Suppression of early growth response factor-1 with egr-1 antisense oligodeoxynucleotide aggravates experimental duodenal ulcers

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Duodenal ulcers are two to four times more prevalent than gastric ulcers, and this form of “peptic ulcer” disease affects 5–10% of the United States population at least once in their lifetime (14, 38). In addition to the role of Helicobacter pylori, about one-third of ulcers are H. pylori negative and are probably drug induced (7, 9). In any case, the molecular mechanisms of ulcer development and healing remain poorly understood. Animal models of duodenal ulcer disease [e.g., induced by cysteamine or propionitrile, because aspirin-like drugs produce only gastric erosions or ulcers in rodents (8, 39, 43)] are needed to gain insight into the early preulcerogenic molecular and biochemical changes in the pathogenesis of duodenal ulceration. Most of the pathogenesis research has been performed with the duodenal ulcerogen cysteamine, which produces perforating ulcers in the rat proximal duodenum in 24–48 h (8, 39, 43). The cysteamine-induced solitary or “kissing” duodenal ulcers usually heal in 2–3 wk. The repair process includes the proliferation of endothelial (i.e., angiogenesis), epithelial, and connective tissue cells, and it is regulated by growth factors (4, 41, 45–47). Our laboratory was the first to report that an intragastric administration of bFGF, PDGF, or VEGF or that gene therapy with either naked DNA or adenoviral vectors of VEGF and PDGF significantly accelerated chronic duodenal ulcer healing in rats (40, 41, 45, 46). One of the very early events in experimental duodenal ulceration is the rapid, local release of endothelin-1 (ET-1) followed by increased expression of transcription factor early growth response factor-1 (Egr-1) in the nuclear fraction of the duodenal mucosa (21, 42, 46). Our microarray gene expression studies (19, 21, 40) revealed the consistent activation of egr-1 and Sp1 as well as bFGF or PDGF genes after cysteamine administration.

The zinc finger transcription factor Egr-1 is an immediate-early gene product that interacts with consensus GC-rich promoter regions to regulate the transcription of a diverse set of genes in response to mitogenic and nonmitogenic stimuli such as growth factors, shear stress, mechanical injury, or hypoxia (1, 18, 23, 31, 33, 36). These stimuli induce the activation of ERK1/2, members of the MAPK family, leading to enhanced Egr-1 transcriptional function and activation of target genes (17, 27, 29). In turn, Egr-1 induces the expression of several target genes, including bFGF, PDGF-A, PDGF-B, and VEGF receptor-1/Flt-1 (3, 35, 50, 52).

Recently, we (20) demonstrated that cysteamine induced hypoxia in the proximal duodenum, stabilized hypoxia-inducible factor-1α (HIF-1α) expression, enhanced HIF-1α transcriptional activity, and increased the transcriptional interaction of HIF-1α with Egr-1. HIF-1α is a hypoxia-inducible transcription factor that enhances promoter activity of VEGF gene and VEGF synthesis (22). Because Egr-1 regulates the expression of genes involved in the proliferative response to a variety of stimuli, targeting this transcription factor may reduce proliferation or repair after injury. An antisense egr-1 oligonucleotide abolished the stimulation of protein synthesis induced by ET-1 in myogenic cell line (24). Inhibition of Egr-1 syn-

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thesis in smooth muscle cells by antisense egr-1 oligonucleotide decreased serum-inducible \[^{1}\text{H}\]thymidine incorporation as well as the stimulation of cell proliferation and repair after injury. Egr-1 induction was critical for PDGF-induced mitogenic signaling in mesangial cells, and inhibition of Egr-1 using antisense egr-1 oligonucleotide in vivo decreased cell proliferation in experimental glomerulonephritis (32). BKS-2 (B cell lymphoma) cells treated with antisense egr-1 oligodeoxyribonucleotide underwent growth arrest and apoptosis, suggesting that the expression of Egr-1 is important not only for the growth of but also for the survival of BKS cells (10).

In the present study, we tested the hypothesis that egr-1 plays a critical role in multifactorial duodenal ulcerogenesis because its protein, transcription factor Egr-1, regulates the expression of angiogenic growth factors like bFGF, PDGF, and VEGF. We investigated the specificity of the cysteamine effect compared with its chemical analog ethanolamine on the time-dependent expression of egr-1 gene and protein and synthesis and activity of bFGF, PDGF, VEGF, and ERK1/2 in the duodenal mucosa before the morphological development of ulcers. Furthermore, we studied whether inhibition of Egr-1 expression by egr-1 antisense oligodeoxyribonucleotide might affect the cysteamine-induced duodenal ulcer formation in vivo and the expression of bFGF, PDGF, and VEGF in the ulcerated rat duodenal mucosa.

MATERIALS AND METHODS

Animal experiments. This study was approved by the Animal Study Subcommittee of the Veterans Affairs Medical Center in Long Beach, CA. Duodenal ulcers were induced in female Sprague-Dawley rats (180–210 g) by an intragastric administration of cysteamine HCl (Aldrich; Milwaukee, WI). Rats had unlimited access to food and water. Randomized groups of rats (n = 5–7) were given either saline or cysteamine HCl (25 mg/100 g) by gavage once and euthanized 0.5, 1, or 2 h later. In other groups, cysteamine was given twice (4-h interval), and rats were euthanized 6 h later. Animals from the next group received three cysteamine treatments at 4-h intervals, and rats were euthanized 12, 24, or 48 h after the first dose of cysteamine. Because our previous structure-activity studies (42, 43) demonstrated that nonulcerogenic analogs could be used as appropriate controls to separate ulcerogenic and toxic effects, we performed additional experiments in which groups of rats were given the nonulcerogenic structural analog of cysteamine (HS–CH\(_2\)–CH\(_2\)–NH\(_2\))–HCl, i.e., ethanalamine (HO–CH\(_2\)–CH\(_2\)–NH\(_2\))–HCl (21.47 mg/100 g), which was used as an internal control to correct variability in starting ulcer areas. All experiments were repeated seven times.

Western blot analysis. The loading controls were performed by using a mouse monoclonal anti-phospho-ERK1/2 (Tyrr204) antibodies (Santa Cruz Biotechnology; Santa Cruz, CA; dilution 1:500). The loading controls were treated with secondary antibodies and visualized using the ECL detection system (Amersham Biosciences). The density of protein bands in Western blots was assessed by a digital imaging system, Eagle Eye II (Stratagene; Austin, TX). Each experiment was repeated four times.

Statistical analysis. The statistical significance of changes was determined by the nonparametric Mann-Whitney U-test. Differences resulting in P values of <0.05 were considered to be statistically significant.

RESULTS

Early increase of egr-1 mRNA and protein expression in the duodenal mucosa of cysteamine-treated rats. The results demonstrated that the duodenal ulcerogen cysteamine increased egr-1 gene activity in the duodenal mucosa by 3.8- and 12.4-fold at 0.5 and 12 h after the administration of cysteamine, respectively (Fig. 1A). The time-dependent increased expression of the egr-1 gene was followed by elevated Egr-1 protein expression in the duodenal mucosa in the early stage of cysteamine-induced duodenal ulcers (Fig. 1B).

Comparison of Egr-1 expression induced by cysteamine and structurally related ethanolamine. Ethanolamine is a toxic analog of the duodenal ulcerogen cysteamine. To establish the
specificity of Egr-1 in duodenal ulcers, we compared Egr-1 expression in the duodenal mucosa of rats treated with cysteamine or ethanolamine. These results demonstrated that the nonulcerogenic analog of cysteamine, ethanolamine, did not change the expression of Egr-1 in the early stage of ulcer development (Fig. 1C).

**Effect of cysteamine on bFGF, PDGF, and VEGF expression.** The first dose of cysteamine did not change the expression of bFGF in the duodenal mucosa. The expression of bFGF was slightly elevated at 6 h and significantly increased after three doses of cysteamine at 12 h. Enhanced bFGF expression was detected at the time of duodenal ulcer appearance (24–48 h) after cysteamine (Fig. 2A). The expression of PDGF and VEGF was enhanced soon after first dose of cysteamine and seemed to peak at 12 h after cysteamine (Fig. 2, B and C).

**Antisense egr-1 oligonucleotide aggravated cysteamine-induced duodenal ulcers.** To determine the role of Egr-1 in cysteamine-induced duodenal ulceration, rats were pretreated with saline or phosphothioate-modified antisense egr-1 or scrambled control oligodeoxynucleotides either at 0.02 or 0.2 mg/rat 30 min before each cysteamine administration. Scrambled control and the small dose of antisense egr-1 oligonucleotides did not alter the size of cysteamine-induced duodenal ulcers. Scrambled control oligodeoxynucleotides did not cause any change in duodenal ulcer appearance in cysteamine-treated rats compared with control saline-pretreated animals, implicating no toxicity of the oligodeoxynucleotide. The larger antisense egr-1 oligonucleotide dose aggravated duodenal ulcer formation and increased the size of ulcers from 8.1 ± 1.8 to 20.7 ± 4.0 mm² (% < 0.01; Fig. 3, A–C). Histological evaluation revealed mostly superficial ulcers in the proximal duodenum of rats treated with scrambled control and cysteamine, whereas deep ulcers penetrating into the Brunner glands, often with perforation, were seen in rats given cysteamine and pretreated with egr-1 antisense oligonucleotide (Fig. 3C).
Fig. 3. Effect of egr-1 antisense oligodeoxynucleotide on cysteamine-induced duodenal ulcers in rats. A: gross appearance of duodenal ulcers 48 h after cysteamine administration. Animals were pretreated with either saline (S; n = 10), scrambled control oligodeoxynucleotide (SC; n = 7), or antisense egr-1 (phosphothioate-modified) oligodeoxynucleotide at 0.2 mg/rat (AS; n = 10). B: light microscopy of duodenal ulcers in rats pretreated with either saline (showing superficial ulcer; left), scrambled control oligodeoxynucleotide (similar ulcer size; middle), or antisense egr-1 (phosphothioate-modified) oligodeoxynucleotide at 0.2 mg/rat before cysteamine (deep ulcer; right) with hematoxylin and eosin staining. C: duodenal ulcer crater dimensions were measured in millimeters, and the ulcer areas were calculated using the ellipsoid formula. Sizes of duodenal ulcers are expressed as means ± SE. **P < 0.01.

Time-dependent effect of antisense egr-1 oligonucleotide on Egr-1 protein expression in the rat duodenal mucosa. The Western blot results demonstrated that the expression of Egr-1 at 6 h after cysteamine in rats pretreated with saline or scrambled egr-1 oligodeoxynucleotide was 56% or 70% higher than in saline-treated control animals (Fig. 4, A and B). There were no differences in Egr-1 expression in rats that were given saline or scrambled oligonucleotide before cysteamine. Antisense egr-1 oligodeoxynucleotide inhibited Egr-1 protein expression by 70%, 35%, and 32% at 6, 12, and 24 h, respectively, after cysteamine.

Cysteamine activates ERK1/2 in the rat duodenal mucosa. We measured ERK1/2 expression and activity in the same samples from the duodenal mucosa of rats treated with only saline and rats pretreated with saline, scrambled control oligonucleotide, or antisense egr-1 oligonucleotide before each cysteamine dose. Figure 4C demonstrates that the expression of ERK1 (p44) and ERK2 (p42) kinases was not changed in rats treated with saline or pretreated with saline, scrambled control oligodeoxynucleotide, or egr-1 antisense oligodeoxyxynucleotide before cysteamine. ERK activation was measured using a phosphospecific anti-ERK antibody, which specifically recognizes ERK after catalytic activation by phosphorylation (Tyr204). The results demonstrated that cysteamine activated ERK1/2 kinases at 6, 12, and 24 h in the duodenal mucosa of rats pretreated with saline or scrambled control compared with rats given saline only (Fig. 4D). Cysteamine treatment did not change the level of total ERK (Fig. 4C), suggesting that ERK activity in our model was the result of catalytic phosphorylation by MEK rather than by changes in total ERK protein expression. Interestingly, we detected an inhibition of ERK phosphorylation in the duodenal mucosa of all rats pretreated with egr-1 antisense oligonucleotide compared with saline-treated or scrambled control-pretreated animals (Fig. 4D).

Egr-1 antisense oligonucleotide decreased the expression of Egr-1 mRNA and protein in the duodenal mucosa of rats with formed ulcers. In our model of duodenal ulceration, cysteamine leads to the development of severe ulcers at 48 h after treatment. The egr-1 antisense oligonucleotide significantly aggravated duodenal ulcer formation, and we measured the changes in egr-1 mRNA and protein expression at 48 h after cysteamine treatment. To investigate the effect of antisense egr-1 oligonucleotide on egr-1 mRNA, we performed RT-PCR analysis. Scrambled oligodeoxynucleotide did not change egr-1 mRNA expression compared with saline-treated rats at 48 h after first dose of cysteamine, whereas the antisense egr-1 oligodeoxyxynucleotide inhibited egr-1 mRNA expression in the duodenal mucosa (Fig. 5A). The antisense egr-1 oligodeoxynucleotide inhibited Egr-1 protein expression in the duodenal mu-
cosa by ~50%, whereas protein levels did not markedly change in the gastric and jejunal mucosa (Fig. 5).

**Effect of antisense egr-1 oligonucleotide on expression of bFGF, PDGF, and VEGF in the duodenal mucosa of rats treated with cysteamine.** Western blots of bFGF expression showed that scrambled control oligonucleotide (two different doses) and the low dose of antisense egr-1 oligonucleotide (0.02 mg/rat) did not alter bFGF protein expression in the rat duodenal mucosa at 48 h. Antisense egr-1 oligonucleotide, at 0.2 mg/rat, however, decreased bFGF expression in the duodenal mucosa by 2.3-fold (Fig. 6A). PDGF and VEGF protein levels in the duodenal mucosa in rats pretreated with antisense egr-1 oligonucleotide were also significantly decreased, e.g., the expression of PDGF in the duodenum was decreased by antisense egr-1 oligonucleotide by 43–50%. The VEGF level in the ulcerated duodenal mucosa was only slightly decreased by 0.02 mg/rat antisense egr-1 oligonucleotide but was significantly decreased (55%) by the 0.2 mg/rat dose of antisense egr-1 oligonucleotide.

**DISCUSSION**

This study demonstrated, for the first time, that egr-1 antisense oligonucleotide significantly aggravated experimental duodenal ulcers and inhibited the expression of egr-1 mRNA and protein and bFGF, PDGF, and VEGF synthesis in the rat duodenal mucosa. Ethanolamine, a nonulcerogenic analog of cysteamine, did not alter Egr-1 expression in the duodenal mucosa. Thus these are specific, duodenal ulcerogenic effects of cysteamine, because these are not shared by the toxic analog ethanolamine, underlining a critical role of Egr-1 in duodenal ulceration. Furthermore, Egr-1 antisense oligonucleotide did not affect Egr-1 protein expression in the stomach or jejunum. Our results also demonstrated that cysteamine activated Egr1/2 kinases without any changes in protein expressions in the rat duodenal mucosa.

The Egr-1 transcription factor belongs to hypoxia-inducible transcription factors, and recently we (20) demonstrated that cysteamine induced hypoxia only in the proximal duodenum but not in the stomach. The cysteamine-induced Egr-1 transcriptional activity, as we (19) demonstrated previously, might result in increased expression of bFGF and PDGF in the duodenal mucosa, because the promoter region of bFGF and PDGF genes contains the Egr-1 binding site (3, 18). Our results show that cysteamine enhanced bFGF and PDGF synthesis in preulcerogenic stages, and the decreased Egr-1 expression in the duodenal mucosa might be the cause for the inhibition of
Recently, we demonstrated that cysteamine enhanced the ability of HIF-1α/H9251 to interact with Egr-1. The increase of HIF-1α binding to hypoxia-responsive elements of the VEGF gene was shown in response to hypoxia (22). Cysteamine increased the expression and transcriptional activity of HIF-1α/H9251 (20) and enhanced the transcriptional interaction of HIF-1α/H9251 with Egr-1. This increased interaction in preulcerogenic stages apparently might participate in mechanisms of enhanced VEGF expression in the duodenal mucosa, e.g., the downregulation of Egr-1 expression and its interaction with HIF-1α/H9251 might suppress VEGF synthesis. Cysteamine is a reducing aminothiol and changed the redox state in the rat proximal duodenal mucosa (20). This ability of cysteamine might also participate in the activation of redox-sensitive transcription factors including Egr-1 and HIF-1α, increase their protein-protein interaction through reducing cysteine residues, and enhance the activation of their target genes bFGF, PDGF, and VEGF.

The transcription factor Egr-1 has nucleotide recognition elements in the promoters of many pathophysiologically relevant genes. The mechanisms underlying the inducible expression of Egr-1 are not clear. Egr-1 was expressed in endothelial cells from the wound edge within minutes of injury (34). Early Egr-1 expression in small intestinal epithelial IEC-6 cells, derived from fetal rat duodenal crypt cells after monolayer injury, was ERK dependent, and inhibition of ERK activity decreased Egr-1 mRNA expression (5). Overexpression of Egr-1 protein was prevented with the MAPKK inhibitor PD-98059, indicating that MAPK ERK is involved in the regulation of Egr-1 expression in renal cells (11). Hypoxia-reoxygenation induces ERK1/2 phosphorylation as well as transactivation of the transcription factors NF-κB and Egr-1 in pulmonary artery endothelial cells (6). Hypoxia caused significant remodeling in the pulmonary artery characterized by thickening of the pulmonary arterial wall and increases in tissue mass and total RNA, JNK, ERK, and p38 kinase tyrosine phosphorylations and their activities. Hypoxia also upregulated VEGF mRNA and PDGF receptor mRNA levels in the pulmonary artery with a time course that correlated with the activation of ERK and upregulated egr-1 mRNA (16).

Cysteamine-induced ERK activation in the duodenal mucosa in the preulcerogenic stage may participate in the increased Egr-1 expression and DNA binding activity, resulting in enhanced synthesis of growth factors in the preulcerogenic duodenal mucosa. Our study also demonstrates that the ini-

Fig. 5. Effect of antisense egr-1 oligodeoxynucleotide on egr-1 mRNA and Egr-1 protein expressions in the duodenal mucosa of cysteamine-treated rats. A: RT-PCR transcript analysis of egr-1 gene expression in the rat duodenal mucosa 48 h after cysteamine. Animals were pretreated 0.5 h before each dose of cysteamine with saline, scrambled control oligodeoxynucleotide, or antisense egr-1 (phosphothioate-modified) oligodeoxynucleotide at 0.2 mg/rat. B: representative Western blot analysis of Egr-1 expression in the proximal duodenal, gastric, and jejunal mucosa 48 h after cysteamine administration. Animals were pretreated 0.5 h before each cysteamine dose with saline, scrambled control oligodeoxynucleotide, or antisense Egr-1 (phosphothioate-modified) oligodeoxynucleotide at 0.2 mg/rat.

Fig. 6. Representative Western blot analysis of bFGF (A), PDGF (B), and VEGF (C) expressions in the proximal duodenal mucosa 48 h after administration of the duodenal ulcerogen cysteamine. Animals were pretreated 0.5 h before each dose of cysteamine with saline, scrambled control oligodeoxynucleotide, or antisense egr-1 (phosphothioate-modified) oligodeoxynucleotide at 0.02 or 0.2 mg/rat. Data depicted are means ± SE of 3 rats/group. *P < 0.05.
bition of Egr-1 synthesis induced by antisense egr-1 oligonucleotide leads to a partial decrease in ERK phosphorylation at 6, 12, and 24 h after cysteamine administration. Egr-1 might participate in the early increase of growth factor synthesis in the preulcerogenic mucosa. Growth factors (e.g., PDGF and bFGF) activate ERK (26–28, 30). Thus the inhibition of Egr-1 expression might decrease growth factor synthesis and result in decreased ERK activity and profound inhibition of growth factor synthesis. This may explain our observation that egr-1 antisense oligonucleotide delayed not only growth factor synthesis but also inhibited ERK phosphorylation induced by cysteamine.

Studies (33, 34) with antisense egr-1 oligodeoxynucleotides have suggested that Egr-1 plays a key regulatory role in smooth muscle cell proliferation and repair after injury. These findings demonstrate that inducible Egr-1 expression after injury is dependent on the release and paracrine action of growth factors. A DNA enzyme targeting egr-1 mRNA has been shown to inhibit vascular smooth muscle proliferation and regrowth after injury (35). A protective function of inducible Egr-1 expression in cell survival was shown after ultraviolet irradiation and egr-1 antisense oligonucleotide reduced the cell growth rate (13). Damage of IEC-6 cell monolayers resulted in rapid and significant increases in Fos and egr-1 mRNA levels, and direct interference with egr-1 by the expression of a dominant negative mutant led to a significantly reduced in vitro monolayer restitution (5).

The angiogenic growth factors VEGF, PDGF, and bFGF may play a role in ulcer development and healing not only by regulating angiogenesis but also by promoting the survival of different cells, e.g., lowering the synthesis of growth factors may increase the susceptibility to apoptosis. VEGF significantly reduced the apoptotic chemotherapeutic damage in endothelial cells, and overexpression of a VEGF dominant-interfering mutant abrogated the protective effect of VEGF (48). VEGF and bFGF synergistically enhanced endothelial cytoprotection (25). bFGF, produced by hearts subjected to ischemia, is a protective factor against myocardial cell apoptosis (15). FGF-2 is a potent cell survival factor and protected (48). VEGF and bFGF synergistically enhanced endothelial cell survival after ultraviolet irradiation and egr-1 antisense oligonucleotide reduced the cell growth rate (13). Damage of IEC-6 cell monolayers resulted in rapid and significant increases in Fos and egr-1 mRNA levels, and direct interference with egr-1 by the expression of a dominant negative mutant led to a significantly reduced in vitro monolayer restitution (5).

The importance of growth factor synthesis in the proximal duodenum was demonstrated recently in mice lacking epidermal growth factor (EGF), transforming growth factor-α, and amphiregulin (49). Triple-null neonates displayed spontaneous duodenal lesions. Adult EGF−/− mice displayed more severe lesions in response to cysteamine treatment compared with wild-type counterparts.

Our study demonstrates that egr-1 is a key gene in the multifactorial mechanisms of cytoprotection and healing of duodenal ulcers because its transcription factor product Egr-1 regulates the expression of angiogenic growth factors. Namely, egr-1 antisense oligonucleotide significantly aggrivated experimental duodenal ulcers and inhibited the expression of Egr-1 mRNA and protein as well as the synthesis of bFGF, PDGF, and VEGF in the rat duodenal mucosa. Thus the egr-1 gene and Egr-1 transcription factor seem to be important molecules in the pathogenesis of experimental duodenal ulceration.

**REFERENCES**


**GRANTS**

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