Advances in Brief

Helicobacter pylori VacA Toxin Up-Regulates Vascular Endothelial Growth Factor Expression in MKN 28 Gastric Cells through an Epidermal Growth Factor Receptor-, Cyclooxygenase-2-dependent Mechanism

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Abstract

Purpose: Helicobacter pylori causes gastric damage and is involved in gastric carcinogenesis. Vascular endothelial growth factor (VEGF) plays a major role in gastric mucosa damage and is overexpressed in gastric cancer. We investigated: (a) whether H. pylori, and in particular H. pylori VacA toxin, affected VEGF expression in gastric epithelial cells in culture; and (b) the signal transduction pathway involved in any effect exerted by H. pylori.

Experimental Design: MKN-28 cells were incubated with un inoculated BCF (control) or with BCF obtained from VacA-producing wild-type H. pylori 60190 strain or from its isogenic mutant 60190Δv, specifically lacking vacA gene in the presence or absence of ZD 1839, a selective inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, PD098059, a selective inhibitor of mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase, the kinase responsible for ERK phosphorylation, or SC-236, a selective inhibitor of cyclooxygenase (COX)-2 for 24–48 h.

Results: (a) Toxigenic H. pylori up-regulated VEGF mRNA and protein expression and caused a 2.5-fold increase in VEGF release compared with control, whereas nontoxigenic H. pylori did not; (b) H. pylori VacA toxin-induced up-regulation of VEGF was counteracted by selective inhibition of EGFR tyrosine kinase; (c) toxigenic H. pylori activated the ERK/MAP kinase cascade, and inhibition of MAP kinase activation counteracted H. pylori-induced VEGF up-regulation; (d) toxigenic H. pylori up-regulated COX-2 expression, and this effect was counteracted by blockade of EGFR tyrosine kinase; and (e) COX-2 selective inhibition counteracted H. pylori-induced up-regulation of VEGF.

Conclusion: (a) H. pylori up-regulates VEGF expression in gastric epithelial cells; and (b) this effect is specifically related to VacA toxin and seems to depend on the activation of an EGFR-, MAP kinase-, and COX-2-mediated pathway.

Introduction

Helicobacter pylori is the major causative agent of chronic superficial gastritis and peptic ulcer disease in humans (1). Moreover, epidemiological and interventional studies in humans, as well as experimental studies in rodents, strongly indicate that H. pylori infection increases the risk for developing adenocarcinoma of the distal stomach (2–4).

H. pylori-related gastroduodenal disease depends on the inflammatory response of the host and release of a number of bacterial virulence factors (5). In particular, a M1 ~90,000 cytotoxin (VacA) that causes vacuole formation in epithelial cells in vitro is produced by 60% of wild-type isolates of H. pylori (6). Essentially, all H. pylori strains possess the vacA gene, and nearly all strains secrete a VacA product, but those that have the cytotoxin phenotype (encoded by vacA alleles belonging to the type s1 family) are referred to as Tox+. An increasing body of evidence indicates that, in western countries, H. pylori strains producing a vacuolating VacA are preferentially associated with the development of peptic ulcer disease or gastric cancer (7).

VEGF,4 the most well-characterized angiogenic factor (8), plays a major role in the multistep process leading to the reconstruction of normal mucosa architecture by stimulating the

4 The abbreviations used are: VEGF, vascular endothelial growth factor; COX, cyclooxygenase; BCF, broth culture filtrate; MAP, mitogen-activated protein; RT-PCR, reverse transcription-PCR; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFR, epidermal growth factor receptor.
process of angiogenesis, which ensures that healing tissues receive an adequate supply of nutrients (9). Moreover, VEGF plays a pivotal role in tumor-associated microvascular angiogenesis (10) and has been demonstrated to be overexpressed in human gastric carcinomas (11–13).

We have shown previously that *H. pylori* up-regulates the expression of EGF-related growth factors and COX-2, the inducible isoform of the enzyme responsible for prostaglandin production (14), in human gastric epithelial cells in vitro (15, 16), as well as in human gastric mucosa in vivo (17, 18). In addition, COX-2 products (i.e., prostaglandins) are known to stimulate VEGF production (19). Whether *H. pylori* affects VEGF expression is not known.

This study was therefore designed to evaluate whether *H. pylori* alters VEGF expression in gastric epithelial cells in vitro and to assess the role of *H. pylori* vacuolating cytotoxin VacA in any such effect. In addition, we sought to dissect out the signal transduction pathway involved in any effect exerted by *H. pylori* and to assess the role of VacA because it was not observed with an isogenic mutant specifically lacking this effect. Whether *H. pylori* VacA-induced up-regulation of VEGF might participate in the pathogenesis of *H. pylori*-related gastric carcinogenesis.

**Materials and Methods**

**Materials.** Clinical grade ZD1839 (Iressa) was provided by AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). MAP kinase inhibitor PD98059 was from Calbiochem (La Jolla, CA). SC-236 was from Pharmacia (Upsala, Sweden).

**Bacterial Strains and Growth Conditions.** We used the urease-positive *Tox* type 1a/m1 vacA allele Caga* wild-type *H. pylori* strain 60190 (ATCC 49503) and its isogenic mutant 60190:v1 in which vacA gene was disrupted by insertional mutagenesis (Ref. 20; both strains kindly provided by T. L. Cover and M. J. Blaser, Nashville, TN). Bacteria were grown in brucella broth (DIFCO Laboratories, Detroit, MI) supplemented with 1% Vitox (Oxoid, Basingstoke, United Kingdom) and 5% FCS (Life Technologies, Inc., Paisley, United Kingdom) for 24–36 h at 37°C in a thermostatic shaker under microaerobic conditions. As described previously (21, 22), when bacterial suspensions reached 1.2 absorbance units at 22°C on a spectrophotometer, the supernatants were sterilized by passage through a 0.22-μm pore-size cellulose acetate filter (Nalgene, Rochester, NY) to obtain the BCFs. The presence or absence of VacA in *H. pylori* BCFs was verified by SDS-PAGE followed by immunoblotting with an anti-VacA rabbit polyclonal antisera, as described previously (15). In all of the experiments, BCFs were used at a dilution of 1:3, and uninoculated broth filtrate served as a control.

**Human Gastric Epithelial Cells in Culture.** We used the MKN 28 cell line. This cell line derives from a well-differentiated human gastric tubular adenocarcinoma and shows gastric-type differentiation (23). MKN 28 cells were grown as monolayers in DMEM Ham’s nutrient mixture F-12 (1:1; Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Inc.) at 37°C in a humidified atmosphere of 5% CO2.

**VEGF Concentration in the Conditioned Media.** The concentration of VEGF in the conditioned media from MKN 28 cells was measured using a commercially available sandwich ELISA kit (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer’s instructions, as described previously (24). Assays were performed on serum-free medium collected after 24 or 48 h of cell incubation. Results were normalized for the number of producing cells and reported as pg/106 cells.

**Western Blot Analysis.** Total cell protein extracts (50 μg of total proteins/lane) were resolved by electrophoresis using 7.5 or 12.5% SDS-PAGE gels (Bio-Rad Laboratories, Milan, Italy) as appropriate, transferred to BA 85 0.45 μm PROTAN nitrocellulose filters (Schleicher & Schuell, Inc., Dassel, Germany), blocked with 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA) in phosphate buffer solution 1X with 0.1% Tween, and incubated with appropriate primary antibodies. Peroxidase-conjugated antirabbit or antimouse or antigato secondary antibodies (Amersham International, Buckinghamshire, United Kingdom) were incubated at the dilution of 1:2000. Immuno reactive proteins were visualized by enhanced chemiluminescence (Amersham International) as described previously (25). Immunoblot analysis using anti-β-actin antisera was performed as a control for protein loading. The primary antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA) include: (a) antihuman VEGF (mouse monoclonal, sc-7269, used at the dilution of 1:1000); (b) antihuman COX-2 (rabbit polyclonal sc-7951, used at the dilution of 1:1000); (c) antihuman actin (goat polyclonal sc-1615, used at the dilution of 1:1000); and (d) anti-ERK-2 (mouse monoclonal sc-1647, used at the dilution of 1:1000).

**RNA Extraction.** Total RNA was isolated from MKN-28 cells using an RNA extraction reagent, TRizol (Invitrogen Life Technologies, Inc., Grand Island, NY), according to the standard acid-guanidium-phenol-chloroform method (26).

**RT-PCR.** RT-PCR analysis was performed on total RNA as described previously (17, 27). First-strand complementary DNA was prepared using 200 units of RT (Supertranscript RT, Life Technologies, Inc., Gaithersburg, MD), 1 μg of total RNA as template, and 10 pmol/liter random hexamers in the presence of 0.1 mmol/liter dTT, 0.5 mmol/liter deoxyxynucleotide triphosphate-lanthium salt (Pharmacia, Milan, Italy), and 20 units of RNase inhibitor (Promega, Madison, WI). The reaction profile was 37°C × 10 min, followed by 42°C × 60 min. To control for contamination by genomic DNA, all RNA samples were run in duplicate with or without the addition of RT. RT-PCR coamplification of VEGF/GAPDH transcript was performed using VEGF (sense, TGGATCCTGAGACTTCTGCTGTC; antisense, TCACCAGCTTGGCTTGTACAT) and GAPDH (sense, CACCATCTTCCAGGACGG; antisense, TCACCCTCACAGTTCCTGGCA)-specific primers (provided by PRIMM Srl, Milano, Italy, and used at the final concentration of 2
Control or BCFs from Tox H. pylori strains of MKN 28 cells were incubated for 24 or 48 h with uninoculated BCF (Control) or BCFs from Tox H. pylori or Tox H. pylori-induced up-regulation of VEGF; cells were incubated for ≤48 h with uninoculated BCF (control) or BCF from Tox H. pylori. B, effect of the selective EGFR tyrosine kinase inhibitor ZD1839 on Tox H. pylori-induced up-regulation of VEGF protein expression; cells were incubated for 48 h with uninoculated BCF (control), Tox H. pylori BCF, or Tox H. pylori BCF in the presence or absence of ZD1839 (1 μM). At the end of the incubation time, cells were lysed, and immunoblot analysis was performed using an anti-VEGF monoclonal antibody. Immunoblot analysis using anti-β-actin antiserum was performed as a control for protein loading. Arrows, VEGF and β-actin immunoreactive bands. Representative Western blots of three independent experiments are shown.

Fig. 2 Tox+ H. pylori BCF up-regulates VEGF protein expression in MKN 28 cells through an EGFR-mediated pathway. A, time course of Tox+ H. pylori-induced up-regulation of VEGF; cells were incubated for ≤48 h with uninoculated BCF (control) or BCF from Tox+ H. pylori. B, effect of the selective EGFR tyrosine kinase inhibitor ZD1839 on Tox+ H. pylori-induced up-regulation of VEGF protein expression; cells were incubated for 48 h with uninoculated BCF (control), Tox+ H. pylori BCF, or Tox+ H. pylori BCF in the presence or absence of ZD1839 (1 μM). At the end of the incubation time, cells were lysed, and immunoblot analysis was performed using an anti-VEGF monoclonal antibody. Immunoblot analysis using anti-β-actin antiserum was performed as a control for protein loading. Arrows, VEGF and β-actin immunoreactive bands. Representative Western blots of three independent experiments are shown.

Results

H. pylori Stimulates VEGF Release from MKN 28—Role of VacA Cytotoxin and of EGFR Activation. After 24- or 48-h incubation with uninoculated BCF (control) or BCFs obtained from Tox+ H. pylori 60190 strain, we observed a 2.5-fold increase in VEGF concentration in the conditioned media of MKN 28 cells (Fig. 1). This effect was not observed after incubation with the Tox− isogenic mutant of the bacterium specifically lacking vacA, thus implying that the effect is related to the production of the VacA cytotoxin (Fig. 1). To assess whether H. pylori-induced increase in VEGF release might be contributed to by the activation of the EGFR, we incubated MKN 28 with BCF from Tox+ H. pylori strain in the presence or absence of the selective EGFR tyrosine kinase inhibitor ZD1839 (1 μM). The stimulating effect on VEGF release exerted by Tox+ H. pylori strain was completely counteracted by selective EGFR tyrosine kinase inhibition (Fig. 1). Comparable effect was observed using bacterial suspensions from the same H. pylori strains (data not shown). Therefore, the subsequent experiments were carried out using H. pylori BCFs only.

Tox+ H. pylori Up-Regulates VEGF Protein and mRNA Expression in MKN 28 Cells through an EGFR-mediated Pathway, whereas Tox− H. Pylori Does Not. To determine whether Tox+ H. pylori up-regulated VEGF protein expression, protein cellular extracts from Tox+ H. pylori-treated or untreated (control) MKN 28 cells were analyzed by Western blot using an antiserum specific for VEGF (Fig. 2). A VEGF immunoreactive peptide of M, ~21,000 was detected in control untreated cells, and incubation with Tox+ H. pylori BCFs caused a time-dependent increase in the expression of VEGF (Fig. 2A). We then sought to evaluate whether this effect was also ob-
H. pylori and VEGF Expression in Human Gastric Cells

Selective COX-2 Blockade Counteracts Tox+ H. pylori-induced Up-Regulation of VEGF Expression in MKN 28 Cells.

Selective COX-2 blockade counteracts Tox+ H. pylori-induced up-regulation of VEGF expression in MKN 28 cells. COX-2 is involved in the regulation of VEGF expression, and selective COX-2 blockade can inhibit this up-regulation.

Discussion

H. pylori infection causes gastritis and gastroduodenal mucosal ulceration and is associated with an increased risk for development of adenocarcinoma of the distal stomach in humans (32, 33). Specific virulence factors produced by the bacterium, such as the vacuolating toxbin A, contribute to gastroduodenal mucosal injury and impair the healing process of the damaged mucosa (5, 22). In addition, host response to the infection and environmental factors are thought to be involved in the pathogenesis of H. pylori-related gastroduodenal disease (32, 33). In particular, an increase in the proliferative activity of gastric epithelial cells without a corresponding increase in apoptosis has been suggested to be implicated in H. pylori-related gastric carcinogenesis (34, 35).

Polypeptide growth factors play a crucial role in the maintenance of gastric homeostasis (36). They are involved in the protection and healing of the gastric mucosa (37–39) and modulate the balance between proliferation and apoptosis in normal...
H. pylori infection causes up-regulation of a number of EGF-related growth factors in vitro and in vivo (15, 17). Additionally, we have recently demonstrated that H. pylori up-regulates the expression of COX-2 in gastric mucosal cells in vitro and in human gastric mucosa in vivo (16, 18). Whether H. pylori alters the expression of VEGF is not known. We found that H. pylori bacterial suspension or BCF increased the release of VEGF from MKN 28 cells, causing a 2.5-fold increase in the concentration of VEGF in the conditioned media. In addition, H. pylori up-regulated VEGF mRNA and protein expression in MKN 28 gastric mucosal cells. To assess the role of the vacuolating toxin VacA in H. pylori-induced up-regulation of VEGF, we studied whether an isogenic mutant H. pylori strain specifically lacking vacA (i.e., nontoxicigenic) exerted similar effect to that observed with its toxigenic wild-type parental strain. We found that only toxigenic H. pylori strain up-regulated VEGF expression, whereas its tox− isogenic mutant did not, thus implying that VEGF up-regulation strictly depends on the integrity of a cytotoxic phenotype.

H. pylori infection transactivates EGFR through up-regulation of EGF-related growth factor expression (41). Activation of the EGFR then initiates a cascade of intracellular signaling pathways, including activation of ERK/MAP kinase cascade, ultimately leading to the activation of the transcription factor activator-protein 1, which plays a crucial role in cell proliferation and transformation (42). Moreover, activation of the MAP kinase cascade is known to be associated with up-regulation of VEGF expression (29, 43). We therefore evaluated whether H. pylori was able to activate the ERK/MAP kinase cascade in MKN 28 cells and whether the inhibition of H. pylori-mediated ERK/MAP kinase activation had any effect on H. pylori-induced up-regulation of VEGF. Tox+ H. pylori BCF activated in a time-dependent manner ERK-2 phosphorylation, and this effect was counteracted by specific inhibition of EGFR tyrosine kinase by ZD1839 or MAP kinase by PD098059, respectively. Additionally, activated specific inhibition of EGFR tyrosine kinase by ZD1839 or MAP kinase by PD098059 abrogated H. pylori toxin-mediated increase in VEGF expression, thus suggesting that H. pylori-related up-regulation of VEGF expression is mediated by the activation of EGFR and MAP kinase cascade.

COX-2 plays a major role in regulating the balance between cell proliferation and apoptosis (44) and activates angiogenesis through up-regulation of VEGF expression (19, 30, 31). We have recently shown that H. pylori up-regulates COX-2 expression in MKN 28 cells (17). In addition, we have shown
previously that *H. pylori* up-regulates the expression of heparin-binding EGF-like growth factor and amphiregulin, members of the EGFR family of ligands (15), which in turn up-regulate COX-2 expression in MKN 28 cells (17). We therefore sought to investigate first whether EGFR blockade counteracted *H. pylori*-induced up-regulation of COX-2 expression and, then, whether specific COX-2 inhibition had any effect on *H. pylori*-dependent up-regulation of VEGF. We found that the EGFR tyrosine kinase inhibitor ZD1839 inhibited *H. pylori*-induced increase in COX-2 protein levels in MKN 28 cells, thus indicating that *H. pylori*-induced up-regulation of COX-2 expression depends on the activation of an EGFR-related pathway. Our finding is in agreement with a recent report showing that bile acids up-regulate COX-2 expression in cholangiocarcinoma cells through activation of the EGFR and MAP kinase cascade (45). Moreover, SC-236, a selective COX-2 inhibitor (46, 47), almost completely abolished *H. pylori*-induced up-regulation of VEGF expression, thus suggesting that COX-2 expression is necessary for *H. pylori* to increase VEGF expression in gastric mucosal cells.

In conclusion, this study shows for the first time that *H. pylori* increases the expression of VEGF in gastric mucosa cells in *vivo*. This effect is strictly VacA dependent because it was observed with the parental toxigenic wild-type *H. pylori* strain but not with its isogenic mutant specifically lacking the cytotoxin VacA. That EGFR tyrosine kinase and MAP kinase inhibition counteracted *H. pylori*-related increase in VEGF expression indicates that activation of the EGFR and MAP kinase cascade are necessary events for *H. pylori* to up-regulate VEGF expression. Finally, that *H. pylori*-induced COX-2 up-regulation was counteracted by inhibition of EGFR activation and that blockade of COX-2 activity abrogated *H. pylori*-induced VEGF up-regulation suggests that *H. pylori*-induced up-regulation of VEGF in MKN 28 cells might be mediated by COX-2 through the activation of EGFR-related events.

The identification of *H. pylori*-specific signaling pathways leading to the induction of cell cycle and/or angiogenesis mediators may be relevant as to the understanding of the mechanism of *H. pylori*-associated gastric carcinogenesis and might be of interest for therapeutic intervention to overcome *H. pylori*-associated gastric cancer. We postulate that *H. pylori*-induced gastric mucosal injury triggers the compensatory overexpression of EGFR-related polypeptide growth factors, which, through the activation of the MAP kinase cascade, stimulate cell proliferation, inhibit apoptosis, and stimulate COX-2 expression. This, through the increased production of prostaglandins, leads to further activation of the EGFR (48) and to increased expression of angiogenetic VEGF. All these events may ultimately favor the progression from chronic gastritis to adenocarcinoma in the multistep model of gastric carcinogenesis (49). One may therefore envision the chemopreventive use of a specific EGFR tyrosine kinase inhibitor alone or in combination with a selective COX-2 inhibitor (50), in those *H. pylori*-infected subjects who are resistant to conventional therapeutic regimens and potentially at risk for developing adenocarcinoma of the distal stomach.

**References**


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