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

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Running title: Egr-1 inhibition in experimental duodenal ulceration.

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Abstract

Previously we demonstrated that cysteamine releases endothelin-1 in the rat duodenal mucosa, followed by increased expression of the early growth response factor – 1 (egr-1). We hypothesized that egr-1 is a key mediator gene in the multifactorial mechanisms of duodenal ulcer development and healing because its protein, transcription factor product Egr-1 regulates the expression of angiogenic growth factors. We wanted to determine the effect of egr-1 antisense on cysteamine-induced duodenal ulcers, as well as on the expression of bFGF, PDGF and VEGF of which synthesis is modulated by Egr-1. An antisense to egr-1 was used to inhibit the synthesis of Egr-1 and to determine its effect on ulcer formation in the rat model of cysteamine-induced duodenal ulceration. Real-time RT-PCR and Western blotting were used to assess the expression of egr-1 mRNA, Egr-1 protein as well as ERK, bFGF, PDGF and VEGF. The antisense egr-1 inhibited the expression of egr-1 mRNA and Egr-1 protein, and increased the duodenal ulcer size from 8.1±1.8 in controls to 20.7±4.0 mm² (p<0.01). Cysteamine induced phosphorylation of both ERK1/2, enhanced the synthesis of bFGF, PDGF and VEGF in the pre-ulcerogenic stages of duodenal ulceration while the egr-1 antisense markedly decreased the expression of these growth factors in the duodenal mucosa. We also demonstrated that the Egr-1 expression relates to the ulcerogenic effect of cysteamine since these actions were not exerted by the toxic analog ethanolamine. Thus, Egr-1 seems to play a critical role in duodenal ulceration because Egr-1 downregulation aggravates the experimental duodenal ulcers, most likely through the transcriptional inhibition of bFGF, PDGF and VEGF synthesis.

Keywords: Cysteamine-induced duodenal ulcer, transcription regulation, angiogenic growth factors.
Introduction

Duodenal ulcer is 2-4 times more prevalent than gastric ulcer and this form of “peptic ulcer” disease affects 5-10 % of the US population at least once in their lifetime (14, 38). In addition to the role of *H. 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, since aspirin-like drugs produce only gastric erosions or ulcers in rodents (8, 39, 43)) are needed to gain insight into the early pre-ulcerogenic, 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 ulcer in the rat proximal duodenum in 24-48 hr (8, 39, 43). The cysteamine-induced solitary or “kissing” duodenal ulcers usually heal in 2-3 weeks. The repair process includes the proliferation of endothelial cells (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 intragastric administration of bFGF, PDGF, 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 ET-1 followed by increased expression of transcription factor Egr-1 in the nuclear fraction of duodenal mucosa (21, 42, 46). Our microarray gene expression studies revealed the consistent activation of egr-1 and Sp1 as well as bFGF or PDGF genes after cysteamine administration (19, 21, 40).
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 non-mitogenic stimuli such as growth factors, shear stress, mechanical injury or hypoxia (1, 18, 23, 31, 33, 36). These stimuli induce the activation of extracellular signal-regulated kinase 1/2 (Erk1/2), members of the MAP kinase family, leading to enhanced Egr-1 transcriptional function and activation of the target genes (17, 27, 29). In turn, Egr-1 induces the expression of several target genes, including bFGF, PDGF-A, PDGF-B, VEGF receptor-1/Flt-1 (3, 35, 50, 52).

Recently, we demonstrated that cysteamine induced hypoxia in the proximal duodenum, stabilized hypoxia-inducible factor-1 α (HIF-1α) expression, enhanced the HIF-1α transcriptional activity and increased the transcriptional interaction of HIF-1α with Egr-1 (20). HIF-1α is a hypoxia-inducible transcription factor, which enhances promoter activity of VEGF gene and VEGF synthesis (22). Since 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 synthesis in smooth muscle cells by antisense egr-1 oligonucleotide decreased serum-inducible $^{3}$Hthymidine incorporation, as well as the stimulation of cell proliferation and repair following 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 oligodeoxynucleotides 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 the 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 cysteamine effect in comparison with its chemical analog ethanolamine on the time-dependent expression of egr-1 gene and Egr-1 protein, synthesis of bFGF, PDGF, VEGF and ERK1/2 activity in the duodenal mucosa before the morphologic development of ulcers. Furthermore, we studied whether inhibition of Egr-1 expression by egr-1 antisense oligodeoxynucleotide 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 VA Medical Center in Long Beach. Duodenal ulcers were induced in female Sprague-Dawley rats (180-210g) by 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 hr later. In other groups cysteamine was given twice (4 hr interval) and rats were euthanized 6 hr later. Animals from next group received three times of cysteamine treatment at 4 hr intervals and rats were euthanized 12, 24 or 48 hr after the first dose of cysteamine. Since our previous structure-activity studies demonstrated that non-ulcerogenic analogs could be used as appropriate controls to separate ulcerogenic and toxic effects (42, 43), we performed additional experiments in which groups of rats were given the non-ulcerogenic structural analog of cysteamine (HS-CH2-CH2-NH2)-HCl, i.e., ethanolamine (HO-CH2-CH2-NH2)-HCl 21.47 mg/100 g, once, two
or three times (as above) and animals were euthanized. In the ulcer studies, all rats were given
cysteamine three times at 4 hr interval and rats were euthanized 48 hr after first dose. Groups of
rats (n=7-10) were pretreated with saline, antisense egr-1 (phosphothioate-modified and HPLC
purified oligodeoxynucleotides 5’-GCGGGGTGCAGGGGCACACT-3’; -118 to -90), or
respective scrambled control (5’-AGGCTGGCTGCCGGAGCGA-3’; Research Genetics, Inc.,
Huntsville, AL or MoleculA, Sterling, VA), subcutaneously at 0.02 mg/rat (n=7) or 0.2 mg/rat
(n=10), 30 min before every dose of cysteamine. This sequence of egr-1 antisense according to
other studies induced the prominent inhibition of Egr-1 synthesis and cell proliferation (2, 12).

Gross and histologic evaluation of duodenal ulcer. The rats were euthanized by CO₂
inhalation and cervical dislocation. The duodenal ulcer crater dimensions were measured in
millimeters, and the ulcer areas were calculated using the ellipsoid formula. The opened
stomachs with 2.5 cm duodenum were fixed in 10% formalin. Sections were embedded in
paraffin and stained with hematoxylin and eosin. The mucosal scrapings of 2.5 cm of proximal
duodenum, glandular stomach and jejunum from some animals were harvested and frozen (-80°
C) for study of gene and protein expressions.

Real time RT-PCR. The total RNA isolation kit (Clontech Lab. Inc., Palo Alto, CA) was used
for total RNA extraction and purification. Real-Time reverse transcriptase (RT) PCR
experiments were performed using TaqMan gene expression assays (Applied Biosystems Inc.,
Forest City, CA) for selected genes such as egr-1. For reaction volume of 20 ul, 1 ul of reverse
transcription product was used with TaqMan Universal PCR Master Mix and Gene Expression
Assay Mix. The following cycles were used on a BioRad iCycler Real-Time PCR machine: 2
min at 50°C, 10 min at 95°C and 40°C cycles of two steps: 15 sec at 95 °C and 1 min at 60 °C.
All assays were repeated with highly reproducible results using RNA from different rats. The
level of target gene mRNA (egr-1) measured by threshold cycle number was compared to GAPDH, which is used as an internal control to correct variability in starting mRNA concentration. The fold changes of treated groups over control group were calculated.

*Reverse transcription-PCR.* RT-PCR was performed with a SuperScript™ one-step RT-PCR kit (Gibco, Invitrogen Corp., Carlsbad, CA). From each sample one µg of total RNA was amplified with the following primers (Sigma Genosys, Woodlands, TX) Egr-1: 5’-GAGCCGAGCGAACAACCCTACGAGCACCTG-3’ (sense) and 5’-GCGCCGAGGATGAAGAGGTCGGAGGATTGG-3’ (antisense), producing a 253-bp product; and internal control GAPDH 5’-ACCACAGTCCATGCCATCAC-3’ (sense) and 5’-TCCACCCACCCTGTTGCTGTA-3’ (antisense) producing a 452 bp fragment. The RT-PCR conditions were: 1 cycle at 50°C for 30 min and 94°C for 2 min; 30 cycles (95°C for 1 min, 58°C for 1 min and 72°C for 1 min). The PCR products were separated on 1.5 % agarose gels.

*Western blotting.* Equal amounts of proteins (25, 50 or 100 µg) which were extracted from duodenal, gastric or jejunal mucosa in a buffer containing protease and phosphatase inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), 40 µg/ml leupeptin, 40 µg/ml aprotinin, 20 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate) were subjected to 7.5, 10 or 12 % SDS-PAGE analysis. Proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, England) and then incubated for 1-3 hr at 20°C with rabbit polyclonal anti-Egr-1, ERK1, ERK2, FGF-2, PDGF-B, VEGF or mouse monoclonal anti-phospho-ERK1/2 (Tyr-204) antibodies, (Santa Cruz Biotech. Inc., Santa Cruz, CA, dilution 1:500). The loading controls were performed by using mouse monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EnCor Biotechnology Inc., Alachua, FL, dilution 1:2000). Blots were treated with secondary antibodies
and visualized using the ECL detection system (Amersham Biosciences, Buckinghamshire, England). The density of protein bands in Western blots was assessed by a digital imaging system, Eagle Eye II (Stratagene, Austin, Texas). Each experiment was repeated four times.

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

Results

Early increase of egr-1 mRNA and Egr-1 protein expressions 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 hr after administration of with cysteamine, respectively (Fig. 1A). The time-dependent increased expression of 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 ulcer we compared Egr-1 expression in the duodenal mucosa of rats treated with cysteamine or ethanolamine. These results demonstrated that the non-ulcerogenetic 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 hr and significantly increased after three doses of cysteamine at 12 hr. Enhanced bFGF expression was detected at the time of duodenal ulcer appearance 24-48 hr after cysteamine (Fig. 2A). The expression of PDGF and VEGF was enhanced soon after first dose of cysteamine, and seemed to peak at 12 hr after cysteamine (Fig. 2B,C).

Antisense egr-1 aggravated cysteamine-induced duodenal ulcers. To determine the role of Egr-1 in cysteamine-induced duodenal ulceration, rats were pretreated with saline, 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 small dose of antisense egr-1 did not alter the size of cysteamine-induced duodenal ulcer. Scrambled control oligodeoxynucleotides did not cause any change in duodenal ulcer appearance in cysteamine-treated rats in comparison with the control saline-pretreated animals, implicating no toxicity of the oligodeoxynucleotide. The larger antisense egr-1 dose aggravated duodenal ulcer formation and increased the size of ulcers from 8.1±1.8 to 20.7±4.0 mm² (p<0.01) (Fig. 3A,B,C). Histologic evaluation revealed mostly superficial ulcers in the proximal duodenum of rats treated with scrambled control and cysteamine, while deep ulcers penetrating into the Brunner glands, often with perforation, were seen in rats given cysteamine and pretreated with egr-1 antisense (Fig. 3C).

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

**Cysteamine activates ERK1/2 in the rat duodenal mucosa.** We measured ERK1/2 expression and activity in the same samples from duodenal mucosa of rats which were treated with only saline, and rats pretreated with saline, scrambled control oligo or antisense egr-1 before each cysteamine dose. Figure 4C demonstrates that the expression of the ERK1 (p44) and ERK2 (p42) kinases was not changed in rats treated with saline, or pretreated with saline, scrambled control or egr-1 antisense oligodeoxynucleotide before cysteamine. ERK activation was measured using a phosphospecific anti-ERK antibody, which specifically recognizes ERK after catalytic activation by phosphorylation (Tyr 204). The results demonstrated that cysteamine activated ERK1/2 kinases at 6, 12 and 24 hr in the duodenal mucosa of rats pretreated with saline or scrambled control in comparison to rats which were 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 compared to the saline or scrambled control pretreated animals (Fig. 4D).

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

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

Discussion

This study demonstrated for the first time that egr-1 antisense significantly aggravated the experimental duodenal ulcers and inhibited the expression of egr-1 mRNA, Egr-1 protein and bFGF, PDGF, VEGF synthesis in the rat duodenal mucosa. Ethanolamine, a non-ulcerogenic analog of cysteamine, did not alter Egr-1 expression in the duodenal mucosa. Thus, these are specific, duodenal ulcerogenic effects of cysteamine since these are not shared by the toxic analog ethanolamine, underlining a critical role of Egr-1 in duodenal ulceration. Furthermore, egr-1 antisense did not affect Egr-1 protein expression in the stomach or jejunum. Our results
also demonstrated that cysteamine activated ERK1/2 kinases without any changes in its protein expressions in the rat duodenal mucosa.

Egr-1 transcription factor belongs to hypoxia-inducible transcription factors, and recently we demonstrated that cysteamine induced hypoxia only in the proximal duodenum but not in the stomach (20). The cysteamine-induced Egr-1 transcriptional activity as we demonstrated previously (19) might result in increased expression of bFGF and PDGF in the duodenal mucosa, because the promoter region of bFGF and PDGF genes contain the Egr-1 binding site (3, 18). Our results show that cysteamine enhanced bFGF and PDGF synthesis in the pre-ulcerogenic stages, and the decreased Egr-1 expression in the duodenal mucosa might be the cause for the inhibition of synthesis of these growth factors. Recently we demonstrated that cysteamine enhanced the ability of HIF-1α to interact with Egr-1. The increase of HIF-1α binding to hypoxia responsive elements of promoter region of VEGF gene was shown in response to hypoxia (22). Cysteamine increased the expression and the transcriptional activity of HIF-1α (20), and enhanced the transcriptional interaction of HIF-1α with Egr-1. This increased interaction in the pre-ulcerogenic stages apparently might participate in mechanisms of enhanced VEGF expression in duodenal mucosa, e.g., the downregulation of Egr-1 expression and its interaction with HIF-1α 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 PD98059 indicating that MAPK ERK is involved in the regulation of the Egr-1 expression in renal cells (11). Hypoxia-reoxygenation induces ERK 1/2 phosphorylation, as well as transactivation of the transcription factors NFkappaB and Egr-1 in pulmonary artery endothelial cells (6). Hypoxia caused significant remodeling in the pulmonary artery characterized by thickening of pulmonary arterial wall and increases in tissue mass, total RNA, JNK, ERK, and p38 kinase tyrosine phosphorylations and their activities. Hypoxia also upregulated VEGF mRNA and PDGF receptor mRNA levels in 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 pre-ulcerogenic stage may participate in the increased Egr-1 expression and DNA binding activity, resulting in enhanced synthesis of growth factors in the pre-ulcerogenic duodenal mucosa. Our study also demonstrates that the inhibition of Egr-1 synthesis induced by antisense egr-1 leads to partial decrease in ERK phosphorylation at 6, 12 and 24 hr after cysteamine administration. Egr-1 might participate in the early increase of growth factor synthesis in the pre-ulcerogenic mucosa. Growth factors (e.g., PDGF, bFGF) activate ERK (26, 27, 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 delayed not only growth factor synthesis but also inhibited ERK phosphorylation induced by cysteamine.
Studies with antisense egr-1 oligodeoxynucleotides suggested that Egr-1 plays a key regulatory role in smooth muscle cell proliferation and repair following injury (33, 34). These findings demonstrate that inducible Egr-1 expression after injury is dependent on the release and paracrine action of growth factors. 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 following ultra-violet irradiation and egr-1 antisense reduced cell growth rate (13). Damage of IEC-6 cell monolayer resulted in rapid and significant increases in Fos and egr-1 mRNA levels, and direct interference with egr-1 by 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 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 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 was a potent cell survival factor and protected human neuroblastoma cells from NO-mediated apoptosis even without affecting cell proliferation (51).

The importance of growth factor synthesis in 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 in comparison with wild-type counterparts.

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

Grants

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References


Figure legends

Figure 1. egr-1 gene and protein expressions in the pre-ulcerogenic stages of cysteamine-induced duodenal ulceration in rats. (A) The increased expression of egr-1 mRNA after cysteamine performed by Real-Time RT-PCR. The level of target gene mRNA (egr-1) measured by threshold cycle number was normalized by GAPDH, which is used as an internal control to correct variability in starting mRNA concentration. The fold changes of treated groups over control group were calculated. (B) Time-dependent expression of Egr-1 protein in the duodenal mucosa after cysteamine and (C) after its toxic analog ethanolamine. GAPDH detection was used as additional control. Assays were repeated three times with highly reproducible results using RNA or protein from three different rats in one group.

Figure 2. Representative Western blotting of bFGF, PDGF and VEGF expressions in the rat duodenal mucosa after cysteamine. Time course of the expressions of bFGF (A), PDGF (B) and VEGF (C) in the proximal duodenal mucosa of control rats and rats after cysteamine treatment. The data depicted are the mean+S.E.M. of three rats in every group, *P<0.05.

Figure 3. Effect of egr-1 antisense oligodeoxynucleotide on cysteamine-induced duodenal ulcers in rats. (A) Gross appearance of duodenal ulcers 48 hr after cysteamine administration. Animals were pretreated with either saline (n=10), scrambled control (n=7) or antisense egr-1 (phosphothioate-modified) oligo, 0.2 mg/rat (n=10). (B) Light microscopy of duodenal ulcers in rats pretreated with either saline (showing superficial ulcer, left), scrambled control (similar ulcer size) or antisense egr-1 (phosphothioate-modified) oligo, 0.2 mg/rat before cysteamine (deep
ulcer, right), (hematoxylin and eosin staining). (C) The duodenal ulcer crater dimensions were measured in millimeters, and the ulcer areas were calculated using the ellipsoid formula. Size of duodenal ulcers, expressed as means ±SEM. ** P < 0.01.

**Figure 4.** Time-dependent expression of Egr-1 protein, ERK1 (p44) and ERK2 (p42) kinases and phosphorylated ERK1/2 in the duodenal mucosa of rats pretreated with saline, scrambled control oligo or antisense egr-1 before cysteamine in comparison with rats treated with saline only. (A) Representative Western blots of the time course of duodenal Egr-1 protein expression in saline-treated animals and in rats pretreated with saline, scrambled control or egr-1 antisense (0.2 mg/rat) 0.5 hr before cysteamine. (B) Quantified data of time-dependent Egr-1 protein expression in control duodenal mucosa (saline treatment only) or in duodenal mucosa of rats pretreated 0.5 hr before each dose of cysteamine with saline, scrambled control or egr-1 antisense (phosphothioate-modified) oligo, 0.2 mg/rat. Assays were repeated three times. The data depicted are the mean+S.E.M. of three rats in every group, *P<0.05. (C) Representative Western blots of ERK1 (p44) and ERK2 (p42) kinase in the same groups of animals. (D) Detection of ERK1/2 activation in pre-ulcerogenic duodenal mucosa of animals pretreated with saline, scrambled control oligo or egr-1 antisense before cysteamine in comparison with the rats treated with saline only. ERK activation was measured using a phosphospecific anti-ERK antibody, which specifically recognizes ERK after catalytic activation by phosphorylation (Tyr 204).

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

**Figure 6.** Representative Western blots of bFGF (A), PDGF (B) and VEGF (C) expressions in
the proximal duodenal mucosa 48 hr after administration of the duodenal ulcerogen cysteamine.
Animals were pretreated 0.5 hr before the each dose of cysteamine with saline, scrambled control
or antisense egr-1 (phosphothioate-modified) oligo, 0.02 or 0.2 mg/rat. The data depicted are the
mean+S.E.M. of three rats in every group, *P<0.05.
Fig. 1

Panel A: Graph showing egr-1 mRNA expression (fold change) over time with Cysteamine treatment. The x-axis represents time (Saline, 0.5, 12 hr) and the y-axis represents the fold change in egr-1 mRNA expression.

Panel B: Western blot analysis showing Egr-1 and GAPDH expression levels under different conditions. The conditions include Saline and Cysteamine treatment at 0.5, 2, 6, 12, and 24 hours.

Panel C: Western blot analysis showing Egr-1 and GAPDH expression levels under different conditions. The conditions include Saline and Ethanolamine treatment at 0.5, 2, 6, 12, and 24 hours.
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