Activation of VEGF and Ras genes in gastric mucosa during angiogenic response to ethanol injury

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Activation of VEGF and Ras genes in gastric mucosa during angiogenic response to ethanol injury. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G1345-G1355, 1999.—Our previous studies demonstrated that ethanol injury triggers the angiogenic response in gastric mucosa bordering necrosis. The present study was aimed to determine whether vascular endothelial growth factor (VEGF) (a potent angiogenic peptide selectively acting on endothelial cells) and Ras (a mediator of cell proliferation and a putative regulator of VEGF expression) are involved in gastric angiogenesis after ethanol injury. We studied the angiogenic response and expression of VEGF and Ras in gastric mucosa after ethanol injury. Ethanol damage triggered 1) angiogenesis in the gastric mucosa bordering necrosis, 2) significant increases in VEGF mRNA and protein expression, and 3) significant increases in the expression of Ki-ras mRNA and Ras proteins. Neutralizing anti-VEGF antibody significantly reduced (by greater than threefold) the angiogenic response to ethanol-induced injury. Moreover, mevastatin, an inhibitor of Ras activation, completely blocked the induction of VEGF expression in cultured primary endothelial cells. Because, in other tissues, VEGF is one of the most potent angiogenic factors and VEGF expression is dependent on Ras, our data indicate that Ras and VEGF are involved in gastric mucosal angiogenesis after ethanol injury.

Ethanol; oncogenes; neutralizing antibody; competitive reverse transcriptase-polymerase chain reaction; inhibitor; vascular endothelial growth factor

BLOOD FLOW THROUGH MICROVESSELS, i.e., capillaries, arterioles, and collecting venules, is essential for supplying the gastric mucosa with oxygen and nutrients as well as for the removal of toxic metabolic products (16). Endothelial cells lining gastric mucosal microvessels are major and early targets of acute injury by ethanol (34, 37, 41). Injury of the microvascular endothelial cells by ethanol leads to the microvascular stasis and ischemia that result in focal deep necroses, e.g., mucosal erosions (23, 34, 37, 38, 41). The repair of such injury requires not only restoration of the surface epithelium, glandular epithelial cells, and connective tissue but also, most importantly, a reestablishment of the microvascular network, crucial for delivery of oxygen and nutrients to the area. Although our knowledge regarding the mechanisms of gastric mucosal injury and restitution of the surface epithelium has advanced rapidly in recent years (3, 19, 26, 33, 38), repair of the microvascular network has not been explored except in our previous experiments. These studies demonstrated that angiogenesis, i.e., formation of new microvessels, occurs in the gastric mucosa acutely injured by ethanol. We have characterized some morphological features of this process (36, 39, 42).

The angiogenic response involved in wound repair results from the stimulation of endothelial cells by growth factors [including acidic (aFGF) and basic fibroblast growth factor (bFGF)] to migrate, proliferate, and form the endothelial tubes and capillary structures that undergo transformation into capillary vessels and lead to the restoration of the microvascular network (1, 13, 21). The importance of bFGF in promoting dermal wound healing has been established (4). We have previously demonstrated, in a preliminary study, that ethanol-induced injury triggers the angiogenic response in the gastric mucosa bordering necrosis and also activates expression of bFGF and its receptors in this area (36).

Another growth/permeability factor, vascular endothelial growth factor (VEGF), which is involved in wound healing (6), has been shown to stimulate normal angiogenesis as well as the angiogenesis that underlies tumor metastasis (14, 18, 30). VEGF is the only growth factor that acts predominantly on endothelial cells upon binding to specific receptors Flt-1 and Flik-1 (KDR) (9, 12, 24).

Several studies have shown that VEGF expression is upregulated by oncogenic Ras in transfected cell lines (such as NIH/3T3 fibroblasts and lines derived from epidermal keratinocytes and intestinal epithelial cells), suggesting a role for Ras in both VEGF regulation and angiogenesis in these models (20, 22, 25). However, the expression and roles of VEGF and Ras have not been extensively studied in the repair of acute gastric mucosal injury and, to our knowledge, have never been investigated with regard to ethanol-induced gastric mucosal injury. Therefore, the aim of this study was to determine whether acute gastric mucosal injury by ethanol triggers the expression of VEGF and Ras at both the transcriptional and translational levels and to establish their relationship to the angiogenic response in the rat gastric mucosa after ethanol-induced injury.

MATERIALS AND METHODS

This study was approved by the Subcommittee for Animal Studies of the Long Beach Department of Veterans Affairs Medical Center (Long Beach, CA). Sprague-Dawley rats (weight range 250–300 g) were used in the experiments. Fifty-four rats fasted for 24 h received, intragastrically, either...
1.5 ml of the indicated concentrations of ethanol (25, 50, or 100%) or 1.5 ml H2O (controls). At 3, 6, and 24 h after ethanol administration, rats were anesthetized, their stomachs were excised, and the animals were then euthanized. The stomachs were then opened along the greater curvature, rinsed with 0.9% NaCl, and examined visually. The macroscopically visible hemorrhagic mucosal erosions were photographed in a standardized manner and evaluated by image analysis as described earlier (38). The area of hemorrhagic erosion was expressed as a percentage of total glandular area (38). Gastric tissue (1.5–2 mm in width) from nonhemorrhagic areas immediately adjacent to the necrotic lesions was excised and either immediately frozen in liquid nitrogen and stored at −80°C for RT-PCR and immunoblotting or fixed in 4% paraformaldehyde for immunohistochemistry.

RNA isolation and RT-PCR. Frozen tissue specimens were homogenized with a Polytron homogenizer (Kinematica, Littau, Switzerland) in 4 M guanidinium isothiocyanate, and total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform method (8). RT was carried out using a GeneAmp RNA PCR kit and a DNA thermal cycler (Perkin-Elmer, Norwalk, CT), which were also used for PCR. An amount of 0.3 µg of total RNA was used as the template to synthesize cDNA with 2.5 units of Moloney murine leukemia virus RT in 10 µl of buffer containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 5 mM random hexamer, and 1.4 U/µl of ribonuclease inhibitor. RT was performed at room temperature for 20 min and then at 42°C for 15 min, at 99°C for 5 min, and at 5°C for 5 min. The resulting cDNA was precipitated and resuspended at 1.5 µg/µl.

PCR amplification of the cDNA was performed using either primers that recognize all four isoforms of VEGF mRNA (5) for VEGF or Ki-ras (28). Primers that recognize β-actin were used with the same cDNA preparations as an internal control for quantifying mRNA. The primers for VEGF were 5'-CCCTGGTGAACTCTTTCCAGGAGTACC-3' (sense) and 5'-GAAAGCTCATCTCTCCATAGTGGCTGGC-3' (antisense). The primers for Ki-ras were 5'-TGAGTATAAATCGTGGTAGTGGG-3' (sense) and 5'-GGTGGAATCCTTCAATGATTAGTAG-3' (antisense). The primers for β-actin were 5'-TTGTAGAAACCTGGAATGATTAGTAG-3' (sense) and 5'-GATCCTGACTTCTGATGTTG-3' (antisense). The primers for β-actin were purchased from Clontech, Palo Alto, CA. PCR was performed with 2.5 µg of cDNA (2 µl) in 50 µl of buffer containing 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 50 mM KCl, 0.2 mM each of deoxyribonucleoside triphosphates, 0.4 µM of each primer, and 2 units of Taq DNA polymerase. For VEGF, the amplification was performed for 33 cycles of 1 min at 94°C for denaturing, 1 min at 55°C for annealing, and 2 min at 72°C for extension. For Ki-ras, the amplification was performed for 35 cycles of 1 min at 94°C for denaturing, 1 min at 57°C for annealing, and 2 min at 72°C for extension. Alliquots (9 µl) of the products were subjected to electrophoresis on a 1.25% agarose gel, and the DNA was visualized by ethidium bromide staining. The gel was then photographed under ultraviolet transillumination.

For the quantitative assessment of the PCR products, a video image analysis system (Image-1/FL, Universal Imaging, Westchester, PA) was used. The Image-1 system can distinguish density on a scale of 0–255 units. Each measurement was standardized by subtracting the background intensity in average.

Immunoblot analysis. Frozen specimens of gastric tissue were homogenized with a Polytron homogenizer (Kinematica) in a lysis buffer containing 62.5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.4% deoxycholic acid, 1% Nonidet P-40, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, and 0.05 mM aminobenzesulfonyl fluoride. The homogenates were then centrifuged (14,000 rpm for 10 min at 4°C). The protein concentration of the homogenate was determined by the bicinchoninic acid protein assay using a commercial kit (BCA protein assay reagent, Pierce Chemical, Rockford, IL). Equal amounts of protein from the tissue homogenates were mixed with 4× sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.65 mM dithiothreitol, and 0.06% bromphenol blue. The samples were then boiled for 2 min and loaded onto 15% acrylamide gels and electrophoresed. The separated proteins were then transferred onto nitrocellulose membranes (Hybond ECL, Amersham Life Science, Arlington Heights, IL), and the membranes were blocked in buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20, and 5% milk for 1 h at room temperature before incubation with primary antibody for either VEGF (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) or Ras (mouse monoclonal, Oncogene Science, Uniondale, NY) at a concentration of 1:100 for 1 h at room temperature on a platform rocker. The membranes were then washed four times in buffer without milk and incubated with either anti-rabbit IgG peroxidase conjugate (Sigma Chemical, St. Louis, MO) or anti-mouse IgG peroxidase conjugate (Transduction Laboratories, Lexington, KY) at room temperature for 1 h on a platform rocker. The signal was visualized by the enhanced chemiluminescence method using ECL Western blotting detection reagents (Amersham). Quantification of the signal was performed by densitometry scanning using an LKB 2222-020 Ultro Scan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).
Assessment for angiogenesis using immunofluorescence staining for vimentin. Angiogenesis was assessed using fluorescence staining for vimentin, a major component of intermediate filaments in mesenchymal cells (41). In the rat gastric mucosa, vimentin is located predominantly in endothelial cells lining the mucosal microvascular network (39). When deep mucosal injury occurs, the microvascular network is destroyed and vimentin fluorescence is extinct (17). Conversely, the restoration of mucosal microvessels during the process of angiogenesis is reflected by the appearance of sprouting tubes of endothelial cells strongly positive for vimentin (39). The staining for vimentin as an indicator of endothelial cells was further confirmed in this study by the staining of similar gastric specimens for factor VIII-related antigen. Gastric specimens were fixed in 4% paraformaldehyde for 4 h and subsequently transferred to 0.5 M sucrose in phosphate-buffered saline for 24 h. They were then frozen at −80°C until cutting. Cryostat sections (10 µm thick; J ung Cryocut 1800, Leica, Deerfield, IL) were digested with 0.1% trypsin (Sigma) at 37°C for 10 min and incubated overnight with an antibody specific for rat vimentin (mouse monoclonal, Dako, Carpinteria, CA) or factor VIII-related antigen (rabbit polyclonal, Dako). For control studies, to determine nonspecific secondary antibody binding, cryostat gastric sections were incubated overnight with PBS instead of the primary antibody. After washing with PBS, sections were incubated for 30 min with fluorescein-conjugated anti-mouse or anti-rabbit immunoglobulin (Sigma) diluted 1:100. Immunofluorescence was evaluated using a Nikon Optiphot epifluorescence microscope with B filter composition (Nikon, Garden City, NY). Angiogenesis was assessed quantitatively by counting (under ×400 magnification) the number of mucosal microvessels demonstrating sprouting endothelial tubes. Counting was performed on coded slides in 20 different randomly selected fields of mucosal erosions. At least 100 microvessels per specimen were counted.

Effect of neutralizing anti-VEGF antibody on angiogenesis. Sprague-Dawley rats (weight range 175–200 g) fasted for 24 h were injected intravenously with either 200 µg of a goat polyclonal VEGF neutralizing antibody (R&D Systems, Minneapolis, MN) or 200 µg of normal polyclonal antibody of the same isotype (controls) in 500 µl of PBS. Concurrently, the rats received, intragastrically, 1.5 ml 50% ethanol. Twenty-four hours after ethanol administration, the animals were anesthetized, their stomachs were excised, and the animals were euthanized. The stomachs were then opened along the greater curvature, rinsed with 0.9% NaCl, examined visually, and photographed in a standardized fashion as described (38). The area of macroscopic necrosis was measured and expressed as a percentage of total glandular area (38). Angiogenesis was then assessed quantitatively, as described above, by counting (under ×400 magnification) the number of mucosal microvessels demonstrating sprouting endothelial tubes.

Effect of Ras inhibition on VEGF expression in vitro. Rat primary aortic endothelial cells were plated in 100-mm tissue culture dishes and grown until ~80% confluent in DMEM supplemented with 10% fetal bovine serum. For the determination of Ras activation, cells were serum-starved for 16 h and metabolically labeled for an additional 8 h in serum-free, phosphate-free DMEM containing 200 µCi/ml 32PO4. The cells were incubated during the final 4 h with vehicle (controls) or 25 µM mevastatin to prevent Ras localization to the plasma membrane, thus inhibiting Ras activation. Then 10 ng/ml bFGF was added and the cells were further incubated for 5 min. Ras activation was determined according to Downward et al. (10). Briefly, cells were washed with ice-cold PBS and lysed on ice in 1 ml of lysis buffer [50 mM HEPES (pH 7.5), 500 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 0.5% deoxycholate, 0.05% SDS, 1 mM EGTA, 10 mM benzamidine, and 10 µg/ml each of aprotonin, leupeptin, and soybean trypsin inhibitor]. Ras proteins contained in the cell lysates were immunoprecipitated with rat monoclonal anti-Ras antibody (Y13-259, Santa Cruz Biotechnology). The guanine nucleotide bound to the Ras proteins were eluted in 16 µl of 2 mM EDTA, 5 mM dithothreitol, 1 mM GTP, 1 mM GDP, and 0.2% SDS at 68°C for 20 min and fractionated by thin-layer chromatography. Quantification was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The percentage of GTP bound to Ras (as an indicator of Ras activation) was calculated as [counts per minute (cpm) in GTP/(cpm in GTP + cpm in GDP)] normalized for moles phosphate in each nucleotide. For determination of VEGF expression, primary endothelial cells were plated as above until ~80% confluent. The cells were then serum starved for 24 h and incubated for 4 h with 25 µM mevastatin or vehicle (controls). The cells were further incubated for 5 min with 10 ng/ml bFGF. The cells were washed with serum-free DMEM and incubated in this medium for an additional 1 h (for mRNA expression) and 3 h (for protein expression). The cells were then washed twice with ice-cold PBS and lysed on ice with either 1 ml of 4 M guanidinium isothiocyanate for total RNA isolation, as described under RNA isolation and RT-PCR or 300 µl of the lysis buffer as described under Immunoblot analysis. RT-PCR and Immunoblot analysis for VEGF expression were performed as described above.

Statistical analysis. Results are expressed as means ± SD. Student’s t-test was used to determine statistical significance between control and experimental groups. A P value of <0.05 was considered statistically significant. Comparisons of data between multiple groups were performed with ANOVA.

RESULTS

Assessment of mucosal injury and the angiogenic response of gastric endothelial cells to ethanol-induced injury. Administration of 100% ethanol caused severe mucosal injury reflected as macroscopically visible hemorrhagic necrotic bands, identical to those described previously (19, 34, 37, 38). The necrosis involved 34 ± 3%, 38 ± 4%, and 42 ± 4% of the total mucosal area at 3, 6, and 24 h, respectively, after ethanol administration. Histological examination demonstrated that the necrotic bands correspond to deep hemorrhagic erosions, as described in our previous papers (37, 38). Within the erosions all mucosal structures, including the gastric glandular epithelium and the microvascular network, were destroyed as reflected by immunofluorescence staining for vimentin (an intermediate filament protein; Fig. 1, A and B), which is strongly expressed in endothelial cells lining mucosal microvessels of normal gastric mucosa (Fig. 1, A and B). Immunofluorescence staining of gastric mucosal sections for vimentin at 24 h after ethanol administration clearly demonstrated angiogenesis reflected by the presence of numerous tubes of migrating endothelial cells (Fig. 1C). Staining gastric mucosal sections for both vimentin and factor VIII-related antigen demonstrated their colocalization in endothelial cells (data not shown). Quantitative analysis demonstrated that, at 24 h, 9 ± 1% of the microvessels in the mucosa bordering necrosis showed sprouting...
endothelial tubes, a hallmark of the angiogenic process (Fig. 1C).

Neutralizing anti-VEGF antibody impairs the angiogenic response of gastric endothelial cells to ethanol-induced injury. To assess the contribution of endogenous VEGF to the angiogenic response that occurs in the gastric mucosa within 24 h of ethanol-induced injury, a “neutralizing” anti-VEGF antibody was administered intravenously concurrently with the intragastric administration of 50% ethanol. Twenty-four hours after administration of anti-VEGF and ethanol, macroscopically visible hemorrhagic necrotic bands were clearly present. The necrosis involved 18 ± 3% of the total mucosal area. The necrosis resulting 24 h after the concurrent administration of 50% ethanol with preimmune IgG (controls) involved significantly (P < 0.025) less of the total mucosal area (4 ± 3%), indicating that anti-VEGF antibody delays healing. Angiogenesis, at 24 h after the concurrent administration of anti-VEGF antibody and 50% ethanol, was assessed as described in MATERIALS AND METHODS. Immunofluorescence staining of gastric mucosal sections for vimentin clearly demon-

strated significant impairment of angiogenesis, as reflected by the severe reduction in tubes of migrating endothelial cells (Fig. 2B) compared with controls receiving preimmune IgG concurrent with 50% ethanol (Fig. 2A). Quantitative analysis demonstrated that administration of the neutralizing anti-VEGF antibody resulted in a threefold reduction vs. controls (without neutralizing antibody) in the percentage of the microvessels showing sprouting endothelial tubes in the mucosa bordering necrosis (2.9 ± 1.2% vs. 9 ± 1.3%, P < 0.0001).

Expression of VEGF mRNA is increased following ethanol-induced gastric mucosal injury. VEGF mRNA expression after ethanol-induced gastric mucosal injury was determined quantitatively. Competitive RT-PCR, in which a DNA fragment of known concentration
Expression of VEGF protein is increased after ethanol-induced injury to the gastric mucosa. Immunoblot analysis demonstrated that ethanol-induced injury of the gastric mucosa also results in increased expression of the secreted isoform of VEGF protein, VEGF165, at 3, 6, and 24 h in the mucosa bordering necrosis. A protein band of ~20 kDa, corresponding to VEGF165, was found to be significantly increased at each of the three time points over the controls, with the greatest increase being at 3 h (367% (P < 0.001) compared with 318% (P < 0.0003), and 385% (P < 0.0001) at 3, 6, and 24 h, respectively, compared with controls (Fig. 4E).

Expression of Ki-ras mRNA is increased after ethanol-induced injury to the gastric mucosa. Because VEGF has been shown to be upregulated by oncogenic Ras in vitro (20, 22, 25), we studied the level of gastric Ki-ras mRNA expression in the mucosa bordering necrosis in response to ethanol-induced injury. RT-PCR demonstrated that the gastric mucosa (bordering necrosis) of rats that received ethanol had increased expression of Ki-ras mRNA to 163% (P < 0.05) and 190% (P < 0.02) at 3 and 6 h, respectively, compared with the controls (Fig. 5A, top). Although this analysis indicated that the increased expression of gastric Ki-ras mRNA resulting from ethanol-induced injury remains twofold greater at 24 h compared with the controls, upon normalizing for β-actin mRNA as shown in Fig. 5A, bottom, the P value was outside the range of statistical significance (P = 0.097). Quantitative data for Ki-ras mRNA expression are presented in Fig. 5B.

Expression of Ras protein is increased after ethanol-induced injury to the gastric mucosa. Immunoblot analysis demonstrated that expression of gastric Ras protein is also increased as a result of ethanol-induced injury. A protein band of ~21 kDa was detected by a monoclonal anti-Ras antibody, which recognizes all four mammalian isoforms of Ras and was found to be increased to 840% (P < 0.05) and 241% (P < 0.005) at 3 and 6 h, respectively, over the controls as shown in Fig. 5B. By 24 h, the level of gastric Ras protein expression had normalized in gastric mucosal tissue of rats treated with ethanol to that of the controls (Fig. 5A). Quantitative data for Ras protein expression are presented in Fig. 5B.

The increased expression of both VEGF and Ki-ras mRNA and VEGF and Ras protein resulting from ethanol-induced injury to gastric mucosa is dependent on ethanol concentration. To determine whether the increased expression of VEGF and Ras, at both the transcriptional and translational levels, resulted from

![Image](https://via.placeholder.com/50x50?text=Image)
ethanol-induced injury to the gastric mucosa in a dose-dependent manner, the effect of three concentrations of ethanol on VEGF and Ras expression was investigated. Although VEGF mRNA expression increased to 153% in the gastric mucosa at 3 h after administration of 25% ethanol (Fig. 7A), this increase was not statistically significant compared with controls \((P > 0.09)\). However, administration of 50% ethanol resulted in deep necrosis in the gastric mucosa at 3 h and a significant increase to 359% \((P < 0.03)\) in VEGF mRNA expression in the gastric mucosa bordering the necrosis compared with controls (Fig. 7A). Administration of 100% ethanol resulted in an increase to 556% \((P < 0.005)\) in VEGF mRNA expression in the mucosa bordering the necrosis compared with controls (Fig. 7A). VEGF protein expression was also not significantly altered in the gastric mucosa at 3 h after administration of 25% ethanol (Fig. 7B). However, administration of 50% and 100% ethanol did result in significant increases in expression of VEGF \(_{165}\) in the mucosa bordering necrosis to 286% \((P < 0.005)\) and 380% \((P < 0.001)\), respectively, at 3 h (Fig. 7B). Expression of the larger, nonsecreted form of VEGF was not significantly altered at 3 h after administration of either 50% or 100% ethanol compared with controls (data not shown). Administration of 25% ethanol did result in a slight but significant \((P < 0.04)\) increase in Ki-ras mRNA expression in the gastric mucosa to 131% at 3 h compared with controls (Fig. 8A). Expression of Ras protein was not significantly different in the gastric

Fig. 4. Western blot analysis of VEGF protein expression in gastric mucosa after ethanol injury compared with gastric mucosa of controls. **A:** analysis of VEGF protein expression was performed as described in MATERIALS AND METHODS. Level of VEGF protein from gastric mucosa samples of control animals is shown together with that of gastric mucosa samples from animals at each time point after intragastric administration of 100% ethanol. **B:** quantitative data of VEGF protein expression (shown in **A**) obtained by densitometric scanning using values of peak area. Values are means ± SD. For each time point, \(n = 6\).

Fig. 5. RT-PCR analysis of Ki-ras mRNA expression in gastric mucosa after ethanol injury compared with gastric mucosa of controls. **A:** top: RT-PCR using specific primers for Ki-ras was performed as described in MATERIALS AND METHODS. Samples from control animals are shown together with samples from each time point after intragastric administration of 100% ethanol. **A:** bottom: RT-PCR of same reverse transcription products in **A** top, using specific primers for \(\beta\)-actin as internal control for total mRNA. **B:** quantitative data for Ki-ras mRNA expression (shown in **A**) using a computerized video analysis of amplified PCR products. Each signal was normalized against corresponding \(\beta\)-actin signal, and results are expressed as Ki-ras/\(\beta\)-actin. Values are means ± SD. For each time point, \(n = 6\).
Ethanol, after intragastric administration, penetrates deeply into the gastric mucosa because of high lipid solubility and, at concentrations of 50–100%, causes microvascular damage and hemorrhagic lesions (23, 34, 37, 38, 41). Ethanol-induced injury to the gastric mucosa is a time-related process in which disruption or exfoliation of the gastric surface epithelium is followed by necrosis of deeper mucosal layers, including the mucosal proliferative zone and the microvasculature (23, 37, 38, 41). Angiogenesis is a prerequisite for the healing of ethanol-induced deep gastric mucosal damage. Our previous study has shown that ethanol-induced injury to gastric mucosa triggers an angiogenic response as well as an increase in bFGF, a known angiogenic factor, in the mucosa bordering necrosis (36, 39).

The present study demonstrates for the first time that ethanol-induced injury to the gastric mucosa activates VEGF gene expression as reflected by increases in VEGF at both the transcriptional and translational levels. VEGF is the only known growth factor to act predominantly on endothelial cells, which are one of the few cell types to express the receptors for VEGF (9, 12, 24). Although not yet characterized for gastric mucosa, in various other tissues VEGF induces angiogenesis by acting both as an endothelial mitogen and by enhancing microvascular hyperpermeability, which stimulates the formation of a fibrin-rich extracellular matrix promoting endothelial cell migration (5). VEGF has been implicated in epidermal wound healing (6) and in the accelerated healing of experimental duodenal ulcers and ulcerative colitis (27, 32). VEGF may also play a role in the angiogenesis involved in the healing of chronic gastric ulcers, which represent a distinctly different type of injury from acute ethanol-induced necrosis (35). In that study, VEGF expression, which was found predominantly in epithelial cells of
normal gastric mucosa, was shown to be induced in gastric fibroblasts of ulcer margins and ulcer beds (35).

Our finding that VEGF expression is increased in the gastric mucosa in response to ethanol injury strongly suggests the importance of VEGF as a mediator of the angiogenesis crucial for the repair of gastric mucosal erosion. This rapid increase in the expression of VEGF was associated with an angiogenic response to ethanol-induced injury as demonstrated in the present study, 24 h after ethanol administration, by the endothelial cell migration and tube formation, in the mucosa bordering necrosis.

We have previously demonstrated that administration of exogenous VEGF enhances angiogenesis and accelerates the healing of chemically induced lesions in the upper and lower gastrointestinal tract (27, 32, 40). The importance of VEGF in the angiogenic response to ethanol-induced gastric mucosal injury is further supported by demonstration in this study that angiogenesis was significantly inhibited by administration of a neutralizing anti-VEGF antibody. Moreover, the extent of macroscopic injury remained greater than fourfold that of controls, indicating that neutralization of endogenous VEGF significantly delays healing of mucosal erosions. The finding that administration of the neutralizing anti-VEGF antibody led to a threefold reduction in the angiogenic response (compared with controls receiving normal antibody), but did not completely inhibit it, can be explained by the presence of other angiogenic factors such as BFGF, which is increased in response to ethanol-induced injury (36). Our data show that gastric VEGF expression at both the transcriptional and translational levels increases early in response to ethanol-induced injury, becoming maximal within 3 h after intragastric ethanol administration. These increases in VEGF mRNA and protein expres-
The finding that intragastric administration of 25% ethanol did not result in increased VEGF expression is not surprising. It has long been known that ethanol at this concentration can produce exfoliation of the surface epithelial cells but does not result in necrotic injury of the deeper mucosa or of microvessels. In fact, this concentration of ethanol has a cytoprotective action against the necrotizing effect of higher concentrations of ethanol administered subsequently (7). The finding that intragastric administration of 50% ethanol results in significant increases in both VEGF mRNA and protein in the gastric mucosa bordering necrosis is clinically relevant because alcoholic beverages produced for human consumption can reach this concentration.

The present study also demonstrates for the first time that ethanol-induced injury to the gastric mucosa activates ras gene expression, increasing Ras at both the transcriptional and translational levels. As with VEGF expression, the increase in Ras mRNA and protein expression in response to ethanol-induced injury was dose dependent on ethanol concentration. Several studies have shown that VEGF expression is upregulated by oncogenic Ras in transfected cell lines, suggesting that one of the roles of oncogenic Ras in the growth of solid tumors is the induction of angiogenesis (20, 22, 25). In the present study, we have demonstrated in primary rat endothelial cell culture that the expression of VEGF induced by bFGF is mediated through Ras activation. In addition, the induction of VEGF by hypoxia was shown to be blocked by the Ras inhibitor, RasN17, in untransformed NIH/3T3 cells, suggesting that nononcogenic Ras may play a role in mediating this induction (22). Furthermore, upregulation of VEGF expression has been shown to be induced by myocardial ischemia in vivo, suggesting a link between tissue hypoxia-induced VEGF expression and the angiogenic response to ischemia resulting in coro-
mucosal erosion, possibly through angiogenesis. We and is normalized to control levels by 24 h are consistent. Our data demonstrating that the increase in Ras protein expression in response to ethanol-induced injury, has not been previously investigated. Our data demonstrating that the increase in Ras protein expression in response to ethanol-induced injury is maximal within 3 h after ethanol administration and is normalized to control levels by 24 h are consistent with an early involvement of Ras in the repair of mucosal erosion, possibly through angiogenesis. We currently have no explanation for why Ki-ras mRNA expression continues to increase at 6 and 24 h after ethanol administration. The decreased expression of total Ras protein (which includes H-Ras and N-Ras, as well as Ki-Ras) at these time points, compared with the expression at 3 h after ethanol administration, implies differential regulation at the transcriptional and translational levels.

Because the induction of VEGF by hypoxia has been shown to be blocked by Ras inhibition, and is thus dependent on Ras, and because data in the present study indicate that, in rat endothelial cells, the expression of VEGF induced by bFGF is mediated through the Ras pathway, the increase in Ras expression resulting from ethanol-induced gastric mucosal injury is likely to cause upregulation of VEGF expression and thus the observed angiogenic response.

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