TGFα and EGF more rigorously, we are presently comparing the effect of recombinant human forms of TGFα and EGF on EtOH- and ASA-induced damage to the rat gastric mucosa.

Levels of endogenous rat TGFα protein have been measured by a sensitive and specific rat RIA in the gastric mucosa and juice as well as in the plasma (14.4±4.8 pg/μg DNA, 0.3±0.1 total ng, 88 pg/ml, respectively) (Dempsey, P. J., and R. J. Coffey, unpublished observations). 15 min after i.p. administration of TGFα (50 μg/kg), plasma levels of human TGFα were 12±2.0 ng/ml, as determined by a human TGFα RIA that does not recognize rat TGFα (Dempsey, P. J., and R. J. Coffey, unpublished observations). Therefore, the doses used in this study are pharmacological ones. This must, however, be tempered by the realization that the effective concentration of the endogenously produced TGFα at the cell surface might be far greater than that measured in the gastric tissues or plasma.

The biological effects of TGFα are mediated through binding to the TGFα/EGFr (62). Accumulating evidence indicates that PLC-γ1 is a substrate for the tyrosine kinase of the TGFα/EGFr (28, 29). The present study demonstrates for the first time in vivo in nonneoplastic tissues that systemic administration of TGFα results in a time-dependent increase in tyrosine phosphorylated PLC-γ1 (Fig. 10). Additional biological relevance of this finding derives from the observation by Konda et al. in which prostaglandin protection of isolated guinea pig chief cells against EtOH has been shown to be via an increase in diacylglycerol (63), which is derived from the PLC-γ1–induced breakdown of the phosphoinositide (64), thus implicating activation of PLC-γ1 as a mediator of gastric protection.

Prostaglandins are known to play in important role in the protection of gastric mucosa (24). Therefore, we studied whether TGFα protection might be mediated by endogenous prostaglandins. TGFα, at a protective concentration, did not stimulate PGE_2 production by the rat gastric mucosa nor did it counteract the INDO-induced depletion in gastric PGE_2 (Fig. 11). Furthermore, TGFα protection was not prevented by pretreatment with INDO in a concentration which decreased gastric tissue levels by > 70% PGE_2 (Fig. 12). Therefore, TGFα, like EGF (1, 2), does not seem to protect gastric mucosa through stimulation of endogenous prostanoid synthesis. A PGE_1 analogue (i.e., misoprostol) recently has been demonstrated to be effective in preventing ASA-induced injury to the human gastric mucosa (65) and approved by the FDA specifically for use in the prevention of nonsteroidal antiinflammatory drug–induced gastric mucosal damage. The observation that TGFα, at a non-antisecretory dose, protects the gastric mucosa against ASA-induced damage and appears to act independently of prostaglandins, leads us to suggest that TGFα might prove of use, alone or in combination with a prostaglandin derivative, to attenuate gastric injury induced by nonsteroidal antiinflammatory drugs (66).

Recently, protection by EGF against stress-induced gastric lesions in the rat has been shown to be mediated in part by an increase in the activity of ODC (30), the rate-limiting enzyme in the biosynthesis of polyamines (67), which also play a role in gastric mucosal protection (68). Pretreatment with TGFα caused a 1.5-fold increase in ODC activity in the gastric mucosa. However, DFMO, a specific and irreversible inhibitor of ODC (69), at a concentration which significantly inhibited basal as well as TGFα-stimulated ODC activity, did not counteract the protective effect of TGFα (Fig. 13). Therefore, it is unlikely that the protective effect of TGFα is mediated through an ODC-related pathway.

Sulfhydryl compounds protect gastric mucosa against damage induced by different ulcerogens in vivo (31–33) and in vitro (70). In particular, glutathione, the most abundant thiol in cells (34), has been shown to play an important role in gastric mucosal protection (71–73), even though this has been questioned by other authors (74). Our study indicates that TGFα does not stimulate glutathione synthesis in the rat gastric mucosa nor does it prevent the depletion of glutathione gastric tissue levels induced by phorone (Fig. 14). However, pretreatment with the sulfhydryl group alkylator NEM, at a concentration which did not increase EtOH injury, partially (40%) prevented TGFα-induced protection (Fig. 15). NEM, at a concentration that significantly increased EtOH injury, almost completely (76%) abolished the protective effect of TGFα (Fig. 15). The inhibition of the protective effect of TGFα by NEM does not seem to be accounted for by interference with the binding of TGFα to its receptor. In fact, NEM up to 5 μM did not interfere with TGFα binding to the TGFα/EGFr in A431 cells (Romano, M., and R. J. Coffey, unpub-

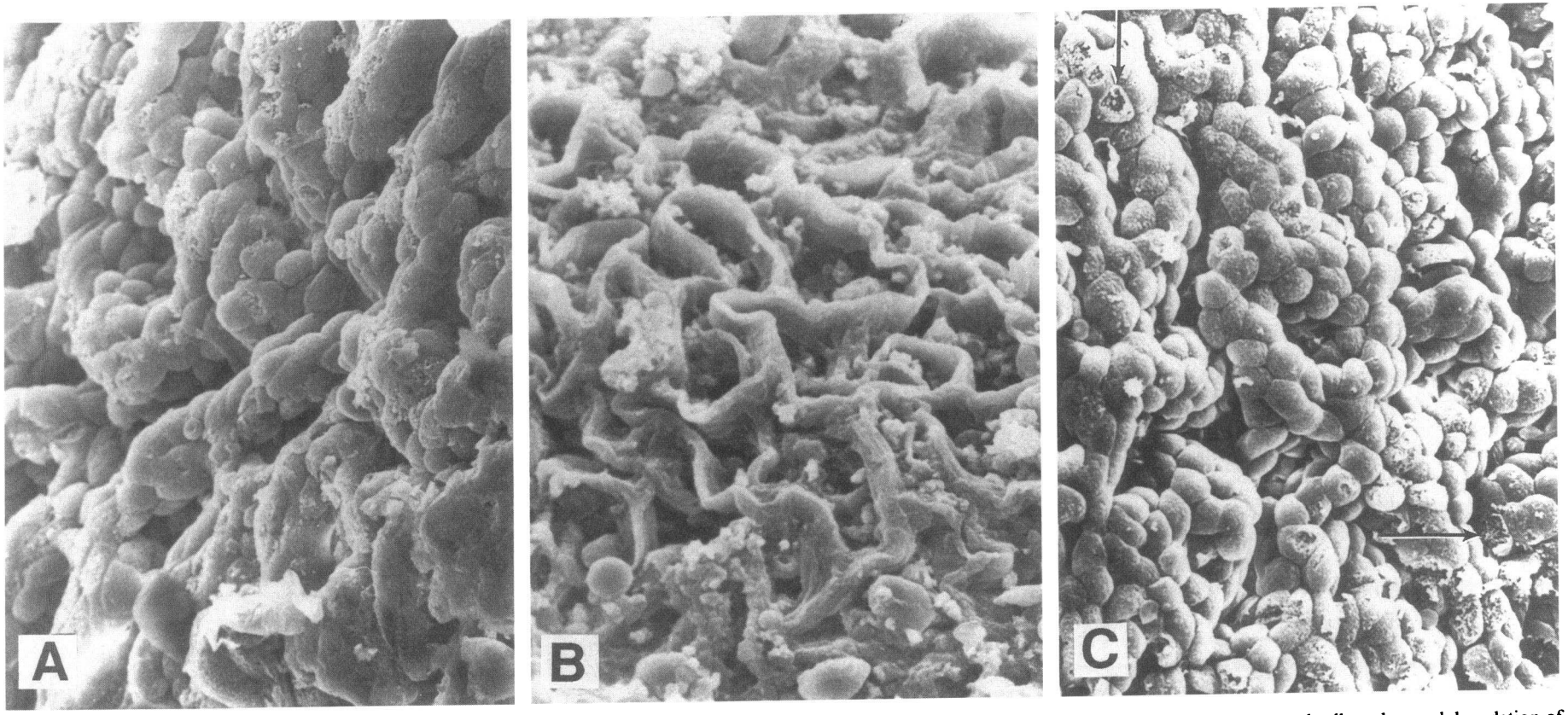

Figure 9. (*A–C*) Effect of TGFα on EtOH injury by scanning electron microscopy. *A* represents normal gastric mucosa. EtOH completely disrupted surface mucosal cells and caused denudation of lamina propria in normal saline-treated animals. (*B*) Pretreatment with systemic TGFα (200 μg/kg) partially prevented damage to superficial epithelium. (*C*) The arrows indicate cells with some degree of damage. Specimens were taken from normally appearing areas located between hemorrhagic bands. ×800.

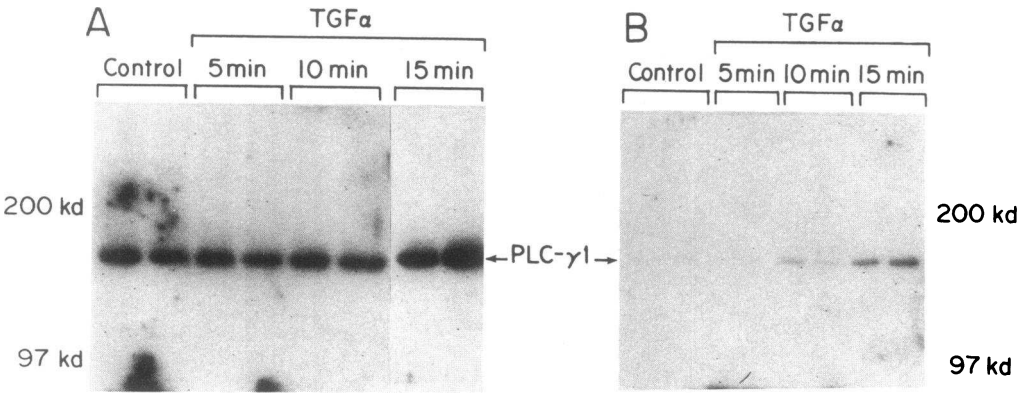

Figure 10. Effect of systemic TGFα on tyrosine phosphorylation of PLC-γl. Tyrosine phosphorylation of PLC-γl was determined in the scraped gastric mucosa 5, 10, and 15 min after i.p. administration of TGFα (200 μg/kg) or normal saline (control) by immunoprecipitation with an antiphosphotyrosine antibody followed by Western blot analysis using an antibody to PLC-γl. (*A*) Total cytoplasmic PLC-γl; (*B*) cytoplasmic PLC-γl after immunoprecipitation of 2 mg of cytosolwith an antiphosphotyrosine antibody.

lished observation). Moreover, NEM, at the concentrations that partially reversed TGFα's protection, did not affect glutathione tissue levels. Therefore, TGFα's protection may be mediated partly by nonprotein sulfhydryls other than glutathione or through a protein-bound, sulfhydryl-sensitive metabolic pathway (75). However, the possibility exists that the reversal of TGFα protection by NEM is caused by an increase in the microvascular permeability which renders the gastric mucosa more vulnerable to the damaging effect of EtOH (76).

In the attempt to elucidate further the mechanism of TGFα protection, we studied the effect of TGFα on insoluble (adherent) gastric mucin. TGFα dose- and time-dependently stimulated adherent gastric mucus (Fig. 16). At 15 min from TGFα administration, there was a sevenfold increase in insoluble mucin, which is consistent with the time frame of TGFα-induced mucosal protection. However, the role of gastric mucus as a protective barrier on the gastric mucosa is controversial (77–79). Adherent mucus is in fact permeable to damaging agents such as EtOH and ASA (80) which gain access through the gel to the superficial epithelial cells. On the other hand, removal of the gelatinous layer of mucus and cellular debris which formed after exposure of the gastric mucosa to 70% EtOH inhibited the protection against a rechallenge with the same necrotizing agent (81). We postulate that the TGFα-induced increase in the adherent mucus gel layer covering the epithelial surface

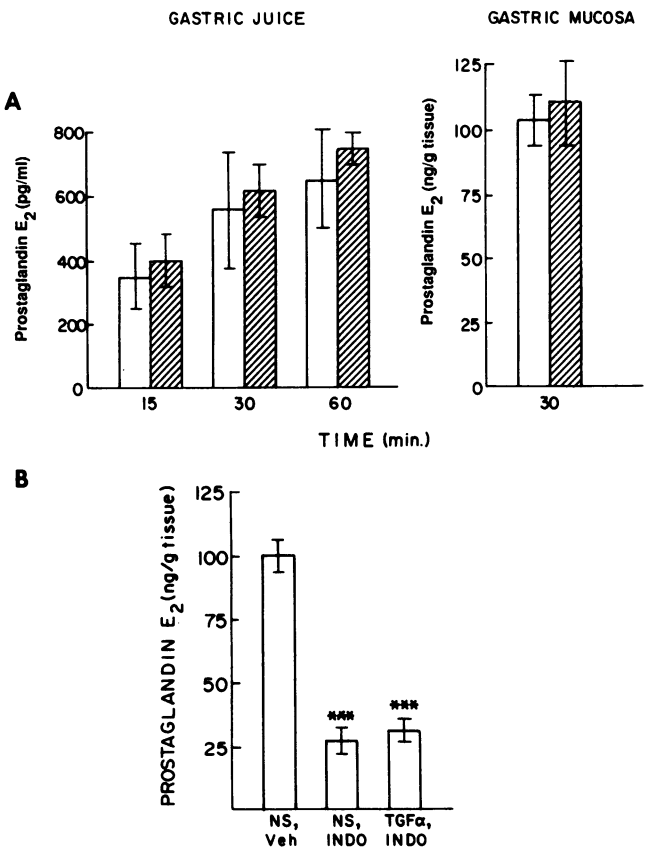

Figure 11. Effect of TGFα on prostaglandin E_2 metabolism in the rat gastric mucosa. (*A*) PGE_2 was measured in the gastric juice and in the gastric mucosa at different time intervals after i.p. administration of TGFα (200 μg/kg) or normal saline. Mean±SE of four animals per each study group. □, Control (normal saline); ■, TGFα 200 μg/kg body wt. (*B*) 30 min after i.p. injection of TGFα (200 μg/kg) or normal saline, rats were given INDO (5 mg/kg) or 5% $NaHCO_3$ (vehicle for INDO) subcutaneously and were killed 1 h later. PGE_2 was measured in the gastric mucosa. Mean±SE of (*n*) animals per each study group. ***$P < 0.001$ vs NS, Veh.

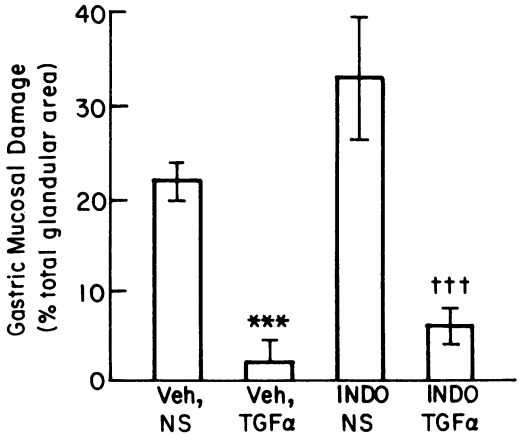

Figure 12. Effect of INDO on TGFα-induced protection against EtOH injury. Rats were pretreated with INDO (5 mg/kg) or 5% $NaHCO_3$ subcutaneously; after 1 h, rats were given TGFα (200 μg/kg) or normal saline i.p. and, 30 min later, both groups received orogastric 100% EtOH. Mean±SE of eight rats per each study group. ***$P < 0.001$ vs Veh, NS; †††$P < 0.001$ vs INDO, NS.

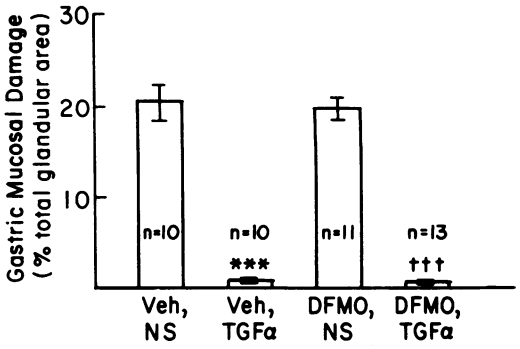

Figure 13. Effect of DFMO on TGFα-induced protection. Rats were pretreated with DFMO (400 mg/kg) or dH_2O (vehicle for DFMO) i.p.; after 4 h rats were given i.p. injection of TGFα (200 μg/kg) or normal saline and, 30 min later, orogastric 100% EtOH. Mean±SE of (n) rats per each study group. ***$P < 0.001$ vs Veh, NS; †††$P < 0.001$ vs DFMO, NS.

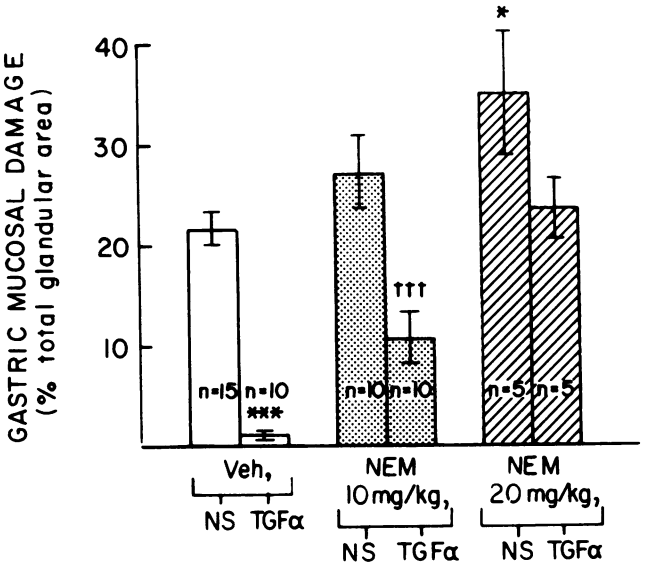

Figure 15. Effect of NEM on TGFα-induced protection against EtOH injury. Rats were pretreated with NEM (10 or 20 mg/kg) or distilled water (vehicle for NEM) subcutaneously; after 10 min, rats were given i.p. injection of TGFα (200 μg/kg) or normal saline and then, after 30 min, both groups received orogastric 100% EtOH. Mean±SE of (n) rats per each study group. Veh, TGFα vs NEM 10 mg/kg, TGFα: $P < 0.01$; Veh, TGFα vs NEM 20 mg/kg, TGFα: $P < 0.01$; NEM 10 mg/kg, TGFα vs NEM 20 mg/kg, TGFα: $P < 0.01$. *$P < 0.05$ vs Veh, NS; ***$P < 0.001$ vs Veh, NS; †††$P < 0.001$ vs NEM 10 mg/kg, NS.

may act as a dilutional barrier to damaging agents, may delay and/or restrict further damage induced by acid and pepsin, and may accelerate early reparative events. An alternate mechanism by which mucin might protect the gastric mucosa is through its ability to scavenge toxic oxygen metabolites (82) which are generated by EtOH and ASA (83). The rapid increase in mucin levels is likely caused by release of preformed mucin. Studies are underway to examine the effect of TGFα on rat gastric mucin mRNA expression and protein production.

In conclusion: (a) systemic TGFα protects the rat gastric mucosa against EtOH-induced microscopic and macroscopic injury; (b) this protective effect is seen also with ASA-induced gastric injury at non-antisecretory concentrations of TGFα; (c) this protection does not seem to be mediated by prostaglandin,

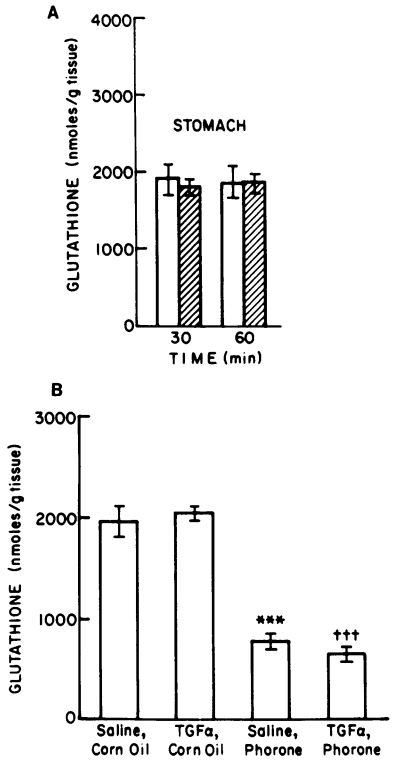

Figure 14. Effect of TGFα on gastric glutathione metabolism. (A) Glutathione was measured in the scraped gastric mucosa 30 and 60 min after i.p. administration of TGFα (200 μg/kg) or normal saline. Mean±SE of four rats per each study group. □, Control (normal saline); ▨, TGFα 200 μg/kg body wt. (B) Rats were pretreated with i.p. injection of TGFα (200 μg/kg) or normal saline; after 30 min rats were given phorone (125 mg/kg) or corn oil i.p. and were killed 1 h later. Glutathione was measured in the scraped gastric mucosa. Mean±SE of four rats per each study group. ***$P < 0.001$ vs saline, corn oil; †††$P <$ 0.001 vs TGFα, corn oil.

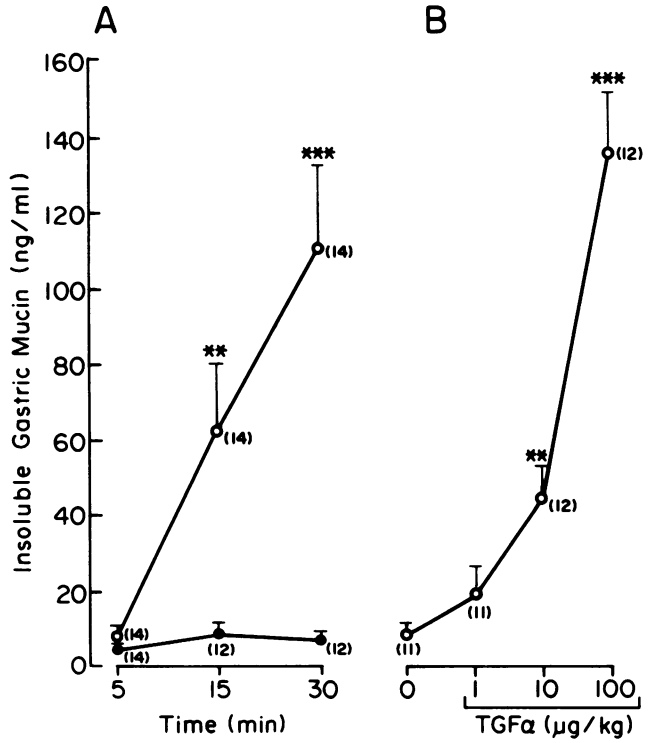

Figure 16. Effect of TGFα on gastric insoluble mucin. Insoluble gastric mucin was measured from lightly scraped gastric mucosa 5, 15, and 30 min after i.p. injection of TGFα (100 μg/kg) or normal saline (control) (A) or 30 min after i.p. injection of TGFα (1–100 μg/kg) or normal saline (control) (B). Mean±SE of (n) rats per each study group; **$P < 0.01$; ***$P < 0.001$ vs normal saline control. ●, Normal saline; ○, TGFα 100 μg/kg.

glutathione, or ODC-related events; (*d*) the protective effect of TGFα is temporally associated with activation of PLC-γ1 and with a significant increase in adherent gastric mucin.

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