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A New Mechanism of Gastric Epithelial Injury Induced by Acid Exposure: The Role of Egr-1 and ERK Signaling Pathways

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ABSTRACT

The molecular mechanisms by which gastric acid causes epithelial injury in the stomach and initiates an inflammatory reaction are poorly understood. We aimed in the present study to investigate the role of the early growth response gene Egr-1 and ERK in gastric epithelial cells following acid exposure, and the signaling pathways involved. Western blotting was used to assess Egr-1 protein levels in AGS cells. A quantitative measurement of acid-induced Egr-1 and ERK translocation was performed using a high content analysis approach. Egr-1 functionality was assessed by transient transfection with Egr-1 antisense oligonucleotide. Exposure of AGS cells to acidic conditions induced Egr-1 protein expression in a pH- and time-dependent manner. Egr-1 expression was markedly increased as the pH was reduced from pH 7.4 to 6.4. High content analysis of Egr-1 activation showed acid-induced Egr-1 nuclear translocation; a maximum observed at 1–2 h followed by a decline to basal levels beyond 4 h. Acidic pH also activated ERK1/2 phosphorylation, whereas ERK1/2 inhibitors PD98059 and U0216 blocked both acid-induced Egr-1 and ERK translocation and expression. Moreover, acid exposure up-regulated VEGF expression, which was inhibited by the Egr-1 antisense oligonucleotide. Our results also demonstrate that exposure to acid induces Egr-1 via MEK-ERK1/2 pathway. These data suggest that Egr-1 activation might play a crucial role in gastric mucosal inflammation and associated epithelial injury. J. Cell. Biochem. 108: 249–260, 2009.

KEY WORDS: ACID; EGR-1; ERK; HIGH CONTENT ANALYSIS; GASTRIC CELLS

The early growth response-1 (Egr-1) transcription factor is the prototype of a family of zinc finger transcription factors involved in the regulation of cell proliferation and differentiation and cell death [Milbrandt, 1987; Sukhatme et al., 1987; Lemaire et al., 1988; Silverman and Collins, 1999]. Egr-1 is inducible in a variety of cell types by a large number of growth factors, cyokines, and injurious stimuli [Lemaire et al., 1988; Sukhatme et al., 1988; Gashler and Sukhatme, 1995]. The target genes of Egr-1 include the cytokine TNF-α, adhesion molecules such as CD44 and ICAM-1, and growth factors and hormones such as PDGF-A/B, TGF-β, FGF-2, and LH-β [Christy and Nathans, 1989; McMahan and Monroe, 1996].

The fact that changes in extracellular pH can modulate gene transcription has been documented in previous studies. We have demonstrated that exposure of gastric epithelial cells to acidic conditions results in increased NF-κB and AP-1 DNA-binding activities [O'Toole et al., 2005]. Other investigators have shown that exposure of macrophages to external acidic environments leads to increased NF-κB binding activity and, further, that this activity was inhibited by treatment with the antioxidants pyrrolidine dithiocarbamate or *N*-acetyl-leucinyl-norleucinol [Bellocq et al., 1998]. Studies by Zabel et al. [1991] have demonstrated that NF-κB could form a complex with DNA within a large pH range. Furthermore, Yamaji et al. [1994] have shown that exposure of renal epithelial cells to acidic conditions leads to transcriptional activation of immediate early genes such as c-Fos and c-Jun. These data point to the importance of the acidic environment as one of the possible factors involved in mucosal inflammation and epithelial injury.

There is clear evidence that gastric acid is involved in gastrointestinal epithelial injury. Recently, Duggan et al. [2006] demonstrated that exposure of esophageal cells to low pH resulted in the co-ordinate expression of multiple genes, including Egr-1. Furthermore, exposure of gastric epithelial cells to *Helicobacter pylori* also induced Egr-1 expression [Abdel-Latif et al., 2004].

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Accumulating evidence suggests that Egr-1 regulates diverse cellular processes during the inflammatory response and in the response to gastric infection. [Christy and Nathans, 1989; McMahan and Monroe, 1996].

The aims of the present study were firstly to investigate the effect of acid exposure on Egr-1 and associated signaling pathways in gastric epithelial cells, and secondly to evaluate the role of acid exposure on ERK1/2, molecules known to be involved in Egr-1 mediated signaling events. We chose to use a high content analysis (HCA) approach to study and quantify the activation and subcellular distribution of Egr-1 and ERK in gastric epithelial cells in response to acid exposure. The HCA combines automated microscopy and image analysis to quantify complex cellular events such as transcription factor translocation, proliferation and apoptosis accurately and quickly in multiple cells at the cell population level. The data that are generated by HCA provides a powerful robust tool for measuring multiple cellular responses that can be hampered by cellular population diversity or below average responses in lumen cell systems. We demonstrate here that acid exposure activates Egr-1 and ERK1/2 in gastric epithelial cells via the MEK-ERK1/2 pathway. Furthermore, we established that acid-induced VEGF expression is Egr-1 dependent.

MATERIALS AND METHODS

MATERIALS

The human gastric cancer cell line AGS was obtained from the European Collection of Animal Cell Cultures (ECACCs, Porton Down, Salisbury, UK). Antibodies to Egr-1, p-ERK1/2, ERK1/2, and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated secondary antibody and Hochest 34442 were from Cellomics, Inc. (Pittsburgh, PA). The p38 MAPK inhibitor SB203580 and the MEK1/2 MAPK inhibitors PD98059 and U0126 were purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO). All tissue culture media and reagents were obtained from GIBCO BRL (Life Technologies, Renfrewshire, Paisley, Scotland).

CELL CULTURE TREATMENT

AGS cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM L-glutamine at 37°C in an atmosphere containing 5% CO_2. For experiments, AGS cells were seeded at a density of 5×10^5 cells/ml medium in 6-well plates and grown to ~80% confluence prior to experiments. The pH of the culture medium was adjusted to the required pH by the addition of 0.1 M HCl. Induction of Egr-1 protein expression was observed in the pH range 7.0–6.0 in the AGS cell line. Routinely, experiments were conducted at pH 6.8, which falls within the induction pH range for Egr-1 protein. The cells were examined for morphological changes under phase contrast microscopy. The colonic epithelial cell lines T84 and HCT116 and the breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the ECACC. The esophageal epithelial cells SKGT-4 were a gift from Dr. David S. Schrump (Thoracic Oncology Section, Surgery Branch, National Cancer Institute, NIH, Bethesda, MD).

PREPARATION OF WHOLE CELL EXTRACTS

AGS cells were harvested by scraping and subsequently washed in ice-cold PBS and collected by centrifugation (1,400 rpm, 5 min). The pellet of cells was resuspended in lysis buffer containing 50 mM Tris (pH 6.8), 2% (w/v) SDS, 0.1 mM PMSF, leupeptin (10 μg/ml), 5% (v/v) 2-mercaptoethanol, 0.1% (v/v) bromophenol blue, and 10% (w/v) glycerol, and then solubilized by boiling for 5 min. The cell extracts were used immediately or stored at −70°C until required. The protein concentration of nuclear extracts was determined by the dye-binding method of Bradford [1976].

SUBCELLULAR FRACTIONATION OF AGS CELLS

AGS cells were harvested and washed in ice-cold PBS. Cell pellets were collected by centrifugation (1,400 rpm, 5 min) and cytoplasmic and nuclear extract fractions were prepared. Both cytoplasmic and nuclear extracts were prepared using the Active Motif Nuclear Extract Kit (Active Motif, Risensart, Belgium) according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS

Equivalent amounts of total cell extracts (50 μg protein) were resolved by 10% SDS–PAGE as described by Laemmli [1970]. Proteins were electrotransferred onto PVDF membrane using a semidry blotting apparatus (Atto). Blots were blocked with 5% (w/v) dried skim milk in PBS for 1 h at room temperature (RT) and then incubated for 1 h at RT with specific primary antibodies (at a dilution 1:1,000). Blots were then incubated with the relevant horseradish peroxidase conjugated secondary antibodies (at a dilution 1:2,000) for 1 h at RT. Immunodetection was performed by enhanced chemiluminescence.

EGR-1/ERK STAINING FOR HIGH CONTENT ANALYSIS

AGS cells (5×10^4 cells/ml) grown in 200 μl in Packard 96 view microplates were incubated in media of different pH values ranging from pH 7.4 to 3.0 for different periods of time. At the end of treatment, the cells were stained Egr-1 or ERK and the cells were scanned in the Cellomics KineticScan HCS Reader and subjected to image analysis. Briefly, the cells were fixed with 100 μl of 4% formaldehyde in phosphate-buffered saline for 10 min at RT, permeabilized with 100 μl of 0.1% Triton X-100 in phosphate-buffered saline for 90 s at RT, and then washed twice with 200 μl of 0.1 M Tris–HCl buffer, pH 7.8. The cells were then incubated for 1 h with 50 μl of anti-Egr-1 or anti-ERK antibody diluted 1:250 in Tris 0.1 M phosphate buffer, pH 7.8. The cells were aspirated and 100 μl of 1× detergent solution was added to each well for 15 min, and washed three times in Tris wash buffer. The cells were then incubated with 50 μl of staining solution containing Alexa Fluor 488-conjugated secondary antibody and Hochest 34442 (Cellomics, Inc.) for 1 h at RT in the dark. The cells were aspirated and 100 μl of 1× detergent solution was added to each well for 15 min, and the cells were washed three times, stored in 200 μl of 0.1 M Tris wash buffer. The plates were scanned in the Cellomics KineticScan HCS Reader.

The system is equipped with emission and excitation filters for selectively imaging fluorescent signals emitted by Hochest

34442 and Alexa Fluor 488, a CCD camera with frame grabber, and a Pentium PC computer and applications software. Data are expressed as the mean ± standard deviation (SD). The nuclear translocation of Egr-1 or ERK in AGS cells was examined using the Cytoplasm to Nucleus Translocation BioApplication. The nuclear boundary detection was performed as described previously [Ding et al., 1998]. Translocation events are measured by the relative distribution of target fluorescence intensities between the cytoplasmic region and the nuclear region of a cell. The difference in fluorescent intensities between the cytoplasmic and nuclear regions is analyzed and indicates the magnitude of translocation from the cytoplasm to the nucleus. The most used parameters in the analysis are mean nuclear intensity (NucInten), mean cytoplasmic intensity (CytInten), and mean cytoplasmic nuclear difference intensity (CytNucDiff).

TRANSIENT TRANSFECTION AND EGR-1 ANTISENSE OLIGONUCLEOTIDE

The Egr-1 antisense oligonucleotide plasmid was designed and prepared as described previously [Du et al., 2000]. The insert of the antisense oligonucleotide directed against nucleotides 131–149 and 2115–2131 of human Egr-1 was subcloned into pcDNA3.1+/zeo at the *Hin*dIII and *Xba*I sites. For control experiments, the sense oligonucleotide with a similar sequence but in the opposite orientation relative to the promoter was used. AGS cells were seeded in 24-well plates at a density of 5×10^5 cells/ml medium and grown to ~80% confluence. The cells were transfected with 5 μg Egr-1 antisense oligonucleotide or Egr-1 sense using GenePORTER. The transfected cells were allowed to recover overnight and then incubated for 8 h at pH 6.8. After the incubation, total cell extracts were prepared and analyzed for VEGF protein levels by Western blotting. To measure transfection efficiency, 1 μg of pSV-β-galactosidase control vector (Promega Corp., Madison, WI) was simultaneously transfected into the cells and β-galactosidase activity was measured.

MORPHOLOGICAL AND CELL PROLIFERATION STUDIES

AGS cells were plated into 96-well plates at 5×10^4 cells per well overnight at 37°C. The cells were incubated in media of different low pH values ranging from 7.4 to 3.0 for 1 and 24 h at 37°C. The cells were examined for morphological changes under phase contrast microscopy and images were captured using Leica DC-100 colour digital camera. Cell proliferation was assessed using the MTT assay (ATCC, VA). AGS cells were also incubated at pH 6.8 for 24 h at 37°C. For inhibition experiments, AGS cells were pre-treated with the ERK1/2 inhibitors PD98059 (50 μM) and U0216 (10 μM) or the VEGFR inhibitor SU5416 (10 μM) for 30 min followed by incubation at pH 6.8 for 24 h at 37°C. At the end of the treatment, 10 μl of MTT reagent was added to each well and the plates were incubated for 4 h at 37°C, followed by 100 μl of detergent reagent for 4 h in the dark. The absorbance was read at 590 nm using a microplate spectorfluorometer reader (Molecular Devices, CA). The results are presented as mean ± SD.

RESULTS

ACID EXPOSURE INDUCES EGR-1 IN GASTRIC EPITHELIAL CELLS

In order to examine the effect of acidic pH on Egr-1 activation in gastric epithelial cells, AGS cells were incubated in media of different pH ranging from 7.4 to 3.0. The pH of the culture medium was decreased from pH 7.4 to 3.0 by the addition of 0.1 M HCl. As the pH decreased from 7.4 to 6.0, induction of Egr-1 expression was observed (Fig. 1A). Greater Egr-1 protein levels were seen at pH 6.8 (Fig. 1B). At pH values <5, Egr-1 expression levels could not be reliably established, whereas the cell viability was significantly compromised, as assessed microscopically and by acridine orange/ethidium bromide staining. The cell death caused by incubating AGS cells at very low pH may explain the lack of Egr-1 activation in gastric AGS cells at pH values ≤5. In order to determine the kinetics of Egr-1 expression by acid, AGS cells were incubated in medium at pH 6.8 for different time intervals ranging from 15 to 240 min and total cell extracts were examined for Egr-1 protein expression by Western blotting. Figure 1C shows that incubation of AGS cells at pH 6.8 resulted in a rapid and transient induction of Egr-1 expression. Immunofluorescence staining of intact AGS cells incubated at pH 7.4 showed largely a predominantly cytoplasmic distribution of Egr-1 (Fig. 1D, upper panel). Following incubation at pH 6.8, large amounts of Egr-1 were found in the nuclei, as demonstrated in Figure 1D (lower panel).

HIGH CONTENT ANALYSIS OF EGR-1 ACTIVATION

To quantify the translocation of Egr-1 in AGS in response to acid, a Cellomics KineticScan HCS Reader was used to measure fluorescence separately both in the cytoplasm and in the nucleus. As seen from Figure 2A,B, upon stimulation, the cytoplasmic Egr-1 staining was notably reduced and nuclear staining increased. The mean cytoplasmic, nuclear staining, and cytoplasmic nuclear difference (CytNucDiff) was shown in five representative un-stimulated (Fig. 2A,C) and acid-stimulated AGS cells (Fig. 2B,D). The cytoplasmic Egr-1 staining in control (intact) AGS cells was decreased by 50% and the translocation of Egr-1 to the nucleus was increased by ~75%. The overall mean cytoplasmic nuclear difference showed ~1.5-fold increase in Egr-1 activation.

KINETICS OF ACID-INDUCED EGR-1 TRANSLOCATION

To quantify the time course of Egr-1 activation-associated translocation, AGS cells were incubated at pH 6.8 for 15–240 min and Egr-1 translocation analyzed by high content image analysis. Figure 3 (panel A) shows images of fluorescently stained Egr-1 after exposure of AGS cells to pH 6.8 over the time course of the experiment. CytNucDiff showed maximal Egr-1 translocation at 1–2 h and yielded a more sensitive signal than cytoplasmic or nuclear intensity (Fig. 3B, a–c). Figure 3C demonstrates the subcellular fractionation of the cytoplasmic and nuclear protein levels of Egr-1 in AGS cells after incubation at pH 6.8 from 15 to 240 min.

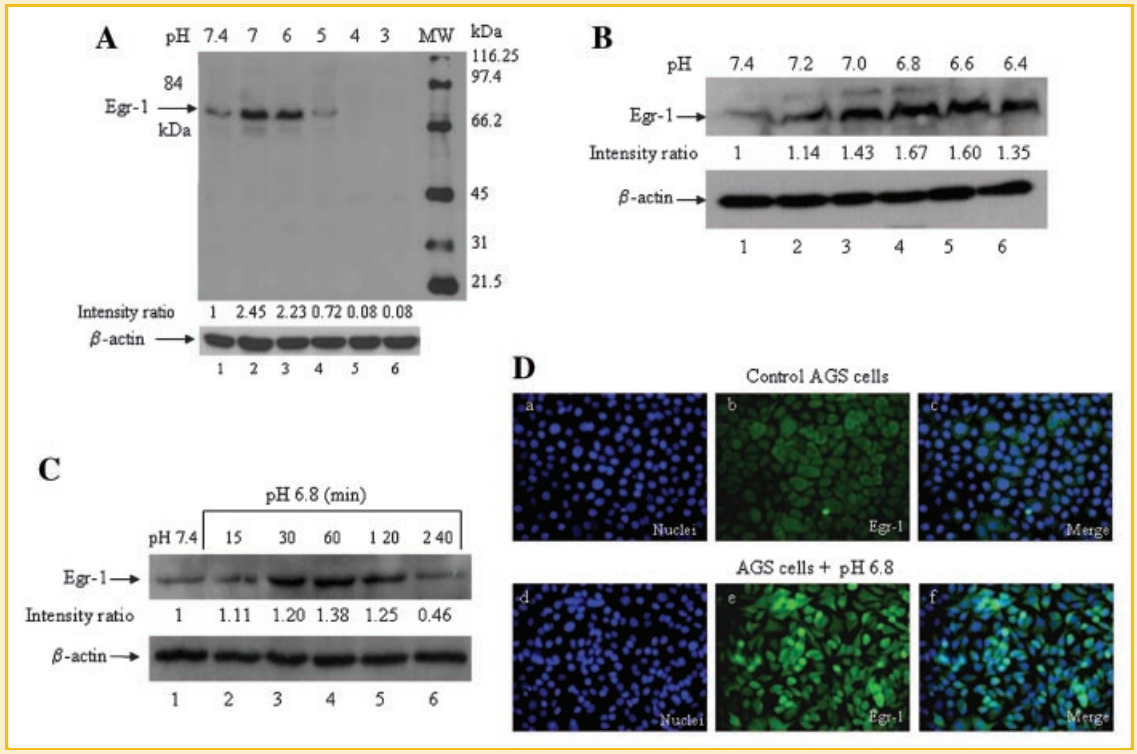

Fig. 1. A: Effect of acid exposure on Egr-1 expression. AGS cells were incubated in media titrated from pH 7.4 to 3.0 for 1 h with 1.0 pH step intervals. Equal amounts of protein in total cell extracts (50 μg) were separated by 10% polyacrylamide gel electrophoresis and transferred onto PVDF membrane followed by Western blot analysis using anti-Egr-1 antibody. MW denotes the molecular weight marker. B: Effect of decremental changes in pH on Egr-1 expression. AGS cells were incubated in media of different pH ranging from pH 7.4 to 6.4 for 1 h. C: Kinetics of Egr-1 induction by acid in AGS cells. AGS cells were incubated at pH 6.8 for different periods of time (0–4 h). β-Actin was used as a loading control. The intensity of Western blot bands was quantified by densitometry and expressed as fold increase over control levels (beneath each lane). The experiments were performed three times and a representative blot is shown. D: Immunofluorescent microscopy showing acid-induced Egr-1 nuclear translocation. Upper panel, control (intact) AGS cells. Lower panel, AGS cells incubated at pH 6.8 for 1 h. Green: Egr-1 staining, blue: nuclei. Magnification is 40×.

ACID INDUCES EGR-1 ACTIVATION IN NON-GASTRIC CANCER CELLS

The effect of acidic conditions on Egr-1 expression was further verified in other cell lines, including colonic epithelial cells T-84 and HCT116, esophageal epithelial cells SKGT-4, and breast cancer cells MCF-7 and MDA-MB-231. The cells were incubated at pH 6.8 for 1 h, and Egr-1 expression was analyzed by immunoblotting and HCA assay. Acid exposure clearly induced Egr-1 expression in all the non-gastric cells examined, as shown by immunoblotting (Fig. 4A) and HCA data (Fig. 4B).

ACID EXPOSURE INDUCES ERK ACTIVATION IN AGS CELLS

Since the extracellular signal-regulated kinases play a critical role in Egr-1 signaling [Guha et al., 2001; Bea et al., 2003; De Sousa et al., 2005], we tested the effect of acid on ERK1/2 (p44/p42) activation by immunoblotting. AGS cells were incubated at pH 6.8 for different periods of time and total cellular extracts were prepared and analyzed for ERK activation. Acidic pH induced ERK1/2 phosphorylation in AGS cells in a time-dependent manner with a peak at 20–30 min and declined to basal levels thereafter (Fig. 5A). Because ERK1/2 are phosphorylated and translocated into the nucleus upon activation, these proteins were studied both at the expression levels of the non-phosphorylated and phosphorylated forms. There were no changes in the non-phosphorylated forms of ERK1/2 (Fig. 5A).

The temporal nature of translocation of ERK from the cytoplasm to the nucleus in AGS cells incubated under acidic conditions was also examined by high content image analysis. Under resting conditions, ERK was clearly absent from the nucleus and predominately distributed throughout the cytoplasm as demonstrated by immunofluorescent imaging (data not shown). The HCA assay clearly demonstrated that upon acid stimulation, a rapid but transient translocation of ERK to the nucleus was observed (Fig. 5B). The subcellular fractionation of the cytoplasmic and nuclear levels of p-ERK in AGS cells after incubation at pH 6.8 from 15 to 240 min are shown in Figure 3C.

To investigate the possible role of the MAPK pathway in Egr-1 signaling in response to acidic pH, AGS were pre-incubated with the p38 inhibitor SB203580 and the ERK1/2 inhibitors PD98059 and U0216 for 30 min followed by 1 h incubation at pH 6.8. The ERK inhibitors PD98059 and U0216 inhibited pH-induced Egr-1 activation (Fig. 5D). Similarly, MAPK inhibitors were also tested for their effects on acid-induced Egr-1 translocation in AGS cells using the HCA technique. The ERK1/2 inhibitors PD98059 and

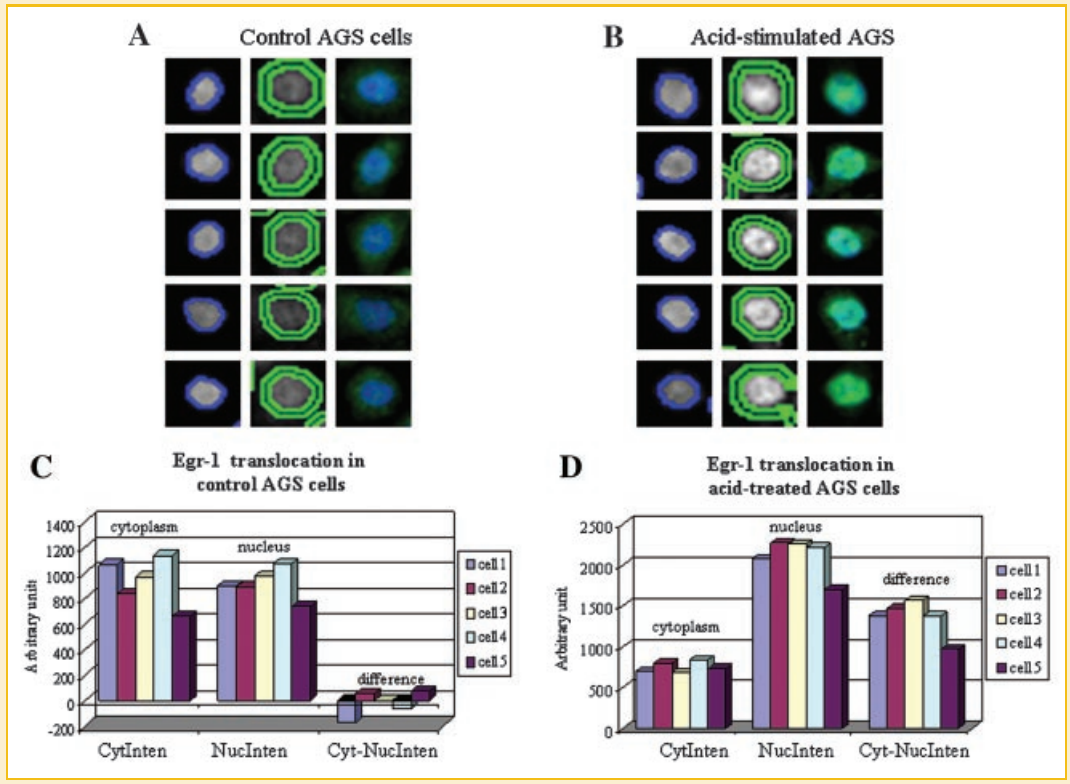

Fig. 2. Schematic diagram showing changes in Egr-1 fluorescence in un-stimulated and acid-stimulated AGS cells taken from Cellomics KineticScan HCA Reader. Five un-stimulated AGS cells and five acid-stimulated cells were analyzed to determine the mean amount of Egr-1 fluorescence in the nucleus and the cytoplasm (A,B). The eroded nuclear ring (blue) together with the cytoplasmic and original nuclear boundaries (green) used to denote the cytoplasmic region for analysis are shown. The same cells as in (A,B) were used for the analysis of Egr-1 fluorescence and the cytoplasmic nuclear difference (CytNucDiff) was determined and plotted (C,D).

U0216 inhibited acid-induced Egr-1 translocation but SB203580 had no effect on Egr-1 activation (Fig. 5E). Furthermore, both PD98059 and U0216 inhibited acid-induced ERK phosphorylation, as demonstrated by immunoblotting (Fig. 5F). These data were further confirmed by the HCA of the inhibitor-treated AGS cells (Fig. 5G).

ACID EXPOSURE AND VEGF REGULATION

As seen from Figure 6A, incubation of AGS cells at pH 6.8 significantly up-regulated VEGF protein expression. We have therefore tested the role of Egr-1 in regulating VEGF expression, as a downstream target for Egr-1. With this purpose, AGS cells were transfected with the Egr-1 antisense (Egr-1/PcDNA3.1+/Zeo) or the Egr-1 sense oligonucleotides, and cell extracts were prepared and analyzed for VEGF expression by immunoblotting. Transfection of AGS cells with the Egr-1 antisense oligonucleotide almost completely inhibited acid-induced VEGF protein expression (Fig. 6B). To further confirm that the ERK1/2 pathway is involved in the acid-induced VEGF expression; we tested whether the induction of VEGF inhibited by the ERK1/2 inhibitors, PD98059 and U0126. Western blot analysis showed that the induction of VEGF by acidic pH was inhibited by pre-treatment with the ERK1/2 inhibitors (Fig. 6C).

MORPHOLOGICAL AND CELL PROLIFERATION STUDIES

Microscopic examinations of AGS cells incubated in media of different pH demonstrated dramatic morphological changes associated with cytotoxicity, particularly at pH $\leq$ 6 (Fig. 7, panel A). At pH values <6, AGS cells became detached and rounded. Additionally, AGS cell proliferation following incubation in media of different pH (pH 7.4–3.0) over 24 h was markedly reduced at pH < 7.4 (Fig. 7B). Moreover, cell growth was also decreased following a 24 h incubation at pH 6.8 (pH value used in most of the experiments) compared to control cells incubated at pH 7.4 (Fig. 7C). Further, we assessed the involvement of ERK1/2 and VEGFR inhibition on acid-induced cell growth suppression using the ERK inhibitors PD098059 (50 μM), and U0126 (10 μM) or the VEGFR inhibitor SU5416 (10 μM). Pretreatment of AGS cells with the ERK1/2 inhibitors, PD98059 (50 μM) and U0126 (10 μM) or the VEGFR inhibitor SU5416 (10 μM) enhanced cell growth suppression following incubation at pH 6.8 for 24 h (Fig. 7D).

DISCUSSION

The role of gastric acid in causing gastric inflammation and mucosal injury is well documented. However, the molecular mechanisms involved in acid-induced inflammation and epithelial cell injury are unclear. The present study demonstrates for the first time that

exposure of gastric cells to acidic conditions induced the expression of the early growth response (Egr-1) transcription factor with downstream induction of VEGF. We have also shown that acid exposure induces phosphorylation of ERK1/2 and inhibition of MEK-ERK1/2 suppressed acid-induced Egr-1 activation.

We have developed a novel quantitative method for determining Egr-1 and ERK activation in gastric cells in response to acid using a HCA approach for Egr-1 and ERK translocation between the cytoplasm and the nucleus in intact cells. The present study uses the highly informative and unbiased HCA method to quantify Egr-1 and ERK activation in gastric cells by measuring the immunofluorescently labeled Egr-1 and phosphorylated ERK in both cytoplasm as well as the nucleus at a single cell population as well as a whole well population. Cellular analysis of Egr-1

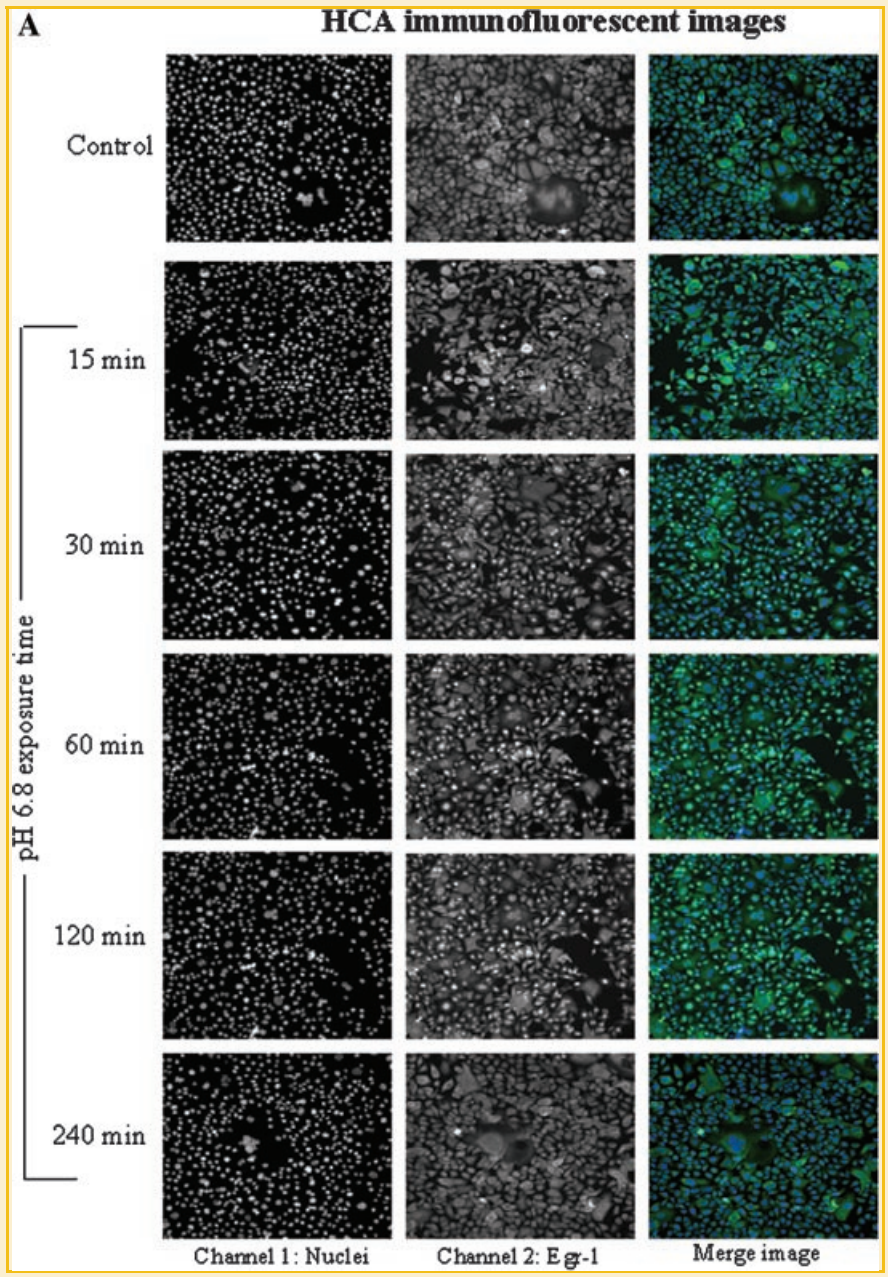

Fig. 3. Time course of Egr-1 nuclear translocation. A: Immunofluorescent images of Egr-1 translocation taken from Cellomics KineticScan HCA reader. AGS cells were incubated at pH 6.8 for the indicated times at 37°C. The cells were fixed and stained with anti-Egr-1 antibody and Hoechst 33342 and analyzed by the Cellomics HCA Reader. B: HCA graphical representation showing nuclear (a), cytoplasmic (b), and cytoplasmic nuclear difference (c) distribution profiles of Egr-1 translocation. C: Subcellular fractionation of AGS cells and Egr-1 protein levels. AGS cells were incubated in media of pH 6.8 for different periods of time (0–4 h), then the cytoplasmic and nuclear fractions were examined for Egr-1 levels by immunoblotting. The experiment was performed three times and a representative blot is shown with the level of expression quantified by densitometry.

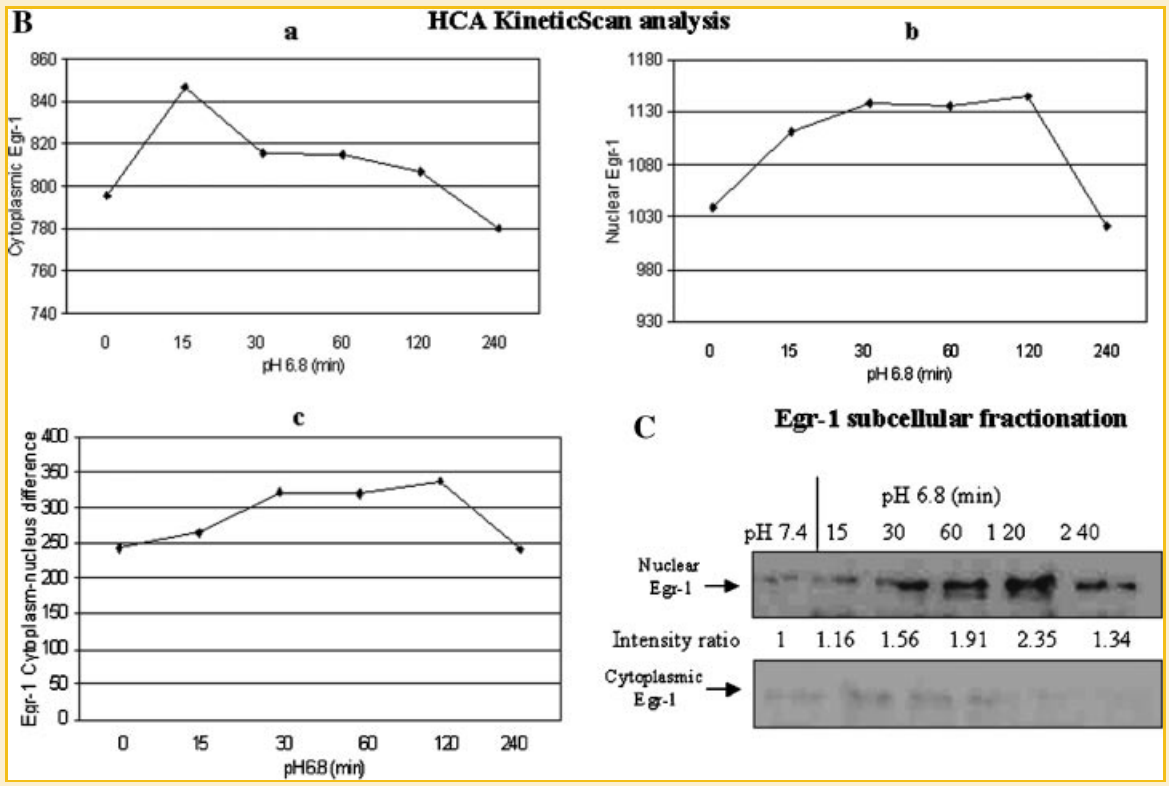

Fig. 3. (*Continued*)

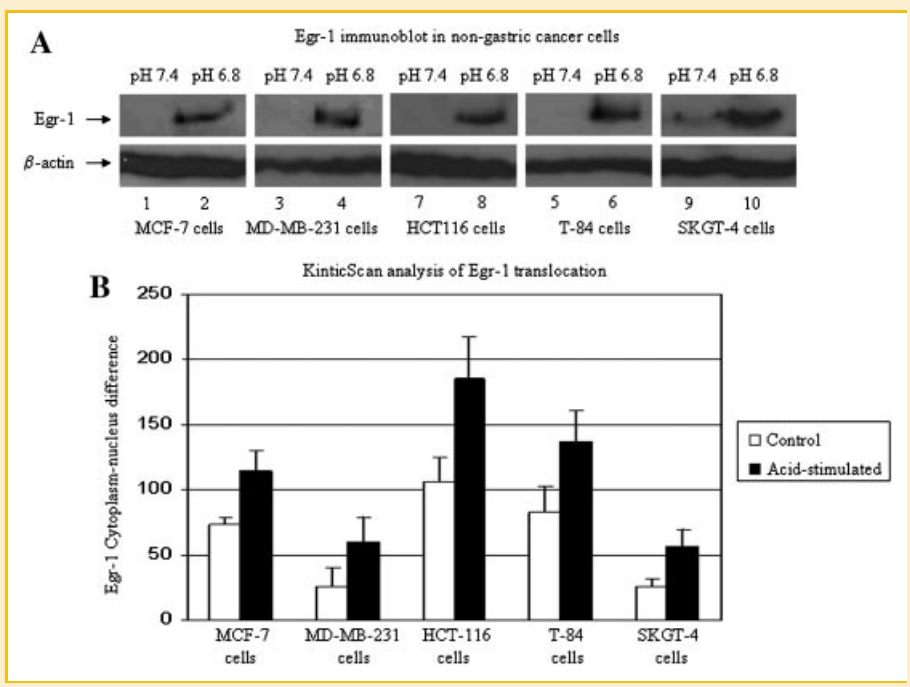

Fig. 4. The effect of acid on Egr-1 activation in non-gastric cells. Colonic epithelial cells T-84 and HCT116, esophageal epithelial cells SKGT-4, and breast cancer cells MCF-7 and MDA-MB-231 were incubated at pH 6.8 for 1 h or left un-treated. Egr-1 expression was analyzed by immunoblotting (A) and high content analysis (B). A representative Western blot and graph of the cytoplasmic nuclear difference is shown. The blot was probed with anti-β-actin to check for equal protein loading.

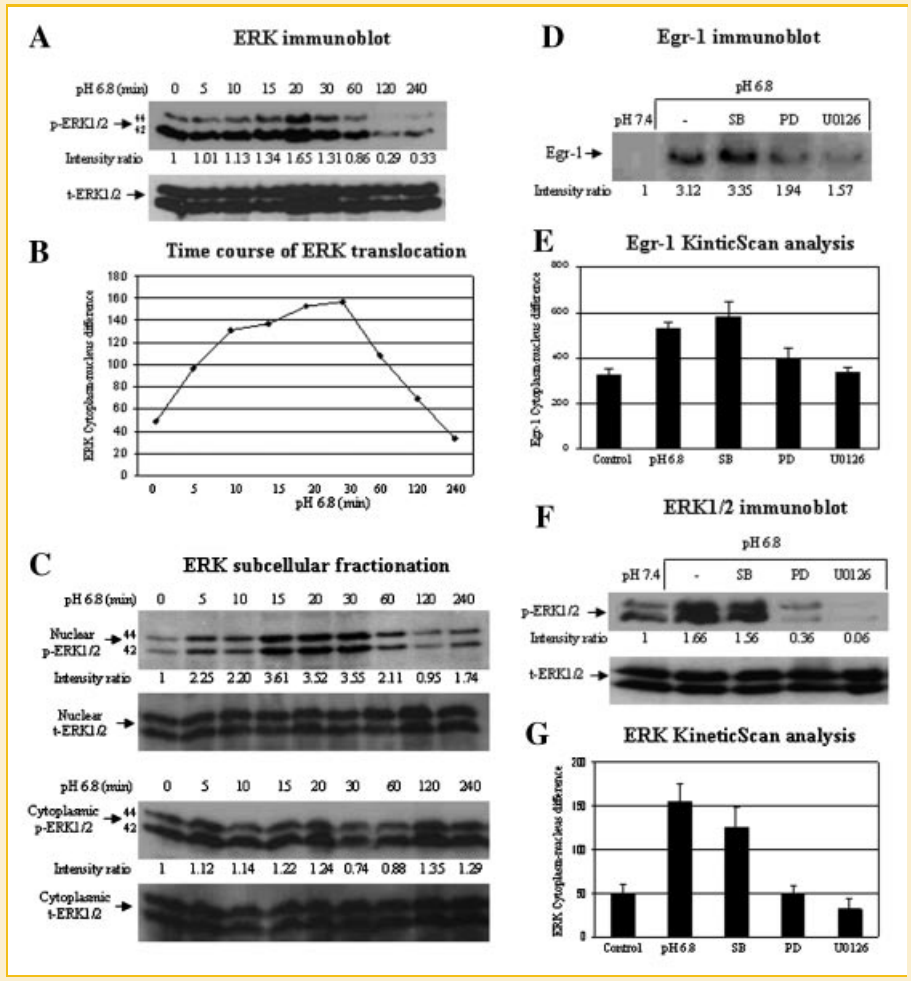

Fig. 5. Acid induces ERK activation in AGS cells. A: Immunoblotting analysis of ERK activation. The activity of phosphorylated ERK1/2 protein expression was analyzed by Western blotting on total cellular extracts using anti-phospho-ERK1/2 antibody. Total ERK1/2 was used as a loading control. B: Time course analysis of ERK translocation. Graphical representation of the Cellomics kineticScan data showing cytoplasmic nuclear difference of ERK translocation. C: Subcellular fractionation of AGS cells and p-ERK protein levels. AGS cells were incubated in media of pH 6.8 for different periods of time (0–4 h), and then the cytoplasmic and nuclear fractions were examined for p-ERK levels by immunoblotting. Total ERK1/2 was used as a loading control. The experiment was performed three times and a representative blot is shown. D: Effect of MAPK inhibitors on acid-induced Egr-1. AGS cells were pre-incubated for 30 min with the MAPK inhibitors SB203580 (10 μM), PD98059 (20 μM), and U0216 (10 μM) under resting conditions prior to subsequent 1 h incubation at pH 6.8. Total cellular extracts were prepared and analyzed by Western blotting for Egr-1 expression. A representative gel is shown of three independent experiments with similar results. β-Actin was used as a loading control. E: Inhibition of Egr-1 translocation by MAPK inhibitors. AGS cells were treated as described above prior to image acquisition and HCA analysis to determine Egr-1 nuclear translocation. The cytoplasmic nuclear difference is shown. F: Immunoblotting of ERK inhibition. AGS cells were pre-incubated for 30 min with the MAP kinase inhibitors SB203580 (10 μM), PD98059 (50 μM), and U0216 (10 μM) prior to incubating the cells at pH 6.8 for an additional 30 min. The activity of phosphorylated ERK1/2 protein expression was analyzed by Western blotting on total cellular extracts using anti-phospho-ERK1/2 antibody. Total ERK1/2 was used as a loading control. G: Inhibition of ERK translocation. AGS cells were treated as described above prior to image acquisition and analysis to determine ERK nuclear translocation. Image analysis of the cells was performed and the cytoplasmic nuclear difference is shown. Densitometry was used to quantify the amount of protein in each lane where appropriate.

translocation showed that exposure of gastric cells to acid induced Egr-1 translocation in a time- and pH-dependent manner. Maximal Egr-1 translocation was observed at 1–2 h. This fast activation highlights the potential impact of acidic conditions on gastric epithelium. A decline in Egr-1 activation to basal levels beyond 4 h likely reflects the cell adaptation mechanisms following exposure of gastric cells to acidic conditions. Image analysis of fluorescently labeled ERK demonstrated a rapid accumulation of p-ERK in the nucleus at 5 min; peaked at 30 min before falling to basal levels by 60 min.

Egr-1 is inducibly expressed in a variety of cell types by diverse stimuli, and is functionally implicated in the regulation of a wide range of cellular effects including the inflammatory and immune responses [Sukhatme, 1990; Gashler and Sukhatme, 1995; McMahan and Monroe, 1996]. The biological function of Egr-1 has been linked to multistage carcinogenesis, in malignancies such as prostate cancer [Eid et al., 1998]. Tumor progression is impaired in transgenic mouse models of prostate cancer lacking Egr-1 [Abdulkadir et al., 2001]. A role for Egr-1 has also been proposed during multistage carcinogenesis in mouse skin [Riggs et al., 2000].

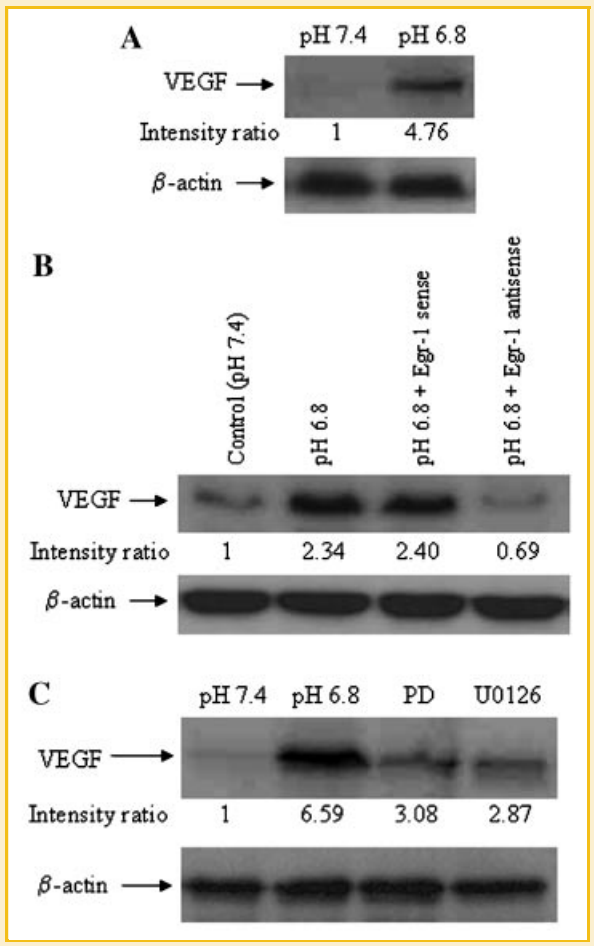

Fig. 6. A: Acid induces VEGF expression in AGS cells. AGS cells were incubated at pH 6.8 for 1 h and total cellular extract were analyzed by Western blotting for VEGF expression using anti-VEGF antibody. B: Egr-1 antisense oligonucleotide inhibits acid-induced VEGF expression AGS cells were transfected with Egr-1 antisense oligonucleotide or the control vector (PcDNA3.1+/Zeo) for 48 h and following incubation of AGS cells at pH 6.8, total cell lysates were prepared and analyzed for VEGF expression. C: ERK1/2 inhibitors block acid-induced VEGF up-regulation. AGS cells were pre-treated with PD98059 (50 μM) or U0126 (10 μM) for 1 h, and then incubated at pH 6.8 for 1 h. Total cell extracts were prepared for Western blot analysis. The representative result of three independent experiments with similar results is presented. Densitometry was used to quantify the amount of protein in each lane where appropriate. β-Actin was used as a loading control.

Furthermore, a role for Egr-1 in esophageal tumorigenesis has been demonstrated in microarray studies showing up-regulation of Egr-1 mRNA and Egr-1 protein in esophageal epithelial cells in response to acid [Duggan et al., 2006]. We have earlier shown that in vitro infection of gastric epithelial cells with *H. pylori* induces Egr-1 and this transcription factor plays a critical role in regulating *H. pylori*-induced adhesion molecule and apoptotic protein expression [Abdel-Latif et al., 2004].

In an attempt to define the signaling pathways regulating acid-induced Egr-1 activation in gastric epithelial cells, the effects of acidic conditions on ERK activation were examined. Upon activation, ERK1/2, a key molecule for many cellular responses, translocates into the cell nucleus, where it triggers the Egr-1 transcription factor, and subsequently modulates gene expression. We show here that exposure of gastric epithelial cells to acidic conditions induced phosphorylation of ERK1/2, and that the ERK1/2 pathway is involved in acid-induced Egr-1 activation. Exposure of AGS cells to acid resulted in transient (peak at ~20–30 min) activation of ERK1/2. The ERK1/2-dependent regulation of Egr-1 by acid was in agreement with the results of several studies investigating the signaling pathways mediating Egr-1 activation in cell culture models, which established a critical role for the ERK1/2 pathway in Egr-1 signaling [Christy and Nathans, 1989; Guha et al., 2001; Bea et al., 2003; Abdel-Latif et al., 2004; De Sousa et al., 2005]. In agreement with these data, we found the MEK-ERK1/2 inhibitors PD08959 and U0126 inhibited Egr-1 protein production and nuclear translocation; whereas the p38 inhibitor SB203580 had no effect.

Our findings highlight that even minor changes in pH can have profound effects on gene expression and cell phenotype. Furthermore, acid-induced transcription factor activation was found not to be restricted exclusively to gastric cells. Exposure of colonic, esophageal, and breast cancer cells to reduced pH also resulted in induction of Egr-1 expression. There is increasing evidence indicating that an acidic environment contributes to the regulation of expression of many genes during the inflammatory process. Previously, we demonstrated that acid induces NF-κB and AP-1 binding activities in gastric epithelial cells and this induction was mediated through MEK1-ERK1/2 dependent signaling pathways [O'Toole et al., 2005]. In this study, we have demonstrated that lowering the extracellular pH from 7.4 (resting pH of the medium) to pH 6.0 activates Egr-1 transcription factor in AGS cells. In addition, exposure of AGS cells to media of pH $\leq$ 6.0 resulted in rapid cell damage with a concomitant reduction in Egr-1 activation. Furthermore, there was a marked decrease in cell proliferation under acidic conditions. Contrary to that, the proportion of non-viable cells was not significantly altered when the cells were incubated at pH 6.8 for 1 h (the pH value at which the experiments were conducted). Thus, the activation of Egr-1 under acidic conditions may be a common theme in the course of inflammatory responses and subsequent carcinogenesis in a variety of disease states.

The extracellular microenvironment of tumors differs from that of most normal tissues. Several studies showed that the factors most influential in the control of the gene expression are those associated with tumor microenvironment [Boyer and Tannock, 1992; Lee and Tannock, 1998]. Many tumors have a relatively acidic extracellular pH, although the intracellular pH of tumor cells remains normal due to the efficient maintenance of a large proton gradient across the membrane. O'Toole et al. [2005] demonstrated that AGS cells exposed to acidic conditions can regulate their intracellular pH. It is known that the stomach is protected from the deleterious effect of gastric acid by several mechanisms [Bhaskar et al., 1992]. Under certain circumstances, gastric acid causes mucosal inflammation and tissue injury due to impairment of the mucosal defense mechanisms. However, the mechanisms involved in acid-induced inflammation and mucosal injury are unclear. Our current efforts are focused on gene regulation and cellular

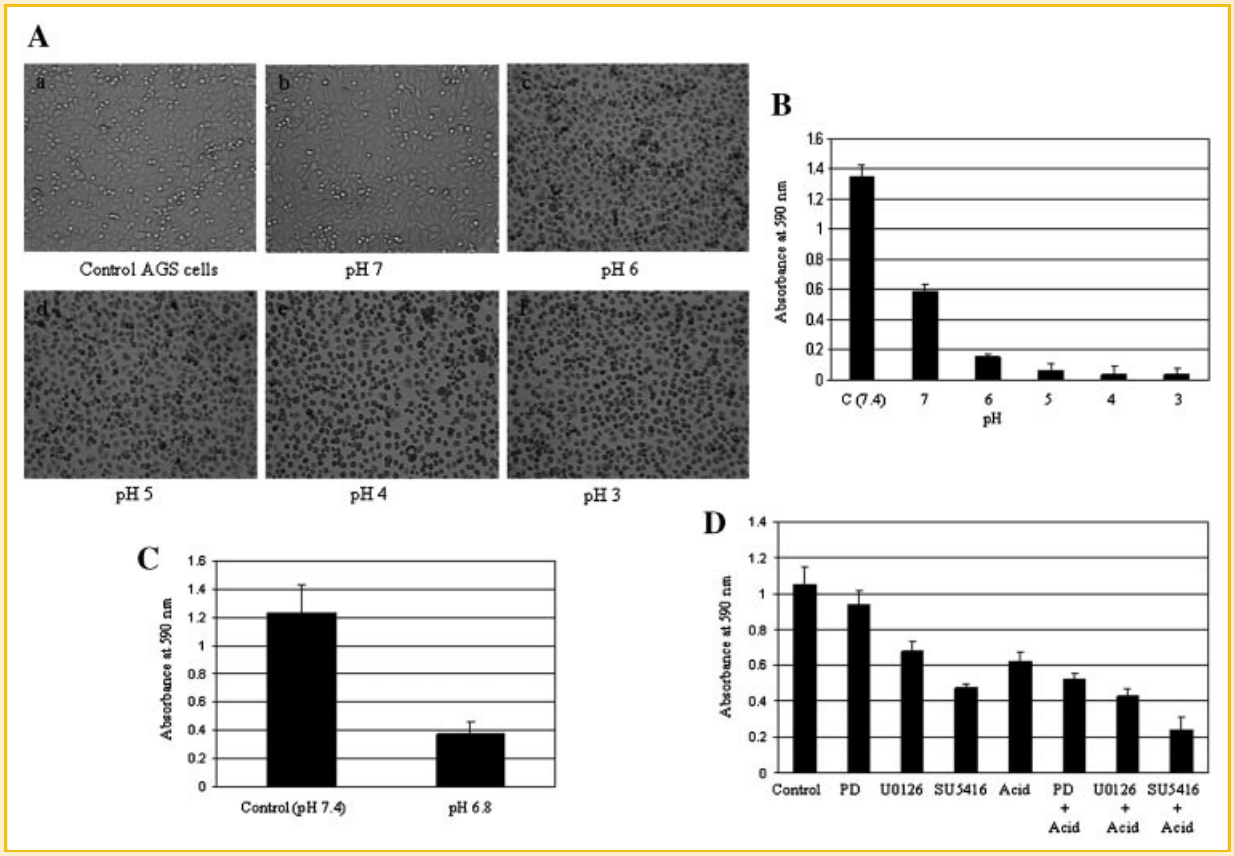

Fig. 7. pH-dependent-morphological changes and cell proliferation. A: Morphological changes of AGS cells incubated at low pH. AGS cells (1×10^5 cells/ml) were cultured in 96-well plates overnight at 37°C. AGS cells were incubated in media of different low pH values ranging from pH 7.4 to 3.0 for 24 h and then cells examined by contrast microscopy (corresponding pH values are shown below each photograph). Cell proliferation assessed by MTT assay at varying pH values (B) or pH 6.8 (C). AGS cells (1×10^5 cells/ml) grown in 96-well plates were incubated at varying pH values (pH 7.4–3.0) (B) or pH 6.8 (C) over 24 h at 37°C. D: Effect of ERK and VEGFR inhibitors on gastric cell growth. AGS cells were treated with the ERK inhibitors PD98069 (50 μM), U0126 (10 μM), or the VEGFR inhibitor SU5416 (10 μM) for 30 min prior to incubation at pH 6.8 for 24 h at 37°C. MTT reagent was added at 10 μl/well to the cultured cells, and the absorbance was read at 590 nm using a microplate reader. Data are presented as the mean ± SD of at least three independent experiments.

events occurring due to acid exposure and subsequent mucosal inflammation.

In conclusion, the exposure of gastric epithelial cells to a reduction in the pH of the extracellular environment resulted in a rapid increase of Egr-1 transcriptional activity involving the MEK-ERK1/2 pathway. The data presented here suggest that acidic conditions are necessary and sufficient to alter this transcription factor activity within gastric cells and other cell types, resulting in transcription of several genes during the inflammatory process similar to what has been previously demonstrated in esophageal cells [Duggan et al., 2006]. Egr-1 plays a critical role in duodenal and vascular injury via the regulation of many pathophysiologically relevant genes like the angiogenic growth factors VEGF, PDGF, and bFGF [Szabo et al., 1998, 2001; Vidal et al., 2000]. Gene expression studies in an experimental duodenal ulcer model demonstrated that the administration of duodenal ulcerogen cysteamine resulted in an early (0.5–2 h) increase in the expression of Egr-1 that is followed (12–24 h) by up-regulation of angiogenic growth factors (e.g., VEGF, bFGF, PDGF) [Szabo et al., 2007]. Khomenko et al. [2006] reported that Egr-1 down-regulation aggravated experimental duodenal ulcers through the transcriptional inhibition of bFGF, PDGF, and VEGF synthesis in a rat duodenal mucosa. Furthermore, functional analysis of the human VEGF promoter revealed that the sequence between −961 and −683 bp upstream of the transcription start site is responsible for the transcriptional activation of the VEGF by acidic pH in U87 MG cells [Xu et al., 2002]. Shortening the promoter to −683 bp significantly decreased acidic pH induction. In agreement with the role of Egr-1 in inflammation, we demonstrated that acidic pH induced VEGF expression and the use of Egr-1 antisense oligonucleotide blocked VEGF expression in gastric cells. The results of our studies suggest that acid exposure triggers both Egr-1 and NF-κB/AP-1 activation in gastric epithelial cells, which in turn, induce the expression of multiple genes including VEGF during the inflammatory response (Fig. 8). It is likely that exposure to pH 6.8 stimulates Egr-1 and ERK expression (and VEGF) because exposure to this pH induces injury, and in response to the insult, the production of VEGF would assist tissue repair. On the other hand, this repair mechanism is inactive at lower pH (<5.0) hence this pathway is not triggered. Milani and Calabrò [2001] suggested that VEGF might contribute to the

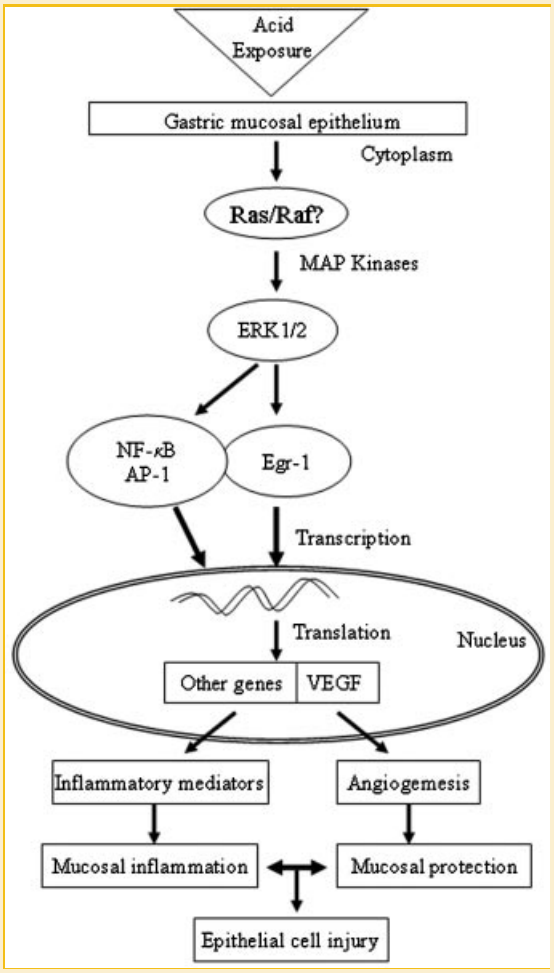

Fig. 8. A schematic diagram showing acid-induced Egr-1 signaling pathways in gastric cancer cells.

development of the angiogenic response together with other growth factors, and thus improve mucosal resistance by increasing vascular permeability, that leads to diluting gastrotoxic agents and reducing the area of the hemorrhagic lesions. Moreover, there is evidence supporting the gastroprotective role of VEGF in gastric mucosal injury [Matsui et al., 2002]. Future studies will decipher how the cells sense the acidic pH signal and examine other signaling pathways, which may be implicated in gastric epithelial injury.

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