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Epidermal growth factor receptor activation protects gastric epithelial cells from *Helicobacter pylori*-induced apoptosis

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Abstract

Background & Aims—*Helicobacter pylori* infection disrupts the balance between gastric epithelial cell proliferation and apoptosis, which is likely to lower the threshold for the development of gastric adenocarcinoma. *H. pylori* infection is associated with EGF receptor (EGFR) activation through metalloproteinase-dependent release of EGFR ligands in gastric epithelial cells. Since EGFR signaling regulates cell survival, we investigated whether activation of EGFR following *H. pylori* infection promotes gastric epithelial survival.

Methods—Mouse conditionally immortalized stomach epithelial cells (ImSt) and AGS cells, as well as wild-type and kinase-defective EGFR (EGFR^{wa2}) mice, were infected with the *H. pylori* cag + strain 7.13. Apoptosis, caspase activity, EGFR activation (phosphorylation) and EGFR downstream targets were analyzed.

Results—Inhibiting EGFR kinase activity or decreasing EGFR expression significantly increased *H. pylori*-induced apoptosis in ImSt. Blocking *H. pylori*-induced EGFR activation with a heparin-binding (HB)-EGF neutralizing antibody or abrogating a disintegrin and matrix metalloproteinase-17

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(ADAM-17) expression increased apoptosis of *H. pylori*-infected AGS and ImSt, respectively. Conversely, pretreatment of ImSt with HB-EGF completely blocked *H. pylori*-induced apoptosis. *H. pylori* infection stimulated gastric epithelial cell apoptosis in EGFR^{wa2}, but not in wild-type mice. Furthermore, *H. pylori*-induced EGFR phosphorylation stimulated PI3K-dependent activation of the anti-apoptotic factor Akt, increased expression of the anti-apoptotic factor Bcl-2, and decreased expression of the pro-apoptotic factor Bax.

Conclusions—EGFR activation by *H. pylori* infection has an anti-apoptotic effect in gastric epithelial cells that appears to involve Akt signaling and Bcl family members. These findings provide important insights into the mechanisms of *H. pylori*-associated tumorigenesis.

Introduction

Helicobacter pylori, a Gram-negative bacterium that selectively colonizes the gastric mucosa, increases the risk of developing gastric adenocarcinoma and gastric non-Hodgkin's lymphoma in humans and animal models.^{1, 2} Host responses that mediate *H. pylori*-associated disease include disruption of the balance between gastric epithelial cell proliferation and apoptosis, which can manifest as increased proliferation and either increased or decreased apoptosis at different stages of infection.^{3, 4} Increased proliferation that is not balanced by compensatory increases in apoptosis in advanced stage of infection may heighten the risk for development of gastric neoplasia.⁵ Thus, understanding the mechanism through which *H. pylori* regulates cell fate is critical for establishing therapeutic approaches to potentially prevent *H. pylori* infection-induced gastric tumorigenesis.

Both *in vivo* and *in vitro* studies have demonstrated that several *H. pylori* virulence factors stimulate gastric epithelial apoptosis, such as products of the *cag* pathogenicity island,^{4, 6} a secreted vacuolating toxin (VacA),⁷ and lipopolysaccharide.⁸ The cellular mechanisms by which *H. pylori* induces apoptosis include activation of Fas⁹ and cytokine receptors, such as tumor necrosis factor (TNF) receptor¹⁰ and induction of spermine oxidase (SMO) activity,¹¹ which lead to Caspase activation and apoptosis in gastric epithelial cells.

H. pylori has also been reported to be associated with attenuated apoptosis following infection,¹² and chronically colonized humans and Mongolian gerbils harboring *H. pylori*^{cag+} strains develop increased gastric epithelial cell proliferation without an associated increase in apoptosis, suggesting that increased proliferation in the absence of a concomitant increase in apoptosis may contribute to increased risk for gastric cancer with *cag+* strains.^{13–15} However, the mechanism through which *H. pylori* exerts anti-apoptotic effects remains unclear.

Recent reports show that *H. pylori* induces up-regulation of EGF receptor (EGFR) expression¹⁶ and EGFR activation by heparin-binding (HB)-EGF release from gastric epithelial cells.^{17, 18} EGFR is a member of the ErbB family which consists of four tyrosine kinase receptors, EGFR (ErbB1), and ErbB2–4. These four receptors are essential for modulating cell survival, proliferation, and differentiation in many tissue types.¹⁹

However, there is no report that has identified the role of EGFR activation in the pathogenesis of *H. pylori* infection. Therefore, the purpose of this study was to define gastric epithelial cellular responses regulated by EGFR activation following *H. pylori* infection. Our studies using *in vitro* cell culture and *in vivo* animal models show that transactivation of EGFR by *H. pylori* protects gastric epithelial cells from apoptosis and that this anti-apoptotic response is mediated by PI3K-dependent activation of Akt and Bcl family members. Thus, our studies demonstrate that activation of EGFR may represent a previously unrecognized molecular regulatory step in determining the effects of *H. pylori* on regulation of gastric epithelial cell homeostasis. Since EGFR has been implicated in a number of epithelial cancers and serves as

a target for cancer therapy,^{20, 21} results in this report provide a novel focus for further investigations into the mechanisms of *H. pylori*-associated tumorigenesis

Materials and Methods

Information regarding inhibitors, antibodies, reagents, cell treatment, gastric epithelial cell isolation, cellular lysate preparations, immunohistochemistry, and apoptosis assays are provided in the Supplemental Data.

H. pylori culture

The *H. pylori* *cag*⁺ rodent adapted strain 7.13 was grown in *Brucella* broth with 5% FBS for 24 h.²² Bacteria were identified as *H. pylori* by urease and oxidase activity and Gram's stain morphology. *H. pylori* were washed with PBS and cell culture medium before addition to cells.

Cell culture, retroviral transduction, and transient transfection of siRNA

Mouse conditionally immortalized stomach epithelial cells (ImSt) were isolated from the gastric epithelium of transgenic mice with a temperature-sensitive mutation of the simian virus 40 (SV40) large tumor antigen gene (tsA58) fused to the promoter of the mouse H-2K^b class I gene (*H-2K^b-tsA58* mice).²³ A disintegrin and matrix metalloproteinase-17 (ADAM-17)^{-/-} ImSt were isolated from the gastric epithelium of ADAM-17 null heterozygous mice crossed with the *H-2K^b-tsA58* mice.²⁴

ImSt were maintained in RPMI 1640 medium containing 10% FBS, 5 U/ml of murine IFN- γ and 100 U/ml penicillin and streptomycin on collagen-coated plates and grown under permissive conditions at 33°C with 5% CO₂. Prior to all experiments, cells were transferred to 37°C (non-permissive) conditions and cultured in medium containing 0.5% FBS, without IFN- γ or antibiotics for 16–18 hours.

ADAM-17^{-/-} ImSt reconstituted to express wild-type (wt) ADAM-17 or catalytically-inactive E406A mutant ADAM-17²⁵ were prepared as described before.²⁶ Briefly, C-terminally HA-tagged murine wt and E406A ADAM17 were cloned into the pBM-IRES-PURO retroviral vector. All cDNA constructs were verified by DNA sequencing. For retroviral transduction, ADAM17^{-/-} ImSt were seeded into tissue culture flasks and cultured for 24 h before incubation with virus stock for 12 h in the presence of 4 μ g/ml polybrene. Cells were then grown for 48 h in normal cell culture medium before passage into medium containing 5 μ g/ml puromycin.

ImSt were transiently transfected with either non-targeting siRNA or mouse EGFR SMARTpool siRNA at 70% confluence using Lipofectamine 2000.²⁷ After 24 h of transfection, cells were cultured in medium containing 0.5% FBS for 16–18 hours before treatment.

AGS cells were grown in RPMI 1640 medium containing 10% FBS and 100 U/ml penicillin and streptomycin at 37°C with 5% CO₂. Prior to all experiments, cells were cultured in medium containing 0.5% FBS without antibiotics for 16–18 h.

Mice and *H. pylori* infection and gastric tissue preparations

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University. C57BL/6^{wa2} mice with a naturally occurring EGFR kinase defective mutation (Val to Gly mutation at residue 743 in the kinase domain)²⁸ were obtained from Dr. David Threadgill (University of North Carolina, Chapel Hill). PCR primers specific for the EGFR sequence containing the point mutation were used for genotyping (sequences available upon request). Mice (8–10 weeks old, 25–30g) were

anesthetized and then infected orally with *H. pylori* (10^9 cfu) or broth only in a total volume of 500 μ l for 24 h.²⁹

Gastric tissue was fixed in 10% neutral-buffered formalin and paraffin-embedded before sectioning. The gastric mucosa was scraped into homogenization buffer and tissue was lysed.³⁰

Apoptosis assays

Apoptosis in cell lines was detected using ApopTag *In Situ* Apoptosis Detection Kits (TUNEL).^{31, 32} For Annexin V-FITC staining, attached cells were dissociated using Accutase and double stained with Annexin V-FITC and propidium iodide.³³ The percentage of apoptotic cell populations was measured by flow cytometry.¹¹

Apoptosis was detected in gastric tissue sections using ApopTag™ *In Situ* Oligo Ligation (ISOL) Kit, and observed by DIC microscopy.^{31, 32} Apoptotic cells were quantified by counting the absolute number of positive stained cells in at least 500 gastric glands.

Flow cytometric analysis of active caspase-3 and Bcl-2

ImSt were fixed with 0.1% paraformaldehyde and incubated with a rabbit anti-active caspase-3 antibody and a mouse anti-Bcl-2 antibody, and were further stained using FITC-conjugated anti-rabbit and APC-conjugated anti-mouse antibodies, respectively.³³ The percentage of active caspase-3 and/or Bcl-2 positive staining cell populations was measured by flow cytometry.

Statistical analysis

Statistical significance in each study was determined by one-way ANOVA followed by Newman-Keuls analysis for multiple comparisons using Prism 5.0 (GraphPad Software, Inc.). A *p*-value < 0.05 was defined as statistically significant. All data presented are representative of at least three repeat experiments and are presented as mean $\pm$ S.E.M.

Results

Inhibition of *H. pylori*-stimulated EGFR kinase activity and protein expression increases apoptosis in ImSt

H. pylori induces up-regulation of EGF receptor (EGFR) expression¹⁶ and EGFR transactivation¹⁸ in gastric epithelial cells. Phosphorylation of Y1068 on EGFR mediates activation of the ERK/MAP and results in recruitment of the p85 subunit of PI3K to the EGF receptor, leading to activation of Akt.³⁴ One significant role of EGFR activation is to promote cell survival. Therefore, we studied the effect of inhibiting EGFR tyrosine kinase activity on *H. pylori*-stimulated apoptosis in ImSt. *H. pylori* were co-cultured with ImSt in the presence or absence of the EGFR tyrosine kinase inhibitor, AG1478, and EGFR Y1068 phosphorylation and apoptosis were analyzed. As expected, *H. pylori*-induced EGFR activation was inhibited by the EGFR tyrosine kinase inhibitor (Figure 1A). However, the expected increase in apoptosis initiated by *H. pylori* was further increased by blocking EGFR tyrosine kinase activity, as detected by Annexin V staining and flow cytometry (Figure 1B and C) and TUNEL assay (Figure 1D and E).

To investigate a potential role for EGFR in suppressing *H. pylori*-induced apoptosis, we used siRNA directed against EGFR to reduce EGFR expression in ImSt (Figure 2A). Consistent with the results of experiments in which EGFR kinase activity was inhibited, loss of EGFR expression significantly enhanced *H. pylori*-stimulated apoptosis, compared to non-targeting control siRNA transfection (Figure 2B). Taken together, these results demonstrate that EGFR

expression and EGFR tyrosine kinase activity enhance survival of gastric epithelial cells infected by *H. pylori*.

Loss of EGFR kinase activity enhances *H. pylori*-induced gastric epithelial cell apoptosis *in vivo*

Our *in vitro* data indicate that EGFR activation plays an anti-apoptotic role in *H. pylori*-infected cells. To determine whether these effects could be extended *in vivo*, wild type (wt) or EGFR kinase defective (EGFR^{wa2}) mice on a C57BL/6 background were infected with *H. pylori*. Mice were sacrificed 5 h or 24 h after infection and tissue was fixed and prepared for immunohistochemistry or mucosal scrapings were prepared for Western blot analysis. The presence of *H. pylori* in both wt and EGFR^{wa2} mice was confirmed by immunostaining (Figure 3A). *H. pylori*-induced EGFR Y1068 phosphorylation was detected in gastric mucosal lysates isolated from wt, but not in EGFR^{wa2} mice, despite similar levels of bacterial infection (Figure 3B). Importantly, immunostaining showed that *H. pylori*-stimulated EGFR Y1068 phosphorylation was present in wt, but was absent in EGFR^{wa2} mice (Figure 3C), which is consistent with our *in vitro* findings using ImSt (Figure 1A).

To determine if EGFR mediates *H. pylori* regulation of cell survival *in vivo*, we performed ISOL and anti-active caspase-3 staining to detect apoptosis. *H. pylori* infection for 24 h did not stimulate apoptosis in the gastric epithelium of wt mice. However, apoptosis was induced by *H. pylori* infection in EGFR^{wa2} mice (Figure 3D). Thus, these data indicate that survival of gastric epithelial cells in mucosa infected by *H. pylori* requires functional EGFR *in vivo*.

We next examined the relationship between EGFR-regulated apoptosis and proliferation in *H. pylori*-infected mice. Interestingly, apoptotic cells were limited to the epithelial cell layer of the lower one third of the gastric glands (Figure 3D). However, proliferating gastric epithelial cells were localized to the upper one third of the gastric glands, and no difference in gastric epithelial cell proliferation rates was detected in wt mice after 24 h of *H. pylori* infection compared to non-infected mice (SD Figure 1). Therefore, no overlap was present between proliferative and apoptotic cell populations. Given the evidence that both gastric epithelial cells with *H. pylori*-activated EGFR and apoptotic gastric epithelial cells localized to the lower one-third of gastric glands (Figure 3C and D) and no proliferation was stimulated by *H. pylori* in this zone, these data suggest that *H. pylori*-activated EGFR after 24 h infection exerts an anti-apoptotic, but not a proliferative role, in gastric epithelial cells.

Metalloproteinase activity and EGFR ligand release are required for suppressing *H. pylori*-induced gastric epithelial cell apoptosis through activation of EGFR

Activation of Src family protein kinases, leading to metalloproteinase-dependent ligand release, serves as one of the mechanisms by which EGFR is transactivated.³⁵ Previous studies have reported that *H. pylori* activated EGFR is mediated by stimulation of HB-EGF release from gastric epithelial cells,^{17, 18} since ADAM-17 can regulate HB-EGF cleavage and ligand binding,^{36, 37} we determined whether ADAM-17 is involved in *H. pylori*-regulated EGFR transactivation and apoptosis. In ADAM-17^{-/-} ImSt, *H. pylori* failed to activate EGFR (Figure 4A) and stimulated increased apoptosis (Figure 4B–C). Infection of wt ADAM-17, but not the catalytically-inactive E406A mutant ADAM-17 restored EGFR activation (Figure 4A) and reduced apoptosis induced by *H. pylori* (Figure 4B–C), indicating a requirement for ADAM-17 in the *H. pylori*-induced EGFR activation-dependent cell survival phenotype.

Since HB-EGF is a ligand generated by ADAM-17 to activate EGFR³⁸ and *H. pylori* stimulates production of HB-EGF, we next determined whether inhibition of HB-EGF altered *H. pylori*-stimulated EGFR activation and apoptosis. Pretreatment of ImSt with HB-EGF neutralizing antibody blocked *H. pylori* activation of EGFR (Figure 5A) and increased

apoptosis (Figure 5B). Importantly, pretreatment of AGS cells with HB-EGF completely blocked *H. pylori*-stimulated apoptosis (Figure 5B), suggesting that HB-EGF is not only required for EGFR activation, but also for gastric epithelial cell survival in response to *H. pylori* infection.

Akt and Bcl-2 family members are targets of EGFR activation that prevent *H. pylori*-stimulated apoptosis in gastric epithelial cells

One of the major downstream signaling targets of EGFR is PI3K. Akt is the principal mediator of PI3K-regulated signaling pathways for promoting cell survival. Akt is also an anti-apoptotic target for EGFR transactivation by TNF.²⁷ Therefore, we tested whether EGFR is required for activation of Akt by *H. pylori*. *H. pylori*-induced AKT and EGFR activation was blocked in ADAM-17^{-/-}ImSt, and these responses were reconstituted in cells expressing wt, but not catalytically-inactive E406A ADAM-17^{-/-} (Figure 4A). Furthermore, HB-EGF neutralizing antibody inhibited Akt and EGFR activation by *H. pylori* (Figure 5A). *H. pylori* infection-induced Akt activation in ImSt was inhibited by blocking EGFR or PI3K kinase activities using an EGFR kinase inhibitor AG1478 or a PI3K kinase inhibitor wortmannin, respectively (Figure 6A). Importantly, pretreatment of ImSt with wortmannin enhanced *H. pylori*-induced apoptosis (Figure 6B).

Since Akt has direct effects on the apoptotic machinery through regulating expression of several factors, including a pro-apoptotic protein, Bax, and an anti-apoptotic protein, Bcl-2,³⁹ we studied expression of the Bcl family member Bcl-2 in gastric epithelial cells. Bcl-2 and active caspase-3, markers for apoptosis, were assessed in the same cells by flow cytometry. Blocking EGFR kinase activity by AG1478 decreased the population of ImSt with *H. pylori*-stimulated Bcl-2 expression. However, the total population of cells with active caspase-3 was enhanced by AG1478 (Figure 7A–B), due to a large increase in Bcl-2⁻/active caspase-3⁺ cells (left upper quadrant, Figure 7A)

We also investigated Bcl-2 and Bax expression in the gastric mucosa of mice infected with *H. pylori*. Infection with *H. pylori* decreased Bax expression and increased Bcl-2 expression in wt mice, but these effects were not observed in the EGFR^{wa2} mice (Figure 7C). In fact, the ratio of Bax:Bcl-2 was reversed in EGFR^{wa2} mice, compared to wt mice (Figure 7D), which represents an increased apoptotic response in the EGFR^{wa2} mice upon *H. pylori* infection. This regulatory effect of *H. pylori* on Bcl-2 and Bax was confirmed in gastric epithelial cells isolated from the stomachs of mice infected with *H. pylori* (SD Figure 2). Thus, these results suggest that Bax and Bcl-2 are downstream targets of EGFR transactivation that promote an anti-apoptotic response in *H. pylori*-infected gastric epithelial cells.

Discussion

In this study, we have demonstrated that activation of EGFR is a key regulatory step in promoting an anti-apoptotic response in gastric epithelial cells infected with *H. pylori* *in vitro* and *in vivo*. Furthermore, our data provide evidence that *H. pylori*-induced EGFR activation results in activation of Akt/PI3K kinase activity and regulation of Bcl family members which promote gastric epithelial cell survival. These novel findings indicate that activation of EGFR may be a previously unrecognized “molecular switch” determining the survival response of *H. pylori*-infected gastric epithelial cells. We speculate that EGFR activation may function to inhibit apoptosis caused by activation of Fas⁹ and cytokine receptors¹⁰ and SMO activation that leads to generation of H₂O₂, which results in cytochrome c release and caspase-3 activation^{11, 33} (Figure 8).

EGFR is a member of the ErbB family which consists of four tyrosine kinase receptors, EGFR (ErbB1), and ErbB2-4. These receptors promote cell survival, proliferation, migration, and

differentiation in many tissue types.¹⁹ EGFR and other ErbB family members can be activated by direct interaction with EGF-like ligands or transactivated by various extracellular stimuli, such as TNF,²⁷ which initiate formation of homo- and/or hetero-dimers and increased kinase activity.⁴⁰⁻⁴¹ Therefore, we recognize that other ErbBs may also be potential targets of *H. pylori* infection. In fact, *H. pylori* stimulates ErbB3 tyrosine phosphorylation, but not ErbB2 or 4 in ImSt (data not shown), thus, *H. pylori* may increase EGFR/ErbB3 heterodimer formation. Since ErbBs have been shown to couple EGFR to the PI3K/Akt pathway in gefitinib-sensitive non-small cell lung cancer cell lines,⁴² EGFR activation by *H. pylori* may be amplified by heterodimer formation with ErbB3. These studies are currently underway in our laboratory.

Given a requirement for metalloproteinase activity in *H. pylori*-initiated HB-EGF release for EGFR transactivation, ADAM-17 is an ideal candidate enzyme for regulation of this pathway.^{18, 43} Our data show that ADAM-17 is required for preventing *H. pylori*-induced apoptosis (Figure 4). ADAM-17 is the first ADAM to have a defined physiological substrate, which is the precursor transmembrane form of TNF. ADAM-17 is required for the release of soluble TNF and at least 3 EGFR ligands, HB-EGF, amphiregulin (AR), transforming growth factor (TGF)- α .^{36, 44} Previous studies have shown that *H. pylori* stimulates AR release from gastric mucosal cells.⁴⁵ Thus AR and TNF release may be another ligand for *H. pylori* activation of EGFR. TNF production is increased in a number of gastrointestinal disorders, including *H. pylori*-induced gastritis,⁴⁶ and TNF transactivates EGFR to promote cell survival in colon epithelial cells.²⁷ Although ADAM-17 is ubiquitously present in the human gastrointestinal tract, its expression and catalytic activity are increased in ulcerative colitis,⁴⁷ and it regulates TNF levels in rodent models of colitis.⁴⁸ However, while ADAM-17 is a target of drug development for inflammatory conditions,⁴⁹ the disorganized and inflamed nature of the gastrointestinal tract seen in ADAM-17-deficient mice suggests this metalloproteinase may also play important roles in gut epithelial homeostasis, perhaps through regulation of EGFR ligands.^{36, 44, 50, 51} Therefore, a better understanding of the function of ADAM-17 during *H. pylori*-induced gastric epithelial injury may provide insights into its potential role in gastric adenocarcinoma.

Elucidating the down-stream targets of EGFR activation by *H. pylori* is important for understanding the cellular responses induced by *H. pylori*. We show that *H. pylori*-initiated EGFR activation is required for activation of an anti-apoptotic signal, Akt/PI3K. In fact, since the submission of this current manuscript another report has now demonstrated specific reduction of Akt via siRNA increases apoptosis in response to *H. pylori* infection of human gastric epithelial cells (Nagy et al., *Journal of Infectious Diseases*, In press). However, inhibition of EGFR does not block *H. pylori*-induced pro-apoptotic signals, such as p38 (Figures 4 and 5), or JNK activation.¹⁸ This evidence supports a model whereby pro-apoptotic signals become physiologically predominant in cells that do not express EGFR or lose EGFR kinase activity. We show that *H. pylori*-initiated EGFR activation is required for Akt/PI3K and ERK1/2 MAPK activation in gastric epithelial cells (Figures 4-5). Interestingly, although *H. pylori*-stimulated ERK1/2 MAPK activation in ImSt requires EGFR (Figures 4A and 5A and 18), inhibition of ERK1/2 MAPK activation does not enhance *H. pylori*-induced apoptosis (Figure 6B). These data suggest that Akt is a potential target of EGFR activation for preventing *H. pylori*-stimulated apoptosis in gastric epithelial cells. However, another group reported a role of *H. pylori*-induced ERK1/2 MAPK activation in prevention of apoptosis.⁵² This difference in the role of *H. pylori*-activated ERK1/2 MAPK may depend on host and/or bacterial strain differences.

In addition to PI3K-dependent Akt activation up-regulation of Bcl-2 and down-regulation of Bax expression (Figure 7), cyclooxygenase 2 (COX-2) may serve as another downstream target of EGFR activation by *H. pylori* that can induce anti-apoptotic responses in gastric epithelial

cells. The COX-2 level in gastric epithelial cells is up-regulated by *H. pylori* infection,^{53, 54} and COX-2 inhibitors increase *H. pylori*-induced apoptosis.⁵⁵ We have also found that transactivation of EGFR is required for *H. pylori* up-regulation of COX-2 expression (unpublished observation).

In summary, our study demonstrates that *H. pylori*-stimulated EGFR activation exerts an anti-apoptotic role in gastric epithelial cells *in vitro* and *in vivo* through its downstream targets, Akt and Bcl2 family members. *In vivo* chronically infected humans and Mongolian gerbils harboring *cag*⁺ strains have increased gastric epithelial cell proliferation without an associated increase in apoptosis, suggesting that increased proliferation in the absence of a concomitant increase in apoptosis may contribute to increased risk for gastric cancer.^{14, 15} Additionally, the lack of gastric epithelial cell apoptosis with acute *H. pylori* infection *in vivo* that we have attributed to EGFR transactivation may contribute to the ability of *H. pylori* to colonize, as there may be less neutrophil infiltration that would otherwise be recruited to engulf cellular debris and may serve to defend against *H. pylori* infection.⁵⁶ Important questions regarding the role of EGFR transactivation in the progression from gastric infection to neoplasia and the interaction of this pathway with the recently reported links between EGFR activation and macrophage recruitment⁵⁷ and activation⁵⁸ need to be addressed to fully understand the preventive and therapeutic implications of the findings on gastric carcinoma development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADAM-17/TACE	A disintegrin and matrix metalloproteinase-17/tumor necrosis factor α converting enzyme
EGFR	EGF receptor
HB-EGF	heparin-binding-EGF
ISOL	<i>in situ</i> oligo ligation
ImSt	immortalized stomach epithelial cells

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Figure 1. EGFR kinase activity is required for survival of gastric epithelial cells infected with *H. pylori*

ImSt were infected with the wt *H. pyloricaga*⁺ strain 7.13 (bacteria:cells = 100:1) for 90 min (A) or 24 h (B–E) pretreated for 30 min with AG1478 (150 nM) or not pretreated. A: EGFR Y1068 phosphorylation was detected by Western blot analysis of cellular lysates using an anti-EGFR-phospho (P)-Y1068 antibody. An anti-EGFR antibody was used as a loading control. Band density was quantified using ImageJ software. The relative density was calculated by comparing density of EGFR-P-Y1068 to the density of EGFR. The relative density in the control group (no AG1478 and no *H. pylori*) was set as 1.0. The relative densities in the treated groups were compared to that of control. B and C: Cells were stained with Annexin V-FITC and PI, and flow cytometry was performed. The percentage of cells in each quadrant is shown (C). The upper right quadrant represents late apoptosis, and the lower right quadrant represents early apoptosis. D and E: Cells were fixed for TUNEL assay, and apoptotic nuclei were labeled with FITC (green) and total nuclei were labeled with DAPI (blue). FITC and DAPI labeled images were taken from the same field. Apoptosis was determined by counting at least 500 cells. The percentage of cells undergoing apoptosis is shown (E). Data in this and subsequent figures are representative of at least three separate experiments. * $p < 0.05$ vs cells alone, ** and *** $p < 0.001$ vs cells alone, § and §§ $p < 0.001$ vs cells infected with *H. pylori*, + $p < 0.001$ vs cells treated with AG1478.

Figure 2. Suppression of EGFR expression promotes apoptosis in *H. pylori*-infected gastric epithelial cells

ImSt transfected with EGFR siRNA or non-targeting siRNA were infected with *H. pylori* for 24 h. A: EGFR expression levels were analyzed by Western blot analysis of cellular lysates using an anti-EGFR antibody. An anti-ERK1/2 antibody was used as a loading control. B: Apoptosis was detected using TUNEL assay as described in Figure 1. Apoptosis was determined by counting at least 500 cells. The percentage of cells undergoing apoptosis is shown. *, **, and *** $p < 0.001$ vs uninfected cells in each cell line, § $p < 0.001$ vs non-transfected cells infected with *H. pylori*.

Figure 3. Loss of EGFR kinase activity promotes gastric epithelial cell apoptosis in mice infected with *H. pylori*.

Wt and EGFR^{wa2} (EGFR kinase defective) mice on a C57BL/6 background were infected with *H. pylori* (10⁹ cfu/mouse) for 5 or 24 h. Paraffin-embedded stomach tissues (A, C–D) and gastric mucosal lysates (B) were prepared. A: Immunostaining with anti-*H. pylori* antiserum was performed to detect the presence of *H. pylori* (arrowheads). B: EGFR Y1068 phosphorylation was detected by Western blot analysis of gastric mucosal lysates as described in Figure 1. C: EGFR Y1068 phosphorylation was detected using immunostaining with anti-EGFR Tyr1068 and FITC-labeled secondary antibody. Green represents positive labeling (L: Luminal side). D: Apoptosis was detected by ISOL staining and active caspase-3

immunostaining using an anti-active caspase-3 antibody. Apoptotic nuclei were labeled with peroxidase and developed with DAB and observed using DIC microscopy. Brown nuclei (arrows) represent ISOL or activated caspase-3 positive staining cells. n = 5–7 mice for each condition.

Figure 4. ADAM-17 mediates *H. pylori*-induced activation of EGFR and is required for an anti-apoptotic effects

Cells were infected with *H. pylori* for 90 min (A) or 24 h (B and C). EGFR Y1068 phosphorylation was analyzed by Western blot analysis of cellular lysates (as described in Figure 1), activation of Akt using anti-phospho (P)-Ser 473 Akt, ERK1/2 MAPK activation using anti-phospho (P)-Thr183/Tyr185 ERK1/2, and p38 using anti-phospho (P)-Tyr180/182 p38 antibodies. B–C: Cells were fixed for TUNEL assay and apoptotic nuclei were labeled with peroxidase and developed with DAB and observed using DIC microscopy. Brown staining represents apoptotic nuclei (B). Apoptotic cells were enumerated by counting at least 500 cells. The percentage of cells undergoing apoptosis is shown (C). *, **, *** and ****p < 0.001 vs

uninfected cells in each cell line. § and # $p < 0.001$ vs ImSt and wt ADAM-17 transfected cells infected with *H. pylori*.

Figure 5. *H. pylori* -induced gastric epithelial cell apoptosis is enhanced by blocking HB-EGF binding to EGFR, but is prevented by HB-EGF pretreatment

AGS cells were pretreated for 30 min with an anti-HB-EGF neutralizing antibody (3 μ g/ml) or HB-EGF (10 ng/ml), as indicated, and then infected with *H. pylori* for 90 min (A) or 24 h (B). A: Cellular lysates were analyzed by Western blot analysis to detect EGFR, Akt, ERK1/2 MAPK and p38 activation as described in Figures 1 and 4. B: Apoptosis was detected using TUNEL assay as described in Figure 4. Apoptotic cells were enumerated by counting at least 500 cells. The percentage of cells undergoing apoptosis is shown. * $p < 0.001$ vs cells alone, ** $p < 0.001$ vs cells treated with HB-EGF antibody only. § $p < 0.001$ vs cells infected with *H. pylori*.

Figure 6. Akt is a target of EGFR for survival of gastric epithelial cells infected with *H. pylori*. ImSt were infected with *H. pylori* 90 min (A) or 24 h (B) in the presence or absence of 30 min pretreatment with AG1478 (150 nM), a PI3K inhibitor, wortmannin (100 nM), or MEK inhibitor, PD98059 (10 μ M). A: Cellular lysates were collected for detecting EGFR, Akt and ERK1/2 MAPK activation by Western blot analysis as described in Figures 1 and 4. B: Cells were fixed for TUNEL assay as described in Figure 4. Apoptosis was determined by counting at least 500 cells. The percentage of cells undergoing apoptosis is shown. *, **, and *** $p < 0.001$ vs uninfected cells in each inhibitor treatment group. § $p < 0.001$ vs ImSt infected with *H. pylori*.

Figure 7. Role of EGFR in regulating anti-apoptotic and pro-apoptotic proteins in gastric epithelial cells

A and B: ImSt were pretreated for 30 min with a EGFR kinase inhibitor, AG1478 (150 nM) or not pretreated, and then infected with *H. pylori* 24 h. Cells were fixed and stained using antibodies against active caspase-3 and Bcl-2 and flow cytometry was performed.

Representative dot plots (A) show the presence of a population of cells that are both low Bcl-2 and active caspase-3 (left lower quadrant), high Bcl-2 and low active caspase-3 (right lower quadrant), low Bcl-2 and high active caspase-3 (left upper quadrant), and high Bcl-2 and active caspase-3 (right upper quadrant). Percentage of Bcl-2 or active caspase-3 cells are shown (B).

C and D: Mice were infected with *H. pylori* (10^9 cfu/mouse) for 24 h. C: Gastric mucosal lysates were prepared for Western Blot analysis to detect relative Bax and Bcl-2 protein expression levels using anti-Bax and anti-Bcl-2 antibodies, respectively, and EGFR phosphorylation and actin levels were determined as described in Figure 1. D: The relative densities of Bax and Bcl-2 protein bands on Western blots were compared to determine the protein expression ratio of Bax to Bcl-2 in each group. The ratio in broth treated mice was set as 100%, and fold change of the ratios in *H. pylori*-infected mice were compared to the broth treated group. N = 5–7 mice for each condition. * $p < 0.05$ vs cells alone, ** $p < 0.001$ vs cells alone, *** $p < 0.001$ vs cells treated with AG1478, § $p < 0.001$ vs cells infected with *H. pylori*. @ $p < 0.001$ vs broth treatment in wt mice. # $p < 0.001$ vs broth treatment in EGFR^{wa2} mice.

Figure 8.

A schematic model of mechanisms for determining the fate of gastric epithelial cells infected with *H. pylori*.